“OVIDIUS” UNIVERSITY OF CONSTANȚA
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DOCTORAL THESIS
ABSTRACT

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EXPLOITATION OF *SEMPERVIVUM RUTHENICUM* KOCH PLANT SPECIES IN DENTAL MEDICINE PRACTICE

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INTRODUCTION

Since the beginning of medicine, plant remedies were used as the primary therapy for a plethora of diseases; these remedies were used empirically as their exact composition was unknown at the time.

Typically bioactive plant compounds are produced as a result of the secondary metabolism [1]. Each living organism, including bacteria, animal or vegetal organism are comprised of billions of cells which in turn contain chemical compounds necessary for their survival and function. All the compounds found in a biological system can be split into two major groups: the first is comprised of primary metabolites and encompasses chemical compounds needed for the growth and development of an organism, such as carbohydrates, amino acids, proteins and lipids. The second group encompasses secondary metabolites which are different from the first group as their purpose involves the organism's survival capacity and the interaction with the organism's local environment [2].

The secondary metabolites of various plant species have significant biological effects on the human orgasm, being considered bioactive compounds. Thus, a simple definition of of bioactive plant compounds is that they represent plant chemicals that elicit pharmacological or toxicological effects on both humans and animals [1].

Lately, a very important research goal for the pharmaceutical industry was using medicinal plants and bioactive compounds. Despite the common myth that all phytocompounds are safe, these chemicals present the same risks as synthetic bioactives. Thus, the determination of side effects, optimal dosing and exact chemical structure of these compounds are necessary.

Laboratories from all over the world have discovered thousands of plant based compounds with *in vitro* antibacterial effects and many of those compounds are submitted for animal trials in order to determine their toxicity. However, the lack of standardization concerning tge extraction methods and the evaluation procedures make the results hard to compare between different laboratories.

The oral cavity is comprised of multiple surfaces, each covered in a plethora of bacteria, representing the bacterial biofilm. Some of these microorganism are involved in oral diseases like dental cavities and periodontal diseases. For example, it was estimated that at least 35% of all adults with ages between 30 90 years in the United States of America suffer from

periodontitis. Furthermore, 3 oral bacterial species were found to be involved in systemic diseases such as bacterial endocarditis, pneumonia, infantile osteomyelitis and cardiovascular diseases [4].

Over 700 bacterial species (Gram positive, Gram negative and archaea) were identified in the human oral cavity, most of them being associated with dental plaque, making the oral microbial community one of the most complex flora of the human organism.

I have chosen the subject of this thesis based on the observation of the general trends in the research area of natural compounds, both in the pharmaceutical and medical field. Also, I was motivated by the acknowledgement of fast developing microbial resistance to the existing antibiotics, which led to multiresistant strains that are increasingly hard to eliminate. In my opinion, this phenomenon can be staved off by the discovery of new compounds that still affect bacteria, that can lead to a better antibacterial therapy and to obtaining chemical derivatives of these compounds that can ensure antimicrobial efficiency for longer periods of time.

The doctoral thesis is divided in a *GENERAL PART* and *PERSONAL STUDIES.* The accessed references are presented in citing order.

This research would not have been possible without the involvement of the scientific coordinator, Prof. univ. dr. Badea Victoria, whom I would like to thank. I would also like to thank the collective of the Faculty of Pharmacy Constanta, without whom I could not complete this research. Lastly, I would like to thank my family and friends who supported me during the years of research for this thesis.

Key words: *Sempervivum ruthenicum,* HPLC, DPPH, antioxidant.

GENERAL PART

In the first part of the thesis a short history of medicinal plants is presented, highlighting the importance of natural remedies in the course of human history as well as the importance of research in this field, due to the high potential of identifying new molecules which can serve as both bioactive and precursors. The second chapter refers to the bacterial resistance to antibiotics and the frequency of these occurrences in Europe. This chapter also highlights the mechanisms that cause bacterial resistance and the need for new therapeutic agents. The third chapter presents one of the consequences of bacterial colonization, oxidative stress. This chapter details the genesis of free radicals in the oral cavity and highlights the effects of oxidative stress both in the pathogenesis and the physiopathology of some oral diseases as well as systemic diseases. The fourth chapter describes the anatomy of the *Sempervivum* genus highlighting some particularities of *Sempervivum ruthenicum* Koch. The fifth chapter details the secondary metabolism of the plant species *Sempervivum ruthenicum*. This chapter also reviews the current literature on the subject of *S. ruthenicum* Koch as well as the pharmaceutical applications of the bioactives produced by this plant.

STUDY I.

WORKING HYPOTESIS – the extracts made from *Sempervivum ruthenicum* Koch contain phytocompounds which can be used in dental medicine.

STUDY SCOPE – obtaining extracts from the plant material, identifying and quantifying the compounds with pharmacological actions.

This study describes the harvesting and preparation of extracts made from the leaves of *Sempervivum ruthenicum* Koch, highlighting the morphology of the collected specimens, as well as the techniques used in order to obtain the plant extracts. The plant species chosen for this research is *Sempervivum ruthenicum* Koch, known in folk medicine as "hen and chicks". This is a rare plant, found in the rocky arrid terrain of the Dobrogea region, Romania. The plant has characteristic yellow inflorescence with a distinctive red spot at the base of the petals.

To obtain the plant extracts from *Sempervivum ruthenicum* Koch, I used both fresh plant material as well as dry plant material. In both cases, 3 types of extract were made, using absolute ethilic alcohol, a hydroethanolic solution (50% m/m) and double distilled water. For the fresh plant extracts, the drying loss was taken into account.

WORK METHOLODOGY

Total phenolic content – The Folin-Ciocalteu Method (F-C) is the simplest method available for determining the total phenolic content of plant extracts. To determine the total phenolic content from the extracts, I used the method proposed by Shirazi et. Al. [93], with slight alterations.

Total flavonoid content – The spectrophotometric evaluation based on the formation of a aluminum complex is a routine procedure to determine the total flavonoid content of the samples. In this study I used method proposed by Piyanete et. Al [96]. Quercetin was used as standard, and the total flavonoid content was calculated as quercetin equivalents.

High performance liquid chromatography analysis – To isolate, identify and quantify the bioactive compounds from the plant extracts, a standardized HPLC method was used to determine the polyphenolic compounds, described by the USP 30-NF25 [97]. Briefly a Agilient 1200 chromatogram was employed, with a quaternary pump, DAD, thermostat, degassing system and autosampler. The working conditions included:

- C18 chromatographic column, 250 mm x 4.6 mm; 5 µm (Zorbax XDB);
- Mobile phase (gradient elution):
    - Solution A – phosphoric acid 0.1%
    - Solution B – acetonitrile;
- Flow: 1.5 mL/min;
- Injection volume: 20 µL;
- Analysis time: 20 minutes.

The identification and quantification of the bioactives from the sample extracts were carried out by comparison with internal standards.

Total antioxidant activity - The DPPH evaluation is a simple method, used for measuring a extract's ability to scavenge free radicals and to determine the total antioxidant activity. This method can be employed to quantify antioxidant in biological systems. Also, this method is relatively simple and can be applied to measure the antioxidant capacity of a sample as well as the radical scavenging ability. The method employed in this study was proposed by Ravichandran et. al. [99] with slight alterations. Briefly, a DPPH 4% solution was prepared in absolute methanol, and its absorbance was measured at 517 nm. All determinations were carried out in triplicate, and the results are expressed as median ± standard deviation. All data was subjected to ANOVA ($p<0.05$). The results were processed with Microsoft Excel 360 (Microsoft Office 2016).

RESULTS AND DISCUSSIONS

The results of the HPLC analysis revealed a variety of polyphenolic compounds like free acids and flavonoids. The results found are similar to other studies [61-67]/ Polyphenols in their

free acid form were found in all types of plant extracts although a higher degree of variety could be observed in the hydroethanolic extracts, due to the solvents higher efficiency.

Regarding the quantification of the polyphenolic acids, a vast difference could be observed between the extracts made from fresh plant product and the extracts made from dry plant product. In the case of fresh plant extracts, smaller amounts of polyphenolic acids could be observed, as well as the lack of compounds like caffeic acid, cinnamic acid, chlorogenic acid and Z-resveratrol.

All other bioactive constituents mentioned in the literature could be observed in this analysis with the exception of sedoheptulose, which could not be identified due to the chromatographic conditions.

Astragalin was the most prominent flavonoid identified, being found in all the plant extracts. The most complex phytochemical profile could be observed for the extracts PVUET50 and PVPET50, due to the high efficiency of the solvent mixture. Although a thorough examination of the polyphenolic profile was conducted, the limitations of HPLC standards caused the omission of some bioactives in the plant extracts.

After the total antioxidant assay of the extracts, the results suggest a high antioxidant activity. It can be observed that the dry plant extracts have the highest antioxidant capacity and the smallest inhibitory concentrations for 50% of the free radical. Regarding the extracts, the highest scavenging activity was found for the hydroethanolic extract made from dry plant material.

Regarding the extracts prepared form fresh plant material, the results showed a lower antioxidant activity. The highest antioxidant activity was seen for the hydroethanolic extract, which was followed by the ethanolic extract. Inhibition concentrations for 50% of the free radical were the lowest for the hydroethanolic extract although significantly higher than for the dry plant extracts.

STUDY II

WORKING HYPOTHESIS – The extracts obtained form *Sempervivum ruthenicum* Koch contain bioactive compounds with *in vitro* antibacterial and anti-inflammatory effects.
STUDY SCOPE – Using the plant extracts to prove *in vitro* antibacterial and anti-inflammatory activities.

This study was conducted in order to determine the antibacterial and antifungal activities of the plant extracts by diffusimetric methods, against pathogens found in the oral cavity. The pathogens used are involved in cariogenesis. Also, the study followed *in vitro* assays to determine the anti-inflammatory action via the inhibition of xanthin oxidase and the thermal denaturation of albumin.

WORK METHODOLOGY

Studies regarding the antibacterial effects of plant extracts obtained from *Sempervivum ruthenicum* Koch – to carry out this experiment, three bacterial species were isolated from the human oral cavity. *Staphylococcus aureus, Staphylococcus citreus* and *Streptococcus sanguis* (viridans) were the bacterial specimens selected for this research. The bacteria were identified by using Api© 20 Strep and Api© 20 Staph kits (bioMereaux, France). The plates used for the isolation of the bacterial colonies were blood agar (REF 6378, Bio-Rad, Dubai) and Mueller Hinton (REF 63824, Bio-Rad, Dubai). All the instruments used were previously sterilized and the turbidity of the bacterial suspensions was tested using a UV-VIS Varian Cary 50 spectrophotometer (Agilient Technologies). The antibacterial activity of the plant extracts was assayed using an adapted diffusimetric method [109]. The principle of the employed method is based on a direct proportional relationship between the level of sensibility of the bacteria and the size of the inhibition area around the tested sample. The adaptation employed in this study consists in replacing antibiotic tablets with Whatman Paper saturated with the sample s0olution. Each identified bacterial strain was inoculated in freshly prepared broth, with a concentration expressed as turbidity of 0.5 Mac Farland [109]. The suspensions were seeded onto Mueller Hinton agar plates with a sterile buffer. The filter papers were saturated with 10 μL

plant extract with a concentration of 500 mg/mL. The samples were then applied to the surface of the seeded agar and incubated for 24 hours at a constant temperature of 36.5 ± 0.5^{o}C. After 24 hours, the agar plates were used to determine the area of inhibition. All experiments were run in triplicate and the results were expressed as median ± standard deviation. The inhibition areas were measured by using AutoCAD 2018, taking the paper filter area as the reference point. All data was subjected to statistical analysis by employing Microsoft Excel 360 software.

Studies regarding the antifungal effects of plant extracts obtained from *Sempervivum ruthenicum* Koch – in this study, a reference strain of *Candida albicans* (ATCC 10231) was employed, which was grown on a Sabouraud agar for 96 hours at a constant temperature of 32^{o}C. The colonies formed were used to confirm the pathogens identity. The plant extract antifungal activity was assayed using the previously described diffusimetric method [109]. The inhibition areas were evaluated after 24 and 48 hours of incubation. All experiments were run in triplicate using the same methodology as previously described.

Xanthin oxidase inhibition assay – the method used in this study was proposed by Isa et al [118]. The method involved using the plant extract at different known concentrations to determine the inhibition of xanthin oxidase spectrophotometrically by determining the quantity of uric acid formed in its presence. Allopurinol was used as a positive control due to being an competitive inhibitor of xanthin oxidase at small doses.

Inhibition of albumin thermal denaturation assay – determining the percentage of thermal denaturation of albumin is a frequently encountered method for screening plant extracts [121-129]. To determine the thermal denaturation of albumin inhibition capacity the method proposed by Kahn et al was employed [24]. The inhibitory concentrations for 50% inhibition were calculated by interpolation, using the equation resulted from plotting the inhibition percentage vs the concentration of the plant extracts.

RESULTS AND DISCUSSIONS

A strong correlation was found between the total polyphenolic content determined via the F-C method, the polyphenolic content determined via HPLC, the total flavonoids determined via HPLC and the antibacterial activities of the plant extracts against *Staphylococcus aureus.* There are also significant correlations between the phytochemical profile and the antibacterial activities against *Staphylococcus citraeus*, however a single correlation could be drawn between the HPLC determined polyphenolic content and the antibacterial activities against *Streptococcus sanguis*.

The inhibition areas observed for *Candida albicans* were small in comparison to the results against the bacterial species. Only two plant extracts showed antifungal activities, however there was limited efficacy. The small size of the inhibition areas obtained against *Candida albicans* led to the decision to discontinue other assays on this microorganism.

All plant extracts inhibited xanthin oxidase, suggesting a favorable anti-inflammatory activity which complements the antioxidant action [117]. The hydroethanolic plant extracts presented the smallest inhibitory concentrations for 50% of xanthin oxidase, although the IC50 values were higher than those presented by allopurinol. Even though other species from the *Sempervivum* genus were studied, no other references to this assay were found in the literature, representing a novel approach for this plant species.

The results showed that all plant extracts had a significant inhibitory effect on the thermal denaturation of albumin at high doses (1000μg/mL), with values rivaling the used standard (aspirin). Also, it is worth noting that the hydroethanolic extract produced from dry plant material had a similar value to the standard suggesting a high content of compounds responsible for this activity. In contrast, the water extracts presented the least efficient inhibiting action. Surprisingly, the ethanolic extract produced form fresh plant material presented a high inhibitory effect at all tested concentrations with a IC50 value smaller than that of the employed standard (192.14 ± 4.433 μg/mL).

STUDY III

WORK HYPOTHESIS – the hydroethanolic extracts produced from both fresh and dry plant material can be formulated as oral mucoadhesive patches.
STUDY SCOPE – The formulation and *in vitro* evaluation of oral mucoadhesive patches loaded with bioactive compounds from the plant extracts.

This study details the formulation and pharmaceutical evaluation of oral mucoadhesive patches loaded with hydroethanolic plant extracts. After the pharmaceutical assay of the formulations, the patches were evaluated for oxidative stress reduction on the oral cavity by employing 48 healthy smoking and nonsmoking volunteers.

WORK METHODOLOGY

Formulation and *in vitro* evaluation of mucoadhesive oral patches loaded with *Sempervivum ruthenicum* Koch hydroethanolic plant extracts - Both extracts were submitted to solvent elimination by employing a rotary evaporation IKA RV!0 at a constant temperature of 70^{o}. After the complete elimination of the solvent, dry plant extracts were obtained, which were pulverized and stored in a desiccator for further use. The polymers employed for the formulation of the oral patches consisted of gelatin, pectin obtained from apple, polyvinyl pyrrolidone and methylcellulose. All polymers were purchased from Sigma and were of analytical grade purity. The oral patches were prepared according to the method proposed by Hashemi [137] with slight alterations. The pharmaceutical evaluation of the obtained patches included: mass and content uniformity, swelling index, mucoadhesive strength and *in vitro* release profile.

***In vivo* antioxidant activity assay -** the study included 48 48 clinical healthy volunteers with ages ranging between 20 and 35 years. All volunteers expressed their written, free informed consent according to the Declaration of The World Health Association in Helsinki (revised in 200, Edinburg), with the approval of the Bioethics Commission of the “Ovidius” University of Constanta, request no. 17712/12.11.2018.

The inclusion criteria were:

- Ages ranging between 18 and 35 years;
- Clinically healthy;
- Smoker or non-smoker;
- No known allergic reactions to the components of the formulation.

The plant extracts were tested for alkaloids by employing the Dragendorff reaction, which was negative in all cases. Also, after the HPLC screening no trace amounts of alkaloids were quantified. The literature does not cite compounds with toxic potentials for this plant genus or adverse reactions associated with *Sempervivum* consumption by humans or animals [58-60, 102, 104].

The volunteers were split into two equal groups based on their smoking status. Each group was randomly assigned to 3 sub-groups based on the type of oral mucoadhesive patch administered: without plant extract (control group), with plant extract obtained from dry plant material (PVU) and with plant extract obtained from fresh plant material (PVP).

The volunteers were instructed not to commence their daily oral hygiene routines and to refrain from chewing gum, using mouthwash and oral drops. Also, the volunteers from the smoking group did not consume tobacco-based products in the day of the study, until the end of the evaluation. The volunteers consumed 350 mL of water each hour for the duration of the experiment.

The saliva samples were harvested in sterile flasks at fixed time intervals. The mucoadhesive patches were applied to all patients in the inferior anterior region of the mouth by applying gentle pressure for 20 seconds. After collecting the samples, they were subjected to centrifugation by employing a Eppendorf 5415C Centrifuge, at 13,000 rpm for 10 minutes with the purpose of eliminating any sediments. The total antioxidant capacity of the saliva was assayed employing the DPPH free radical scavenging ability according to the above-mentioned method.

RESULTS AND DISCUSSIONS

After applying the mucoadhesive patches an initial drop of antioxidant activity was recorded for all subgroups, followed by a constant rise of the DPPH scavenging ability up to

25.7% of the initial values. By comparing the type of extract used, a slight rise in the scavenging ability of the patches could be observed for the volunteers who received PVU patches. The oral residence time exceeded the total release time determined *in vitro*, however, significant correlations could be drawn between the DPPH scavenging ability and the *in vitro* release profile.

FINAL CONCLUSIONS

1. The results from the HPLC analysis revealed high concentrations of polyphenols bioactives with therapeutical potential, such as free polyphenolic acids, flavonoids, heterosides and flavonols.
2. All plant extracts have DPPH scavenging abilities. The hydroethanolic extracts presented the highest degree of free radical scavenging.
3. The plant extracts poses *in vitro* antibacterial activities, with the highest efficiency recorded for the hydroethanolic dry plant extract.
4. The studied extracts have a low antifungal effect against *Candida albicans.*
5. The plant extracts poses xanthin oxidase inhibitory effects which correlate to the total flavonoid content.
6. The hydroethanolic fresh plant extract is the strongest xanthine oxidase inhibitor in this study.
7. All studied plant extracts have an inhibitory effect on the thermal denaturation of albumin, which correlate to their flavonoid content.
8. The hydroethanolic dry plant extract is the strongest albumin thermal inhibitor in this study.
9. The hydroethanolic extracts obtained from *Sempervivum ruthenicum* Koch can be incorporated into a polymeric matrix in order to formulate high quality mucoadhesive patches.
10. The mucoadhesive patches loaded with dry plant extract led to the highest rise of saliva total scavenging ability in smoking and non-smoking volunteers.

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