"CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY BUCHAREST
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CONSIDERATIONS ON THE USE OF LASER IN ENDODONTIC THERAPY

PHD THESIS SUMMARY

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INTRODUCTION

The issue addressed in the doctoral thesis is related to a number of fundamental aspects in the field of laser technology for biomedical applications and falls between the main directions of research programs promoting the scientific understanding of lasers used in dentistry.

An important area, both from the perspective of potential profitability and population health, is the development of biocompatible therapeutic alternatives with high functionality and reduced side effects in medical applications, enabling high-performance therapies and successful treatments.

The non-invasive treatment of oral diseases, a major objective of modern dentistry, encourages the intense study of laser systems that can currently offer new therapeutic possibilities, with the potential to establish themselves as a better alternative or as an adjuvant factor in conventional therapy. I chose to conduct my doctoral research in this field, both due to my personal interest in laser therapy and for what this new technique represents in the complex approach to endodontic treatments. Endodontic treatment is performed by combining physical and chemical agents, and one of its goals is to eliminate pulp residues, bacteria and bacterial products from the root canal, at the same time with deep dentin disinfection and the tubular network, essential elements for the long-term success of the treatment. Due to the large number of microorganisms showing increased resistance to conventional treatments, the development of new antimicrobial products becomes a necessity.

My personal concern regarding the success of endodontic treatments led me towards an in-depth study of the applicability of the 940 nm diode laser and the in vitro evaluation of its efficiency on the viability of some microbial species frequently encountered in the etiopathogenesis of endodontic infections. By studying the synergistic bactericidal effect of the laser with solutions of natural plant extracts activated with this type of 940 nm diode laser of high power, I tried to identify the antimicrobial potential of some plant extracts treated with the 940 nm diode laser in order to obtain new irrigation solutions, biocompatible, with potential applications in endodontics.

My doctoral thesis is structured in two parts, the general part and the personal part and includes seven chapters in which studies from literature and personal contributions are presented regarding the effects that the use of laser systems has, as an adjuvant in dental treatment and especially in endodontic treatment.

The first three chapters are dedicated to the general part and refer to the current state of knowledge in the complex field of lasers and their use in dentistry and endodontics, while the next four chapters highlight the general methodology of experimental research, the actual studies in

accordance with the proposed research objectives, followed at the end by general conclusions, personal contributions and annexes.

CURRENT STATE OF KNOWLEDGE

CHAPTER 1. GENERAL NOTIONS ABOUT LASER

The first chapter of the thesis is an introductory one in which a brief incursion into the addressed problem is presented, general notions about laser and a series of elements related to the generation of laser light. The characteristics of laser devices are presented, namely the components, properties, classification, types of action and effects produced by lasers in interaction with target tissues.

CHAPTER 2. LASER'S ACTION MECHANISM AT THE LEVEL OF BIOLOGICAL STRUCTURES

The detailed description of aspects regarding the current state of knowledge in the field of lasers in terms of the action mechanism at the level of biological structures, namely at the level of soft and hard tissues, cellular response as well as dosing and safety of laser use, is the subject of the second chapter.

CHAPTER 3. LASER APPLICATIONS IN DENTISTRY AND ENDODONTICS

In dentistry, lasers have become an instrument applied in many procedures, including soft tissue surgery, decontamination, ensuring anti-inflammatory effects, cavity preparation, caries prevention, decontamination of carious lesions and precise removal of them, as etching agents and antibacterial agents. They can be successfully used in endodontic therapy and in the treatment of periodontal diseases (Shanthala BM and colleagues, 2017). Laser-assisted treatments have the potential to establish themselves as a better alternative to conventional therapy.

Research and clinical use, after the initial interest for all possible therapeutic uses of lasers in endodontics, is reduced to cleaning and decontaminating the root canal system.

The frequent use of the diode laser in endodontic treatment has increasingly generated studies in recent years that have evaluated the antibacterial efficiency of the 940 nm diode laser, at different output powers.

Furthermore, in chapter three, information related to the use and applications of lasers in dentistry and the diode laser in endodontics are synthesized, whether used alone or combined with classic and alternative irrigation solutions.

PERSONAL CONTRIBUTIONS

CHAPTER 4. GENERAL METHODOLOGY OF SCIENTIFIC RESEARCH

4.1 Research Purpose and Objectives

The purpose of my doctoral thesis research was to evaluate, through conducted studies, the in vitro efficiency of the high power diode laser with a wavelength of 940 nm on microbial viability, the effect and potentiation of the antimicrobial effect of some plant extracts, to be used as adjuvants in the sanitization of root canals, by irradiation with a laser beam, as well as demonstrating the biocompatibility of these natural products before and after irradiation with a laser beam with a wavelength of 940 nm.

In the first study of doctoral scientific research, we aimed to assess the efficiency of the high-power diode laser, with a wavelength of 940 nm, and photodynamic antimicrobial therapy on microbial viability, namely on the two species frequently involved in primary or recurrent endodontic infections, particularly virulent, namely Enterococcus faecalis and Candida albicans, which are, after all, common microorganisms.

In the second study of doctoral scientific research, I aimed to investigate in vitro the antimicrobial activity of 7 alcoholic and hydroalcoholic plant extracts from walnut (Juglans regia), sage (Salvia officinalis) and echinacea (Echinacea purpurea), before and after exposure to the action of some laser beams emitted by the 940 nm diode laser, at 2 variants of output power of 3W and respectively 5W, from the perspective of establishing efficiency in endodontic treatment.

The third in vitro study aims to comparatively assess the potential toxicological implications of plant extracts from walnut (Juglans regia), sage (Salvia officinalis) and echinacea (Echinacea purpurea), and the possibility of mitigating the harmfulness outside the root canal and enhancing some recovery and healing reactions, at the cellular level, following activation by irradiation with a laser beam with the same output power variants of 3W and 5W.

These alternative irrigation solutions based on natural products, natural plant extracts and extracts activated with a laser beam generated by the diode laser with a wavelength of 940 nm, can contribute to root canal disinfection, with the possibility of mitigating the harmfulness outside of it and enhancing some recovery and healing reactions at the level of periapical tissues.

4.2 Materials Used in the Study

4.2.1 Laser Device Used

The frequent use of the diode laser in endodontic treatment has increasingly generated studies in recent years that have evaluated the antibacterial efficiency of the diode laser with a wavelength of 940 nm, at different output powers. In addition to the bactericidal effect, the diode laser has a biostimulatory effect, which is of great importance in terms of healing periapical tissues.

I chose to use the diode laser with a wavelength of 940 nm because its clinical application in endodontics has been less studied and also because of the reduced cost, greater versatility and easy portability due to its compact size. In the conducted studies, I used the 940 nm wavelength diode laser, Biolase Epic X (USA).

4.2.2 Natural plant products and solvents used in the conducted studies

In the first study, phenothiazine dyes were used as photosensitizers, namely a 5mg/ml solution of methylene blue (MB) and a 1% toluidine blue solution (TB), substances chosen for their effectiveness in photodynamic therapy (PDT), due to the presence of the cationic charge.

In the next two studies carried out, plant extracts obtained at the Faculty of Biology, University of Bucharest, prepared from walnut (Juglans regia), sage (Salvia officinalis) and echinacea (Echinacea purpurea) were used.

From walnut (Juglans regia), fruits were used, and 4 alcoholic and hydroalcoholic extracts were made in methanol (100% methanol and water:methanol 1:1 v/v, on which 5g of plant were put) and 4 alcoholic and hydroalcoholic extracts in ethanol (100% ethanol and water:ethanol 1:1 v/v on which 1g of plant was put) from the following components: pericarp and mesocarp (green walnut peel), endocarp (woody part of the walnut), seed coat (the coat covering the seed), woody walls inside the seed that separate the cotyledons and lobes. Green peel fragments were lyophilized before use.

From sage (Salvia officinalis), a hydroalcoholic extract was made in ethanol from sage leaf (Salvia officinalis herb) (extract 1:5, ethanol 96%:water = 35:65 ratio).

From Echinacea purpurea (aboveground parts) the product used for testing in the conducted studies was the ready-made echinacea tincture containing E. purpurea 20g and 70% v/v ethanol for 100g solution. From all the prepared plant extracts, the following samples were chosen with the corresponding codes:

N5 – methanol extract from lyophilized pericarp and mesocarp of Juglans regia

N7 – ethanol extract from endocarp of Juglans regia

N11 – methanol extract from seed coat of Juglans regia

N19 – water:methanol extract from pericarp and mesocarp of Juglans regia

N20 – water:methanol extract from inside the seed that separates the cotyledons and lobes of Juglans regia

S – water:ethanol extract from Salvia officinalis

E – Echinacea tincture

3W – the tested extract treated with 3W laser beam

5W – the tested extract treated with 5W laser beam

The 7 untreated extracts represent the control, and the variants analyzed were obtained by treating the plant extracts with a beam of the diode laser with a wavelength of 940 nm, in pulsed mode (CP2), with output laser beam powers of 3W and respectively 5W. The same stock solution from each extract was used in all relevant determinations.

4.2.3 Cell Cultures

The cell cultures used in the conducted studies were represented by: the reference bacterial strain, Enterococcus faecalis ATCC 29212 (American Type Culture Collection), derived from ATCC accredited cultures; the standard bacterial strain, Candida albicans ATCC 10231 (American Type Culture Collection); a L929 cell culture line, with fibroblast type morphology (ATCC L929).

4.3 Methodology

In the first study, the influence of the diode laser was tested on standard strains of Enterococcus faecalis ATCC (American Type Culture Collection) 29212 and Candida albicans ATCC (American Type Culture Collection) 10231. From these, a 0.5 standard McFarland suspension was prepared in physiological serum.

After irradiation, serial dilutions were made from which 10 µL were inoculated in triplicate, and the number of colony-forming units per mL (CFU/mL) was determined to establish the viability of the microorganisms. For each test, 5 replicas were conducted for E. faecalis and 4 replicas for C. albicans.

To evaluate the influence of the irradiated microbial suspension volume on the viability of the microorganisms, the same microbial suspension of C. albicans was analyzed in parallel, divided into different containers, specifically 900 µL distributed in a container with a final volume of 1.5 mL and 100 µL distributed in a container with a final volume of 0.2 mL.

The same reference bacterial strain used, Enterococcus faecalis ATCC 29212 (American Type Culture Collection), in the research carried out in the second study was inoculated on Mueller Hinton agar and TSB (triptone soy broth) at 37°C.

For the third study, fibroblasts (ATCC L929) were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum.

To obtain extracts from walnut fruits, alcoholic and hydroalcoholic extracts in methanol and ethanol (100% methanol and water: methanol 1:1 v/v, over which 5 g plant was added; 100% ethanol and water: ethanol 1:1 v/v over which 1 g plant was added), microwave extraction was used with the Ethos Start D extractor (1 hour at 100°C).

The hydroalcoholic extract in ethanol from sage leaf (Salvia officinalis herba herba) was made as follows: sage leaves (Salvia officinalis herba) were cleaned of impurities, washed, and left to dry at room temperature, then ground with a laboratory mill and mixed with the hydroethanolic solution (extract 1:5, ratio of 96% ethyl alcohol:water = 35:65).

Extraction was done by microwave with the Ethos Start D extractor (1 hour at 100°C). The extracts obtained were dried in a rotavapor, and the resulting product was taken up in DMSO (dimethyl sulfoxide) to a final concentration of 20 mg/mL.

From Echinacea purpurea (i.e., the aboveground parts), the product used for testing in the conducted studies was a prepared Echinacea tincture containing E. purpurea 20 g and 70% v/v ethyl alcohol for 100 g solution.

To test the influence of the diode laser on microbial viability, in the first study, the microbial suspension (0.5 standard McFarland) in physiological serum, was exposed to the laser beam using different intensities, contact times, and application tips according to the device's preset programs, namely IP (infected pockets), PP (periodontal pockets), AU (aphthous ulcer).

The surgical handpiece, which evenly distributes the laser energy in the treated area, was used in non-contact mode because it used at a variable distance of up to 100 cm does not change the energy of the irradiation over a spot with a 1 cm diameter. The handpiece is most commonly used for biostimulation and anti-inflammatory treatment. For the tests carried out in this study, the E3, E4 tips were used for the surgical piece.

After irradiation, from the suspensions used, serial dilutions were made from which 10 µL were inoculated in triplicate, and the number of colony-forming units per mL (CFU/mL) was determined to establish the viability of the microorganisms. For each test, 4 and 5 replicas were performed.

For the second study, the laser with a wavelength of 940 nm was set for the Infected Pockets program and activations were made of selected plant extracts for testing (N5, N7, N11, N20, S, E) at different output power intensities at the tip of the laser beam fiber, starting with 1W, 1.5W, 2W, 2.5W, 3W, 4W, and 5W, using different tips for activation (E2, E3, E4) and varying exposure times respectively from 10s to 30s, 45s, 60s, 180s with successive repetitions and retries for each experiment.

For the third study of the research, three test tubes were prepared for each selected plant extract for testing plus the bacterial strain: control, lot irradiated with 3W laser beam power, lot irradiated with 5W laser beam power.

Irradiation was performed using the uninitiated E4 tip, for 30 seconds, and the preset program (IP)Infected Pockets , in CP2 (Comfort Pulse) mode, with a pulse duration of 1 millisecond.

The working technique for activating the prepared extracts consisted of inserting the tip vertically into the test tubes, with light circular movements made from bottom to top, along the entire length of the test tube.

In the preliminary tests and trials carried out with the initiated E4 tips, cauterization of the solution was noticed, which is why uninitiated tips were used.

The evaluation of the antibacterial effect of the solutions represented by the plant extracts activated by irradiation with a laser beam with output power intensities of 3W and 5W was performed using the adapted Kirby-Bauer method.

The quantitative determination of the antimicrobial activity of the analyzed extracts was performed by the method of serial microdilution in a liquid medium (Mueller Hinton), with the aim of determining the minimum inhibitory concentration (MIC), that is, the minimum amount of the tested compound capable of inhibiting the growth of microbial cells.

The influence of the tested compounds on the development of microbial biofilms on an inert substrate was carried out in 96-well plates in which binary dilutions of the extracts were placed, in contact with a bacterial inoculum with a density of 10^6 CFU/mL. The intensity of the resulting colored suspension was read spectrophotometrically (Flex Station 3) at a wavelength of 492 nm.

4.4 Research data processing

The collected data were centralized and statistically processed using Microsoft Office Excel/Word 2013 and IBM SPSS Statistics v20, 22.0 (Statistical Package for the Social Sciences for Windows). The quantitative variables were expressed as average values with standard deviations (SD).

The charts were created with GraphPad Prism 5.04.

Differences between determined values were analyzed using the Mann Whitney test, a statistical test used to evaluate differences between two independent samples regarding the level of any feature, measured quantitatively, which allows for the detection of value differences of a parameter between small samples.

The level of statistical significance (p) was set at a maximum of 0.05. Values of $p < 0.05$ were

considered statistically significant. The levels of statistical significance were *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

The statistical analysis and interpretation of the results and their comparison with data from similar specialty studies allowed me to establish the conclusions of my scientific research.

CHAPTER 5. IN VITRO ASSESSMENT OF THE EFFICIENCY OF DIODE LASER AND PHOTODYNAMIC THERAPY ON MICROBIAL VIABILITY

5.1 Introduction

The human oral cavity is a suitable environment for the unlimited formation of natural microbial biofilm. However, in the distorted balance of oral health, infectious pathogens can gain access to dental tissues and the gingival area (Takahashi N, Nyvad B, 2011; Grzech-Leśniak K et al., 2017, 2019).

Managing infections caused by bacteria and fungi is a viable challenge in various medical fields, including dentistry. The development of laser medicine has provided a range of new therapy modalities capable of eliminating pathogenic organisms, alternative methods for managing their drug resistance.

In endodontic therapy, the removal of pulp residues and bacterial populations from the root canal system is an important objective since infections at the root canal level are caused by microorganisms that have penetrated the dental pulp and managed to colonize and survive at that level. (Wong et al., 2021).

Although biomechanical preparation can efficiently eliminate microorganisms, more than 50% of the wall surface may remain intact during instrumentation, due to the anatomical complexity of the root canal system (Stuart CH et al., 2006). Eradicating persistent bacteria in remote areas of the root canal system is a major challenge in the different treatment techniques used and is crucial for long-term preservation of endodontically treated teeth. During a canal infection, the microenvironment of the canal favors the selection of a few bacterial species, among which particularly virulent are Enterococcus faecalis and Candida albicans, otherwise common, important microorganisms, found in teeth with refractory endodontic infection.

E. faecalis is a facultative anaerobic, gram-positive coccus, resistant to therapy, which contributes to the failure of endodontic therapy by forming biofilm (Eslami L.M. et al., 2019).

For this reason and due to the fact that it is found in the recurrent failure of endodontic treatment due to virulence, its control is of utmost importance and finding new tools to help eliminate E. Faecalis has

become increasingly necessary (Castilho A L et al., 2013).

C. albicans is a fungus identified in 21% of primary endodontic infections and in 18% of retreatment cases (Eslami L M et al., 2019). Candida can survive in extreme environments by forming biofilms and using its physicochemical properties to adapt to local conditions (Silva Garcez A et al., 2006; De Souza EB et al., 2008).

An innovative approach to disinfection in endodontics is the use of lasers. The potential bactericidal effect of laser irradiation can be efficiently used for further cleaning of the root canal system, a widely studied effect using different lasers such as: the CO2 laser, Nd:YAG, diode laser, and Er:YAG (Tunér J et al., 2010; Silva Garcez A et al., 2006; De Souza EB et al., 2006, 2008, 2010).

For this reason, and due to the adverse effects of drugs on the physical properties of dentin and bacterial resistance, the diode laser and photodynamic therapy (PDT) have been introduced as tools in endodontic therapy to favor the disinfection of the complex system of root canals. Photodynamic therapy (PDT) can specifically target microorganisms without damaging host tissues and has the benefits of immediate action, access to complex areas, such as cracks and bifurcations, preventing bacteremia in immunocompromised patients, and relieving symptoms.

Antimicrobial photodynamic therapy (APDT) is a two-step procedure that involves the application of a photosensitizer, followed by laser irradiation of the sensitized tissues, which will generate a toxic photochemical reaction on the target cells, as a result of which pathogenic microorganisms will be eliminated. The photosensitizer has the ability to selectively accumulate in tissues and to interfere with biological substances. This is a chromophore-based compound that can absorb photons from incident light, thus producing and releasing reactive oxygen and free radicals. Methylene blue (MB) and toluidine blue (TB) are photosensitizing substances that have been widely studied for their effectiveness on planktonic bacteria and have been tested against microbial biofilm structures (Bhavya K et al., 2022).

Antimicrobial photodynamic therapy is a safe, efficient, and easily implementable alternative, and its spectrum of activity covers bacteria, fungi, viruses, and protozoa, which gives it superiority over conventional therapies (Wainwright M et al., 2017). There are studies that report the antimicrobial and bactericidal effects of laser application, thus offering a promising treatment modality (Seyedmousavi S et al., 2014).

The aim of the research in this study was to explore and highlight the influence of diode laser irradiation with a wavelength of 940 nm (high power diode laser) and the effect of photodynamic therapy (PDT) on the viability of standard strains of Enterococcus faecalis (ATCC 29212) and the influence of the diode laser with a wavelength of 940 nm on the viability of standard strains of Candida albicans (ATCC

10231) by varying the conditions and modes of laser beam application.

5.2 Material and method

The influence of the diode laser was tested on standard strains of Enterococcus faecalis ATCC 29212 and Candida albicans ATCC 10231, respectively.

A 0.5 standard McFarland suspension was prepared in saline, which was subjected to the action of the laser beam applied using the Epic Biolase diode laser, with a wavelength of 940 nm and a maximum power of 10W, with settings determined in laboratory research. Different intensities, contact times, and application tips were used according to the device's preset programs, namely IP (infected periodontal pockets), PP (periodontal pockets), AU (aphthous ulcer). For the tests performed in this study, the E3, E4 tips and the surgical piece were used.

After irradiation, from the suspensions used, serial dilutions were made, from which 10 µL were inoculated in triplicate, and the number of colony forming units per mL (CFU/mL) was determined to assess the viability of the microorganisms. For each test, 4 and 5 replicates were made.

To assess the influence of the volume of the microbial suspension of C. albicans tested, the same microbial suspension distributed in different containers was analyzed in parallel: 900 µL distributed in a container with a final volume of 1.5mL and respectively 100 µL distributed in a container with a final volume of 0.2 mL. For their effectiveness in photodynamic therapy (PDT), due to the presence of a cationic charge, we used the following phenothiazinium dyes as photosensitizers in the study: a solution of 5mg/ml of methylene blue (MB) and a 1% toluidine blue (TB) solution.

In the statistical analysis, the data presented are mean ±SD (standard deviation) and the graphs were made with GraphPad Prism 5.04.

Differences between determined values were analyzed using the Mann Whitney test. P values < 0.05 were considered statistically significant. Statistical significance levels were *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** $p < 0.0001$.

5.3 Results and Discussions

E. Faecalis suspensions were tested using the 940 nm diode laser, with the pre-set IP program (infected periodontal pockets), on 3 lots, the differences being in the number of irradiations, the pause between them was 10 seconds, power of 2.5 W, continuous mode, with E3 tip.

Irradiation of an E. faecalis suspension with the same type of tip, E3, but with different contact times,

by the number of irradiations, influences to a small extent the bacterial viability.

In the next testing batch, the statistical analysis of the obtained colony-forming units did not reveal a significant difference between the different irradiations with the E4 tip. Compared to the first batches tested, a higher efficiency is observed for the E3 tip, under the same irradiation conditions, only with a different application tip.

The antibacterial effects of the laser in patients treated for oral cavity diseases have been confirmed in previous research, where the diode laser, using the pulsed mode, was applied with power settings over 2 W, to induce a photothermal effect (de Souza EB et al., 2008).

In the next experiment, changing the irradiation program, from IP (infected periodontal pockets), continuous mode (CW) to PP (periodontal pockets), pulsed mode CP2 (interval between pulses of 2 milliseconds), with E4 tip, made a statistical difference when the irradiation time was increased to 15 seconds and we used a power of 3W.

Photoactivated disinfection is recommended as an adjunct procedure for eliminating residual bacteria from the root canal after standard endodontic debridement. The effectiveness of photodynamic therapy (PDT) depends on the microorganism, the type of photosensitizer, and the light used. Methylene blue (MB) and toluidine blue (TB) are commonly used as photosensitizers, phenothiazinium dyes effective in photodynamic therapy due to their cationic charge.

When exposed to light with a certain wavelength, the photosensitizer can react either with oxygen or with other biomolecules, to create free radicals, reactions that will lead to cell death; at the same time, photodynamic therapy targets the microorganisms without collateral damage to human cells and tissues (Usacheva MN et al., 2001).

We evaluated the antimicrobial effect of the laser beam on the suspension of E. faecalis after the application of a photosensitizing substance respectively a solution of methylene blue (MB) with a concentration of 5mg/mL and one of toluidine blue (TB) of 1%. For each of them, two treatment modalities were tested:

variant 1 - a 15 sec irradiation, 3W power, pulsed mode CP2, E4 tip

variant 2 - biostimulation (biostimulation with surgical piece), 180s, 4W power, 720J energy.

The analysis of the results showed that the application of the parameters corresponding to variant 2 determines a decrease in the number of bacteria.

The application of a photosensitizing substance increases the inhibitory effect of the laser beam, but it was observed that in the case of toluidine blue the number of CFU is lower compared to methylene blue.

Considering the positive effect of photodynamic therapy on the viability of E. faecalis where we used

toluidine blue as a photosensitizer, we tested the effect of the 940 nm diode laser in the presence of different volumes, respectively 1/100 and 1/10, of toluidine blue at a concentration of 5mg/mL, using the same irradiation parameters: biostimulation, power 4W, energy 720J, for 180s.

The evaluation of the results showed that a larger volume, a proportion of 1/10 of toluidine blue photosensitizer, leads to a decrease in the number of viable bacteria.

With the following batches of study we experimented with applying a laser beam at different power intensities. The E. faecalis suspension after a 3-minute contact at room temperature with the solution of toluidine blue (5mg/mL) in a 1/100 proportion (1/100=10 microL) was irradiated with the following parameters: I1, I2, I3 with an irradiation of 30 seconds, pulsed mode CP2, program IP, tip E4, changing the power to 2W, 3W, and 4W and we noticed that a power of 3 and respectively 4W results in a decrease in the number of viable bacteria.

We conducted tests on the action of the laser by applying the same contact time, but different powers and tips and found that tip E3 is more efficient compared to E4 when testing for 30 seconds at a power of 5W, pulsed mode CP2, preset program IP (infected pockets), after applying a solution of toluidine blue (1/100=10 microL) at a concentration of 5mg/mL.

The inhibitory effect of the laser in the presence of a photosensitizer (toluidine blue) is influenced by the action time. Thus, the use of the same type of tip, program, and intensity, but with a change in contact time, showed that a longer contact time results in a decrease in the number of viable cells.

Strains of Candida are occasionally found in primary root canal infections, but occur more frequently in filled teeth with treatment-refractory lesions. Candida albicans is the most widespread fungal species, a microorganism that has an affinity for dentin and is resistant to some drugs applied in root canals, for example, those based on calcium hydroxide.

The increase in cases of infection caused by Candida strains and consequently the excessive use of antimicrobial drug treatments, has favored in recent decades the emergence of resistance of these species to conventional antifungal agents. The study of additional methods for controlling these microorganisms, such as laser irradiation and photodynamic therapy, becomes essential.

Thus, we analyzed in vitro the effect of the laser beam with a wavelength of 940 nm on the standard strain of C. albicans ATCC 10231.

A series of work batches were made, varying the parameters of the used diode laser by changing the power (2W, 3.5W, 5.5W) and the tip for applying the laser beam (E3, E4), using the preset program aphthous ulcer (AU).

In an initial test, the results showed that in cases 3 and 1, where powers of 2W and 5.5W were used,

with tips E4 and E3, there is a decrease in the number of viable cells compared to the control, respectively a greater decrease in microbial viability at a power of 5.5 W, with tip E3.

Using the same preset program AU (aphthous ulcer) but varying the power intensity, the number of irradiations, and the type of tips, the determination of the number of CFU/mL showed that among the tested variants, V5 and V6, inhibit microbial growth, with better efficiency for a power of 6.5W, using the uninitiated tip (V5: 6.5W, 2 irradiations of 20 sec with a 10 sec pause between them, AU program).

During the analyses, the efficiency of a single-stage irradiation (a single irradiation), for 30 seconds with the same type of tip, but changing the power intensity, was also tested.

Among the tested variants, it was observed that applying the laser at a power of 4.5W in variant 9 results in a significant reduction in the viability of C. albicans from a statistical point of view.

To evaluate the influence of the volume of the tested microbial suspension, the same microbial suspension distributed in different containers was analyzed in parallel, namely 900 µL distributed in a container with a final volume of 1.5 mL and respectively 100 µL distributed in a container with a final volume of 0.2 mL, considering that in the root canal the workspace is restricted, and the irradiated space is limited.

Testing different volumes with the same parameters did not reveal statistically significant differences regarding the reduction of the microbial load.

Discussions

The results obtained in the study carried out, in terms of lower efficiency in completely reducing microbial viability through the exclusive use of the laser, are in agreement with studies published by Moshonov et al., in which Nd:YAG laser irradiation was compared with NaOCl efficiency in root canal system disinfection. The authors found that Nd:YAG laser irradiation significantly reduced the number of bacteria, while NaOCl irrigation also efficiently disinfected the root canals.

Similar to the results of our study, Jha D et al., (2006) demonstrated the inability of laser and rotary instruments to achieve complete disinfection of root canals on their own.

Christo JE et al., (2016) highlight in the same sense that NaOCl solutions and Er:YAG laser irradiation were effective against all microorganisms, with NaOCl treatment being statistically superior to laser treatment.

Various laser systems were examined for use as adjuncts to the current disinfection methods used in root canal treatment to increase the effectiveness of NaOCl in cleaning the canal system, including the lateral canals (Christo JE et al., 2016). The authors used 810 nm diodes with power output settings of 2.5W and 5 seconds per cycle and with a cycle repeat 4 times at an interval of 2 seconds. Disinfection of root canals

experimentally contaminated with Enterococcus faecalis with diodes was compared to disinfection with sodium hypochlorite, chlorhexidine, and the combination of diodes with 2% chlorhexidine solution. Diodes as canal disinfectant and the combination of diodes with 2% chlorhexidine solution showed the highest level of antimicrobial effectiveness against Enterococcus faecalis (Olivi G et al., 2011, 2013).

The data can be considered in accordance with the personal study considering the different laser wavelength lengths used and the different settings applied for testing.

Similar results to our study were communicated following the experiments conducted by Kreisler M (2003) who investigated the bactericidal effect of a semiconductor laser used in combination with NaOCl/hydrogen peroxide (H2O2) irrigation or only with saline solution and found that only the use of the laser did not lead to a significant bactericidal reduction compared to its use together with irrigation solutions.

In the parameters of the study conducted by Preethee T et al., (2012), it was concluded that the 908 nm diode used in conjunction with conventional chemomechanical techniques demonstrated significant elimination of E. faecalis in the apical third of the root canal dentin. Unidirectional analysis of variation showed statistically significant differences between laser irradiated groups, non-irradiated groups, and the control group, emphasizing the efficiency of the laser in activating irrigation solutions and less as a singular effect on E. Faecalis, in accordance with the results obtained in personal research, thus underlining that the efficiency of the laser must be supported by the use of irrigation solutions.

Udart et al. (2011) tested the bactericidal effect of the 940 nm wavelength on E. faecalis biofilm. In their study, they found that this wavelength has a very weak antibacterial effect on bacterial biofilm and concluded that the bactericidal effect of the 940 nm laser comes from its thermal effect. For this effect to be strong enough to disinfect the root canal, the laser would have to be applied at a sufficiently high power, and the temperature in the root canal dentin would have to rise to about 70°C, a situation which could destroy periodontal tissues. The increase in efficiency on microbial viability was demonstrated in our personal research along with the increase in power intensity.

Studies were conducted to establish the efficiency of the 940 nm diode laser on smear layer removal using various setting characteristics. Thus, Maden M et al. (2013) show that by using 13 cycles of 10 seconds of exposure associated with lavage with 15% EDTA, followed by 2.5% NaOCl, the best results are obtained, while a study by Schlafer et al. (2010) shows that at a photo-activated exposure of 30 seconds, the number of viable pathogens in the root canals is reduced.

In the research conducted within our personal study, we noted a reduction in the number of colony-forming units following an increase in the number of irradiations and exposure time.

Alfredo E et al., in 2008, evaluated the temperature increase after irradiation inside the canal with a

980 nm diode laser for 30 seconds, with different power settings (1.5 W, 3.0 W, 5.0 W), under conditions of humidity and drying of the canal analyzed in the cervical, medium, and apical third. The authors concluded that the application of 1.5 W and 3.0 W, for 20 seconds, represents a safe therapeutic modality in endodontic therapy. Da Fonseca A. et al., in 2012, evaluated the temperature change in mandibular incisors using an 810 nm laser diode, at different power settings (1.5W, 2.0W, 2.5W, 3.5W, 3.0 W). The authors reported that only at 3.5 W there was an increase in temperature by more than 7 degrees (critical value assumed within the study). They recommended using the laser at a power lower than 3W for endodontic treatment.

In accordance with the recommendations from the specialized literature, the power values used for our personal research, which demonstrated a decrease in microbial viability, were 3W for 15 seconds for both tested bacterial strains.

Hmud et al. (2010) confirm the possibility of using infrared lasers (940 nm and 980 nm) to activate the action of irrigants in endodontic treatment, using a power of 4 W at 10 Hz and 2.5 W at 25 Hz, respectively. The authors warn about the lack of affinity between these wavelengths and water; (Tilakchand M et al., 2018; Kuzekanani M et al., 2019; Constanza M et al., 2020).

Given that the final goal of canal therapy is the elimination of as many bacteria as possible from the root canal, studies have been conducted (Hendi SS et al., 2021) aiming to compare the antibacterial effects of a 940 nm wavelength diode laser and silver nanoparticles and the synergistic effects of both techniques on Enterococcus faecalis.

Although the diode laser showed a significant difference in the number of bacterial colonies before and after intervention, it alone was not efficient enough to eliminate the bacteria. According to the results, the use of silver nanoparticles, along with the appropriate concentration and time, eliminated more bacterial colonies than the negative control group. The simultaneous use of these with the diode laser showed that laser irradiation cannot increase the antibacterial effect of silver nanoparticles. Ultimately, sodium hypochlorite is still the gold standard in root canal disinfection (Hendi SS et al., 2021).

Mustafa M B et al., (2020), conducted an in vitro study to test and evaluate the bactericidal effect of the 940 nm diode laser. In this study, the antibacterial effect of the laser and 5.25% NaOCl separately and combined was tested. Laser radiation from a 940 nm wavelength diode laser at a power of 1.3 W showed a small antibacterial effect on bacterial biofilm when used without NaOCl, data that agrees with the results obtained by us by applying the laser beam even at a higher power of 2.5 W. In conclusion, irradiation with the 940 nm diode laser has a reduced antibacterial effect on E. faecalis because the photonic energy is poorly absorbed by gram-positive bacteria (Udart M et al., 2011).

From these studies, we can draw the conclusion that it is difficult to compare the results due to the

differences in wavelength and different settings, but the use of diode lasers is of interest due to its small size, reduced cost and significant contribution it brings to the sterilization of root canals.

Photodynamic therapy is considered a supplement to traditional canal disinfection protocols and can be combined with conventional mechanical instrumentation techniques and antimicrobial chemical therapy (Hamblin MR et al., 2004).

Garcez et al., (2008, 2010, 2011), compared the efficacy of antimicrobial photodynamic therapy, standard canal therapy and combined treatment for eliminating bacteria present in infected canals and showed that canal therapy reduced bacteria by 90%, only antimicrobial photodynamic therapy reduced 95% of bacteria and the combination of the two procedures resulted in a 98% greater reduction, an aspect demonstrated in personal research by increasing the inhibitory effect of the laser beam in the presence of the two types of photosensitizer.

Photodynamic therapy has several advantages over antibiotics because it offers precise targeting, and has triple local specificity by: (1) predominant association/absorption of photosensitizers by target cells compared to non-target cells, (2) pharmacodynamic inertia of non-irradiated photosensitizing substances, as well as (3) irradiation limited to the area of the infected zone (Garcez et al., 2010).

Consequently, systemic toxicity is largely absent outside the irradiated area, an area full of photosensitizer. Compared to antibiotics, resistance does not develop against photodynamic therapy and photosensitizers, and repeated treatment using this technique has not led to the selection of resistant strains (Mohammadi Z et al., 2017). These reasons underlie the potential use of photodynamic therapy in combating resistant strains in a minimally invasive and patient-friendly manner.

Meire et al., (2009), compared the antimicrobial efficacy of 2 high-power lasers (Er:YAG and Nd:YAG) with that of NaOCl action on Enterococcus faecalis. They concluded that the use of both systems resulted in a weak reduction in the number of bacteria.

According to George and Kishen (2007, 2008), referring to Enterococcus faecalis, APDT can destroy the functional integrity of bacterial cell walls, DNA, and cell membrane proteins. The volume of damage on these targets is influenced by the photosensitizer used during APDT.

Thus, according to our results, toluidine blue showed a greater antimicrobial photodynamic effect for E.faecalis than methylene blue, and a greater effect at a higher volume, 1/10 of toluidine blue. Using laser powers of 3 and 4 W led to a decrease in the number of colony-forming units, and using a power of 5W, in CP2 pulse mode for 30 seconds, in the presence of toluidine blue, was the most efficient for reducing the number of viable bacteria. Longer application time increases efficiency and in the same vein, at the same irradiation time, the number of irradiations influences the decrease in microbial viability.

Considering that not all photosensitizers have the same photodynamic effect against different microorganisms, Soria-Lozano P et al., (2015), compared in vitro the photodynamic effect of methylene blue (MB), Bengal rose (RB) and curcumin (CUR) in combination with white light, on the microorganisms S. mutans, S. sanguis, and C. albicans. They concluded that the effectiveness of photodynamic therapy depends on the microorganism, photosensitizer, and light used, thus, Bengal rose showed a greater antimicrobial photodynamic effect for Streptococcus species, while methylene blue was more efficient for Candida species. However, in our study, no antimicrobial effects were observed when the strains were exposed separately to the light source.

Studies on the photobactericidal efficacy of methylene blue and toluidine blue have shown inconsistent results in the literature. For the most part, a higher bactericidal activity of toluidine blue is in line with the results obtained in our own research.

The aim of the study by Eslami LM et al., (2019), was to compare the antimicrobial effects of calcium hydroxide, triple antibiotic paste, photodynamic therapy in the presence of toluidine blue, light-emitting diode, and 940 nm diode laser on the biofilm of Enterococcus faecalis and Candida albicans in the root canal system of ex-vivo human teeth. Following the study, they found that treatment with antibiotic paste, photodynamic therapy, and LED reduced the biofilm thickness compared to the control group and other experimental groups. Since photodynamic therapy is a single-visit treatment and does not cause microbial resistance, it is the preferred method and compared to the laser it has several advantages, such as: more effectiveness due to the wider spectrum, feasibility, safety, and accessibility, results consistent with the reduction of microbial viability obtained in our study in the presence of toluidine blue under the influence of action time, power, and number of irradiations.

Souza RC et al. (2010) evaluated the specific effects of photodynamic therapy using methylene blue, toluidine blue, and malachite green as photosensitizers and only low-power laser irradiation on the viability of C. albicans, concluding that there was a reduction in the number of C. albicans colony-forming units. These results, as well as those communicated by Souza SC et al. (2006), and Giroldo LM et al. (2009), are in agreement with those of the study conducted in this research, namely that the application of a 940 nm diode laser at a power of 4.5 W determines a statistically significant reduction in the viability of C. albicans.

Wilson and Mia (1993) studied the photosensitization of Candida species following the use of toluidine blue and light generated by the helium-neon (He-Ne) laser (632.8 nm and 66.36 J/cm2) and found a reduction in the number of CFU/ml of 77% for C.albicans. Similarly, Giroldo et al. (2009) reported a significant decrease in C.albicans growth when methylene blue was combined with the

application of a diode laser (wavelength 684 nm and power 28J/cm2). The study conducted as part of my personal research also highlighted increased efficiency following the application of the laser along with the modification of the number of irradiations, the application time, and the applicator tips which influence the fiber diameter and the peak power intensity, specifically when a power of 6.5 W and the uninitiated E4 tip of the 940 nm wavelength diode laser were used.

Studies conducted by Usacheva et al. (2001) evaluated the bactericidal efficacy and found that the reduction in the number of CFU/ml of C. albicans increased with the increasing energy density of the applied laser, from 15.8 J/cm2 to 39.5 J/cm2. This is in agreement with the results of my study.

Maver-Biscanin et al. (2005) demonstrated the fungicidal effect of low-power laser light in the absence of a photosensitizer. In our study, a statistically significant reduction in CFU/ml was recorded at an irradiation for 30 seconds with a power of 4.5 W.

Seyedmousavi et al. (2014) observed that using energies of 10 J, at wavelengths of 685 and 830 nm produced statistically significant effects, in vitro, on the pathogenicity of C.albicans (p value ≤ 0.05). In turn, Maver-Biscanin et al. (2005), in their in vivo and in vitro study for the reduction of C. albicans species, performed irradiations of the treatment areas with different exposure times, 5 min (830 nm, 3.0 J/cm2, 60 mW) and 10 min (685 nm, 3.0 J/cm2, 30 mW). In contrast to the above studies, we found a statistically significant reduction in the number of CFU/ml of C. albicans, in vitro, by applying two irradiations, with an exposure time of 20 seconds, a 10-second break between irradiations, and a power of 5.5 W but also by applying a single irradiation, for 30 seconds, with a power of 4.5 W.

The current data suggest that laser light at certain wavelengths may have some positive effects on reducing C. albicans infection; however, there is a clear need for further research and clinical studies.

The differences between our study and the results reported in the specialized literature may be due to the lack of protocols, predefined parameters for the use of laser therapy and photodynamic therapy, which affects the reliability of the comparison between the results obtained in different studies. In addition, the concentration, physiological state of the microorganisms, incubation time, type of photosensitizer, as well as exposure time, number of irradiations, power, and energy density, along with the characteristics related to the preset programs of the used laser, can influence the results of endodontic therapy that uses only the application of laser technology or photodynamic therapy.

5.4 Conclusions

Irradiating the microbial suspension with the diode laser highlighted that the contact time, via the number of irradiations, slightly influences microbial viability, whereas under the same irradiation conditions, the E3 application tip demonstrated greater efficiency compared to the E4 tip.

With an increased irradiation time of 15 seconds, a power intensity of 3W, and a pulsed application mode (CP2), statistically significant differences were observed in reducing the number of viable bacteria.

Although the diode laser showed a significant reduction in the number of bacterial colonies after application, even after increasing the time and applied power, it alone was not efficient enough to completely eliminate the bacterial strains used in the studies, namely E. faecalis and C. albicans.

As we have previously stated, the effectiveness of photodynamic therapy depends on the microorganism, the photosensitizer, and the characteristics and parameters of the laser used. According to our results, toluidine blue showed a higher antimicrobial photodynamic effect for E. faecalis than methylene blue, and a larger effect with an increased volume, namely when we used a greater proportion, of 1/10 of toluidine blue. In the presence of toluidine blue, using laser powers of 3W and 4W led to a reduction in the number of colony-forming units. Under the same irradiation conditions, specifically a time of 30 seconds, at a power of 5W in pulsed mode CP2, the use of the E3 tip was the most efficient in reducing the number of viable bacteria. It was also highlighted that in the presence of toluidine blue, using the same irradiation conditions, a longer application time increases efficiency and similarly, at the same irradiation time, the number of irradiations positively influences the reduction of microbial viability.

It can be concluded that, concerning the viability of C.albicans, the same application time conditions for the laser beam, number of irradiations, using the same tip, only changing the power, and respectively the batch in which a power intensity of 4.5W was used, led to a reduction in microbial viability. The decrease in viability of C.albicans was also achieved when power variations revealed a scenario in which a higher power of 5.5W with the E3 tip was used.

In the variants where the power and the type of tip used were modified, we obtained statistically significant decreases in the number of colony-forming units at powers of 6.5W and 7.5W, but the highest efficiency was at the power of 6.5W when the uninitiated tip was used. Testing different volumes with the same laser parameters did not reveal statistically significant differences regarding the reduction of microbial load.

CHAPTER 6. STUDY ON THE EVALUATION OF ENHANCING THE ANTIMICROBIAL EFFECT (QUALITATIVE AND QUANTITATIVE) OF SOME NATURAL PRODUCTS, USED AS ADJUVANTS IN ENDODONTIC THERAPY, BY IRRADIATING THEM WITH A BEAM OF THE 940 NM DIODE LASER

6.1 Introduction

The essential objective for the success of endodontic treatment is the thorough cleaning of the root canal system, by removing the infected pulp tissue, the present bacteria, and the remnants of contaminated dentin before sealing it, along with the disinfection of deep dentin and the tubular network to prevent infection and promote long-term healing.

The non-surgical endodontic procedure is based on biomechanical instrumentation, canal irrigation, and medication for disinfecting the root canal system. Various instrumentation techniques have proven their effectiveness in decreasing bacterial load without achieving their total eradication. Intracanal irrigants, such as NaOCl, require direct contact along the surface of the dentin to exert their bactericidal effect. Also, due to surface tension, these solutions fail to penetrate the dentin deeply enough, compared to the ability of pathogenic microorganisms to penetrate dentin (Gomes-Filho J et al., 2008).

At the same time, the complex anatomy of the root canal system allows bacteria to survive harsh conditions. Among the microorganisms involved in the failure of endodontic treatment that can be cultured from root canals subjected to retreatment, the most commonly encountered is Enterococcus faecalis, a gram-positive bacterium, similar to streptococci, capable of forming intra and extra radicular biofilms. These biofilms partially protect these bacteria from the immune defense, endodontic procedures, and drugs, making them resistant to phagocytosis, antibodies, and antimicrobial agents. E. faecalis has various mechanisms that allow it to survive in unfavorable environments, for example, the ability to develop at an alkaline pH, high salinity, resistant to temperatures between 10° - 60°C. This bacterium can survive in the presence of irrigants and the solutions used in canal treatment, which is why there is increasing interest in efficient and economical alternative products as treatment options for oral diseases.

Plant extracts and plant-based products are promising alternatives to synthetic chemicals used as root canal irrigation solutions. The use of plant-based alternatives for root canal treatment is becoming increasingly popular due to their beneficial properties, availability, lack of side effects, extended storage time, profitability, minimal toxicity, and the lack of recorded microbial resistance so far, (Arora S et al., 2021).

Laser technology, used together with classic irrigation solutions and natural extract-based ones, improves the cleaning ability, the removal of debris and the smear layer from root canals, thus making the decontamination of the endodontic system more efficient.

The use of the diode laser with a wavelength between 810 and 940 nm has become increasingly

frequent, in combined therapy consisting of irrigation with solutions and irradiation with the 940 nm diode laser, as well as activation of solutions with laser, an efficient treatment option for reducing E. faecalis as well as other bacterial flora from the root canal system.

Based on these observations, *the purpose* of this study was to evaluate in vitro, qualitatively and quantitatively the antimicrobial activity and the influence on the development of microbial biofilms, of 7 alcoholic and hydroalcoholic plant extracts (from walnut, sage, and echinacea), before and after exposure to the action of laser beams emitted by the diode laser with a wavelength of 940 nm, at 2 variants of the output power at the tip of the fiber, of 3 W and respectively 5 W from the perspective of efficiency in endodontic treatment, the tooth's lifespan depending largely on the correctness and efficiency of the treatment.

6.2 Materials and Methods

From the walnut (Juglans regia), the fruits were used to create 4 alcoholic and hydroalcoholic extracts in methanol (100% methanol and a water:methanol ratio of 1:1 v/v, over which 5g of plant material was added) and 4 alcoholic and hydroalcoholic extracts in ethanol (100% ethanol and a water:ethanol ratio of 1:1 v/v, over which 1g of plant material was added) from the following components: pericarp and mesocarp (the green shell of the walnut), endocarp (the woody part of the walnut), the seed coat (the coat covering the seed), the woody walls inside the seed that separate the cotyledons and lobes. The green shell fragments were freeze-dried before being used. To obtain the extracts from the walnut fruits, microwave extraction with Ethos Start D extractor was used (1 hour at 100°C).

From the sage (Salvia officinalis), a hydroalcoholic extract was made from the sage leaf (Salvia officinalis herba) (extract 1:5, ratio of 96% ethyl alcohol:water = 35:65). The hydroalcoholic extract in ethanol from the sage leaf (Salvia officinalis herba herba) was made as follows: the sage leaves (Salvia officinalis herba) were cleaned of impurities, washed and left to dry at room temperature, then crushed with a laboratory mill and mixed with the hydroethanolic solution (extract 1:5, ratio of 96% ethyl alcohol:water = 35:65).

The extraction was performed by microwave with Ethos Start D extractor (1 hour at 100°C). The obtained extracts were dried on a rotary evaporator, and the resulting product was taken up in DMSO (dimethyl sulfoxide) at a final concentration of 20 mg/mL.

From the above-ground parts of the echinacea (Echinacea purpurea), a pre-prepared echinacea tincture was used for testing in the studies performed. The Echinacea tincture contains E. purpurea 20g and 70% v/v ethyl alcohol for 100g solution.

From the prepared plant extracts, the following samples were selected for testing with the corresponding codifications: N5 - methanol extract from freeze-dried pericarp and mesocarp of Juglans regia N7 - ethanol extract from endocarp of Juglans regia N11 - methanol extract from seed coat of Juglans regia N19 - water:methanol extract from pericarp and mesocarp of Juglans regia N20 - water:methanol extract from inside the seed separating the cotyledons and lobes of Juglans regia S - water:ethyl alcohol extract from Salvia officinalis E - Echinacea tincture 3W - test extract treated with 3W laser beam 5W - test extract treated with 5W laser beam.

The 7 untreated extracts represent the control (witness), and the analyzed variants were obtained by treating the plant extracts with a laser beam with a diode with a wavelength of 940 nm, in pulsed mode (CP2), with beam powers at the output of 3 W and respectively 5 W.

The same stock solution from each extract was used in all the determinations made.

The bacterial strain used in this study was Enterococcus faecalis ATCC 29212 (American Type Culture Collection) which was sown on Mueller Hinton agar medium and TSB (tryptone soy broth) at 37°C.

To determine the potentiating effect of the diode laser, a part of the quantity obtained from the 7 extracts was treated with a diode laser beam with a wavelength of 940 nm, in pulsed mode, with beam powers at the output through the optical fiber of 3 W and respectively 5 W.

From each plant extract chosen for testing plus the bacterial strain, 3 test tubes were prepared: control, lot irradiated with 3W laser beam, lot irradiated with 5W laser beam. I performed the irradiation using the E4 tip, uninitiated, for 30 seconds and the preset program Infected Pockets, in CP2 pulsed mode (Comfort Pulse), with a pulse duration of 1 millisecond.

The work technique for activating the prepared extracts consisted in the vertical introduction of the tip in the test tubes, with slight circular movements executed from bottom to top, along the entire length of the test tube.

The qualitative assessment of the antimicrobial activity was performed by the adapted Kirby-Bauer method. A bacterial suspension adjusted to a density of 1.5x108CFU/mL (Colony Forming Units), was sown in fabric on plates with Mueller Hinton agar medium. The tested extracts were deposited in 10 µL spots. After sowing, the plates were left to rest at room temperature for the solution droplet to adsorb into the medium, after which they were incubated at a thermostat (37°C), for 24 hours. The antibacterial effect was quantified by the appearance of an inhibition halo (a clear area) around the spot which was measured with a ruler.

The quantitative determination of the antimicrobial activity of the analyzed extracts was made by

the serial microdilution method in liquid medium (Mueller Hinton), in order to determine the minimum inhibitory concentration (MIC), i.e. the minimum quantity of the tested compound capable of inhibiting the growth of microbial cells.

For this purpose, 96-well plates were used in which binary serial dilutions of the extracts were made. In the first well, 90 µl of liquid culture medium and 90 µl of extract were pipetted. From this, 90 µl were transferred to the second well, and from the second well, 90 µl were transferred to the third and so on until the last well, from which 90 µl were discarded.

Subsequently, the wells were inoculated with 10 µl microbial suspension with a density of 106 UFC/mL. For each test, a microbial culture control (a row of wells containing respectively culture medium inoculated with microbial suspension) and a sterility control of the medium (negative control) were also conducted.

After incubating the plates at 37°C for 24 hours, the obtained results were analyzed by macroscopic examination.In the growth control well, the medium was cloudy as a result of microbial growth. The obligatory sterility control well did not show any bacterial growth; the liquid remained clear, transparent.

The extract concentration corresponding to the last well where growth or culture development was observed represented the minimum inhibitory concentration (MIC) for the respective compound.

The evaluation of the microbicidal activity of the extracts was carried out by inoculating a volume of 10 µL from the wells used to determine the MIC, to ascertain the minimum bactericidal concentrations (MBC), expressed as the smallest concentration at which the tested extract had a lethal effect on bacteria.

The study of the influence of the tested compounds on the development of microbial biofilms on inert substrate was conducted in 96-well plates with a flat bottom, where binary dilutions of the extracts were put into contact with a bacterial inoculum having a density of 10^6 CFU/mL.

After incubating at 37˚ C for 24 hours, the plates were emptied and washed three times with sterile saline solution to remove bacteria that did not adhere to the well walls. Bacteria adhered to the plate material were fixed for 5 minutes with methanol, then stained with a 1% crystal violet alkaline solution for 15 minutes. The staining solution was removed, and the plates were washed under running tap water. After drying at room temperature, the microbial biofilms formed on the plastic plates were resuspended in 33% acetic acid, and the intensity of the colored suspension was read spectrophotometrically at 492 nm.

6.3 Results and discussions

The qualitative evaluation of the antimicrobial activity is a method that highlights the phenotypic resistance of the tested strains. Antimicrobial substances diffuse in the agar and inhibit the germination and

development of the tested microorganisms, forming inhibition zones.

The qualitative evaluation of the inhibitory effect of walnut, sage, and echinacea extracts has revealed differences between the inhibition zone values obtained for the control and the extracts treated with a 3 W and 5 W laser beam.

It is observed that the irradiation of the extracts with the 3 W beam resulted in larger inhibition zone diameters compared to those of the untreated extracts and those irradiated at the power of 5 W. Hydroalcoholic extracts from walnut fruit, specifically the water:methanol extract from the inner cotyledon wall (N20) and the water:methanol extract from lyophilized pericarp and mesocarp (N19), and the hydroalcoholic extract from sage did not show antimicrobial activity.

The quantitative determination of the inhibitory effect of the analyzed extracts using the microdilution method highlighted that treating the plant extracts with a laser beam enhances the antibacterial effect. Extracts from the seed coat (N11), from the inner cotyledon wall (N20), from the pericarp and mesocarp (N5, N19) obtained from walnut fruits, as well as extracts from sage and echinacea treated with the laser beam have exerted an inhibitory effect at lower concentrations compared to the untreated extract.

Comparing exposure to the two output powers from the fiber tip, it was observed that by activating the solutions with the 5 W laser beam, the efficiency of all tested extracts is increased.

The most effective of the tested extracts was the one obtained in methanolic solution from the seed coat of walnut fruit (N11), and the most evident difference between the treated extracts and control was recorded for formulas N7 (ethanol extract from J. regia endocarp) and S (S. officinalis extract).

As we mentioned, in our study, the bactericidal effect of irradiated extracts was also tested. The most effective extract was from walnut seed coat, followed by sage.

Similar to other tests conducted, an enhancement of the antimicrobial effect was observed following the activation of testing solutions by applying a 5W laser beam, with the exception of echinacea tincture.

Biofilms formed by Enterococcus faecalis strain are inhibited by the untreated extracts used in the research, with the best evaluation for the methanol extract from the J. regia seed coat (N11).

It is noted that by pre-treating and activating with a 5 W fiber tip laser beam, concentrations below 1% of the extracts obtained from walnut seed coat (N11) and sage (S) inhibit the biofilms formed by the Enterococcus faecalis strain.

In the literature, studies that obtained similar results regarding the antibacterial effect of some of the natural extracts used in this research, as well as for other plant extracts, can be found.

Thus, Vieira et al., (2020), analyzed the antimicrobial effect of a hydroethanolic extract from green walnut bark, on a series of bacteria, including E. faecalis, and determined the MIC value at 20 mg/mL, while the bactericidal concentration was more than 20 mg/mL, an antibacterial effect also confirmed by the results obtained through personal research carried out.

Deshpande et al., (2011), reported that the walnut extract proved to be the most effective among the extracts, as an antimicrobial against oral microflora, differentiating from the results of my research by using walnut bark extract and its efficiency on the saliva microbial flora, E.faecalis not being mentioned.

In another study, by Alkhawajah A M, (1997), the walnut bark extract was tested and showed broad-spectrum antimicrobial activity in a dose-dependent manner; it was reported that it inhibits the growth of several pathogenic microorganisms, such as Gram-positive bacteria (Staphylococcus aureus and Streptococcus mutans), Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa) and Candida albicans, however, this extract was not tested on E. faecalis (Noumi E et al., 2010, 2011).

The evaluation of the antimicrobial activity of the ethanolic and aqueous extracts from walnut bark against four species of oral bacteria in the saliva of patients with dental caries (Zakavi et al., 2013) showed that they significantly inhibit the growth of oral bacteria, and the tincture (ethanolic extract) had a greater efficacy than the aqueous extract as an antimicrobial against pathogenic oral microflora, a study that is similar to the results obtained in my personal research regarding the increased effectiveness of alcoholic extracts. The S. officinalis extract with a concentration of 50mg/mL was effective against a number of 50 strains isolated from the oral cavity and reference (De Oliveira et al., 2019).

The results of my research are similar in terms of the possibility of using sage extract for endodontic lavage, with the results obtained by Mehraban A et al., (2016), who evaluated the antimicrobial effect of the aqueous, ethanolic and hydroalcoholic extracts from the root of Salvia chorassanica, an indigenous Iranian species, against Staphylococcus aureus, Enterococcus faecalis, Salmonella typhimurium and Escherichia coli, results suggesting that the extracts of Salvia chorassanica have a considerable antimicrobial capacity against the studied strains "in vitro" and can be used as an alternative to antibiotics.

In a study evaluating the antimicrobial effectiveness of S. officinalis against E. faecalis infection in root canals, the antimicrobial activity of the methanolic extract of S.officinalis was evaluated, as an irrigation solution against E. faecalis compared to the conventional irrigation solutions (NaOCl and CHX) currently used in the root canal, a study that presented that the extract of S. officinalis has a much lower antimicrobial effectiveness compared to NaOCl and CHX (Guneser MB et al., 2016).

In the research on the antimicrobial activity of the tested extracts, in the case of Echinacea tincture,

we noticed a moderate, even reduced response, without improvement, especially after its activation with a laser beam. There are studies that argue that through the constituents of the Echinacea composition, alkylamides and polyacetylenes, derivatives of caffeic acid and polysaccharides, it contributes to the increase of the production and activity of white blood cells (lymphocytes and macrophages) being efficient in gingivitis and periodontal disease in combination with sage, mint, and chamomile (Modarai et al., 2009).

The analysis of the obtained results highlights the fact that the diode laser can be used in enhancing the antimicrobial activity of some plant extracts. Our studies confirm the results obtained by other researchers in the sense that the microbial load can be decreased using photodynamic therapy (Garcez et al., 2008; Garcez et al., 2010; Siddiqui et al., 2013; Tennert et al., 2014; Arneiro et al., 2014; Abdelkarim-Elafifi et al., 2021).

The diode laser, along with numerous benefits, can also have some less positive effects still incompletely determined, therefore it is necessary to determine the parameters in vitro before using it in clinical practice.

In the specialized literature, studies have been presented on the antibacterial activity against E. faecalis of some plant extracts such as myrrh extract, licorice extract, Miswak extract, Moringa triphala, Citrifolia juice, and green tea polyphenols to be used as irrigants in endodontic therapy (Salman BN et al., 2017; Tyagi SP et al., 2013).

To discover new compounds, over 2,000 plant extracts have been tested against several microorganisms, including Enterococcus faecalis (Suffredini IB et al., 2006), and Castilho AL et al., (2013), in the study conducted, they aimed to determine the antibacterial activity of 25 plant extracts and their residues against E. faecalis and to determine the chemical profile of the active extracts and residues, by thin layer chromatography. As a result, the extracts obtained from seven plants, species originating from the Amazon tropical forest, have shown significant in vitro activity against E. faecalis, and six of the seven extracts proved to be effective in preventing biofilm formation.

6.4 Conclusions

The plant extracts tested in the study carried out certainly have antibacterial properties.

By irradiation with laser beams of the diode laser with a wavelength of 940 nm, with the two power variants (3 W and 5 W), the action of enhancing the antimicrobial effect of the solutions, plant extracts, that can be used to reduce the bacteria following the irrigation of the root canals, is observed.

The antimicrobial effect of the solutions irradiated with a 5 W power laser beam was superior to

that obtained in the case of the solutions irradiated with a 3 W power laser beam.

The most efficient extract was from walnut seed coat, followed by sage, and the biofilms formed by the Enterococcus faecalis strain were inhibited by concentrations below 1% of the extracts obtained from walnut seed coat and sage, previously treated with a 5 W fiber tip power laser beam.

CHAPTER 7. EVALUATION OF THE BIOCOMPATIBILITY OF NATURAL PRODUCTS, USED AS ADJUVANTS IN THE IRRIGATION AND SANITIZATION OF ROOT CANALS, IRRADIATED WITH A 940 NM DIODE LASER BEAM

7.1 Introduction

Endodontic therapy is based on the cleaning, shaping, and sealing of the endodontic system represented by the root canal to achieve the complete dissolution of residual pulp tissue, elimination of bacteria from the root canal space, and prevention of recontamination after treatment.

Once the root canal is coronally infected, the infection progresses apically, and the microorganisms and their products (endotoxins) cause pulp and periapical lesions. Root canals with primary infections contain a large, polymicrobial bacterial load, with anaerobic bacteria dominating, and Enterococcus faecalis is predominant in persistent infections after root canal treatment and one of the most common organisms that can be cultivated from root canals that are undergoing retreatment.

Irrigation solutions are considered essential and the objectives of irrigation are both mechanical and biological. Therefore, the ideal solutions for endodontic lavage are those that possess good antimicrobial properties against a wide spectrum of microorganisms and that will enhance the outcome of instrumentation procedures, inactivate bacterial virulence factors, such as endotoxins and lipoteichoic acids, disrupt biofilm formation and favor biofilm removal, assist in dissolving pulp tissue residues and removing accumulated hard tissue and the smear layer or prevent their formation, present no adverse effects, both local (on dentin and periapical tissues) and systemic (toxicity, allergic reactions), and present high availability at low costs.

Currently, there is not a single irrigant that meets all the requirements mentioned above, so in practice, there is a need to combine irrigants, but also to find alternative solutions.

As we have experimented in the second study conducted during doctoral research, walnut, sage, and echinacea plant extracts have demonstrated favorable antibacterial properties following the tests

carried out on E. faecalis with an increase in microbicidal activity on the biofilm formed by E. faecalis, as a result of their activation by irradiation with a beam of the diode laser with a wavelength of 940 nm, with the output power at the fiber tip of 3W and 5W, thus demonstrating potential for use in lavage along with mechanical treatment of root canals. Recent studies indicate that, in addition to the bactericidal effect, the diode laser has a biostimulatory effect which is of great importance regarding the healing of periapical tissues. It stimulates cell proliferation and presents an inhibitory effect on inflammation propagation enzymes (Siqueira JF et al., 2000).

The purpose of this in vitro study was to examine the potential toxicological implications of plant extracts from: walnut (Juglans regia), sage (Salvia officinalis), and Echinacea tincture (Echinacea purpurea) and to highlight their biocompatibility.

7.2 Materials and methods

In this study, the same plant extracts were used, prepared from walnut (Juglans regia), from sage (Salvia officinalis) and echinacea (Echinacea purpurea). Walnut fruits, sage leaves and the above-ground parts of echinacea were used, and the preparation method and coding of the samples are the same as those used and previously presented, in the second study. The biocompatibility of the compounds, represented by the prepared plant extracts and plant extracts activated with a laser beam emitted by the 940 nm wavelength diode laser with the output power, at the tip of the fiber, of 3W and 5W, for 30 seconds, was evaluated on ATCC L929 fibroblasts, grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% bovine fetal serum. The cells were seeded at a density of 1.5 x 10^4 cells per well in 200 µl of culture medium, over which the test extracts were placed (1:10 dilution) and were incubated at 37 °C (5% CO2) for 24 hours. The degree of biocompatibility (material cytotoxicity) was evaluated by the Mosmann Tetrazolium Toxicity (MTT) test.

The MTT test is a viability test that allows the quantitative evaluation of living cells in culture. The MTT compound [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is permeable for the membranes of living cells. After metabolizing the MTT compound, formazan crystals soluble in isopropanol are formed, resulting in a solution (purple color) with optical density that can be read spectrophotometrically at 550 nm wavelength. For the MTT test in the test plate with L 929 cells with 96 wells, the rest of the culture medium was removed, the surface was washed with saline solution to remove any trace of bovine fetal serum, which inhibits the MTT compound, an MTT solution of 1mg/ml was prepared and each sample was incubated in the presence of 1 ml MTT solution for 4 h at 37°C and 5%CO2. To be able to read the results, the formed formazan crystals were solubilized with isopropanol. The resulting solution, of purple color, was read at the spectrophotometer at 550 nm. The color intensity is

directly proportional to the number of live cells in the sample. To quantify the cytosolic enzyme lactate dehydrogenase (LDH) that is released into the extracellular environment when the cells are affected (the cell membrane is damaged), the Cytotoxicity Detection KitPLUS (LDH) produced by Roche Applied Science was used. 100µl reaction mix was prepared, which equally contained all the components of the mix, and from the test plate, 50µl of medium were collected in duplicate and transferred to a 96-well plate. After 100 µl was added over each sample, the plate was incubated for 15-20 minutes in the dark. Based on the level of LDH in the culture medium, the color intensity of the pink solution varied directly proportional to the number of dead cells in the sample. It was read spectrophotometrically (Flex Station 3) at a wavelength of 490 nm. To consider a particular sample biocompatible, the optical density values for the MTT tests must be higher than those of the LDH quantification test (more precisely, we say that a sample is biocompatible if the amount of viable, metabolically active cells is greater than that of dead cells).

7.3 Results and discussions

The analysis of the extracts included in the study, in terms of biocompatibility, highlighted the fact that irradiation influences the characteristics of the extracts and their ability to react with the metabolic indicators used in testing. For the ethanol extract from endocarp (N7), it was observed that after irradiation with a 5W laser beam, the number of viable cells considerably increases, the MTT value being much higher than that of LDH (Fig. 7.1). The methanol extract from the seminal tegument of Juglans regia (N11) loses its biocompatibility after irradiation corresponding to the applied intensity, but it does not become cytotoxic. The hydroalcoholic extract, water:methanol, from the wall inside the cotyledon of J. regia after treatment with laser beams with a power of 3W and respectively 5W, presents values of optical densities for the MTT tests higher than those of the LDH quantification test, proportional to the intensity of the beam used (Vasilache A et al., 2021)

Although initially the water:methanol extract from pericarp and freeze-dried mezocarp of J. regia was biocompatible, the treatment with a 5W laser beam, for 30s, determined a high value of LDH absorbance compared to MTT, a value that highlights the presence in a large number of dead cells and respectively the cytotoxicity of the extract.

For the alcoholic extract, in methanol from pericarp and freeze-dried mezocarp of J. regia, an improvement in characteristics was observed, so although initially the extract was cytotoxic, after irradiation, by applying the laser beam with an intensity of 5W, the values recorded for MTT exceeded the values for LDH, the extract being compatible.

The analysis of the obtained results highlights the fact that the diode laser with a wavelength of 940 nm, with the output power of the beam of 3W but especially of 5W, for 30 seconds, potentiated the

biocompatibility of the plant extracts used in the study. This result allows us to appreciate that the 940 nm diode laser can also be used to enhance the biocompatibility of other plant extracts.

The results of this study, by ensuring a high degree of biocompatibility of the tested solutions, following irradiation with the laser beam of the diode laser with a wavelength of 940 nm, confirm the results presented by other researchers in terms of the positive effects obtained using photodynamic therapy (Garcez et al., 2008; Garcez et al., 2010; Siddiqui et al., 2013; Tennert et al., 2014; Arneiro et al., 2014; Abdelkarim-Elafifi et al., 2021).

Also, Spangberg L. and colleagues (1973) and Türkün M. and colleagues (1998) compared in their studies the antimicrobial effect, the chemical properties, and the biocompatibility of the irrigants used in endodontics to establish an ideal solution to be used as an adjunct to canal treatment. The study conducted by Gomes-Filho and colleagues (2008), to compare the reaction of subcutaneous connective tissue in rats injected with a solution or gel of 0.9% chlorhexidine gluconate, 2.5% sodium hypochlorite, 5.25%, and 2%, showed that 0.9% saline solution, 2.0% chlorhexidine solution, and 2.5% NaOCl showed good biocompatibility. The results of the study indicate that 5.25% NaOCl was the most toxic endodontic irrigant for subcutaneous connective tissue at the end of the evaluation period.

Thus, due to their good antimicrobial properties and reduced cytotoxicity, some of the natural products represented by the tested plant extracts, treated with a 5 W intensity laser beam, can be used in treating infections at the root canal level.

7.4 Conclusions

The analysis of extracts prepared from walnut (Juglans regia), sage (Salvia officinalis), and echinacea (Echinacea purpurea), from the point of view of biocompatibility, showed that irradiation influences their characteristics and their ability to react with the metabolic indicators used in testing.

For the ethanolic extract from the endocarp of J. regia, it was observed that after irradiation with a diode laser beam with a wavelength of 940 nm with an output power of 5 W, for 30 seconds, the number of viable cells increases considerably, so the biocompatibility of the product increases.

For the methanol extract from lyophilized pericarp and mesocarp of J. regia, an improvement in characteristics was observed, so that although initially the extract was cytotoxic, after irradiation with a beam of the diode laser with a wavelength of 940 nm, with an intensity of 5 W, the extract becomes compatible. The absence of cytotoxicity and the compatibility of the tested plant extracts offer the possibility of using them in endodontic therapy, as a possible alternative to classic lavage solutions.

Despite high expectations for their implementation in all fields of dentistry, the effects of lasers are still being researched. In the near future, lasers have the potential to emerge as an alternative, a

supplement to conventional therapies.

Additional in vitro and especially in vivo studies are recommended, both for lasers and for simple plant extracts and/or activated with laser light, before use, regarding their effectiveness in root canals against biofilms, their biocompatibility, and their ability to eradicate the dental smear layer.

GENERAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

The synthetic general conclusions boil down to a few essential aspects regarding the applicability of lasers, as follows:

Laser therapy is increasingly used and increasingly popular in the field of dentistry due to the advantages it offers, and the range of procedures performed successfully with lasers on dental tissues is continuously expanding. The lasers currently used in dentistry are small in size, extremely light, portable, and reasonably priced.

Laser applications have improved the prognosis and the outcome of dental treatments, so in the near future, lasers have the potential to assert themselves as an alternative, a supplement to conventional therapies.

Since among the advantages of using laser therapy are the antimicrobial, anti-inflammatory actions and with a stimulatory effect for healing, I proposed to evaluate its use as an adjunct for decontamination in classical endodontic therapy.

Numerous studies on different types of lasers that are used in endodontics demonstrate the benefits that each offers when applied correctly, taking into account the wavelength and optical characteristics of the irradiated tissue, to improve the results obtained following traditional procedures with a revolutionary impact for disinfecting the root canal.

The use of the diode laser in endodontic treatment has become increasingly common in recent years and the antibacterial efficiency of the 940 nm diode laser at different output powers has been evaluated.

The studies conducted as part of the thesis support the fact that the use of laser therapy and photodynamic therapy, adjunct in endodontic decontamination procedures, with a diode laser with a wavelength of 940 nm, shows in vitro antimicrobial activity against C. albicans and E. faecalis.

The irradiation of the microbial suspension with the diode laser showed that the contact time through the number of irradiations slightly influences microbial viability while under the same irradiation conditions the application tip used, shows statistically significant differences in reducing the number of

viable bacteria at an irradiation time increased to 15 seconds, with a power intensity of 3 W and a pulsating application mode (CP2).

Although the diode laser showed a significant reduction in the number of bacterial colonies after application, even after increasing the time and power applied, it alone was not efficient enough to completely eliminate the bacterial strains used in the respective studies, E. faecalis and C. albicans.

As we have mentioned before, the efficacy of photodynamic therapy depends on the microorganism, photosensitizer, and characteristics and parameters of the laser used. According to our results, toluidine blue showed a greater antimicrobial photodynamic effect for E.faecalis than methylene blue, and a greater effect at an increased volume, specifically when we used a larger proportion, of 1/10 of toluidine blue.

In the presence of toluidine blue, using the laser at powers of 3 and 4 W led to a decrease in the number of colony-forming units. The most efficient irradiation condition for reducing the number of viable bacteria was with the following parameters: time 30 seconds, at a power of 5 W in CP2 pulse mode, with the E3 tip.

Using a longer application time increases efficiency, and at the same irradiation time, the number of irradiations positively influences the decrease in microbial viability.

Regarding the viability of C.albicans, at the same conditions of laser beam application time, the number of irradiations, using the same tip, only changing the power (4.5 W) led to a reduction in microbial viability, a reduction that became significant with increasing power to 5.5 W with the E3 tip and at the power of 6.5 W with the uninitiated tip. Testing different volumes with the same laser parameters did not show statistically significant differences in terms of reducing the microbial load.

Thus, the diode laser can be considered an alternative in endodontic treatment for root canal disinfection either singularly or as part of antimicrobial photodynamic therapy.

The combined therapy consisting of irrigation with solutions and irradiation with a 940 nm diode laser, as well as activating the solutions with a laser, represents an efficient treatment option for reducing E. faecalis as well as other pathogenic microorganisms present in the bacterial flora of the root canal system.

The plant extracts tested in the studies carried out certainly have antibacterial properties. By irradiating with laser beams of the diode laser with a wavelength of 940 nm, with the two output power variants at the fiber tip, of 3 W and 5 W, for 30 seconds, the potentiating action of the antimicrobial effect of the solutions used is observed.

The antimicrobial effect of the solutions irradiated with the laser beam with a power of 5 W was

superior to that obtained in the case of the solutions irradiated with a laser beam with a power of 3 W.

The most efficient extract was the one from walnut seed coat, followed by sage, and the biofilms formed by the Enterococcus faecalis strain were inhibited by applying extracts obtained from walnut seed coat and sage, pre-treated with a 5 W laser beam for 30 seconds.

The analysis of the extracts prepared from walnut (Juglans regia), sage (Salvia officinalis) and echinacea (Echinacea purpurea), in terms of biocompatibility, highlighted the fact that irradiation influences their characteristics and their ability to react with the metabolic indicators used in testing.

For the ethanolic extract from the endocarp of J. regia, it was observed that after irradiation with a beam with an intensity of 5 W, the number of viable cells significantly increases, the extract being biocompatible.

For the methanolic extract from the lyophilized pericarp and mesocarp of J. regia, an improvement in characteristics was observed, so that although initially the extract was cytotoxic, following treatment and irradiation with a laser beam with an intensity of 5 W for 30 seconds, the extract becomes compatible.

Laser technology, used in conjunction with classic irrigation solutions and those based on natural extracts, improves cleaning capacity, the removal of residues and the smear layer from the root canals, thus improving the decontamination of the endodontic system, their effect being directly related to the time and amount of irradiation and the level of energy emitted.

Plant extracts and specifically the extracts tested in the thesis, used as such and as laser-activated extracts, have not been researched, so they could be the beginning of a new stage in this modern era of endodontics, and we can hypothesize that some of these phytotherapeutic substances activated and used simultaneously with lasers could be a potential alternative to top irrigation solutions in the field, for the biomechanical treatment of endodontic space.

The use of diode lasers with a wavelength of 940 nm is efficient and safe, as an adjunct, as a complement to endodontic treatment, used at the right time, with preset, safe settings, in order to improve its results.

RECOMMENDATIONS

Plant extracts treated with a laser beam, tested in this thesis, can be an alternative to classic irrigation solutions in endodontic treatment. These plant extracts can be a treatment option in the case of deciduous teeth or young permanent teeth, where sodium hypochlorite is contraindicated.

Further studies are recommended, both for lasers and for simple plant extracts and/or laser-activated, before use, regarding their efficacy in root canals. The development of alternative solutions for irrigating root canals, biocompatible, with high functionality and reduced side effects is an important

objective for performing high-performance therapies and successful treatments.

Future studies will consider determining the in vitro and in vivo effects and other protocols for using the 940 nm diode laser, but also other plant extracts alone or incorporated into different pharmaceutical formulations to determine a potential synergistic effect. Thus, expanding the research area related to the field addressed in the current doctoral thesis could consider the following aspects:

The source, type, and concentration of plant extracts as well as the correlation of the chemical composition and parameters of the solutions used with the characteristics of the preset laser programs.

The synthesis and choice of other materials that include natural extracts with a therapeutic role and with a well-determined synergistic action with the used diode laser.

Performing cytotoxicity tests and expanding microbiological studies to other bacterial strains with increased nosocomial incidence.

We also intend to evaluate the selectivity of both the laser programs used and the essential plant extracts regarding their antimicrobial properties.

The results of the research should be viewed also through the perspective of the limitations associated with in vitro studies. It would be recommended to continue the studies regarding the utility of the diode laser as well as the efficiency and biocompatibility of the tested plant extracts, alone or activated with the laser, in root canal diseases by performing in vivo tests.

PERSONAL CONTRIBUTIONS

My research has contributed to a multi and interdisciplinary approach of the studied theme, interdisciplinary collaboration being essential, as addressing this subject implies corroborating notions of endodontic therapy with pharmacological technique, dental materials technology, and general microbiology laboratory techniques.

The conducted research involved a detailed study of the literature to establish the usage modes, from the perspective of preset programs, of the diode laser with a wavelength of 940 nm, as well as towards the choice of certain medicinal plant extracts of interest.

The research carried out within this thesis represents interdisciplinary studies of originality due to several aspects:

- Studying certain medicinal natural extracts, known for their antimicrobial action, which have not been studied or tested so far on the microbial flora from infected root canals.
- Obtaining improved results through the use of solutions activated with a laser beam, compared with data from the specialized literature.
- Conducting detailed and complex in vitro analyses on samples treated only with laser, on

samples with the selected plant extracts, alone or activated with laser, to determine the multiple beneficial effects with therapeutic potential.

- In vitro determination of the toxicity of the extracts used in the study, alone or activated with the 940 nm wavelength diode laser.

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