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ABSTRACT

CONTRIBUTIONS REGARDING THE ANTIMICROBIAL STUDY OF THE PRUNUS SPINOSA L. AQUEOUS EXTRACT IN ORODENTAL DISEASES

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C U P R I N S

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INTRODUCTION

In the recent years, a tendency to replace drugs with natural remedies has been noticed, due to the fact that a multitude of adverse effects in drugs have been discovered. If the effects of natural remedies and of drugs are the same, the least harmful treatment has to be chosen.

Herbs with antibacterial action could represent an important source of bioactive compounds, which in synergism with already known antibacterial agents, could be exploited in combined therapies.

In recent decades, the emergence of antibiotics resistance has prompted us to seek new effective antibacterial agents. To combat multidrug-resistant microorganisms, scientists are studying the mechanisms of resistance to antibiotics, but also new possibilities for the development of alternative natural medication.

Many aromatic herbs have antioxidant properties, mainly due to the content of phenolic compounds. The compounds of this plant are useful as alternative therapeutic agents or as models for new synthetic substances. Phenolic compounds (flavonoids, phenolic acids, anthocyanins, stilbene, tannins, lignans and lignin) are important for the normal growth and development of plants, and for the development of defense skills against oxidative stress.

Oxidative stress is induced by a number of environmental factors (UV rays, pathogens and pollution), dietary factors and drugs and is involved in the development of over 200 diseases including atherosclerosis, heart failure, Parkinson's disease, Alzheimer's disease, myocardial infarction, cancer and periodontal disease.

Periodontal disease affects approximately 70% of the population in developed and less developed countries and is defined as a chronic inflammatory disease, with occurences in the supporting tissue of teeth (gum, periodontal ligament, alveolar bone). Periodontal disease is a multifactorial disease in which the action of bacteria that form plaque and their association with genetic factors are of special importance in the development and progression of periodontal disease.

More than 700 bacterial species have been identified in subgingival plaque, some of them becoming etiological agents in periodontal disease under the influence of local or systemic factors. The most commonly involved bacterial species are: *Aggregatibacter*

actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia and *Treponema denticola*.

Because *Prunus spinosa* L. species is found in the flora of many countries and is used for its pharmacological effects, for our studies we chose the dried fruits of this species, which were harvested in Tulcea.

The entire previous consideration triggered the idea for this thesis, in which we intended to highlight the possible correlation between antibacterial and antioxidant actions of *Prunus spinosa* L. and the normal or pathological oro-dental status.

CHAPTER 1. THE PRESENT STAGE OF KNOWLEDGE

Prunus spinosa L. is a native shrub, found in all regions of the country, from the plains to the mountains, both on cliffs and rocky hills and sunny coasts [1].

The essential components of fruits are ozes, organic acids, vitamin C, polyphenols, tannins, flavonosids, anthocyanosides, cyanogen heterosides, carotenoids, gumirezines and Ca and Mg salts [1,14,15,16]. Polyphenols are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donors and as singlet oxygen of saturation. Some exhibit chelating properties for metals. In addition, certain polyphenols have antimicrobial activity [33].

Given the known current state of antibiotic resistance and its ascending trend, it is required to identify new and efficient antibacterial compounds. This can be done by global investigation of herbal extracts and by studying their antibacterial, antifungal and antiviral activities.

Plants as a future source of drug production are mentioned in WHO documents, since there is a possibility that some of the plant based products could be used for the development of antibacterial agents [12].

Based on the chemical composition of *Prunus spinosa* L. fruit species and on data from literature concerning its antibacterial action, their use as an alternative to antibiotic treatment involves a comprehensive analysis.

CHAPTER 2. OXIDATIVE STRESS

The human body physiologically produces free radicals, which are responsible for cellular aging, and when they are produced in large amounts, they are involved in disease development. Thus, it is estimated that more than 200 diseases, such as atherosclerosis, cardiovascular disease, diabetes, obesity, cancer and periodontal disease are caused by oxidative stress [30, 66].

Among exogenous sources which induce the formation of reactive oxygen species are: irrational diet, alcohol abuse, exposure to cigarette smoke, air pollution, UV radiation and the administration of some medication.

In the case of oral diseases such as periodontal disease, exposure to cigarette smoke is associated with increased lipid peroxidation; therefore, it can be suggested that the oxidative effects of periodontal disease are compounded by the effects of smoking [61]. It is estimated that cotinine is a biomarker for evaluating smoking, being the primary metabolite of nicotine [79]. Salivary cotinine is correlated with recent exposure to nicotine (3-4 days), with the smoking status (active or constant smoker, passive smoker, nonsmoker) and with cotinine levels in plasma and urine [73].

Due to limited diagnosis possibilities for the periodontal disease (clinical and radiological), the use of biomarkers from oral fluids is one way to optimize the diagnosis and monitoring of the periodontal disease [72].

CHAPTER 3. PERIODONTAL DISEASE

According to the World Health Organization (WHO), periodontal disease is currently identified in 5-20% of middle-aged adults worldwide.

The chronic periodontal disease main feature is the depth of the periodontal pocket that creates a favorable environment for anaerobic and aerobic facultative anaerobic microorganisms; in active periodontal pockets, *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* may develop. Along with *Aggregatibacter actinomycetemcomitans, Capnocytophaga spp.*and *Porphyromonas gingivalis*[82] are found in aggressive periodontal disease.

Taking into account the degree of pathogenicity of microbial species involved in periodontal disease, Socransky has classified them into "bacterial complexes". Most often, these bacterial species associate, causing the specific features of periodontal disease [86].

Several types of biomarkers have been associated with systemic diseases and diseases of the oral cavity, given the fact that they can be detected in saliva and crevicular fluid [90]. These biomarkers are represented by interleukin-1β, interleukin-6 and interleukin-8, tumor necrosis factor-α (TNF-α), etc.

CHAPTER 4. INTERLEUKIN

Defense capabilities of the immune system depend on the cells' ability to communicate with each other through cell-cell direct contact or by secretion of small signaling proteins called cytokines [86]. Depending on the roles and actions, cytokines are divided into: interleukins (IL), tumor necrosis factor (TNF-α, TNF-β), interferons (IFN), colony stimulating factors (C-CSF), chemokines and growth factors (GF-Growth Factors) [94]. IL-6 is an interleukin with pleiotropic functions involved in the regulation of the immune response, in the acute phase response, hematopoiesis and inflammation. It is secreted by the activated B and T lymphocytes, the granulocyte neutrophils, macrophages, fibroblasts, osteocytes, monocytes, mast cells, keratinocytes and even cancer cells [100].

Having various biological functions, it is involved in the proliferation of normal and pathological cells (platelets, keratinocytes, B lymphocytes) and it is responsible for the occurrence of some diseases. It is secreted in large amounts in multiple myeloma and kidney cancer; it induces fever that can be blocked by cyclooxygenase inhibitors and is involved in neuronal differentiation. It was also shown that serum levels of IL-6 are increased in psoriasis, rheumatoid arthritis, cirrhosis, lymphoma, mesangial proliferative glomerulonephritis and carcinomas [94].

Speciality studies have demonstrated the presence of high levels of IL-6, IL-8 and TNF-α in the chronic inflammation of the gingiva, as well as in the crevicular fluid of subjects with periodontal disease [103]. The combination of these substances and clinical parameters in gingivitis and periodontal disease, allows the assessment that IL-6, IL-8 and TNF-α could be used as biomarkers in the evaluation of these types of diseases.

CHAPTER 5. FIRST STUDY - PHARMACOGNOSTIC ANALYSIS OF VEGETAL PRODUCT OBTAINED FROM *PRUNUS SPINOSA* L. FRUITS

5.1 Introduction

The research was based on the chemical and biological characterization of dried fruits of the *Prunus spinosa* L. species harvested from Tulcea. Also, the identified compounds were analyzed from a known pharmaceutical preparation destined for mass usage - the aqueous solution.

5.2 Objectives

The objective was to obtain aqueous solutions from the dried fruits of *Prunus spinosa* L. which would allow the determination of antioxidant activity of this species and also the highlighting of a potential correlation between the content of polyphenols and antioxidant activity of the studied species.

5.3 Materials and Methods

The general methodology consisted of explaining the working methods and use principles typical for medicinal plants according to the tenth edition of the Romanian Pharmacopoeia monograph (FR X) [106] and the third edition of the European Pharmacopoeia (EP 3.0) [108].

The separation, identification and quantification of the phenolic compounds were achieved by a standard HPLC method for determining total polyphenols, according to the monograph USP 30-NF25.

The DPPH method was performed by measuring the neutralizing ability of the diphenylpicrylhydrazyl radical (DPPH) and its transformation in its reduced form, by the examined solutions.

The materials under study were both the powder obtained from the dried fruit of *Prunus spinosa* L. species and its aqueous solution.

5.4 Results

Microscopic examination of the vegetable powder allowed the observation of the following elements: sclereide, calcium oxalate druzes, epicarp with ordered cells, mesocarp (broken cells), dotted and cured vessels.

The presence of these microscopic elements is common in fruit-type vegetable products powder. If the fruits are colored, the pigment can be observed in epicarp cells.

According to the FR X, the plant product showed no impurities from the same plant (stems, bits of branches or hard endocarp) and no foreign materials (fruits of other species or mechanical impurities), so we can say that the product was 100% pure.

After determining the loss through oven drying, we obtained values approximately equal to 11.27% for the vegetal product, represented by pulp obtained from *Prunus spinosa* L. species fruits. These values were within the limits set by the speciality literature of 12-20% for the type of organ called *fructus*.

The content of water-soluble substances in 100 g of vegetable product (g%) obtained after the assay was approximately 58.43%.

From the results of the tests carried out, we can say that the water-soluble active principles are in sufficient amount to justify the use of water as extractive solvent for the extractive solution.

The amount of total polyphenols highlighted in the *Prunus spinosa* L. species was 6.95 g%, while the tannins were 3.38 g%. The results confirmed that the plant product comes under the provisions of the European Pharmacopoeia 3.0 Edition [108].

After HPLC analysis, from the separate peaks that occur frequently in the analyzed samples, chlorogenic acid, caffeic acid and gallic acid were identified in varying concentrations. All compounds detectable by HPLC method are known for their antioxidant and antimicrobial properties [21,24,25,32].

The aqueous extract obtained from fruits harvested in Tulcea County had a high capacity of DPPH radical scavenger (87.30%), which could be compared with standard scavenger capacity with concentrations between 2.5-20 mg/ml, of 93.56-95.60%.

The presence in *fructus* plant product of some active principles groups allowed us to appreciate that the *Prunus spinosa* L. dried fruit harvested from Tulcea could be of interest regarding the therapeutical use.

5.5 Discussion

In some similar studies conducted by Veličković *et al.* on *Prunus spinosa* L., the presence of caffeic acid, which we identified in this study, was not detected [18]. Studies carried out by Rodríguez *et al.* on *Prunus spinosa* L. revealed the presence of higher concentrations of gallic acid (430.38-985.56) [109] compared to those found in this study (81.46). For the same

types of extracts, Radovanović *et al.* have identified, by HPLC method, other types of active ingredients: sirginic acid and p-coumaric acid [19]; gallic acid was identified in the Radovanovic study in a higher concentration (150.21) compared to that found in this study (81.46), while caffeic acid showed a lower concentration in the study of the same author (0.34).

We consider that these different concentration levels of polyphenols found in several studies on the same species, but from different regions of the world, are due to a possible interaction between soil-temperature-precipitation-plants. The antioxidant activity determined by DPPH method in *Prunus spinosa* L. species was studied by many authors. Similar results to those obtained in this study have been presented in the literature by Veličković and collaborators, who identified a value of antioxidant activity between 32.05 and 89.10% [18].

Also, using the same method, Radovanović *et al.* determined for the *Prunus spinosa* L. extract, a free radical scavenger capacity which was much lower than in this study, respectively 27.06% [19].

The antioxidant properties appreciated by assessing the scavenger ability of DPPH radicals is correlated with the content in polyphenols, which may vary depending on the period of harvest, due to different annual climatic conditions.

5.6 Preliminary Conclusions

1. The existence of tannins in the composition of *Prunus spinosa* L. dried fruits harvested in Tulcea may warrant further study of plant material for the evaluation of antibacterial properties.
2. The presence of polyphenols in *Prunus spinosa* L. fruit pulp explains demonstrated antioxidant properties and supports the possibility of using these active ingredients in some diseases caused by free radicals.
3. I estimate that there may be variabilities in the total polyphenols content, respectively the content of tannins in the *Prunus spinosa* L. fruits harvested in Tulcea, depending on climatic conditions.

CHAPTER 6. SECOND STUDY - STUDIES REGARDING THE ANTIBACTERIAL AND ANTIFUNGAL EFFECTS OF *PRUNUS SPINOSA* L. AQUEOUS EXTRACT

6.1 Introduction

Given that the presence of antibacterial and antioxidant potential active principles was assessed and quantified, the second study aimed at assessing these possible effects on various bacterial and fungal species.

6.2 Objectives

We started from the premise of the existence of possible antibacterial and antifungal effects of the aqueous extracts obtained with powder from *Prunus spinosa* L. dried fruit harvested in Tulcea County; if these effects exist, we will evaluate the antifungal and antibacterial efficiency compared to some antibiotics.

6.3 Materials and Methods

Two aqueous extracts, in dilutions of 1:10 and 5:10, were prepared from the *Prunus spinosa* L.dried fructus harvested in Tulcea.

In the first phase, the study was conducted on reference bacterial and fungal strain type ATCC (American Type of Culture Collection) lyophilized, stabilized and viable (each used pellet containing lyophilized microorganism). The reference strains used were: *Staphylococcus* ATCC 25923, *Streptococcus* ATCC 19615, *Enterococcus* ATCC 19433, *Escherichia coli* ATCC 25922, *Pseudomonas* ATCC 27853, *Candida albicans* ATCC 10231.

Antifungal and antibacterial activity for the first phase of the second study was determined by disc diffusion method in a culture medium inoculated with the reference strains that we have mentioned above [111].

For the realization of the second stage of the study, which was focused on comparing the antibacterial action produced by the working solution with some antibiotics, bacterial strains were isolated as follows: *Group B Streptococcus* and *Group A β hemolytic Streptococcus* from the throat, *Staphylococcus aureus, Staphylococcus capitis*from otic secretion, *Streptococcus mutans* from dental caries and *Prevotella spp.* from periodontal pus. Identification of bacterial species has been achieved in the API system. Assessment of the sensitivity level was achieved compared with 4 common antibiotics, such as: Ampicillin, Amoxicillin, Azithromycin, Clarithromycin, according to CLSI standards.

6.4 Results

The results obtained showed that the tested solutions have antibacterial activity, but they do not have antifungal activity and this is demonstrated by the total resistance of *Candida albicans* strain for both concentrations of the aqueous solutions. The antibacterial activity of the studied aqueous extracts is differentiated according to the substance concentration as follows: the 50% concentration of the aqueous extract (14 mm) had a higher antibacterial effect against *Staphylococcus sp.* as opposed to the 10% concentration (11 mm).

The results regarding the testing of the sensitivity effect of *Streptococcus sp.* strain to the action of the solutions suggested that an inhibition area of 14 mm was measured for the 50% concentration while for a 10% concentration the area of inhibition had a diameter of 12 mm.

The aqueous extracts, in both concentrations, had antimicrobial properties on the *Enterococcus sp.* bacterial species. In the case of the 50% concentration extract, an inhibition diameter of 12 mm was measured (with the emergence of resistant mutant colonies), and for the 10% concentration, the diameter of inhibition area was 10 mm.

For *Pseudomonas sp.,* the total resistance has been observed for both concentration levels of the aqueous solution.

The results showed that *Escherichia coli sp.* is sensitive, having an inhibition area diameter of 13 mm for the high concentration level and 12 mm for the low one.

In the second phase of the study, the existence of differences regarding the size of inhibition area diameters for all bacterial strains tested imposed the use of only the 50% concentration solution. The obtained results showed that the aqueous extract of the plant acts differently for the strains tested, compared to the antibiotics used, as follows: antibacterial effect against *Staphylococcus aureus* was of similar magnitude to that of Clarithromycin (7 mm) and smaller than the other three antibiotics. Regarding the *Staphylococcus capitis*, the antibacterial effect was of similar intensity to that of Azithromycin (8 mm) and stronger compared to Clarithromycin.

The study results have shown that the aqueous extracts presented a good antibacterial activity against the strains of *Staphylococcus aureus* and *Staphylococcus capitis* compared to the antibiotics used.

The antibacterial effect against *group B Streptococcus*, *Streptococcus mutans*, *group A β-hemolytic Streptococcus*and *Prevotella intermedia* was lower compared to all the antibiotics used.

6.5 Discussions

Similar studies on the antibacterial activity of *Prunus spinosa* L., but with alcoholic extracts, are referred to in the speciality literature as follows: the ethanolic extract was tested against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* [18] and the methanolic extract was tested on strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* [19, 114].

The results of this study on the antibacterial action of *Prunus spinosa* L. aqueous extract are similar to those cited in the speciality literature [37, 38, 39], taking as standard bacterial strain the *Staphylococcus aureus* strain, which was presented in the range of bacterial strains in all studies cited above [18, 19, 114]; the antibacterial action of *Prunus spinosa* L. aqueous extract was 7 mm in our study and in the studies mentioned above it was higher, respectively 13 ± 0.2 mm in the ethanol extract and 14.2 ± 2.2 mm in the methanol one.

From these figures it appears that the antibacterial activity of *Prunus spinosa* L. aqueous extract, as shown in this personal study, is smaller, but I consider that the higher antibacterial action from the studies of other authors is due to a cumulative antibacterial effect – alcohol with plant extract, which is why I appreciate that you cannot strictly define the precise antibacterial action of the plant in those authors' studies.

In conclusion, I appreciate that this study, based on a technique for obtaining an aqueous extract from the dried fruit of *Prunus spinosa* L. species, can provide more accurate data on the antibacterial effect upon the studied strains.

Very recent studies carried out by Aliyazicioglu *et al.,* in which the antibacterial activity of the aqueous and methanol extracts over *Staphylococcus aureus* strain in comparison with Ampicillin and Streptomycin [115] were evaluated, showed total resistance to both antibiotics and the two types of plant extracts; these results, which are different from those obtained in my personal study, are probably due to different climatic conditions in areas where the harvesting was done.

In the studied literature, there is very little data on the sensitivity of bacterial strains commonly involved in tooth decay (*Streptococcus mutans*) and periodontal disease (*Prevotella intermedia*) to *Prunus spinosa* L. extracts.

Regarding the bacterial species involved in the production of dental caries, results similar to those obtained in this study are cited in literature by Smullen and collaborators, who identified the existence of the antibacterial action of *Prunus spinosa* L. aqueous extracts on the *Streptococcus mutans* strain [116].

In the studied literature we did not find data on the antibacterial properties of *Prunus spinosa* L. species extracts on the *Prevotella intermedia* bacterial species involved in the onset of periodontal disease; only data relating to the antibacterial action of the ethanolic, chloroformic, aqueous ethanolic extracts and aqueous extracts from other plants that contain phenolic compounds are cited [117,118].

6.6 Preliminary conclusions

1. The antibacterial activity of aqueous extracts used can be the result of tannin content, and of a possible interaction between the phenolic components identified in previous studies.

2. The two aqueous solutions obtained in the first stage had antibacterial effects against the tested reference strains (10-14 mm).

3. The diameter of the inhibition area increases with the raising of the concentration of the aqueous solution, and the best result is obtained from the 50% concentration on *Staphylococcus* ATCC 25923 and *Streptococcus* ATCC 19615(14 mm).

4. The *Pseudomonas* ATCC 27853 and *Candida albicans* ATCC 10231 strains were totally resistant to both aqueous concentrations tested.

5. The best antibacterial activity compared to the tested antibiotics was recorded on *Staphylococcus capitis*, the antibacterial action being the same as that of Azithromycin (8 mm) and stronger than that of Clarithromycin (6 mm).

6. On the strain of Staphylococcus aureus, an antibacterial activity identical to that of Clarithromycin (7 mm) and very close to Azithromycin (8 mm) and Ampicillin (9 mm) was noticed.

7. For the *Streptococcus mutans* strain, a level of inhibition area smaller than that of the tested antibiotics was achieved.

8. The results show that the aqueous extract has a moderate antibacterial activity on the species of *group B Streptococcus* and *group Aβ-hemolytic Streptococcus*, which is lower than that of the antibiotics tested.

9. For the *Prevotella intermedia* strains, the lowest level of inhibition area (4 mm) was obtained as compared with the tested antibiotics.

10. Our study shows that the two aqueous extracts obtained from the *Prunus spinosa* L. species may be used in the future for the development of new pharmaceutical products with antibacterial properties, creating the prospect of limiting the resistance and multi-resistance phenomenon to antibiotics, an urgent and actual problem in today's modern medicine practice.

CHAPTER. 7 THIRD STUDY - ANTIOXIDANT EFFECT OF THE *PRUNUS SPINOSA* L. AQUEOUS SOLUTION EVALUATED THROUGH SALIVARY COTININE AND IL-6 BIOMARKERS

7.1 Introduction

The aqueous extract of the *Prunus spinosa* L. species frutis was shown to have antioxidant activity in the first study, which is why the research has been oriented towards the highlighting of these properties in the oral cavity.

7.2 Objectives

1. The evaluation of oxidative stress and the existence of a possible correlation between the salivary cotinine biomarker and the orodental clinical status in smokers, casual smokers and nonsmokers.

2. The evaluation of oxidative stress and the existence of a possible correlation between salivary cotinine, salivary IL-6 and the dentoperiodontal status in subjects with shallow and deep chronic periodontal disease.

I. Evaluation of the oxidative stress level assessed through salivary cotinine biomarker in a lot of healthy orodental subjects

I.7.3 Materials and Methods

Standard questionnaires were designed to collect information on tobacco use in the study group. We collected saliva samples from each subject according to the prospectus content of the *NicAlert*™ kit [119].

In the first phase, we did a rinsing of the oral cavity with blank solution (boiled and cooled water) for 7 days; finally, saliva was harvested again and cotinine levels were evaluated.

The second step involved rinsing the oral cavity for a period of 7 days with the aqueous solution; repeated saliva harvesting was done at the end and salivary cotinine levels were evaluated.

I.7.4 Results

Following the completed self-assessment questionnaires, we made the distribution of smokers/nonsmokers categories in the study group. Then, we achieved the integration of volunteers in the study group according to the kit.

Obvious differences between the two types of statuses could be noticed: the declared one - through questionnaires and the proven one - by salivary cotinine levels quantified as follows: there is a lower percentage of active smokers shown after salivary cotinine dosing as compared with the percentage of those assigned based on questionnaires. Also, there was a greater proportion of occasional smokers compared with the percent obtained through the questionnaires and, for nonsmokers, a smaller percentage than those employed on the basis of questionnaires.

After using the control solution, limited variations were noticed in the growth - 2 samples and in the drop - 2 samples of the salivary cotinine levels before and after rinsing, which can be explained by variations in consumer products containing tobacco and exposure to tobacco smoke among volunteers before and after the test.

After using the aqueous solution, salivary cotinine levels decreased or remained constant, with one exception, the explanation of the phenomenon being related to variations in the consumption of tobacco products and the exposure to tobacco smoke among volunteers before and after testing.

Significant statistical differences ($p > 0.05$; t-test) between the values of salivary cotinine before and after using the control solution were not obtained. The existence of highly significant statistical differences, $p = 0.001$ between salivary cotinine values before and after rinsing with the aqueous solution of *Prunus spinosa L.* demonstrates the effectiveness of the solution in the reduction of the salivary cotinine concentration and thus, in the decreased levels of oxidative stress in the oral cavity.

I.7.5 Discussions

In the literature studied we found no information on the research of the effects of plant extracts over the salivary cotinine level. The only identified data is for the various preparations that contain active principles of other plants (for example plants of the subclass *Magnoliidae*), having the function of lowering the level of nicotine in the body [121]. Also Narotzki and collaborators have demonstrated that the presence of polyphenols in green tea reduces oxidative stress and inflammation in the oral cavity installed as a result of smoking and thus, of the presence of nicotine [122].

Therefore, we can say that our personal study, which reflects decreased levels of salivary cotinine, the primary metabolite of nicotine, after rinsing with the aqueous solution, aligns with other studies in the same direction.

The measurement of cotinine levels in biological fluids has been used in other scientific studies to assess the level of oxidative stress after exposure to tobacco [123]. Thus, multiple analytical techniques were used to measure levels of cotinine in body fluids in smokers and in nonsmokers, including quantitative [123] and also semiquantitative measurements (NicAlert test) [124], which were also used in present study.

My results are similar to those obtained by Nuca *et al.,* who dosed salivary cotinine with the purpose of evaluating passive smoking in nonsmoking adults; I appreciate that the results join the results of other studies and thus allow obtaining new information on the assessment of oxidative stress levels by quantifying this metabolite of nicotine [125,126,127].

My results are consistent with other studies carried out to assess exposure to tobacco and nicotine addiction, which indicates that measuring salivary cotinine, even by semi-quantitative methods as NicAlert ™ Saliva, is a very valuable method for evaluating tobacco consumption [128,129,130].

In this study, questionnaires were used as a standard way of classifying subjects in smokers (steady and casual) and nonsmokers. Cross-sectional studies focused on tobacco, that generally use evaluation of smoking and nicotine addiction through questionnaires, have been addressed in other studies [131,132,133].

I.7.6 Conclusions

1. The results prove that the aqueous solution of *Prunus spinosa* L., has an antioxidant effect demonstrated by the decrease of salivary cotinine.

2. The results suggest that salivary cotinine levels may provide useful information regarding the level of oxidative stress in the orodental cavity.

3. The benefit of *Prunus spinosa* L. aqueous solution on lowering oxidative stress in the orodental cavity of orodental clinically healthy smokers is demonstrated by the differences of the biomarker values quantified before and after rinsing with test solution ($p = 0.001$).

4. The results of this study demonstrate the need to expand the research on the antioxidant action of *Prunus spinosa* L. aqueous solution in the orodental cavity.

II. The evaluation of oxidative stress by salivary cotinine and salivary IL-6 biomarkers in a group of subjects with superficial and deep chronic periodontal disease

II.7.3 Material and Methods

After applying the exclusion criteria, the group consisted of 24 nonsmoker subjects (aged 35-44, from Constanta county), selected from a total of 37 subjects. After the clinical examination, the following parameters were recorded: periodontal pocket depth, based on CPI index (Community Periodontal Index), and gingival bleeding index GI (Gingival Index).

The response rate was 100% for both parts of the study and included:

- Five healthy subjects (CPI = 0, G = 0 and there was no gingival bleeding at the examined teeth);

- 11 subjects with shallow marginal chronic periodontitis (CPI = 2; between 4 and 9 teeth with gingival bleeding);

- 8 subjects with deep marginal chronic periodontitis (CPI = 3/4, from 7 to 12 teeth with gingival bleeding).

Obtaining and collecting biological material (saliva) was made using the same method presented above (Study I). The cotinine kit was presented in the previous study (Study I) and the kit used for salivary IL-6 was produced by Salimetrics USA and it allows the quantitative determination of this biomarker by ELISA (enzyme linked immunosorbent asssay).

The solution used was a 10% aqueous solution of *Prunus spinosa* L. obtained using the same method as in the previous study. The rinsing of the oral cavity has been done for 1 min, twice a day for 10 days, using 10 ml of aqueous solution. After 10 days, the 24 subjects have been **clinically and orodental** evaluated and saliva samples were collected again.

Reading and interpreting the salivary levels of cotinine and IL-6 was performed before and after the interventional study in which the subjects were involved, based on written and freely expressed informed consent.

II. 7.4 Results

Clinical parameters and also the salivary cotinine and interleukin-6 levels were evaluated before and after the use of *Prunus spinosa* L. aqueous solution for a period of 10 days.

In the control group, the results demonstrated that there were no statistical significance between salivary cotinine values before and after rinsing ($p = 0.373$); but we notice the existence

of statistically significant differences between the values of salivary IL-6 before and after the rinse ($p = 0.049$).

Moreover, in the group of patients with superficial chronic periodontal disease there was no significant difference between the values of the periodontal pocket depths before and after the rinse ($p = 0.264$); at the same time, there are highly significant statistical differences between the gingival index values before and after the rinse ($p < 0.0001$).

Between the salivary cotinine values measured before and after rinsing in this group, there is no statistically significant difference ($p = 0.077$).

The results in the group of adults with shallow chronic periodontal disease showed there is a statistically significant difference between the values of IL-6 before and after rinsing ($p = 0.050$).

In the group of patients with deep chronic periodontal disease there were statistically significant differences between the values of the periodontal pocket depths before and after the rinse ($p = 0.049$); also, the results showed the existence of high statistically significant differences between GI values before and after the use of the *Prunus spinosa* L. aqueous solution ($p = 0.002$) and statistical differences between the values of IL-6 before and after rinsing with the study solution ($p = 0.041$).

Within this group, there were no statistically significant differences between the salivary cotinine values before and after the rinse with the study solution ($p = 0.350$).

The results showed that there are statistically significant differences between the values of IL-6 before and after rinsing with the solution of the study ($p = 0.041$) in the group of adults with deep chronic periodontal disease.

To determine the possible correlations between clinical parameters and salivary cotinine and IL-6 biomarkers, we created a unique study group with superficial and deep chronic periodontal disease.

The results obtained showed that there is a very poor correlation ($r = 0.035$) between salivary cotinine (ng/ml) and GI prior to the use of the study solution in patients with periodontal disease.

There was a weak correlation ($r = 0.164$) between salivary cotinine (ng/ml) and GI before the use of the study solution in patients with periodontal disease, between salivary cotinine and

the depth of the periodontal pocket before the use of the study solution (r = 0,218) and between cotinine and periodontal pocket after rinsing (r = 0.319)

Reasonable correlation is obtained between the salivary IL-6 value and the GI before rinsing (r = 0.505), but there are also statistically significant differences (p = 0.027); also the salivary IL-6 value correlates with the gingival index after rinsing (r = 0.454), with statistically significant differences (p = 0.050).

There is a reasonable correlation between IL-6 and periodontal pocket depth before rinsing (r = 0.557), with some statistically significant difference (p = 0.013).

There is also a weak correlation between IL-6 and periodontal pocket depth after rinsing (r = 0.377), but with no statistically significant difference (p = 0.111).

7.5 Discussion

In the speciality literature, we found no studies that aimed to assess the antioxidant role of the *Prunus spinosa* L. species on periodontal disease. The interest in the use of plant extracts in the context of periodontal disease is high and it is demonstrated by the fact that they are cited in literature, but for other plants.

In a study published in 2012, the authors presented the effects of mouthwash obtained by associating plants such as *Centella asiatica*, *Echinacea purpurea* and *Sambucus nigra* in the periodontal disease. In the study, a decrease in the gingival index value was noticed after the completion of therapy in the group of patients who used natural mouthwash compared to the control group that used water as control solution [141].

In another study, the authors show that some volatile components of plants (thymol, eugenol, eucalyptol) produce reductions in the gingival index levels after the treatment period compared to the placebo [142]. The study was conducted in the same way by Karim and collaborators, who have experienced a significant reduction in the gingival index after using Aloe vera plant [143].

In another study, which involved local application of ozonated olive oil, the researchers obtained a high statistical significance (p <0.001) regarding the decrease of the periodontal pocket depth after 8 weeks of treatment compared with the placebo [144].

In the literature, there are studies similar to our own, but on other plants with similar composition to the one found in this study.

There were studies with plants that contains phenolic compounds, such as studies conducted by Nanescu and collaborators, who have evaluated the role of polyphenolic compounds from green tea extract in periodontal disease and obtained changes of some periodontal parameters (lowering of the gingival bleeding index with significantly improved results and the reduction of the depth of periodontal pockets) [145]. The results of this study are consistent with the results of our study, demonstrating the benefits of phenolic compounds in the reduction of oxidative stress in periodontal disease.

The beneficial effect of polyphenols in the periodontal disease was also demonstrated by reducing the salivary IL-6 cytokine in studies done by Zdařilová and collaborators from fruits of *Lonicera caerulea* L. species, whose results are similar to those of the present study [146].

Also, the study by Jeong *et al.* highlighted the role of the lindenenyl acetate compound, isolated from *Linder strychnifolia* species, in reducing IL-6 involved in periodontal disease [147].

The results of my study show that, regarding the two evaluated markers, salivary cotinine and IL-6, within groups of patients with superficial and deep chronic periodontal disease, they may serve as tools for assessing the effect of the therapeutic potential of the solution obtained from *Prunus spinosa* L. species within periodontal disease. This is supported by the fact that there are highly significant statistical differences in the gingival index values before and after rinsing with the study solution, both in patients with superficial periodontitis ($p < 0.0001$) and in patients with deep chronic periodontitis ($p < 0.002$).

Regarding the periodontal pocket depth, we obtained statistically significant differences after using the test solution, only in patients with deep chronic periodontal disease ($p = 0.049$), but weaker than the differences obtained for the gingival index; this was explained by clinicians based on the fact that the gingival index is a clinical parameter that improves before the periodontal pocket depth.

Concerning the two biomarkers studied, salivary IL-6 and cotinine, it is worth noting that by comparison, the IL-6 biomarker is more sensitive in assessing the possible therapeutic effect of the study solution; this affirmation is supported by the fact that there are statistically significant differences in salivary IL-6 values before and after rinsing with the study solution,

both in patients with superficial chronic periodontitis (p = 0.050) and in those with deep chronic periodontitis (p = 0.041).

In addition, salivary IL-6 appears to be an important biomarker for the diagnosis and assessment of the periodontal disease progression under treatment and that salivary IL-6 is correlated, before its use in the test solution, both with the gingival index (r = 0.505) and the periodontal pocket depth (r = 0.557), and after the use of the test solution as follows: with the gingival index (r = 0.454) and with the periodontal pocket depth (r = 0.377). There is also a weak correlation between cotinine and the gingival index and the periodontal pocket depth before and after using the test solution.

7.6 Preliminary conclusions

1. The antioxidant effect of the aqueous solutions studied is demonstrated by the existence of a statistically significant difference regarding the values of the IL-6 biomarker in saliva before and after rinsing in all three groups of subjects (p <0.05).

2. Highly statistically significant differences between the values of the gingival index - as dento-periodonta clinical evaluation parameter quantified before (p <0.0001) and after (p = 0.002) rinsing with the study solution, demonstrates the beneficial role of the solution in improving the orodental clinical status in patients with different clinical forms of periodontitis.

3. There are differences in the periodontal pocket depth before and after rinsing with *Prunus spinosa* L. aqueous solution (p = 0.049), but weaker, demonstrating that the improvement of the periodontal pocket depth requires a longer time therapy.

4. The salivary creatinine biomarker has a low sensitivity level when it comes to the possibility of clinical status assessment before and after rinsing with the study solution in all studied groups (p> 0.05).

5. Our results create the premise for obtaining a pharmaceutical preparation with antioxidant properties in order to protect and improve the dento-periodontal status in the future.

CHAPTER 8. THE THESIS ORIGINALITY AND INNOVATIVE CONTRIBUTIONS

Taking into account the fact that, so far in the speciality literature studied, the microscopic features of *Prunus spinosa* L. species fruits have not been presented, the present thesis brings, as originality, a significant amount of new information regarding the microscopic profile of the dried fruits of this species.

Another element of originality consists in the results obtained regarding the chemical composition, antibacterial and antioxidant action of *Prunus spinosa* L. species fruits harvested from Tulcea and aqueous solutions obtained, which can be added to the known phytochemical and phytopharmacological studies about this species from other regions of the world.

The originality note of the thesis consists in the study of the antioxidant capacity of the *Prunus spinosa* L. aqueous solution using as assessing tools salivary cotinine and salivary IL-6 biomarkers in healthy subjects and patients with superficial and deep periodontal disease.

This thesis is the first research of the antioxidant capacity of *Prunus spinosa* L. species determined by a number of analyses of constituents who belong to the class of phenolic compounds.

Also, the thesis is the first national research on in vivo antioxidant study of *Prunus spinosa* L. species fruit harvested from Tulcea, thus being provided a series of data highlighting the protective effect of the active principles contained in this species on oxidative stress from the orodental cavity.

Given that smoking is an important risk factor in the onset and progression of periodontal disease, the first novelty demonstrated by the results is that the *Prunus spinosa* L. aqueous solution decreases the level of oxidative stress in smokers and nonsmokers orodental healthy subjects, proving the protective effect of the solution in the orodental cavity and the existence of the possibility to prevent the occurrence of periodontal disease to some extent.

The second element of absolute novelty of this thesis is the possibility of using the aqueous extract of *Prunus spinosa* L. to improve various forms of periodontal disease.

The third element of absolute novelty of the thesis is the assessment of the level of oxidative stress after using the *Prunus spinosa* L. aqueous extract by quantifying salivary cotinine and IL-6 biomarkers.

The results were obtained through interdisciplinary studies on the borderline between dentistry, pharmacognosy, pharmacology, microbiology, using modern techniques and equipment that have ensured absolute accuracy of results.

CHAPTER 9. GENERAL CONCLUSIONS

1. Pharmacognostic research carried out on the dried fruits of *Prunus spinosa* L. species have revealed the presence of total polyphenols and tannins, compounds with antibacterial and antioxidant properties.
2. Microbiological studies carried out by using aqueous solutions of dried fruit pulp obtained from species have demonstrated antibacterial effects against all reference strains tested, except *Pseudomonas* and *Candida albicans* strains which were totally resistant.
3. The antibacterial activity of the test solution on the bacterial strains isolated from human pathological products showed that the solution has antibacterial activity comparable to certain antibiotics.
4. The beneficial role of the solution in improving periodontal disease is evidenced by the existence of differences in the value of the gingival index - both in the superficial chronic periodontitis patients ($p < 0.0001$) and those with deep chronic periodontitis ($p = 0.002$).
5. The benefit of this solution is demonstrated by differences in the depth of the periodontal pocket before and after rinsing with the aqueous solution of *Prunus spinosa* L. in patients with deep chronic periodontitis ($p = 0.049$).
6. The *Prunus spinosa* L. aqueous solution used has an antioxidant effect demonstrated by the decrease, after rinsing with the study solution, of salivary cotinine in clinical orodental healthy smokers ($p = 0.001$) and of IL-6 in patients with periodontal disease ($p < 0.05$).
7. The results show that the salivary cotinine biomarker has a lower sensitivity level compared to the salivary IL-6 biomarker regarding the possibility to assess the clinical status before and after rinsing with the solution of the study, both in healthy subjects and in patients with different clinical forms of periodontitis ($p > 0.05$).

8. The results support the inclusion of the salivary IL-6 biomarker in the group of biomarkers that can help monitor various clinical forms of periodontal disease, in the context of using other solutions in order to improve the dento-periodontal clinical status.
9. The results of this study demonstrate the need to expand the research about the antioxidant action of *Prunus spinosa* L. aqueous solution in the orodental cavity.
10. The results of multidisciplinary research conducted within the thesis are aligned with other similar studies through its contribution to knowledge development, being, at the same time, a starting point for obtaining some products that can maintain or improve the orodental health status.

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