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Eco-innovative strategy to valorize Chestnut (*Castanea sativa*) Shells as Active ingredient for Oral Films: A New Approach to Prevent/Treat Oral Mucositis

Ana Sofia Mendes Ferreira

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Department of Chemistry and Biochemistry
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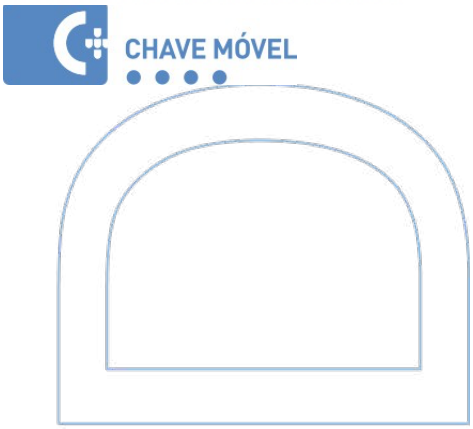
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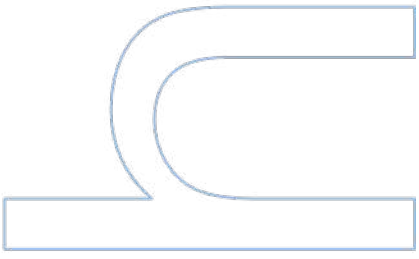
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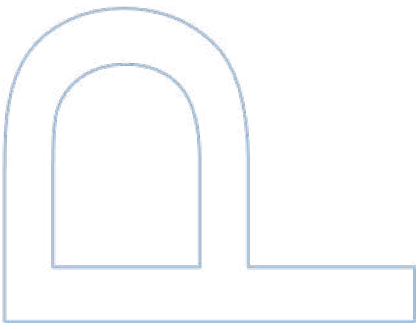
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Ao meu querido pai,

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"Porque os que passam por nós, nunca nos deixam sós, deixam um pouco de si e levam um pouco de nós."

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A dissertation presented to the Faculty of Science from Porto University, Nova School of Science and Technology from Lisboa and Aveiro University for the degree of Doctor in Sustainable Chemistry







Porto, 2024

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Abstract

Food industries generate large quantities of by-products such as seeds, stems, roots, pulp and peels, which are often considered as waste, despite their inherent nutritional value. In recent years, growing concerns about sustainability have led to increased efforts to valorize these by-products. Research has demonstrated that bioactive molecules can be extracted from these materials, which can then be used as innovative industrial raw ingredients. These bioactive compounds have shown potential for a variety of applications, thus promoting the circular economy through sustainable and efficient use of resources within the food industry.

Castanea sativa Mill., commonly known as the European chestnut, is a widely cultivated crop in southern Europe, with significant economic impact in producing countries due its nutrient-rich fruits. Chestnut shells (CS) constitute about 20% of the total fruit weight and are one of the major by-products of the chestnut production chain. CS have been identified as a rich source of valuable molecules, including (poly)phenols, lignans, vitamins, and amino acids. These compounds exhibit exceptional health benefits, such as antioxidant, anti-inflammatory, antimicrobial and anticancer properties, as evidenced by both *in vitro* and *in vivo* assays.

Oral mucositis (OM) is one of the most prevalent side effects of anticancer treatments, including chemotherapy and radiotherapy. It is characterized by acute inflammation, ulceration, and pain, severely impacting patients' quality of life. Studies have linked the oxidative stress pathway to the development of OM, identifying it as a potential therapeutic target. Natural compounds, due to their proven efficacy, safety, and minimal side effects, are being explored as therapeutic phytochemicals.

This thesis aimed to develop an oral film to prevent and treat OM by valorizing CS through a green extraction method, namely Subcritical Water Extraction (SWE).

The optimization of SWE was conducted evaluating the effect of five different temperatures (110, 120, 140, 160 and 180 ºC). The results indicated that the optimal extraction temperature was 110 ºC, which yielded the highest phenolic content, including gallic acid, catechin, epicatechin, protocatechuic acid, rutin and vanillic acid. This extract exhibited superior antioxidant and radical scavenging activities, with IC_{50} values of 4.47 µg/mL and 0.73 µmol TE/mg DW against HOCl and $ROO^{\bullet}$, respectively. Additionally, it demonstrated antimicrobial activity against oral microorganisms such as *Streptococcu*s, *Staphylococcus*, *Enterococcus*, and *Escherichia*. In oral cell lines, namely TR146 and

HSC-3, IC_{50} values of 1325.03 and 468.15 µg/mL were respectively achieved. Based on these findings, the encapsulation of the 110 ºC extract was subsequently investigated.

To overcome the instability and poor water solubility of (poly)phenols, the optimal extract was encapsulated using spray-drying. The process achieved a product yield of 44% and resulted in microparticles with a total phenolic content of 9.73 mg GAE/g, antioxidant activity of 91.76 µmol FSE/g, and antiradical capacity of 7.83 mg TE/g. FTIR analysis confirmed the successful encapsulation. The CS-loaded microparticles revealed a median size of 1.95 µm (Dv_{50}), a moisture content of 4.33% and a swelling capacity of 98.16%. LC-HRMS analysis attested the encapsulation of CS extract (poly)phenols. The thermal properties of the extract were preserved, while the rheological studies revealed that the microparticles exhibited a pseudoplastic behavior. Safety tests demonstrated that the microparticles were non-toxic to oral epithelial cell lines at concentrations up to 1 mg/mL, and the permeation of the CS extract (poly)phenols was confirmed using an *in vitro* buccal model.

Orodispersible films (OFs) offer a promising delivery system for bioactive compounds, addressing the limitations of conventional dosage forms and presenting potential to manage OM. By incorporating CS extract in HPMC E10M OFs via solvent casting, the resulting films exhibited optimal content uniformity, excellent mechanical and physicochemical properties, with important attributes for a suitable oral application, highlighting the great performance of HPMC polymers. Stability testing over 90 days demonstrated that the formulation remained stable at room temperature (25 °C/65% RH) and under accelerated conditions (40 °C/ 75% RH). The CS-loaded films displayed significant antioxidant and antiradical activities (DPPH= 143.42 mg TE/g film; FRAP= 0.142 µmol FSE/g film) and confirmed the antitumoral effects of the CS extract, evidenced by its selective cytotoxicity. LC-ESI-LTQ-Orbitrap-HRMS analysis established the high permeation of bioactive compounds such as sebacic acid, epicatechin, isorhamnetin, protocatechuic acid, and l-tyrosine through *in vitro* and *ex vivo* buccal models. These findings highlight the considerable potential of CS-loaded OFs for the prevention and treatment of OM.

Keywords: cancer, microencapsulation, (poly)phenols, porcine buccal mucosa, solvent casting, spray-drying, subcritical water extraction, orodispersible films.

Resumo

A indústria alimentar gera grandes quantidades de subprodutos, tais como sementes, caules, raízes, polpas e cascas, que frequentemente são considerados resíduos, apesar do seu valor nutricional. Nos últimos anos, têm vindo a aumentar as preocupações com a sustentabilidade, conduzindo a um maior esforço para valorizar esses subprodutos. Diferentes estudos demonstraram que os compostos bioativos podem ser extraídos destes subprodutos e utilizados como ingredientes industriais inovadores numa enorme variedade de aplicações, promovendo a economia circular através da utilização sustentável e eficiente dos recursos dentro da indústria alimentar.

Castanea sativa Mill., conhecido como castanheiro europeu, é amplamente cultivado no sul da Europa, tendo um impacto económico significativo nos países produtores devido as suas sementes, as castanhas, ricas em nutrientes. As cascas da castanha (CS) representam cerca de 20% do peso total da semente e são um dos principais subprodutos da cadeia de produção de castanhas. Estas cascas são ricas em polifenóis, linhinas, vitaminas e aminoácidos, compostos com propriedades benéficas para a saúde, tais como propriedades antioxidantes, anti-inflamatórias, antimicrobianas e anticancerígenas, conforme evidenciado por ensaios *in vitro* e *in vivo*.

A mucosite oral (OM) é um dos efeitos secundários mais prevalentes dos tratamentos anticancerígenos, incluindo a quimioterapia e a radioterapia. Caracteriza-se por uma inflamação aguda, ulcerativa e dolorosa da mucosa oral que afeta severamente a qualidade de vida dos pacientes. O stress oxidativo tem sido associado ao desenvolvimento da OM, identificando-o como um potencial alvo terapêutico. Os compostos naturais, devido à sua comprovada eficácia, segurança e reduzidos efeitos colaterais, têm sido explorados como fitofármacos terapêuticos.

Esta tese visou desenvolver um filme oral para prevenir e tratar a OM, valorizando as cascas de castanha através de um método de extração verde, nomeadamente a Extração com Água Subcrítica (SWE).

Com efeito, otimizou-se a SWE através da avaliação do efeito de cinco temperaturas (110, 120, 140, 160 e 180 ºC). Os resultados indicaram que a temperatura de extração ótima foi de 110 ºC, originando um extrato com maior teor de fenóis, incluindo ácido gálico, catequina, epicatequina, ácido protocatecuico, rutina e ácido vanílico. Este extrato apresentou elevada atividade antioxidante e capacidade de captação de radicais livres, com valores de IC_{50} de 4,47 µg/mL e 0,73 µmol TE/mg DW contra HOCl e $ROO^{\bullet}$, respetivamente. Adicionalmente, demonstrou atividade

antimicrobiana contra microrganismos orais como *Streptococcus*, *Staphylococcus*, *Enterococcus* e *Escherichia*. Em linhas celulares orais, nomeadamente TR146 e HSC-3, este extrato apresentou os melhores valores de IC_{50}, respetivamente 1325,03 e 468,15 µg/mL. Com base nestes resultados, procedeu-se à encapsulação do extrato obtido a 110 ºC.

Para superar a instabilidade e a baixa solubilidade em água dos polifenóis, o extrato ótimo foi encapsulado através do método de *spray-drying*. O processo alcançou um rendimento de produção de 44% e resultou em micropartículas com um teor total de fenóis de 9,73 mg GAE/g, atividade antioxidante de 91,76 µmol FSE/g e capacidade de captura de radicais de 7,83 mg TE/g. A análise FTIR confirmou a realização bem-sucedida da encapsulação. As micropartículas que incorporam o extrato apresentaram um tamanho médio de 1,95 µm (Dv50), um teor de humidade de 4,33% e uma capacidade de absorção de 98,16%. A análise através de LC-HRMS confirmou a encapsulação dos diversos polifenóis. As propriedades térmicas do extrato foram preservadas, e os estudos reológicos revelaram que as micropartículas exibiram comportamento pseudoplástico. Os ensaios de segurança demonstraram que as micropartículas não eram tóxicas para linhas celulares do epitélio oral em concentrações até 1 mg/mL, enquanto a permeação dos diversos polifenóis presentes no extrato de CS foi confirmada num modelo bucal *in vitro*.

Os filmes orodispersíveis (OFs) atuam como um sistema de administração de compostos bioativos, mitigando as limitações das formas farmacêuticas convencionais e apresentando potencial para o tratamento da OM. Ao incorporar o extrato de CS em OFs de HPMC E10M através de *solvent casting*, os filmes resultantes apresentaram um conteúdo uniforme, bem como propriedades mecânicas e físico-químicas ótimas, qualidades importantes para uma aplicação oral adequada. Os ensaios de estabilidade efetuados ao longo de 90 dias comprovaram que a formulação permaneceu estável tanto à temperatura ambiente (25 °C/65% RH) como em ensaios acelerados com condições mais exigentes (40 °C/75% RH). Os filmes que incorporam o CS exibiram atividades antioxidantes e de captação de radicais livres significativas (DPPH=143,42 mg TE/g filme; FRAP= 0,142 µmol FSE/g filme) e confirmaram os efeitos antitumorais do extrato de CS, evidenciados pela sua citotoxicidade seletiva. A análise LC-ESI-LTQ-Orbitrap-HRMS confirmou a alta permeação de compostos bioativos como ácido sebácico, epicatequina, isorhamnetina, ácido protocatecuico e l-tirosina através de modelos bucais *in vitro* e *ex vivo*. Estes resultados destacam o considerável potencial dos OFs com CS para a prevenção e tratamento da OM.

Palavras-chave: cancro, extração de água subcrítica, microencapsulação, mucosa bucal de porco, polifenóis, *solvent casting*, *spray-drying*, filmes orodispersíveis.

List of Publications

Publications in International Peer-reviewed Journals

(included in chapters 1, 2, 3 and 4 of the present thesis)

1. **Ferreira**, **Ana Sofia**; Macedo, Catarina; Silva, Ana Margarida; Delerue-Matos, Cristina; Costa, Paulo; Rodrigues, Francisca. Natural Products for the Prevention and Treatment of Oral Mucositis - A Review. *Int. J. Mol. Sci.* 2022, *23*, 4385. https://doi.org/10.3390/ijms23084385
2. **Ferreira**, **Ana Sofia**; Silva, Ana Margarida.; Pinto, Diana; Moreira, Manuela M.; Ferraz, Ricardo; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. New Perspectives on the Sustainable Employment of Chestnut Shells as Active Ingredient against Oral Mucositis: A First Screening. *Int. J. Mol. Sci.* 2022, *23*, 14956. https://doi.org/10.3390/ijms232314956
3. **Ferreira**, **Ana Sofia**; Silva, Ana Margarida.; Laveriano-Santos, Emily P.; Lozano-Castellón, Julián; Lamuela-Raventós, Rosa M.; Švarc-Gajić, Jaroslava; Delerue-Matos, Cristina; Estevinho, Berta; Costa, Paulo; Rodrigues, Francisca. Development and Characterization of Spray-Drying Microparticles Loaded with Chestnut Shells Extract: New Insights for Oral Mucositis Therapy. *Powder Technol*. 2024, 446. https://doi.org/10.1016/j.powtec.2024.120182
4. **Ferreira**, **Ana Sofia**; Silva, Ana Margarida.; Macedo, Catarina; Laveriano-Santos, Emily P.; Lozano-Castellón, Julián; Lamuela-Raventós, Rosa M.; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. Innovative orodispersible films with chestnut shells extract as active ingredient to prevent oral mucositis: *In vitro* and *ex vivo* studies. *Submitted*

Book Chapter

1. Silva, Ana Margarida; **Ferreira**, **Ana Sofia;** Luís, Ana Sofia; Macedo, Catarina; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. Cosmetic applications of herbal products and nano/microencapsulated herbal active formulation, “Nanotechnology in Herbal Medicine: Applications and Innovations” (Eds. Sabu Thomas, Adebola Oyedeji, Samuel Oluwatobi Oluwafemi and Rose Jaquilin PJ), Elsevier, 2022, 447-490.

List of Presentations in Scientific Meetings

Oral Communications

1. **Ferreira**, **Ana Sofia**; Pinto, Diana; Silva, Ana Margarida; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "Subcritical Water Extraction of chestnut shells: A promising source of bioactive compounds". XVI *Encontro Química dos Alimentos*, 25th October 2022, Castelo Branco (Portugal).
2. **Ferreira**, **Ana Sofia**; Pinto, Diana; Silva, Ana Margarida; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "Sustainable extraction of bioactive compounds from *Castanea sativa* shells". 5th ISEKI-Food e-conference, 23-25 November 2022, online.
3. **Ferreira**, **Ana Sofia**; Silva, Ana Margarida; Pinto, Diana; Ferraz, Ricardo; Moreira, Manuela M.; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "From Green Extraction of Chestnut By-Products to Pharmaceutical Application". XXXI International Conference on Polyphenols, 3-6 July 2023, Nantes (France).
4. **Ferreira**, **Ana Sofia**; Silva, Ana Margarida; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "Oral films incorporating chestnut shells bioactive compounds as delivery systems for the prevention/treatment of oral mucositis". I International Meeting - Molecules4Life, 20-22 September 2023, Vila Real (Portugal).
5. **Ferreira**, **Ana Sofia**; Silva, Ana Margarida; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "Chestnut shells bioactive compounds as an ingredient to delivery systems for the prevention/treatment of oral mucositis". 6th ISEKI-Food e-conference, 22-24 November 2023, online.
6. **Ferreira**, **Ana Sofia**; Macedo, Catarina; Silva, Ana Margarida; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "Natural Products as a new source of bioactive compounds for the prevention/treatment of oral mucositis". Kiwi4Health Seminar, 10th January 2024, online.

Poster Communications

1. **Ferreira**, **Ana Sofia**; Silva, Ana Margarida; Pinto, Diana; Moreira, Manuela M.; Ferraz, Ricardo; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "Sustainable valorization of phenolic compounds from *Castanea sativa* shells for pharmaceutical application". 5º Encontro de Biotecnologia Medicinal/ 2nd Iberian Congress on Medicinal Biotechnology, 31st October 2022, Porto (Portugal).
2. **Ferreira**, **Ana Sofia**; Silva, Ana Margarida; Pinto, Diana; Ferraz, Ricardo; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "From agricultural waste to antioxidant-rich extracts: Green extraction of polyphenols from chestnut shells". 4th SPLC-CRS Young Scientists Meeting, 6-7 February 2023, Porto (Portugal).
3. **Ferreira, Ana Sofia**; Silva, Ana Margarida; Pinto, Diana; Ferraz, Ricardo; Moreira, Manuela M.; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "Potential therapeutic application of chestnut shells in oral mucositis treatment: Green extraction of polyphenols". Dare2Change, 21st March 2023, Porto (Portugal).
4. **Ferreira, Ana Sofia**; Silva, Ana Margarida; Pinto, Diana; Ferraz, Ricardo; Moreira, Manuela M.; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. "Repurposing Chestnut Shells as Antioxidant-Rich Ingredient". I International Congress on Antioxidants, 10-12 May 2023, Barcelona (Spain).

List of Awards and other distinctions

2nd best oral presentation in the 6th ISEKI-Food e-conference. “Chestnut shells bioactive compounds as an ingredient to delivery systems for the prevention/treatment of oral mucositis”. 22-24 November 2023.

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List of Abbreviations and of Acronyms

5-FU	5-Fuorouracil
AAPH	α,α′-azodiisobutyramidine dihydrochloride
ABTS	2,2´-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
ANOVA	Analysis of variance
BMI	Body Mass Index
CAT	Catalase
COX-2	Cyclooxygenase-2
CS	Chestnut Shells
CT	Chemotherapy
CTCAE	Common Terminology Criteria for Adverse Events
DHR	Dihydrorhodamine 123
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential Scanning Calorimeter
DW	Dry Weight
EDS	Energy Dispersive Spectroscopy
EE	Encapsulation Efficiency
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EMA	European Medicines Agency
eNOS	endothelial Nitric Oxide Synthase
ERK	Extracellular signal-Regulated Kinase
FDA	U.S. Food and Drug Administration
FRAP	Ferric Reducing Antioxidant Power
FSE	Ferrous Sulfate Equivalents
FTIR	Fourier-Transform Infrared Spectroscopy
FTMS	Fourier-Transform Mass Spectroscopy
GAE	Gallic Acid Equivalents
GR	Growth Rate
GSH	Gluthathione
GSH-PX	Gluthatione Peroxidase

H_2O_2	Hydrogen peroxide
HCO_3	Hydrogen carbonate
HCST	Hematopoietic Stem Cell Transplantation
HNC	Head and Neck Cancer
HNSC	Head and Neck Squamous Carcinoma
HOCl	Hypochlorous acid
HPLC-PDA	High Performance Liquid Chromatography-Photometric Diode Array
HPMC	Hydroxypropyl methylcellulose
HV	High Voltage
IC_{50}	Half-maximal inhibitory concentration
ICH	International Council of Harmonization
IFN-γ	Interferon-gamma
IL	Interleukin
iNOS	inducible-Nitric Oxide Synthase
ISOO	International Society of Oral Oncology
JNK	c-Jun N-terminal Kinase
KBr	Potassium bromide
LC-ESI-LTQ-Orbitrap-HRMS	Liquid Chromatography-Electrospray Ionization-Linear Trap Quadrupole-Orbitrap-High Resolution Mass Spectrometry
LOD	Limit Of Detection
LOQ	Limit Of Quantification
LPs	Lipid Peroxides
LPS	Lipopolysaccharide
LVER	Linear Viscoelastic Region
MAE	Microwave-Assisted Extraction
MAPK	Mitogen-Activated Protein Kinase
MASCC	Multinational Association of Supportive Care in Cancer
MASWE	Microwave-Assisted Subcritical Water Extraction
MBC	Minimum Bactericidal Concentration
MDA	Malondialdehyde
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MMPs	Matrix Metalloproteinase
MPO	Myelo-Peroxidase

MRJP	Major Royal Jelly Protein
mRNA	messenger Ribonucleic Acid
MRSA	Methicillin-Resistant *Staphyloccoccus aureus*
MTT	3.[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NA	Not applicable
NBT	Nitroblue Tetrazolium chloride
ND	Not Determined
NF-κB	Nuclear Factor-κb
NLS	Nuclear Localization Sequence
NO	Nitric Oxide
NOX	NADPH Oxidase
Nrf2	Nuclear factor erythroid 2-related factor 2
$O_2^{\bullet-}$	Superoxide
OD	Optical Density
OFs	Orodispersible Films
OM	Oral Mucositis
$ONOO^-$	Peroxynitrite
OSCC	Oral Squamous Cell Carcinoma
PBM	Photobiomodulation
PCA	Protocatechuic Acid
PDA	Photodiode Array
PEG	Polyethylene Glycol
PGA	Pyroglutamic Acid
PGE2	Prostaglandin E2
PMS	Phenazine Methosulphate
RH	Relative Humidity
RNS	Reactive Nitrogen Species
$ROO^\bullet$	Peroxyl radical
ROS	Reactive Oxygen Species
RSM	Response Surface Methodology
RT	Radiation
RTOG	Radiation Therapy Oncology Group
SD	Standard Deviation
SEM	Scanning Electron Microscopy

SFE	Supercritical Fluid Extraction
SOD	Superoxide Dismutase
STAT	Signal Transducers and Activator of Transcription
SWE	Subcritical Water Extraction
SWOT	Strengths, Weaknesses, Opportunities, and Threats
TE	Trolox Equivalents
TEER	Trans-Epithelial Electrical Resistance
TGF-β-1	Transforming Growth Factor-β-1
TNF- α	Tumor Necrosis Factor-α
TPC	Total Phenolic Content
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UAE	Ultrasound-Assisted Extraction
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
WD	Working Distance
WHO-OMGS	World Health Organization's Oral Mucositis Grading Scale
XOD	Xanthine Oxidase

Relevance and Motivation

Castanea sativa Mill., a member of the *Fagaceae* family prevalent in southern Europe, produces appreciated fruits that are commonly known as chestnuts and present high economic value. The cultivation of chestnuts has been on the rise due to a growing awareness of their nutritional properties and potential health benefits [1]. Portugal is one of the main worldwide producers, achieving ~27,000 tons of chestnut just in 2020 [2]. During chestnut production huge amounts of by-products are generated, mainly shells that represent 20% of the fresh fruit weight [1].

Various studies have reported the abundance of phenolic compounds in chestnut shells extracts, particularly phenolic acids, such as gallic and ellagic acids, as well as tannins [1-4]. Additionally, the presence of vitamin E and amino acids has been noted [1, 5, 6]. These bioactive compounds are known for their potential antioxidant, anti-inflammatory, and antimicrobial properties. They can also regulate gene expression, enhance endothelial function, and inhibit enzymes such as matrix metalloproteinases [7]. However, the extraction process is crucial for the isolation, identification, and utilization of these bioactive substances. Subcritical Water Extraction is considered the most promising sustainable method for large-scale recovery of natural bioactive compounds, owing to its numerous advantageous including low costs, reduced solvent employment, shorter processing times, higher yields, and minimal risk of constituent degradation [8].

(Poly)phenols have garnered great interest on the last decade due to their biological activities and demonstrated positive effects against cancer and related diseases in human trials [9-12]. Oral mucositis is the most common side effect of cancer treatments such as chemotherapy and radiotherapy, being characterized by the presence of erythematous and ulcerated lesions in the oral mucosa, associated with pain, which compromise the life's quality of the patients.

The standard treatment for oral mucositis is ineffective, providing only brief periods of moderate pain relief, often leading to an increased reliance on opioid analgesics and parenteral or enteral nutrition for patients unable to eat orally [13]. The use of bioactive compounds extracted from natural sources with biological properties (e.g. antioxidant activity) is a promising approach to prevent/treat oral mucositis. However, the bioactive compounds concentration absorbed in the buccal mucosa are often low, avoiding an acceptable therapeutic effect, mainly due to the saliva turnover, tongue and masticatory movements, enzymatic degradation and lack of epithelium permeation [14].

Encapsulation emerges as an effective strategy to address these challenges and enhance the local buccal effect, improving the overall pharmacokinetics of bioactive compounds, extending shelf life and protecting compounds [15]. The carriers should be selected based on the extracts composition in order to ensure a controlled release profile, resulting in steady-state plasma concentration and reduced side-effects. For the oral mucositis treatment, oral films are the most suitable drug delivery system, providing an easy and convenient means of bioactive compounds administration. Orodispersible films are polymeric thin layers that intend to disintegrate and deliver bioactive compounds inside oral cavity, without requiring water [16, 17].

This study aims to take advantage of all these techniques to develop an orodispersible film containing chestnut shells extract for the prevention and treatment of OM.

Thesis objectives

The main goal of this thesis was to develop an oral film incorporating the bioactive compounds extracted from chestnut shells using Subcritical Water Extraction to prevent/treat oral mucositis. In this context, different specific objectives were aimed to be reached:

- Optimization of a SWE procedure of CS under different conditions by Response Surface Methodology (RSM);
- Screening of the extracts total phenolic content (TPC) antioxidant/antiradical activities (DPPH, ABTS, FRAP);
- Identification and quantification of extracts bioactive compounds: phenolics and organic acids (HPLC-PDA);
- Evaluation of extracts Reactive Oxygen and Reactive Nitrogen Species scavenging activities;
- Screening of extracts antimicrobial activity against oral pathogens;
- Encapsulation of extracts into polymeric particles (PPs) and optimization of particulate systems after physical-chemical characterization;
- Development of oral films with PPs considering technological properties;
- *In vitro* safety assessment of extracts, PPs and oral films on buccal cell lines monolayers and 3D models;
- *Ex vivo* permeability and accumulation of extracts, PPs and oral mucositis in porcine buccal mucosa;

Thesis structure and organization

This thesis presents the research conducted over the past four years within the scope and objectives of the approved PhD project SFRH/BD/7519/2020. It is structured in five main chapters, including the publications published during this period. The figures and tables were numbered sequentially throughout the thesis, while the references were grouped into a single final list presented at the end of the thesis. Each chapter is summarized as follows:

CHAPTER 1 – General Introduction: Natural Products for the Prevention and Treatment of Oral Mucositis

This chapter consists of an extensive review paper addressing the potential use of natural products as effective agents in the prevention and treatment of oral mucositis. Initially, this condition is contextualized as a prevalent and debilitating side effect of cancer treatments. Then, pathogenesis, risk factors and current management strategies are presented. Finally, it provides a detailed summary of natural products that have demonstrated positive effects in the prevention and treatment of oral mucositis through experimental animal studies and clinical trials, highlighting their potential application in pharmaceutical formulations.

CHAPTER 2 – Employment of Chestnut Shells as Active Ingredient against Oral Mucositis

This chapter represents the preliminary stage of the experimental work, focusing on the extraction of bioactive compounds from chestnut shells using an environmentally sustainable method, namely Subcritical Water Extraction. It emphasizes the economic significance of *Castanea sativa* and the need to address sustainability by leveraging agro-industrial by-products as valuable sources of bioactive compounds. The extracts obtained were subsequently evaluated for their antioxidant/antiradical and antimicrobial activities, phenolic profile and cellular viability using oral mucosal cell lines, demonstrating their potential use in the prevention and treatment of oral mucositis.

CHAPTER 3 – Incorporation of Chestnut Shells Extract into Spray-Drying Microparticles

This chapter is devoted to the incorporation of the optimal extract of chestnut shells into microparticles. Bioactive compounds were encapsulated by spray-drying to

mitigate the instability and poor solubility of (poly)phenols. The obtained formulations were characterized in terms of morphology and size, moisture content, antioxidant/antiradical activities, spectrometric, thermodynamic and rheological properties, cell viability and permeability through an *in vitro* buccal permeation model, using LC-HRMS analysis.

CHAPTER 4 – Orodispersible Films with Chestnut Shells extract to prevent Oral Mucositis: *in vitro* and *ex vivo* studies

This final experimental chapter details the development of orodispersible films as a potential delivery system for local treatment of oral mucositis, incorporating the chestnut shells extract. The solvent casting films were assessed based on their mechanical, physicochemical properties, antioxidant/antiradical and anticancer activities, as well as the permeation of bioactive compounds through *in vitro* an *ex vivo* models.

CHAPTER 5 – Conclusion and Final Remarks

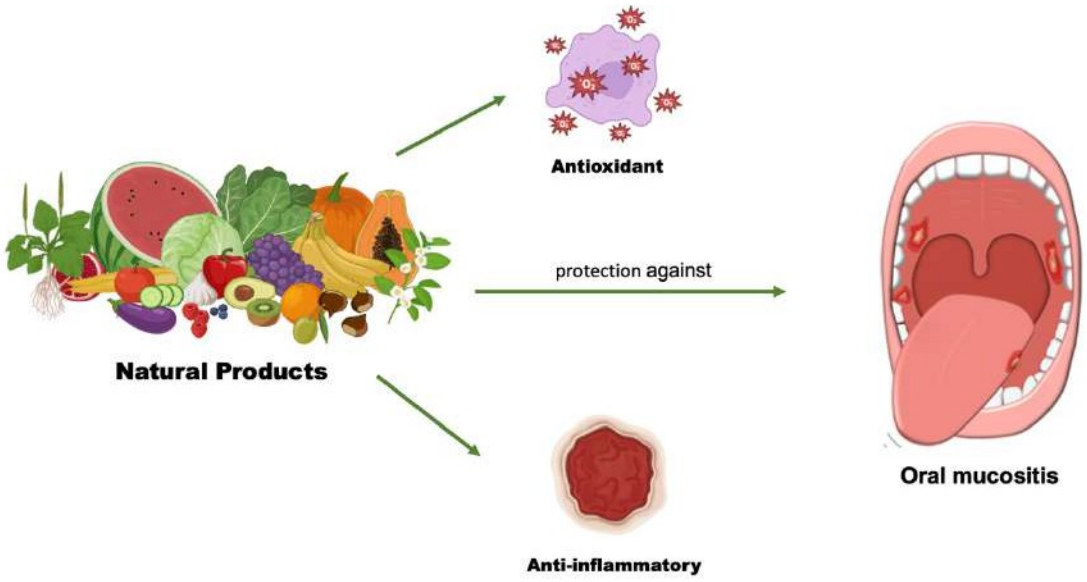
Finally, this chapter summarizes the final remarks on the main results obtained concerning the thesis abstract and outlines indications for future research investigations.

Chapter 1

GENERAL INTRODUCTION: Natural Products for the Prevention and Treatment of Oral Mucositis

The information presented in this chapter was published in the following publication:

Ferreira, Ana Sofia; Macedo, Catarina; Silva, Ana Margarida; Delerue-Matos, Cristina; Costa, Paulo; Rodrigues, Francisca. Natural Products for the Prevention and Treatment of Oral Mucositis - A Review. *Int. J. Mol. Sci.* 2022, *23*, 4385.



Statement of contribution:

The contribution of the first author, Ana Sofia Ferreira, in this work, includes the literature review and writing of the original draft.

1.1. Introduction

Cancer is currently a major public health problem all over the world. In 2020, almost 19.3 million new cases were diagnosed worldwide [18]. Treatment of malignancies with cytotoxic chemotherapy (CT), radiation (RT), or a combination of the two is becoming more effective, as it is associated with short- and long-term adverse effects, including mucositis [19, 20]. This secondary reaction may occur in any area of the gastrointestinal tract's mucosal layer, from the mouth to the anus, with the oral cavity being the most prevalent location. The cytotoxicity is caused by a variety of mechanisms, including inhibition of DNA replication and repair, cell-cycle arrest, DNA damage, and cell death [21]. However, the precise and complex molecular pathways underlying the oral epithelial damage are not completely known [22, 23].

Oral mucositis (OM) is a painful inflammatory and frequently ulcerative disorder of the oral mucosa that severely reduces the patient's quality of life [20, 24, 25]. OM occurs in 20 to 40% of the patients submitted to conventional CT, 80% of patients on high-dose CT, 75 to 100% of patients receiving hematopoietic cell transplants, and practically all patients with head and neck squamous carcinoma (HNSC) undergoing RT [21, 22, 26-28]. Common symptoms of OM include pain, bleeding, ulcers, and difficulty ingesting fluids or solids and speaking, as well as severe complications, such as secondary infections and significant weight loss, which may compromise the treatment of the primary disease and its outcome [19, 29, 30]. In addition, OM may result in the need for enteral or parenteral nutrition [31, 32] and systemic analgesics [33-35], thus increasing hospitalizations [30, 36], the use of resources and higher costs [36, 37], and, in some cases, the risk of sepsis [25, 38]. However, when mucositis progresses, topical analgesics become less effective and systemic opioids may be required [39-41]. Different strategies have been used to attempt the prevention or amelioration of this condition, and some clinical trials were effective [25, 34, 35, 42]. For example, cryotherapy [27, 43] and keratinocyte growth factor [27, 44] demonstrated some benefits in preventing mucositis. Zinc [45, 46] and vitamin E [45, 47, 48] were effective in reducing the severity of OM, but *Aloe vera* [49], amifostine [21, 50], glutamine [45, 48, 51], honey [49, 52-55], photobiomodulation (PBM) therapy [56-58], and antibiotics [38] demonstrated lower evidence of benefits. The studies reviewed were evaluated in patients with different types of cancer who underwent different treatment approaches.

While there are a growing number of innovative anticancer agents, few therapeutic alternatives for the prevention or treatment of oral mucositis have been reported. Most important, the scarce alternatives that have been successfully achieved

are still unsatisfactory [23, 34]. Therefore, the search for alternative compounds obtained from natural sources could be an option and a challenge for this research field. Natural compounds, in contrast to synthetic ones, are often thought to have fewer side effects, are easy to access, and present beneficial bioactive properties (e.g., anti-inflammatory, antioxidant, and antimicrobial properties), making them interesting solutions as promising therapeutics. Aside from the protective results of natural products against toxicity induced by radiation or antineoplastic drugs, one of the most promising preventive measures in patients during therapy may be the employment of natural products. The aim of this review is to provide an overview of the use of natural compounds for the prevention and eventual treatment of OM in cancer patients and their potential applications in drug delivery systems to overcome the specific limitations of the oral cavity environment.

1.2. Oral Mucositis

As previously stated, mucositis is an inflammatory response condition of the oral mucous membrane that is frequently observed in malignant neoplastic patients undergoing CT, RT, or both. This condition develops due to interactions among an oral tissue injury, the oral cavity environment, bone marrow suppression, and innate predisposing factors in the patient [33, 59, 60]. The symptoms of OM, such as oral mucosal atrophy, swelling, erythema and subsequent pain, bleeding, ulceration, difficulty in feeding and even swallowing saliva, or a combination thereof, may be diverse [19, 27, 61]. Difficulties with eating reduce the nutritional intake, resulting in a decline in the patient's nutritional status. This can also seriously affect their speech due to an uncomfortably dry mouth and a decrease or increase in salivation [27, 34, 61]. OM may also be aggravated by injuries induced by sharpened teeth, bruxism, food, and microorganisms [61, 62]. Naturally, additional ulcers provide an easy access point for microorganisms, including bacteria, fungi, and viruses, to enter the bloodstream because of the loss of mucosal integrity, culminating in systemic infections that may cause the treatment for fighting the primary disease to be discontinued or even threaten the patient's survival. Moreover, the dysphagia, xerostomia, and changes in taste caused by OM can increase the systemic symptoms, such as lethargy and anorexia, as well as psychological issues. Consequently, OM is associated with increased resource needs and potentially major economic impacts—depending on its severity—due to the more frequent and prolonged hospitalizations for support and nutritional care and analgesic treatments.

Three tools are available for assessing the severity of OM. The most extensively used is the World Health Organization's Oral Mucositis Grading Scale (WHO-OMGS), which incorporates clinical criteria to evaluate the OM lesion and eating capacity [63]. On the other hand, the Common Terminology Criteria for Adverse Events in its fifth revision (CTCAE v5.0) considers the following factors when assessing the impact of OM: pain intensity, ability to eat, and need for intervention [63]. Finally, Radiation Therapy Oncology Group (RTOG) defines the severity of RT-induced OM using a four-point scale [63]. OM is classified according to these three criteria, as summarized in Table 1.

Table 1 - Available Clinical Scales of Oral Mucositis Assessment. Adapted from [51].

Scale	Grade 0	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 (Life-threatening)	Grade 5 (Death)
WHO	No findings	Oral erythema and soreness; no ulcers	Oral erythema, ulcers; solid diet tolerated	Oral ulcers; liquid diet only	Oral alimentation impossible	NA
CTCAE	None	Asymptomatic or mild symptoms; intervention not indicated	Moderate pain or ulcer that does not interfere with oral intake; modified diet indicated	Severe pain, interfering with oral intake	Life-threatening consequence; urgent intervention indicated	Death
RTOG	No change over baseline	Irritation; may experience mild pain, not requiring analgesics	Patchy mucositis that may produce an inflammatory serosanguinous discharge; may experience moderate pain requiring analgesia	Confluent, fibrinous mucositis; may include severe pain requiring narcotic	Ulceration, hemorrhage, or necrosis	NA

* NA – Not applicable.

1.2.1. Physiopathology of OM

In the last decades, substantial evolution has taken place in the understanding of the complex mechanism behind the development of mucositis [23]. A five-phase model that begins with an (i) initiation involving cell injury, (ii) elevation of inflammatory cytokines, a (iii) primary damage response, and (iv) signaling and amplification of the inflammatory cascade, followed by (v) ulceration and mucosal repair through epithelial proliferation, has been reported by different authors [19, 20, 34, 64]. Thus, OM is characterized by a cascade of events that occur simultaneously and are mechanistically related (Figure 1). Therefore, each factor that drives each phase may constitute a possible therapeutic target [34].

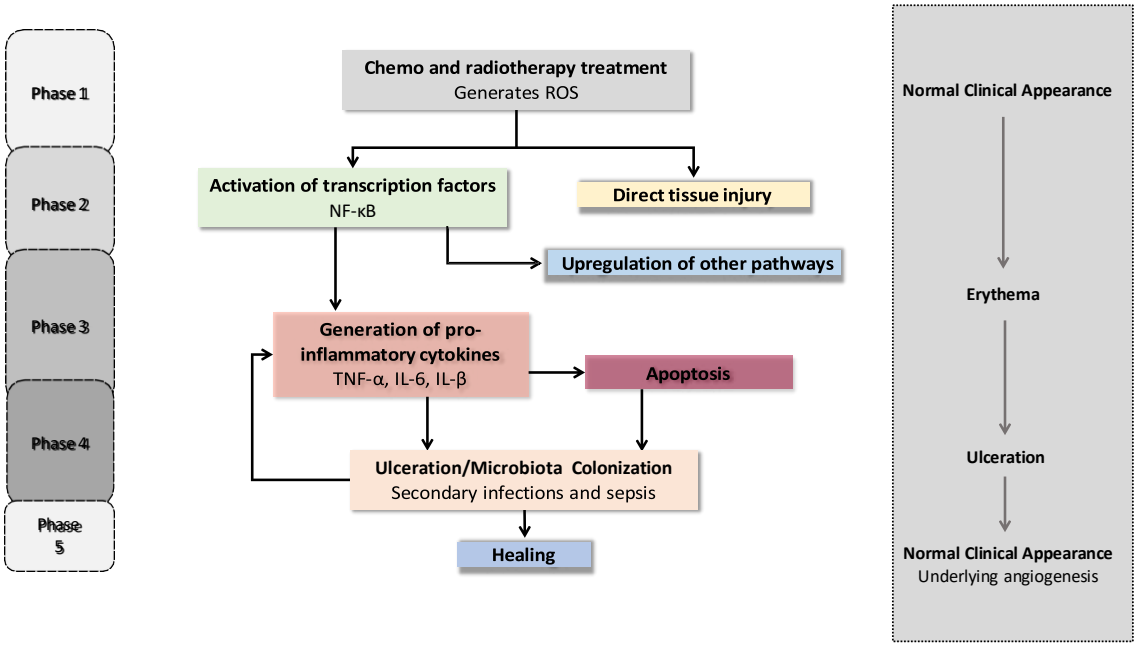


Figure 1 - Diagram representing the mucosal cells and clinical manifestations of oral mucositis.

The mucositis initiation phase—initiation—corresponds to the injury of oral mucosal cells caused by CT and/or RT. This phase begins instantaneously as the antineoplastic treatment is being administered [23, 65-67]. The second phase—upregulation with messenger generation—involves the cytotoxic effect, resulting in the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and DNA damage, leading to basal and suprabasal epithelial cell death [19, 20, 23]. Particularly, when DNA strands breaks, the apoptotic process is activated, with p53 and nuclear factor κB (NF-κB) playing major roles [68, 69]. At this point, inflammatory cytokines, chemokines, and adhesion molecules are generated when NF-κB, the key

mediator of pro-inflammatory gene expression, is activated, which is clinically manifested as mucosal damage. The release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), is mediated through transcription factor activation, and this promotes connective tissue and endothelial damage, limiting the tissue oxygenation and stimulating epithelial basal cell death [19, 33, 68-71]. The third phase—signaling and amplification—is the consequence of tissue damage, apoptosis, enzyme activation, and vascular permeability, which amplify the molecules of the innate immune response as pro-inflammatory cytokines in a positive feedback mechanism, leading to more tissue damage [33, 72]. In the fourth phase—ulceration—clinical signs of mucositis become visible, as the integrity of the mucosa and submucosa is disrupted, causing pain control to be required [20, 34, 65]. In neutropenic patients, the immune cells cannot respond properly, and the ulcerative lesions allow several microorganisms to penetrate into the connective tissue, triggering the production of more pro-inflammatory cytokines and increasing the tissue damage [68, 69]. Bacteremia and sepsis are mostly caused by herpes simplex virus, *Candida albicans*, or other fungal genera, such as *Aspergillus* [28]. Healing usually occurs naturally after the cancer treatment is ceased, and it is marked by epithelial proliferation, migration, and differentiation promoted by the extracellular matrix [19, 20, 67]. The oral mucosa recovers, but the patient remains at risk for recurrent episodes due to residual angiogenesis [33, 34, 67, 73].

CT patients often experience acute symptoms 3–5 days following its administration, with ulcerative lesions appearing a few days later and resolving within 2 weeks [20, 61, 69]. On the other hand, RT mucositis is a chronic condition that lasts up to 7 weeks. The radiation doses range from 2 to 70 Gy per day and cause ulcerations that remain for 3–4 weeks after the treatment is ceased [26, 27, 33]. The lack of taste develops because the oral mucosa is exposed to radiation after few weeks, compromising nutrition and psychological status, while the recovery begins 6–8 weeks after the completion of the treatment [26, 61, 65].

1.2.2. Risk factors

The risk factors of OM can be classified as patient-related, tumor-related, and treatment-related variables, as summarized in Table 2 [34, 62].

In the patient-related factors, gender has been linked to mucositis, since women are associated with a higher risk, which could be due to dosimetric considerations [29, 42, 74, 75]. However, other studies reported the absence of evidence that gender and

OM are correlated [34, 62, 75, 76]. Although age is frequently reported as a mucositis risk factor, there are few consistent reports that link younger and older patients and mucositis severity [62, 75]. Likewise, the effect of body mass index (BMI) on mucositis risk is inconsistent, with data suggesting that a low BMI and a BMI higher than 25 are related with a superior risk, as body composition can affect drug metabolism, as can smoking and poor oral hygiene [33, 62, 75]. Genetic variants, previous treatment, and comorbidities (such as renal dysfunction and diabetes mellitus) have been indicated as possible factors for chronic OM associated with RT [34, 77].

Table 2 - Risk factors related to patients, tumor, and treatment to the development of oral mucositis.

Risk Factor	Criteria	References
Related to patient		
Age	Extremities	[43, 46, 59, 74, 78-80]
Gender	Female	[36, 37, 45, 57]
Body Mass Index (BMI)	Low and high Body Mass Index	[36, 37, 45, 57]
Dental prothesis	Orthodontics and Prothesis	[43, 44]
Education	Lack of health literacy	[41, 81-83]
Oral hygiene	Oral hygiene less than 2 times/day Periodontal disease	[36, 37, 45, 57]
Comorbidities	Diabetes Mellitus, Renal and Hepatic dysfunction	[51, 84, 85]
Leucocytes	Neutropenic patients are immune compromised	[36, 50, 74]
Alcohol	Use of alcohol prior and during treatment	[36, 37, 50, 74]
Smoking	Smoking prior and during treatment increases the severity	[39, 74]
Genetics	Genetic polymorphisms (e.g., TNF-α)	[45, 74]
Mucosal trauma	Sharpened teeth	[39]
Related to tumor		
Types of cancer	Solid tumors have higher risk, mainly those located near oral cavity	[74, 86, 87]
Related to treatment		
Type of treatment	5-fluorouracil, Doxorubicin, Methotrexate, Cisplatin, Vinblastine, Mitomycin, Transtuzumabe, Docetaxel, Melphalan	[45, 51, 84]
Dose	High doses over short periods and its extension	[45, 51, 84]
Type of administration	Intravenous	[36, 45, 51, 74, 84]

In what concerns the tumor's nature, its location, size, and stadium may also influence the grade of OM [62]. For instance, in HNSC patients, the standard protocol includes RT with a specific area and prescription dose, which influences the exposure to radiation and the subsequent mucosal damage [34, 62]. However, in recent years, there was an increased investment in intraoral medical devices that enable the minimization of excessive irradiation of normal tissues [87].

Although the risk factors of OM are not completely understood, the characteristics of anticancer therapeutics (mechanism of action, dose, planning, and number of cycles) are closely associated with the prevalence and severity of the lesions, as their effects accumulate [29, 33, 62]. It is well known that female patients using methotrexate and melphalan have a greater chance of developing this local inflammatory condition [34].

Along with investigating intrinsic patient characteristics, such as pre-existing medical conditions, altered oral dynamics, and general health, age, oral health (hygiene prior to treatment), nutritional status, and liver and kidney function are critical, as they are parameters that a medical team must consider [19, 33]. Aside from that, it is necessary to emphasize that OM is frequently documented only in its advanced phases owing to the requirements for clinical therapy and assistance [30, 31, 36, 88]. Therefore, the search for new active ingredients that could be used in the prevention (and even treatment) of OM is of outmost importance.

1.2.3. Prevention and management of OM

OM management strategies include either preventive or symptom control strategies [25, 33, 40, 42]. The primary key measure in preventing OM is the preservation of tissue during RT treatment planning and the use of RT procedures that conserve the uninvolved oral mucosal surface [35, 89]. Some strategies are addressed in the evidence-based guidelines developed by the Multinational Association of Supportive Care in Cancer and the International Society of Oral Oncology (MASCC/ISOO), which present three categories: a recommendation, a suggestion, and a situation where no guideline is possible [72, 76]. These guidelines can be adjusted at any time to compensate for possible restraints in the clinic and patient choices [76]. Table 3 summarizes the recommended or suggested strategies for most of the groups of cancer patients.

Table 3 - Management of Oral Mucositis Guidelines created by the Multinational Association for Supportive Care in Cancer (MASCC) and the International Society of Oral Oncology (ISOO).

Intervention	Aim	MASCC/ISOO Guideline category	Results	References
Oral care	Prevention	Suggestion	Increases patient's awareness and enhances their compliance with treatment	[39, 73, 76, 90-94]
Oral Cryotherapy	Prevention	Recommendation	Local vasoconstriction that minimizes drug absorption	[44, 76, 82, 85, 95, 96]
Photobiomodulation therapy	Prevention	Recommendation	Promotes wound healing and has anti-inflammatory effect	[57, 60, 75, 76, 97, 98]
Benzydamine mouthwash	Prevention	Recommendation	Anti-inflammatory properties by inhibiting the production of pro-inflammatory cytokines	[76, 79, 80, 84, 99, 100]
Keratinocyte Growth Factor-1 (Palifermin)	Prevention	Recommendation	Proliferation and restoration of epithelial cells	[60, 61, 76]
Glutamine	Prevention	Suggestion	It is used by cells of the immune system	[62, 69, 76, 101]
Honey	Prevention	Suggestion	Inhibits bacterial growth and enhances healing rate	[64, 67, 70, 72, 73, 76, 102]
Patient-controlled analgesia (e.g., Morphine 0.2% mouthwash)	Treatment	Recommendation	Pain management	[76, 83, 103]
Zinc supplements	Prevention	Suggestion	Prevents lipids peroxidation and replaces redox reactive metals	[62, 76, 101]
Doxepin mouthwash	Treatment	Suggestion	In topical application has analgesic and anesthetic properties	[76, 83, 104]
Vitamin E	Prevention	Suggestion	Antioxidant that may protect tissue damage from free oxygen radicals	[62, 64, 76, 82, 101]
Amifostine	Prevention	Suggestion	Reduces DNA strand breaks, recruits ROS scavengers and preserves salivary glands, endothelium, and connective tissue integrity	[38, 68, 76]

Proper oral health and hygiene are essential for mitigating the risk and severity of OM [27, 105]. Before initiating CT or RT, all potential causes of mucosal irritation should be removed, as they may worsen and prolong the development of oral mucositis [105]. Teeth with sharp surfaces must be restored, orthodontics and protheses should be removed, and the maintenance of a stable oral microbiome is also an important aspect. The presence of a balanced nutrition is another variable that may help in the relief of discomfort from mucositis [26, 27, 105]. Alcohol, smoking, and foods that are crunchy, acidic, spicy, or sweetened should be limited or eliminated [85].

As previously stated, OM can make the ingestion process a challenge, as it is normally unpleasant and, in extreme cases, impossible due to painful symptoms; therefore, a liquid diet is the only solution [19, 33]. Therefore, soft and liquid diets may be necessary, and, in the case of patients that cannot tolerate a liquid diet, the solution is parenteral nutrition [35]. The patients' complaints can be reduced with the use of specific mouthwashes with topical analgesics, anesthetics, antibiotics, and steroids [78, 103, 106, 107], as topical analgesics and anesthetics are intended to relieve localized pain [40].

According to the MASCC/ISOO guidelines (Table 3), a benzydamine mouthwash may be useful due to its anti-inflammatory properties, which inhibit the production of TNF-α and IL-1β [63, 70, 76, 78]. However, the use of saline, sodium bicarbonate, and antimicrobial (e.g., chlorhexidine 0.12%) rinses can ameliorate the symptoms of moderate mucositis [70, 101]. In clinical practice, topical analgesics (e.g., morphine, benzocaine, and menthol) are applied to provide temporary relief in some patients, but their concentrations are not well established [39, 108].

Currently, palifermin was the only agent that has been approved by the European Medical Agency (EMA) and the American Food and Drug Administration (FDA) for the prevention of OM in HSCT patients receiving CT and RT. However, on 1 April 2016, the European Commission withdrew the marketing authorization for this drug in the European Union (EU). The withdrawal was at the request of the marketing authorization holder, which notified the European Commission of its decision to permanently discontinue the marketing of the product for commercial reasons. It has also been tested in HNSC patients in terms of its reduction of the state of pathogenic severity [28]. The MASSC/ISOO guidelines also indicate cryotherapy and photobiomodulation (PBM) protocols for more advanced phases. In particular, cryotherapy has been reported to reduce the symptoms of oral mucositis in patients undergoing CT as a result of its vasoconstriction, decrease in the blood flow, and reduction of the local distribution of the chemotherapeutic agent (e.g., fluorouracil (5-FU) and melphalan) [40, 109]. Thirty

minutes of ice chips used prior to the administration CT are the recommended and tolerable period [81, 109].

PBM is another method employed to stabilize and inhibit the development of OM [58, 110]. It has anti-inflammatory effects, diminishes the pain, and improves the healing rate of the basal wound. The energy applied to the specific area must be adapted according to the patient's lesion. To relieve the most common complaints, PBM can be used both prophylactically and therapeutically, that is, it can be used before and after an antineoplastic treatment [57, 94, 111]. Mucositis may also be treated with supplementary vitamins and minerals. For instance, vitamin E, a potent antioxidant, may reduce the grade of mucositis by preventing the damage caused by ROS [19]. A blood test performed in severe OM patients demonstrated a lack of some vitamins (such as vitamins E, A, and D), which inhibited the pro-inflammatory pathways [51]. Different studies also showed that oral zinc supplements may be applied as a prophylactic treatment [33, 46, 86].

Therefore, for most of the strategies recommended or suggested in Table 3, the research in the literature displays minimal evidence or even contradictory results, thus invalidating the definitions of the guidelines [24, 25, 72, 76]. Consequently, the search for new active ingredients with potential therapeutic effects for preventing or treating OM is a challenge. Natural compounds, the majority of which are rich in polyphenols, are an option that should be explored.

1.3. Natural products and their properties for preventing/treating OM

Currently, the protocols and therapeutical agents available from the different authorities have the purpose of ameliorating the OM grade, as mentioned in the previous section, but no treatments with reasonable results have been established [48, 59, 70]. Aside from that, many of these compounds have been associated with adverse effects and high costs [25, 36]. Thus, natural products, such as honey, *Aloe vera*, curcumin, or propolis, are of huge interest for the nutraceutical and pharmaceutical industries, as they are easily accessible and allow more cost-effective treatments with minimal or no toxicity when compared to conventional strategies [90, 112]. Their richness in bioactive compounds with anti-inflammatory, antioxidant, antiseptic, analgesic, and wound-healing properties that may interfere with many cellular signaling pathways could play an important role in the progression of OM and the activity of carcinogenic cells (e.g., HNSC) [28].

1.3.1. Bees products

Honey is a natural product generated by bees and has been used since ancient times in traditional medicine. The huge diversity of studies has shown the multiplicity of beneficial applications of honey based on its antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, antitumoral, antimutagenic, and wound-healing properties [49, 53-55, 100]. The composition of honey is difficult to exactly define, as the components and relative amounts are conditioned by the flora of the geographical area from which honeybees collect pollen [92]. In a general way, honey is a heterogeneous mixture of water, nectar sugars, and glandular secretions produced by honeybees that contain proteins, vitamins, and enzymes [91]. One of the enzymes present is glucose oxidase, which, when in contact with body tissue, may stimulate the production of hydrogen peroxide, which acts as a messenger and promote wound healing and rapid epithelization at low concentrations by stimulating the proliferation of fibroblasts and epithelial cells [91, 93]. It is also suggested that matrix metalloproteases of connective tissue and neutrophil serine proteases may be activated by hydrogen peroxide [113]. Furthermore, the levulose and fructose present in honey may improve local nutrition and promote epithelialization [93, 113]. Honey also has immunomodulatory effects, as it influences the activation of macrophages and the proliferation of B-lymphocytes and T-lymphocytes [95], in addition to decreasing the inflammatory process by inhibiting cyclooxygenase pathway and reducing prostaglandin synthesis [96]. The beneficial effects of honey may also be due to its moisturizing effect, low pH, and viscosity which inhibit the proliferation of bacteria [53].

Charalambous *et al*. conducted a randomized, controlled trial to evaluate the potential effect of thyme honey rinses on HNSC patients [53]. In this study that involved 72 participants, a solution of 20 mL of thyme honey diluted in 100 mL of purified water was given to the patients to gargle in the oral cavity three times per day (15 min before and after the RT session and 6 h later) for 7 weeks, starting from the first day of the fourth week of RT. The results showed a significant improvement ($p < 0.001$) in the patients' quality of life, leading to fewer symptoms and maintenance of the body weight ($p = 0.001$) when compared to saline rinses [53]. Honey mouthwash also proved to be effective in a randomized, single-blind controlled trial that enrolled 53 patients [114]. The honey solution (honey-to-water ratio of 1:20) at 37 °C was gargled and kept in the mouth before and after each meal and before sleeping for 30 s by the treatment group, while the patients in the control group received routine care, such as ingestion of fluconazole capsules, nursing care, and mouth hygiene training [114]. According to the authors, the

solution reduced or eliminated weight loss, leading to some weight gain and preventing and reducing the severity of OM in the acute myeloid leukemia patients receiving CT ($p < 0.001$ at the fourth week of treatment) [114]. In another randomized, controlled trial with a parallel design involving 150 children, Sener *et al*. treated 25 OM patients with honey (with vitamin E as the most effective compound) for 21 days, applying the amount of 1–1.5 g of honey per weight (kg) of the child twice per day (every 12 h) [47]. Honey was found to be more effective in the management of OM ($p < 0.05$) when compared to chlorhexidine, a wide-spectrum antifungal and bactericidal antiseptic solution that is frequently used in oral care [47].

Motallbnejad *et al*. also conducted a randomized single-blind (examiner-blind) clinical trial to evaluate the effect of pure honey on radiation-induced mucositis in a total of 40 patients with head and neck cancer receiving RT [95]. Twenty patients were instructed to rinse and gradually swallow 20 mL of pure honey 15 min before radiation, then again at intervals of 15 min and six hours after radiation, while the control group was advised to rinse with 20 mL of saline before and after radiation. This procedure was repeated weekly from the beginning of the treatment until the end of the RT. The honey-receiving patients exhibited a significant reduction in OM ($p < 0.001$) when compared to the control group [95]. In a unicenter randomized, controlled clinical human study involving 82 patients with head and neck cancer treated with RT over 4–6 weeks, the treatment group was instructed to take 20 mL of Ziziphus honey 15 min before and after the radiation and before sleeping at night, while the control group repeated the process using 20 mL of 0.9% saline [91]. The results showed that the proportion of mucositis (Grades 3 and 4) was lower in the honey-treated group ($p = 0.016$ and $p = 0.032$ for Grades 3 and 4 of mucositis, respectively) than in the control group at the end of 6 weeks of RT [91]. In 2010, Khanal *et al*. conducted a single-blinded, randomized, controlled clinical trial over 6 weeks on 40 oral carcinoma patients receiving RT [92]. Radiation was given once per day for 5 days a week, and the application was performed 15 min before and after radiation and once before going to bed. Honey extracted from beehives of the Western Ghats forests or lignocaine gel 2% (control group) was swished around the oral cavity for 2 min and expectorated. Only one of the 20 patients of the treatment group developed intolerable mucositis ($p < 0.0001$) compared to 15 of the 20 patients of the lignocaine group [92].

Caffeine, a natural alkaloid with hypoalgesic, antioxidant, and anti-inflammatory effects, has also been screened as a potential ingredient to work against oral mucositis [102, 115-117]. In a double-blinded randomized clinical trial involving 75 patients (that randomly fell into three treatment groups) presenting OM after CT, the therapeutic effects

of coffee plus honey were compared with those of topical steroids that are usually used in the treatment of OM after CT [115]. A syrup-like solution was prepared for each treatment group: 300 g of honey plus 20 g of instant coffee for the honey-plus-coffee group; 300 mg of honey for the honey group; the control group was treated with 20 eight-milligram ampoules of betamethasone solution. All groups were instructed to sip 10 mL of the prescribed product and swallow every 3 h for 1 week. While all treatment regimens decreased the severity of the lesions, the best result was achieved in the honey–coffee group ($p < 0.05$), followed by the honey-and-steroid groups [115].

1.3.1.1. Propolis

Propolis is a resinous material produced by bees and is frequently used as natural nutritional supplement [118]. It is composed of a mix of plant buds and exudates, bee enzymes, pollen, and wax, and it has been widely used by different civilizations to treat colds, wounds, and ulcers due to its anesthetic, antimicrobial, anti-inflammatory, antitumor, immunomodulatory, and antioxidant properties [118]. Similarly to honey, the chemical composition of propolis is highly dependent on the diversity of the flora and bee species [104, 119]. It is mainly composed of proteins, amino acids, vitamins (A, B1, B2, B3, and B7), minerals, essential oils, phenolic acids, alcohols, fatty acids, and flavonoids [118, 120-122]. Regarding OM, the bioactivity of propolis is mainly associated with flavonoids, as these molecules are capable of sequestering or inhibiting the formation of free radicals, and they promote immunomodulatory, antioxidant, wound-healing, and anti-inflammatory activities [123]. The anti-inflammatory properties are directly related with the inhibition of the synthesis of prostaglandins and promotion of phagocytic activity [124]. In addition, propolis promotes healing effects in epithelial tissues, while the presence of iron and zinc improves the synthesis of collagen [122].

Akhavan-Karbassi *et al*. conducted a randomized double-blind placebo-controlled trial to evaluate the potential effect of propolis mouthwash on head and neck tumor patients undergoing CT [125]. In the treatment group ($n = 20$), 5 mL of propolis mouth rinse (30% extract) was administered every 8 h for 7 consecutive days. The solution was swished in the patients' mouths for 60 s, gargled, and expectorated. In the control group ($n = 20$), the process was repeated with a placebo mouth rinse. OM, erythema, and eating and drink ability were evaluated. When compared to the control group, the treatment group presented significant improvement in OM, wound healing, and erythema at day 7 ($p = 0.006$), but no significant differences in eating and drinking

ability were observed (p = 0.21). Moreover, 65% of the patients in the propolis group were completely healed by day 7 of the trial [125].

1.3.1.2. Royal jelly

Royal jelly is a secretory product of the cephalic glands of nurse bees that serves as the diet of honeybee larvae in their first 2–3 days, while for the queen, it is the specific food for her whole life period [126]. It is widely used in folk and mainstream medicines and as a dietary supplement due to its antioxidant, anti-inflammatory, hypoglycemic, antibiotic, antitumor, antiallergic, antiaging, immunomodulatory, neurotrophic, hypocholesterolemic, hepatoprotective, hypotensive, and blood pressure regulatory activities [126-134].

Similarly to the aforementioned bee products, the composition of royal jelly is dependent on the geography and climate [135]. It is a complex substance with a unique combination of sugars (mainly glucose and fructose, as well as traces of sucrose, maltose, trehalose, melibiose, ribose, and erlose), proteins (which represent >50% of the dry weight of royal jelly), amino acids, nucleotides, ascorbic acid, phenols, waxes, fatty acids, steroids, and phospholipids [135] . The impact that royal jelly has on OM may be closely related to its anti-inflammatory and wound-healing activities. However, the active compounds of royal jelly and the mechanisms underlying these activities are still largely unknown. *In vitro* studies performed on mice revealed that supernatants of royal jelly suspensions added to a mouse peritoneal macrophage culture stimulated with lipopolysaccharides and IFN-γ efficiently suppressed the secretion of pro-inflammatory cytokines TNF-α, IL-6, and IL-1, which was probably due to protein factors such as Major Royal Jelly Protein 3 (MRJP3) [133]. MRJP2, MRJP3, and MRJP7 are thought to be responsible for the wound-healing bioactivity of royal jelly, as they stimulate cell migration and proliferation [136], along with the antioxidant compounds present in royal jelly, which, when taken orally, lowered the levels of 8-hydroxy-2-deoxyguanosine, a marker of oxidative stress in mouse kidney DNA and serum [137].

Suemaru *et al*. evaluated the effects of royal jelly, honey, and propolis on OM induced with 5-fluorouracil and mild abrasions made on the cheek pouch in hamsters [138]. The bee products were topically administered to the oral mucosa. Royal jelly ointments at 3%, 10%, and 30% improved the recovery from 5-fluorouracil-induced damage in a dose-dependent manner, while the results of ointments of honey at 1%, 10%, and 100% and propolis at 0.3%, 1%, and 3% were not statically different from those of the Vaseline-treated control group [138]. In a more in-depth trial in Golden Syrian

hamsters, the influence of royal jelly on 5-fluorouracil-induced OM was assessed using oral mucosal adhesive films containing royal jelly [139]. The 5-fluorouracil was administered through intraperitoneal injections on days 0 and 2, and the left cheek pouches of hamsters (n = 12 per group) were everted and scratched with a small wire brush on days 1 and 2. Royal-jelly-containing sodium alginate–chitosan films (10% or 30%) were applied to the cheek pouches every day from day 3. Royal-jelly-containing films (both 10% and 30%) improved the recovery from 5-fluorouracil-induced OM, which presented lower erythema and absence of ulceration and abscesses on day 8. They also reduced the myelo-peroxidase (MPO) activity and the expression of pro-inflammatory cytokines. The data suggest that these effects were caused by the anti-inflammatory or antioxidative properties of royal jelly [139]. In humans, the effect of royal jelly on OM in patients with different types of malignancies undergoing RT and CT was evaluated by Erdem *et al*. in a randomized, controlled trial [140]. In this clinical trial that involved 103 patients, all patients received a mouthwash therapy with benzydamine hydrochloride and nystatin rinses. In addition, patients in the experimental group received royal jelly two times per day for a total daily dose of 1 g. Royal jelly was orally swished for 30 s and swallowed. The treatment group showed a mean resolution time of OM that was significantly shorter than that of the control group (OM Grade 1: $p = 0.0001$; OM Grade 2: $p = 0.0001$; OM Grade 3: $p = 0.05$) [140]. In a single-blind clinical trial that involved 13 patients with head and neck cancer receiving CT, 1 g of royal jelly was given three times per day to the treatment group during the RT period [141]. Royal jelly was shown to have a preventive effect on the progression of CT-induced OM from the early phase ($p < 0.001$) [141].

1.3.2. *Spondias mombin*

The leaves of *Spondias mombin*, commonly known as the cashew tree, are a rich source of interesting bioactive compounds, with particular emphasis on tannins, saponins, triterpenes, and flavonoids [142]. Traditionally, the leaves have been used to treat inflammatory pathologies, making them a promising source for the development of new therapeutic agents for OM [142]. Gomes *et al*. assessed the effects of a hydroethanolic extract of *S. mombin* leaves on 5-fluorouracil-induced OM in Golden Syrian male hamsters. The animals were orally pre-treated with the hydroethanolic extract of *S. mombin* leaves (50, 100, or 200 mg/kg) for ten days [142]. The treatment with the highest dose of the extract (200 mg/kg) showed the best healing effect, with hamsters displaying reduced oxidative stress and inflammation and no evidence of

ulceration. Further analysis showed re-epithelialization, absence of hemorrhage, discrete mononuclear inflammatory infiltration, and lower expression levels of different molecules involved in the modulation of inflammation, such as MMP-2, COX-2, TNF-α, NF-κB p50 NLS, iNOS, and IL-1β, as well as an increase in glutathione (GSH) levels [142]. Although the mechanisms behind these effects remain under investigation, the hydroethanolic extract of *S. mombin* leaves is rich in potent antioxidant phenolic phytochemicals, such as ellagic acid (12 mg/g) and chlorogenic acid (19.4 mg/g), which could justify these activities [143]. Studies have demonstrated that chlorogenic acid acts on the reduction of COX-2 expression in macrophages, as well as in the inhibition of the production of pro-inflammatory cytokines, such as IL-1β and TNF-α, and of NF-κB activation [143]. On the other hand, chlorogenic acid was proven to promote wound healing in rats [144], while ellagic acid acted by down-regulating MMP-2 expression and inhibiting NF-κB-mediated transcriptional activation [143]. These activities may justify the results achieved in the previously detailed trial.

1.3.3. *Camellia sinensis*

Camellia sinensis (green tea) is one of the most popular drinks in the world and is widely known for its antimicrobial, antitumoral, antioxidant, and anti-inflammatory activities [106]. Different compounds with therapeutic effects have been discovered in this plant. The majority of the health-promoting properties are associated with polyphenols [145], which represent almost 30% of the fresh-leaf dry weight, including flavandiols, flavonols, flavonoids, and phenolic acids [146]. However, most of the polyphenols present in leaves of *C. sinensis* are catechins, namely, (+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin (EGC), (−)-epicatechin gallate, and (−)-epigallocatechin gallate (EGCG) [147]. Catechins are mainly responsible for the ROS scavenging and antioxidant activities of *C. sinensis* [148, 149]. EGCG, in particular, efficiently inhibits the transcription of NF-κB, resulting in a decrease in the expression of different pro-inflammatory genes [150]. The anti-inflammatory effect of catechins may be due to the activation of endothelial nitric oxide synthase (eNOS) [151, 152].

The effect of green tea on OM was evaluated in oral cancer patients [106]. For that, a single-blind randomized, controlled trial was made with 63 participants. For 6 months, after the tooth-brushing procedure, the intervention group rinsed the mouth with a solution of 5 g of green tea dissolved in 100 mL of water for 60 s, and the control group rinsed the mouth with 100 mL of tap water for the same period. The results demonstrated an improvement in oral health status and the preservation of the oral mucosa at the end

of the follow-up period (6 months), with a higher reduction of the oral health status score in the intervention group than in the control group (p = 0.008) [106]. In another randomized study, the effect of Baxidil Onco® mouthwash (Sanitas Farmaceutici Srl, Tortona, Italy), composed of *C. Sinensis* leaf extract and palmitoyil hydrolyzed wheat protein, was tested in 60 hematologic patients undergoing hematopoietic stem cell transplantation (HCST) [153]. Twenty mL of Baxidil Onco® was used to rinse the mouths of 28 patients four times per day for at least one minute without swallowing, while the remaining 32 patients were treated with standard prophylactic schedules and served as control. The results demonstrated that the incidence, severity, and duration of OM were significantly reduced (p = 0.022) by the oral rinsing with Baxidil Onco® [153].

1.3.4. *Plantago major*

In traditional Persian medicine, *Plantago major* was used as a wound-healing herb, as it possesses a wide range of bioactive properties, such as anti-inflammatory, antiulcerogenic, antioxidant, antimicrobial, analgesic, wound-healing, and immunomodulatory effects [154].

Soltani *et al*. conducted a randomized, double-blind, placebo-controlled clinical trial to assess the effects of *P. major* syrup as a natural agent against OM for 7 weeks [155]. The participants were HNSC patients who were going to receive RT. The 23 patients of the intervention group received 7.5 cc of *P. major* syrup three times per day, starting from three days before the start of RT until the end of it, while the placebo group received 7.5 cc of placebo syrup. The *P. major* syrup was shown to be effective in the reduction of the mucositis and the severity of pain caused by RT (p < 0.001) [155]. A multicenter randomized, controlled trial developed by Cabrera-Jaime *et al*. evaluated the efficacy of *P. major* extract vs. chlorhexidine vs. sodium bicarbonate in the treatment of CT-induced OM in solid-tumor cancer patients with grade II–III mucositis [140]. A total of 45 patients were randomized for one of the treatments, consisting of a 5% aqueous solution of sodium bicarbonate together with (i) an additional dose of 5% sodium bicarbonate, (ii) *P. major extract*, or (iii) 0.12% chlorhexidine. The solutions were applied over 14 days. The differences in healing time and the lower pain levels among the three groups were not statistically significant (p = 0.702) [154].

The properties of *P. major* leaves are dependent on the different compounds present. The leaves are rich in different bioactive molecules, such as aucubin, a glycoside with anti-toxin activity, and ursolic, oleanolic, and α-linoleic acids, which inhibit COX-2-catalyzed prostaglandin production [156-158]. Extracts of *P. major* leaves have

remarkable antioxidant and antiradical capacities due to the presence of baicalein, luteolin, salicylic acid, citric acid, ascorbic acid, apigenin, ferulic acid, benzoic acid, chlorogenic acid, oleanolic acid, and ursolic acids [159, 160]. According to different studies, the bioactivity of *P. major* is due to the decrease in the inflammatory reaction through the modulation of NF-κB, NO, COX-2, and B4 leukotriene (LB4) levels [154].

1.3.5. *Aloe vera*

A. vera is a plant that has been used for medical purposes for thousands of years. It is widely employed for the treatment of various medical conditions, such as oral ulcers, psoriasis, skin burns, and frostbite, since it presents analgesic, liver-protection, antifungal, antidiabetic, anti-inflammatory, antiproliferative, anticarcinogenic, antiaging, and immunomodulatory properties [161, 162]. In addition, it can scavenge free radicals, improve wound oxygenation, promote wound healing, increase collagen formation, and inhibit metalloproteinase and collagenase activity [163-166]. Different studies have shown the potent free-radical and superoxide anion activity of three derivatives from *A. vera*, namely, isorabaichromone, feruoylaloesin, and *p*-coumaroylaloesin [163, 164]. The beneficial effects, assumed to be exerted in the oral cavity, may also be due to its moisturizing effect, which is provided by the polysaccharide components (principally mannose, glucose, xylose, arabinose, galactose, and rhamnose), which provide and sustain moisture in tissues [166]. One of the sugars presents in a higher quantity, mannose-6-phosphate, acted as an active-growth substance and anti-inflammatory agent in *in vivo* studies on mice [166]. The anti-inflammatory effects of *A. vera* extracts are attributable to the inhibitory action on the arachidonic acid pathway via COX-2 inhibition [163, 167], as well as the reduction of leukocyte adhesion molecules and TNF-α levels [168]. *In vitro* and animal assays suggest that *A. vera* promotes wound healing through the reduction of the vasoconstriction and the platelet aggregation at the wound site [165].

An initial assessment of *A. vera*'s potential in preventing RT-induced OM did not yield promising results in a single-institution, double-blind, prospective, randomized trial that involved 58 head and neck cancer patients [169]. The patients were instructed to take a 20 mL swish (*A. vera* solution or placebo) and swallow four times daily, beginning on the first day and continuing throughout the course of RT. However, no significant differences were observed between treatments (p = 0.07) [169]. Better results were achieved in other studies. Mansouri *et al*. evaluated the effect of *A. vera* on CT-induced OM in patients with acute lymphocytic leukemia and acute myeloid leukemia [170]. In

this randomized, controlled clinical trial, 64 patients were divided into an intervention group and a control group. The first group was instructed to wash their mouths with 5 mL of *A. vera* solution for 2 min three times per day for 14 days. The control group repeated the procedure using mouthwashes that are typically recommended by hematologic centers, including normal saline, nystatin, and chlorhexidine. An evaluation of the patients' mouths was performed on days 1, 3, 5, 7, and 14. Even though, regarding the intensity of stomatitis and pain, no significant differences were found between the two groups on the first day, a significant difference was observed in this regard on the other days ($p < 0.001$) [170].

In a similar study, an assessment of the effect of *A. vera* mouthwash on CT-induced OM was performed in a double-blinded randomized clinical trial on 120 patients, who were divided into three groups [171]. Until 2 weeks after the CT sessions, group 1 received tablets with 10 mg of atorvastatin daily plus a placebo mouthwash, group 2 received placebo tablets and *A. vera* mouthwash, and group 3 received placebo tablets and placebo mouthwash. The analysis of the results showed that 50% of the placebo patients (group 3) experienced mucositis, while that value decreased to 2.5% in group 2 ($p < 0.042$), with no significant differences between groups 1 and 3 ($p < 0.674$) [171]. Likewise, the efficacy of *A. vera* use for prevention of CT-induced OM was evaluated in a randomized, controlled clinical trial in 26 children with acute lymphoblastic leukemia [172]. Depending on the treatment group, a 70% *A. vera* solution or a 5% sodium bicarbonate solution was applied twice per day to oral tissues with spongeous sticks. The application started 3 days before the CT therapy. The application of *A. vera* solution showed to be effective in the prevention and reduction of OM severity ($p < 0.001$) [172].

A triple-blind randomized and controlled interventional quality-of-life clinical trial on the efficacy of *A. vera* and a benzydamine mouthwash in the alleviation of RT-induced OM was performed by Sahebjamee *et al*. in a study with 26 head and neck cancer patients [173]. The intervention group rinsed the mouth three times per day with 5 mL of an *A. vera* mouthwash, while the control group repeated the procedure with benzydamine mouthwash. The protocol was applied from the first day of RT until the end of the treatment, demonstrating that *A. vera* mouthwash was as efficient as benzydamine at reducing the severity of RT-induced OM, without differences between them ($p < 0.09$) [173].

1.3.6. *Curcuma longa*

Curcuma longa, also known as turmeric, is an herb that is extensively grown in Asia [174] and is often used culinarily as a spice and in traditional Asian medical treatments for depression, stress, infection, and dermatological diseases [175, 176]. Various compounds were identified in this plant, including polyphenols, sesquiterpenes, diterpenes, triterpenoids, sterols, and alkaloids [175, 177]. Among these, the most studied component of *C. longa* is curcumin, a lipophilic polyphenol extracted from the rhizomes of *C. longa*, which represent 2–5% of turmeric [174, 175].

Due to the antioxidant, anti-inflammatory, and anticancer effects of curcumin, it has an important role in the prevention of depression, cancer, and pro-inflammatory, neurodegenerative, diabetic, autoimmune, and cardiovascular diseases [178-182]. Furthermore, curcumin has antimicrobial, insecticidal, larvicidal, and radioprotective activities [175]. Curcumin mediates its effects through direct or indirect interactions with growth factors, kinases, enzymes, transcription factors, receptors, and proteins that regulate cell proliferation and apoptosis [178, 183-185]. In the case of OM, the beneficial effects of curcumin may be related with the upregulation of TGF-β-1, which promotes re-epithelialization through the stimulation of fibronectin and collagen production by fibroblasts, while increasing the rate of granulation [178, 186, 187]. TGF-β-1 also promotes the removal of dead tissue by enhancing the recruitment of macrophages [187]. Aside from that, curcumin potently inhibits the activation of nuclear factor- κB (NF-κB), but activates others, such as the nuclear factor erythroid 2-related factor 2 (Nrf2) [178, 186, 187]. COX-2, the inducible form of COX, can be selectively induced by mitogenic and inflammatory stimuli, resulting in enhanced synthesis of prostaglandins, such as IL-6. The activation of NF-κB significantly upregulates superoxide dismutase (SOD) expression [178, 186, 187]. Curcumin also enhanced the expression of antioxidant enzymes such as SOD, catalase (CAT), glutathione (GSH), and glutathione peroxidase (GSH-px) through the regulation of Nrf2 [178, 186, 187].

The wound-healing ability of curcumin is accelerated by its antioxidant activity, as it decreases the levels of lipid peroxides (LPs) and increases the activity levels of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [188].

In a placebo-controlled study, an assessment of the tolerability of a curcumin mouthwash for the prevention of OM in pediatric patients undergoing CT was performed in a group of seven pediatric and young-adult patients [189]. In this study, which was developed without a control group for ethical reasons, in addition to the standard preventive oral care consisting of 0.2% chlorhexidine mouthwash for 30 s twice per day,

the patients also used a mouthwash with 10 drops of Curcumall® (a dietary supplement containing turmeric, curcumin and ginger) twice per day during the CT treatment. The researcher concluded that curcumin mouthwash was safe and well tolerated by the patients [189]. The efficiency of curcumin mouthwash in cancer patients undergoing RT and suffering from OM was evaluated in a randomized trial involving 20 patients [190]. The study group used 0.004% curcumin mouthwash diluted at a ratio of 1:5 for 1 min three times per day for 20 days, while the control group was treated with standard preventive oral care using a commercially available 0.2% chlorhexidine mouthwash to be used in a 1:1 dilution for 1 min three times per day for 20 days. Curcumin promoted faster wound healing and better patient compliance in the management of RT-induced OM ($p < 0.001$) [190]. In another double-blind randomized clinical trial, the effects of curcumin encapsulated in nanomicelles on OM in 32 head and neck cancer patients receiving RT were evaluated [191]. During the RT, patients in the treatment group received daily one capsule of SinaCurcumin® (Exir Nano Sina Company, Tehran, Iran), which contained 80 mg of curcumin-loaded nanomicelles. The control group received placebo tablets containing lactose. There were statistically significant differences ($p < 0.05$) between the two groups in the severity of OM, as all of the patients in the placebo group developed OM versus the 32% of the case group [191].

1.3.7. *Olea europaea*

Olive leaf extract is a natural product extracted from *Olea europaea*, which is traditionally used to treat and prevent hypertension and diabetes due to its antioxidant, anti-inflammatory, anticancer, antiapoptotic, antimicrobial, hypoglycemic, and diuretic properties [192-197]. The leaves of *O. europaea* contain a high concentration of phenolic compounds (1450 mg/100 g of fresh leaf), with secoiridoid oleuropein, verbascoside, rutin, luteolin-7-glucoside, and hydroxytyrosol as the main phenolic constituents [198]. Oleuropein is possibly the main active compound promoting the wound-healing activity of olive leaf extract, as it increases collagen fiber deposition and advanced re-epithelialization [199, 200]. Furthermore, it has been demonstrated that oleuropein decreases oxidative stress and inflammation through the modulation of the COX-2, AMPF, eNOS, MAPK, and apoptosis cell signaling pathways in *in vivo* studies on mice [193]. In addition, olive leaf extract also inhibited the aggregation platelets in *in vitro* studies [195].

In 2013, the effect of a mouth rinse containing olive leaf extract on the prevention of severe OM in CT-receiving patients, as well as an estimation of its effect on the

salivary levels of pro-inflammatory cytokines, was assessed in a prospective, randomized, double-blind, placebo-controlled cross-over study design involving 25 cancer patients [192]. The studied drugs (olive leaf extract at 333 mg/mL, benzydamine hydrochloride at 0.15 g/100 mL, or normal saline) were self-administered 3–4 times daily for 14 days, starting on the first day of chemotherapy. The patients were evaluated weekly until 15 days after CT for each cycle. The findings indicated that the olive leaf extract could effectively reduce the OM rates ($p < 0.001$) by decreasing the salivary levels of IL-1β and TNF-α [192]. Briefly, Ahmed *et al*. performed an experimental animal study and a prospective, randomized, double-blind, placebo-controlled cross-over study to evaluate the management of OM with mouthwashes containing olive leaf extract [201]. In the animal study, 45 male albino rats received two intraperitoneal injections of 5-fluorouracil (60 mg/kg) on day 0 and day 2. The first group received normal saline, the second group received olive leaf extract (333 mg/mL), and the third group received benzydamine hydrochloride (0.15 g/100 mL). By the end of the study (day 14), the control group presented ulcerated connective tissue that was not completely covered by epithelium, and there was evidence of necrosis and degeneration. The animals with the olive leaf extract and benzydamine hydrochloride presented a totally re-epithelialized mucosal surface with hyperkeratinization and hyperplasia, while the sub-epithelia were more organized, with decreased cellularity of fibrous tissue [201]. In a clinical study, 62 CT-receiving patients were divided to receive olive leaf extract, benzydamine hydrochloride, or a placebo in the form of a mouth rinse, and the treatment was changed in the next chemotherapy cycle for each patient (cross-over design) [201]. Mouth rinses were self-administered 3–4 times per day for 14 days from the start of the CT. When compared to the benzydamine hydrochloride and the control, the olive leaf extract more efficiently reduced the oral pain, dysphagia, and functional impairment of eating ($p < 0.001$) [201].

1.3.8. *Glycyrrhiza glabra*

Glycyrrhiza glabra, commonly known as licorice, is one of the most important herbal medicines for traditional Chinese medicine and Japanese Kampo medicine [202]. It is traditionally used to relieve inflammation, gastric and peptic ulcers, arthritis, eye and liver disorders, hyperacidity, and sex-hormone imbalance [203-211]. This plant has attracted the attention of the pharmacological field due to its antimicrobial, antiviral, and anti-inflammatory properties [212-215]. The roots of *G. glabra* have been found to possess many secondary metabolites, with numerous pharmacological properties that

contribute to their medicinal use, including flavonoids (such as liquirtin, rhamnoliquirilin, liquiritigenin, and prenyllicoflavone A) and volatile components (including pentanol, hexanol, tetramethyl pyrazine, linalool, and terpinen-4-ol) [216]. The essential oil extracted from the roots of *G. glabra* contains propionic acid, 1-methyl-2-formylpyrrole, benzoic acid, 2,3-butanediol, and ethyl linoleate, among other compounds. The roots of *G. glabra* are also composed of 20% moisture, 3–16% sugars, 30% starch, and 6% ash [217].

The main biologically active components of *G. glabra* are dipotassium glycyrrhizinate, glycyrrhizin, also known as glycyrrhizic acid, and its aglycone, glycyrrhetinic acid [216]. Dipotassium glycyrrhizinate has similar properties to those of corticosteroids, namely, anti-inflammatory, antiallergic, and antibiotic activities, without the side effects of allergic reactions on the skin [218]. This property is due to dipotassium glycyrrhizinate's ability to efficiently inhibit the activity of phospholipase A2 enzyme, which is necessary for several inflammatory processes [219-221]. Moreover, it is able to avoid damage to the extracellular matrix by inhibiting the activity of hyaluronidase enzyme, histamine release, inflammatory chemical mediators, leukotrienes, and prostaglandins [222]. Glycyrrhizic acid inhibits prostaglandin E2 synthesis by suppressing the activity of COX-2, resulting in the augmentation of NO production through the enhancement of iNOS mRNA secretion and indirectly preventing platelet aggregation [221, 223, 224]. The anti-inflammatory activity of glycyrrhizic and glycyrrhetinic acids is realized through cytokines such as 1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, and IL-17, IFN-γ, and TNF-α [225-227]. Moreover, these compounds also present immunomodulatory activity through their interaction with different transcription factors, such as NF-κB, as well as signal transducers and activator of transcriptions (STAT- 3 and STAT-6) [225].

Najafi *et al*. conducted a double-blind clinical trial to evaluate the potential effect of *G. glabra* extract on cancer patients under head and neck radiotherapy [217]. The experimental group received a 50% extract of *Glycyrrhiza* (hydroalcoholic extract) and the placebo group received a brown-colored water. The patients were asked to use 20 cc twice per day for 14 days after the beginning of RT. According to the results obtained, *Glycyrrhiza* extract efficiently decreased the OM, wound size, and irritation ($p < 0.001$) [217]. The effect of *G. glabra* on head and neck cancer patients receiving RT was also evaluated in a small randomized study with six patients who were assigned to receive a licorice mucoadhesive film or a placebo mucoadhesive film [228]. The level of pain and the mucositis severity were significantly lower in the licorice-mucoadhesive-

film-receiving patients in the last 2 weeks of the clinical trial (weeks 3 and 4) ($p < 0.05$) [228].

The efficiency of a *G. glabra* root extract in preventing CT-induced OM in colon cancer patients was evaluated in a double-blind randomized clinical trial that involved 72 patients [229]. The treatment group received 5% licorice root extract, and the control group received a combined mouthwash composed of aluminum, magnesium, diphenhydramine, nystatin powder, and 2% lidocaine. For one week, from the first day of CT, both mouthwashes were used daily, every 8 h, at a dose of 10 cc. The researchers did not observe differences between the two groups in terms of the incidence and severity of OM ($p > 0.05$) [229].

1.3.9. *Matricaria recutita*

The chamomile plant, *Chamomilla recutita* or *Matricaria recutita*, one of the most common medicinal plants, is characterized by flowers with anti-inflammatory, antibacterial, and antifungal properties [230]. It is mainly used to treat different inflammatory conditions of the skin and mucosa, as it promotes faster a wound-healing process in comparison to corticosteroids [230, 231]. *M. recutita* owes its therapeutic activity to chamazulene, α-bisabolol, bisabolol oxides, spiroethers, and flavonoids [230]. Flavonoids—in particular, apigenin-7-glucoside—have been found to be responsible for the anti-inflammatory activity that may be involved in recuperation from OM [232]. Pre-clinical studies showed evidence of the anti-inflammatory action of *M. recutita* through the inhibition of COX-2 and IL-6 production [233, 234].

In a small comparative study with random assignment, dos Reis *et al*. evaluated the efficacy of *M. recutita* infusion cryotherapy for the prevention and reduction of the intensity of OM in gastric and colorectal cancer patients [235]. The study was performed during the first course (5 days) of CT. The patients in the *M. recutita* group received a cup of ice chips made with an *M. recutita* infusion at 2.5%, while the control group received a cup of ice chips made with pure water. The patients in both groups were instructed to swish the ice chips around in their mouths for at least 30 min, starting 5 min before the CT infusion. The *M. recutita* group presented less pain and had no ulcerations when compared to the control group [235]. The effects and the percentage of extract necessary to reduce the incidence and intensity of OM in patients undergoing hematopoietic stem cell transplantation were assessed in a randomized, controlled, phase II clinical trial [231]. All 40 patients received standard oral care, while the treatment group received an additional mouthwash containing a liquid extract of *M. recutita* at

0.5%, 1%, or 2%. When compared with the control group, the *M. recutita* group at 1% (equivalent to 0.108 mg of apigenin-7-glucoside/mL) demonstrated have reduced incidence, intensity, and duration of OM in patients undergoing hematopoietic stem cell transplantation ($p < 0.01$) [231]. Shabanloei *et al*. performed a randomized, double-blind clinical trial between alloporinol and *M. recutita* extract in the prevention of OM in CT-receiving patients [236]. Group 1 received 5 mg/mL of allopurinol, group 2 received a solution of 8 g of *M. recutita* in 50 cc, and the control group received a normal saline solution as a mouthwash. All patients gargled daily, four times per day, for the 16 days following the beginning of CT. The researchers concluded that both the allopurinol and *M. recutita* mouthwashes were effective in reducing post-CT OM, with no significant differences in the mean stomatitis ($p = 0.59$) and stomatitis pain ($p = 0.071$) [236].

1.3.10. *Calendula officinalis*

Calendula officinalis, commonly known as marigold, has been used for centuries as a topical and oral herbal remedy due to its bactericidal, antioxidant, anti-inflammatory, antiseptic, hepatoprotective, and anti-metastatic effects, with applications in blood purification and treatment of herpes, keratolytic radiation dermatitis, wounds, and scars, and as an antispasmodic [237-239]. The main compounds that contribute to its medicinal use are triterpenoids, flavonoids, oleanolic acid, faradiol, glycosides, quinones, tannins, coumarins, carotenoids, saponins, alkaloids, phenolic acids, and amino acids [237]. Triterpenoids provide anti-inflammatory and anti-edematous effects, in addition to stimulating the proliferation of fibroblasts, possibly through the inhibition of COX-2, C3-convertase, and 5-lipoxygenase [240-242]. Flavonoids are reported to have anti-inflammatory, antioxidant, and anti-edematous properties, in addition to their inhibition of lipoxygenase enzymes and mast cells [243].

The potential of *C. officinalis* extract for the healing of 5-fluorouracil-induced OM was studied in hamsters [239]. OM was induced in 60 male hamsters on days 0, 5, and 10 through the intraperitoneal administration of 5-fluorouracil (60 mg/kg). The cheek pouch was scratched with a needle once per day, from day 1 until day 12, when erythematous changes were noted. The treatment of OM started on days 12–17 with the topical application of a gel once a day. The animals were divided into four groups: 12 without treatment as control animals, 15 treated with 5% *C. officinalis* gel, 15 treated with 10% *C. officinalis* gel, and 15 treated with the gel base. The *C. officinalis* gel (5% and 10%) significantly reduced the microscopic and macroscopic scores of OM when compared with the gel base and the control group. Moreover, the animals of the treatment groups gained more weight than those in the gel base and the control groups

[244]. In humans, the effect of *C. officinalis* on OM was evaluated in a placebo-controlled clinical trial with 40 patients with neck and head cancers under RT or concurrent CT [245]. Patients were given 5 mL of either placebo or a 2% *C. officinalis* extract gel mouthwash to be held for at least 1 min in the oral cavity two times per day. Compared to the placebo group, the intensity of OM was significantly lower in the *C. officinalis* mouthwash group at weeks 2, 3, and 6 ($p < 0.048$). According to the same study, the high content of flavonoids and phenolic compounds and the antioxidant activity may be responsible for the protective effect of *C. officinalis* in RT-induced OM [245].

1.3.11. Other Natural Products

In addition to the compounds mentioned above, different experiments were also performed to evaluate the potential of other natural compounds for preventing/treating OM. However, due to the low number of studies published, not only regarding the OM application, but also with respect to the molecular mechanism of action enrolled, a section was not dedicated to them in this review. Table 4 summarizes the different natural products in these circumstances.

Table 4 - Summary of studies with natural products for oral mucositis prevention/treatment.

Name	Properties/Mechanisms	Application	Experimental setting/Model	References
Manuka (*Leptospermum scoparium*) essential oil	Anti-inflammatory, analgesic, antimycotic and antibacterial	Mouthwash	Randomized clinical trial	[73]
Kanuka (*Kunzea ericoides*) essential oil	Anti-inflammatory, analgesic, antimycotic and antibacterial	Mouthwash	Randomized clinical trial	[73]
Indigo root (*Isatis indigotica*)	Anti-inflammatory and antiviral	Mouthwash	Randomized clinical trial	[98]
Rhodiola algida	Immunomodulatory effects	Mouthwash	Randomized clinical trial	[97]
Thymus spp. L	Antiseptic, anti-inflammatory, antimicrobial and antimycotic	Mouthwash	Randomized pilot study	[75]
Eucalyptus	Antibacterial, antiviral, antifungal, anti-inflammatory, analgesic, and antioxidant	Topical gel	Hamsters	[246]
Zizyphus jujuba	Anti-inflammatory, analgesic, and wound healing	Topical gel and dietary	Hamsters	[105]
Zataria multiflora	Carminative, stimulant, diaphoretic, diuretic, antiseptic, anesthetic, antispasmodic, anti-hermitic, antidiarrheal, and analgesic	Mouthwash	Randomized clinical trial	[77]
Carapa guianensis oil	Anti-inflammatory, analgesic and antimicrobial	Topical gel/swab	Randomized clinical trial/ hamsters	[74, 76]
Plantago ovata	Antioxidant, anti-inflammatory and antibacterial	Mouthwash	Randomized crossover clinical trial	[87]
Achillea millefolium	Antimicrobial and anti-inflammatory	Mouthwash	Randomized clinical trial	[88]
Vaccinium myrtillus	Antioxidant, cardioprotective, neuroprotective, anti-inflammatory, and anticarcinogenic	Topical application, gavage administration, mouthwash	Clinical trials, Hamsters	[89, 106, 247]
Carum carvi	Antioxidant, antidiabetic, antifungal, and antimicrobial	Topical gel	Hamsters	[248]
Pistacia atlantica	Antioxidant and anti-inflammatory	Topical gel	Hamsters	[99, 109]
Hypericum perforatum	Antioxidant and anti-inflammatory	Topical gel	Hamsters	[84]
Elaeagnus angustifolia	Anti-inflammatory, analgesic, and wound healing	Topical gel	Hamster	[81]
Trachyspermum ammi	Anti-inflammatory, antiviral, antifungal, antioxidant, and analgesic	Topical gel	Hamsters	[109]
Hippophae rhamnoides	Antioxidant, anti-inflammatory, antimicrobial and anti-ulcerogenic	Gavage administration	Rats	[78, 79]

1.4. Conclusions and Future Perspectives

OM is a common and incapacitating side effect of antineoplastic therapies. The increased knowledge of its pathogenesis allows a better prediction of a patient's risk with the aim of adapting the management protocols and improving the development of new therapies. Nevertheless, standard guidelines for preventing and treating OM do not display significant effectiveness. The interest in natural products as potential therapeutic drugs has increased in recent years, as they have the advantage of being accessible and generating minimal side effects, with potential properties that include anti-inflammatory, antioxidant, antimicrobial, antiulcerative, and wound-healing capacities. In addition, over recent years, there have been multiple efforts to develop naturally based therapies, with natural compounds being tested in model organisms and clinical trials that are currently ongoing. However, the environment of the oral cavity is a complex system that is divided into two functional layers—the epithelium (thick and avascular) and the underlying tissue (vascular)—that are anatomically different, which affects their permeability to drugs and the capacity for maintaining a system for a certain period [249]. The buccal mucosa, which is composed of epithelial cells, provides a large surface area of almost 100 cm^2 [250]. This area is ideal for attaching a drug delivery system, providing a permeability that is 4 to 4000 times higher than that of skin [250-253]. Oral administration provides the advantage of a simple administration that does not suffer from the first-pass metabolism and that is safe and increases the drug availability. In addition, this route has a rapid action, reduced side effects, easy access to the local condition, and great patient compliance [249, 254]. These characteristics make the buccal mucosa an optimal solution for the systemic and local treatment of OM [255]. However, it also has limitations that are associated with a functionalized protective barrier. The presence of saliva and its enzymatic action, as well as the constant mechanical pressure caused by eating and speaking movements, may compromise the penetration of the drug present in the delivery system; as such, the application of mucoadhesive components may be required to solve this issue, but this can compromise the therapeutic effectiveness [249, 251-253].

Due to the characteristics of the oral cavity, it is necessary to develop novel strategies for overcoming topical delivery, such as mucoadhesive dosage forms (e.g., films, tablets). For the treatment of oral diseases, the most suitable formulations investigated were in the form of tablets, films, sprays, mouthwashes, gels, and pastes [41, 253, 256]. Gel and film formulations were evaluated in hamsters with CT-induced mucositis. By the 28th day, the hamsters' mucosa appeared to be healed, as no

erythema or edema was visible. These results proved their efficiency, as the animals' survival was higher than in the control group, and these treatments showed promising potential for a function as an occlusive patch and for delivering therapeutic compounds [255]. Films containing ethanolic propolis extract also presented optimal mucoadhesion capacity, ensuring the release of propolis compounds, a good stability, a high swelling capacity, and antimicrobial effects against *S. aureus* [257]. In addition, the incorporation of nanoparticles in the forms of dosage for buccal drug delivery has recently been encouraged [41, 250, 258]. Furthermore, nanoparticles could transport many therapeutic agents [41, 250]. Functional and biocompatible carriers that display chemical stability are sought in the innovation of buccal drug delivery systems [258-261]. Chitosan is an example of a biopolymer that is biologically safe and bioadhesive, and it has been used in several studies for the development of drug delivery systems, as it has longer retention periods in the oral mucosa [255, 262, 263]. In addition, it inhibits the attachment of *C. albicans* to human oral mucosal cells [255, 262, 263].

A SWOT diagram (Figure 2) was constructed with the aim of summarizing the previously described strengths, weaknesses, opportunities, and threats of employing natural products for the prevention/treatment of OM.

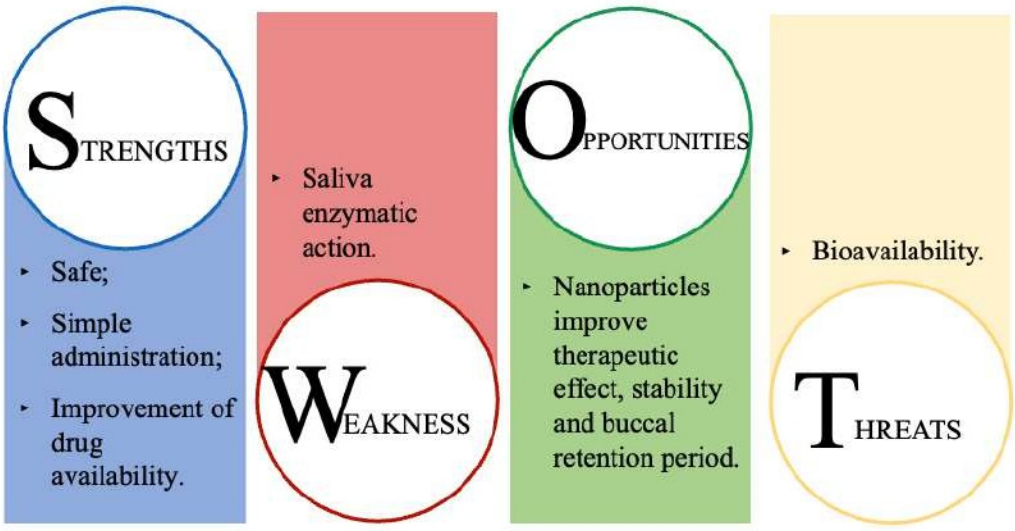


Figure 2 - SWOT analysis for possible use of natural products to prevent/treat oral mucositis.

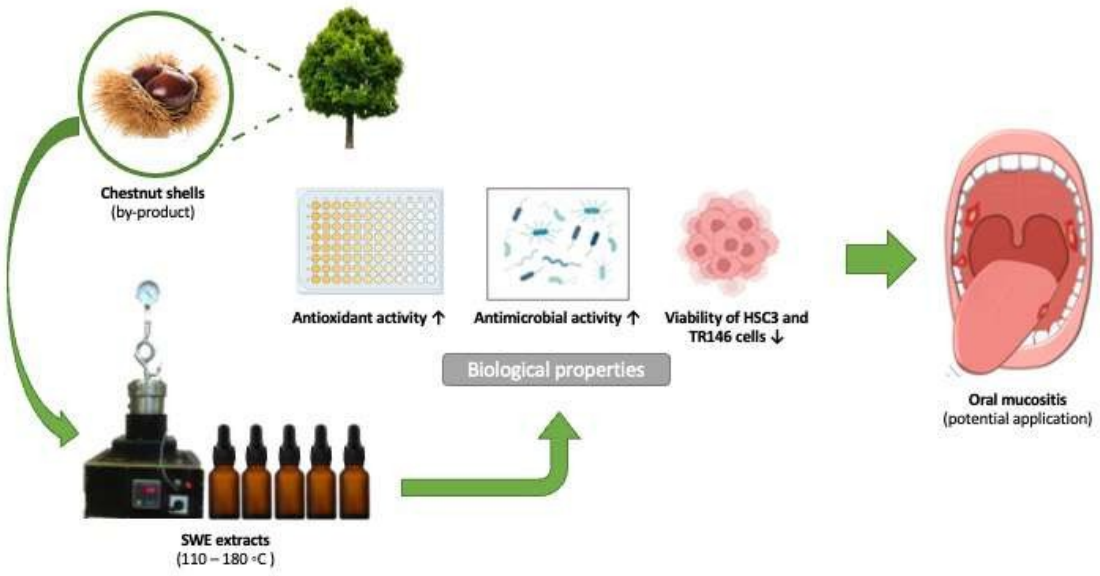
Despite the significant advances made in this area, more investigations are needed to ensure that these formulations reach the pharmaceutical market, and few have been published regarding this topic with natural products.

Chapter 2

Employment of Chestnut Shells as Active Ingredient against Oral Mucositis

The information presented in this chapter was published in the following publication:

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The contribution of the first author, Ana Sofia Ferreira, in this work, includes the literature review, investigation, methodology and writing of the original draft.

2.1. Introduction

Oral mucositis (OM) is one of the most common side effects of antineoplastic treatments, namely chemo- and/or radiotherapy, and is defined as an acute inflammatory, ulcerative, and painful disorder that severely degrades the quality of life of patients [75, 264, 265]. Ulceration of buccal mucosa compromises the natural defensive barrier, allowing oral bacteria and inflammatory cytokines to enter underlying tissues and the systemic blood supply [265]. During the antineoplastic treatments, reactive oxygen species (ROS) are generated, leading to the development of oxidative stress, one of the principal phenomena in the basis of OM development [75, 265]. An imbalance between the body's synthesis of oxidants and antioxidant defenses of human body is known as oxidative stress, which may result in a wide range of diseases, including diabetes, cardiovascular or neurodegenerative diseases, and, at the last step, cancer [266, 267]. Therefore, the oxidative stress pathway could be a therapeutic target against OM, particularly in what concerns natural antioxidants that have a strong potential to prevent and control the generation of free radicals and restore cellular homeostasis [268]. A recent analysis by our research team highlighted the benefits of using natural substances to treat this oral condition [264].

As the pharma-, nutra-, and cosmeceutical industries shift their focus on natural bioactive chemicals, natural product research is increasing due to their unique properties as source of therapeutic phytochemicals, commonly associated with efficacy, safety, and minimal side effects [269, 270]. Among natural products, food by-products arise as a unique opportunity, considering sustainable principles and circular economy questions, such as low cost, abundance, and accessibility. Agro-industries generate large amounts of by-products in the form of shells, skins, flowers, leaves, and pulps, that may be used as raw resources to establish added-value products, a key strategy for a sustainable production [271, 272]. This is the case of chestnut shells. European or sweet chestnut (*Castanea sativa*, Mill.) production has, as its primary purpose, the natural fruit consumption, or the production of frozen chestnut [273, 274]. Portugal is one of the major European producers, with its main distribution coming from the northeast part of the country, covering 35,000 ha, with an estimated fruit production of 1800 kg/ha/year by 2022, expected to rise to about 3000 kg/ha/year in 2030 [274]. The inner and outer shells are generated by chestnut peeling processes, representing almost 10–15% of the whole chestnut weight [273, 275]. Shells are mostly used as fuel and as source of tannins for wine aging, also being a major origin of fermentable sugars for the development of

biofuels, while the extracts are known for their high polyphenol content (with emphasis on phenolic acids, flavonoids, and tannins) [273, 275, 276].

Extraction is the most vital phase for the recovery of bioactive compounds from natural matrices. A great challenge to start implementing eco-friendly and effective extraction methods, rather than the use of standard solvent extraction, has arisen in industry [277, 278]. Greener alternative technologies, such as Subcritical Water Extraction (SWE), are being described as more ecological and efficient to recover valued compounds and produce high-quality extracts [279, 280]. SWE presents a major advantage by using water as solvent, which is heated at a temperature range of 100–374 °C and a pressure state between 10–100 bar to maintain in its liquid form [8, 281]. Under these parameters, water's dielectric constant decreases, exhibiting a behavior like organic solvents and enabling a faster extraction, higher extraction yield, and efficient recovery of both polar and nonpolar compounds [8]. The short extraction time, energy savings, selectivity, discontinuation of organic solvents, as well as the low environmental impact and high cost-effectiveness ratio are positive aspects that should be considered [282, 283]. Nevertheless, some phenolic compounds can be completely or partially degraded at high temperatures, producing new ones. Therefore, temperature is a key parameter in the SWE [8, 282, 284]. In 2019, our research team published the first study that evaluated the subcritical water extraction of chestnut shells, but the influence of temperature was not deeply explored, posing a challenge. Determining the ideal SWE temperature (110–180 °C) to extract bioactive compounds from *C. sativa* shells was the main aim of this study, to produce an optimal extract with promising effects against OM. While the *in vitro* effects on several buccal cell lines and the antibacterial activities against oral pathogens ensure the final OM effects, the phenolic profiles were evaluated to understand the specific compounds responsible for the antioxidant/antiradical activities. To the best of our knowledge, this is the first research that provides insights on how OM can be treated using a food by-product.

2.2. Results and discussion

2.2.1. Extraction yield

Extraction is a critical step in the phytochemical processing pathway to obtain the highest quantity of bioactive compounds from plant sources. To standardize plant products, it is crucial to use an appropriate extraction method [285, 286]. Furthermore, for upscaling reasons, it is crucial to select an appropriate extraction technique and optimize various parameters, such as temperature, sample-to-solvent ratio, pH, and

extraction period [283]. Various extraction techniques such as maceration, infusion and Soxhlet extraction, and alternative and greener methods, such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), and SWE, are available in the market [280, 286-289]. Green extraction techniques are faster and more environmentally friendly in terms of solvent and energy use, leading to a continuously increasing application in recent years. In some circumstances, the green extraction yield is considerably higher than that of the conventional approaches [287, 288, 290, 291]. Table 5 summarizes the extraction yields of the different extracts of chestnut shells obtained at different temperatures (110 °C, 120 °C, 140 °C, 160 °C, and 180 °C).

Table 5 - Extraction yields, total phenolic content (TPC), and antioxidant/antiradical activities evaluated by DPPH, ABTS and FRAP assays of *C. sativa* extracts, prepared by SWE. Results are expressed as mean ± standard deviation (SD) (*n*= 3). Different letters (a,b,c,d,e) in the same column indicate significant differences between extracts ($p < 0.05$).

SWE extracts	Extraction yield (%)	TPC (mg GAE/g DW)	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)	FRAP (µmol FSE/g)
110 ˚C	20.88 ± 0.78	239.53 ± 23.17 [a]	426.88 ± 13.42	148.68 ± 13.20 [a]	4240.38 ± 10.04 [a]
120 ˚C	21.00 ± 1.49	201.75 ± 13.02 [b]	489.49 ± 5.96	228.32 ± 16.48 [b]	4092.98 ± 92.82 [b]
140 ˚C	20.83 ± 0.62	162.52 ± 7.14 [c]	460.21 ± 14.81	232.97 ± 4.23 [b]	3398.98 ± 57.23 [c]
160 ˚C	20.97 ± 1.74	122.31 ± 5.89 [d]	496.15 ± 8.75	220.40 ± 7.83 [b]	2728.06 ± 35.04 [d]
180 ˚C	20.29 ± 0.93	126.19 ± 6.33 [d]	583.47 ± 14.68	256.59 ± 5.55 [b]	2464.32 ± 68.44 [e]

IC_{50}= *In vitro* concentration required to decrease in 50% the reactivity of the studied radical species in the tested media.
GAE= gallic acid equivalent
FSE= ferrous sulphate equivalent.

As it is possible to observe, no significant extraction differences were observed between the extract's yields, with the extract prepared at 180 °C achieving the lowest value (20.29%), oppositely to the extract obtained at 120 °C (21.00%). Cacciola *et al*. (2019) evaluated three different methods (conventional liquid extraction, UAE, and MAE) using water as a solvent to recover bioactive compounds from chestnut shells. According to the authors, the extraction yields obtained for conventional liquid extraction, UAE, and MAE were, respectively, 5.2%, 2.2%, and 3.8% [292], at least four times lower than those shown in Table 5. In another study, Fernández-Agulló *et al*. (2014) reported higher extraction yields for chestnut shells extracted using 50% of organic solvents, namely methanol (12.09%) and ethanol (13.27%), when compared to conventional extraction at 75 °C with water (8.72%) and MAE (10.75%, 10.38% and 5.64%, respectively, for

methanol, ethanol, and water) [293]. The authors demonstrated the influence of temperature on the extraction efficiency, as the extraction yields increased with the augmented extraction temperatures tested [293]. In addition, Pinto *et al*. (2021) determined the efficiency of MAE in extracting *C. sativa* shells and reported extraction yields ranging from 6.52% (25% aqueous extract) to 11.13% (100% ethanol) [294]. The same research group tested different SWE times and stated yields between 6.70 and 9.19% [8].

The influence of sample-to-solvent ratio and mass transport enhancement on chestnut shells extraction was also evaluated in Microwave-Assisted Subcritical Water Extraction (MASWE) using a range of temperatures between 100 °C and 220 °C [295]. According to the authors, it was evidenced an increase of the extract production proportional to the increase of temperature, but minor deviations were observed for the sample-to-solvent ration (1:20–1:30), with exception of the 150 °C extract (322.37 mg/g DW and 416.56 mg/g DW, respectively) [295].

The SWE extraction yields reported in Table 5 were consistent with those determined by Wang *et al*. (2014) for citrus peel [296], Silva *et al*. (2022) for kiwiberry leaves [297], and Rodrigues *et al*. (2019) for papaya seed extraction [298].

2.2.2. Total phenolic content and antioxidant/antiradical activities

Table 5 summarizes the TPC, and antioxidant/antiradical activities of SWE extracts obtained at different temperatures. As shown, the TPC ranged between 126.19 and 239.53 mg GAE/g DW. The highest results were obtained for the extracts prepared at 120 °C (201.75 mg GAE/g DW) and 110 °C (239.53 mg GAE/g DW), and significant differences were noted between the extracts obtained at 120 °C and 140 °C (p= 0.008). Thermal degradation is probably responsible for the decrease in polyphenols content at higher temperatures. Nonetheless, this phenomenon alone cannot explain polyphenol behavior, as in conventional extraction methods, and the polyphenols breakdown can also occur at 80 °C. In fact, the increase in TPC observed at high SWE temperatures could also be attributed to the disruption of lignin–phenolic acid linkages or to the breakdown of lignin itself, resulting in large amounts of phenolic acids [284].

Pinto *et al*. (2021) assessed the phenolic profile, bioactivity, and cell viability of *C. sativa* shells extracted by MAE using different solvents (water and ethanol). The research team concluded that the highest TPC was achieved by the aqueous extract (173.89 mg GAE/g DW) [294]. The same authors tested this matrix by SWE extraction using response surface methodology (RSM) and reported higher TPC values for the

extract prepared at 220 °C (417.30 mg GAE/g DW); however, the phenolic profile was different from that obtained in the present study. According to the authors, the response surface 3D plots of the TPC showed that the temperature of 220 °C was the optimal temperature and the phenolic content at 80 °C is higher than that at 150 °C, probably due to the degradation of tannin [8].

IC_{50} values for the DPPH assay ranged from 426.88 g/mL (110 °C) to 583.47 µg/mL (180 °C). Statistical analysis demonstrated significant differences between the extracts (p= 0.002), which aligns with the TPC results. At lower temperatures the extracts presented higher phenolic content, resulting in higher antioxidant powers.

Regarding the ABTS assay, the IC_{50} results varied between 148.68 µg/mL and 256.59 µg/mL for the extracts obtained at 110 °C and 180 °C, respectively. Significant differences were noted between the extracts obtained at 110 °C, 120 °C, and 140 °C (p < 0.016), as well as between the extracts obtained at 120 °C, 160 °C, and 180 °C (p < 0.05).

According to Table 5, the extract's ability to decrease ferric ions increased with temperature was 180 °C < 160 °C < 140 °C < 120 °C < 110 °C. The extract processed at 110 °C had the highest value (4240.38 µmol FSE/g DW), while the extract produced at 180 °C had the lowest activity (2464.32 µmol FSE/g DW), an almost two-fold difference. Significant differences (p= 0.031) were observed between the extracts obtained at 110 °C and 160 °C.

Fernández-Agulló *et al*. (2014) also used DPPH and FRAP assays to evaluate the antiradical and antioxidant properties of chestnut shells and found that the aqueous extract (75 °C) obtained by conventional extraction produced the best results (IC_{50}= 0.031 mg/mL for DPPH; IC_{50}= 0.284 mg/mL for ABTS). The same extract obtained the highest FRAP (56.23 g GAE/100 g extract), in the accordance with TPC results [293]. In the chestnut shells SWE study, the extract prepared at 220 °C was assessed through DPPH, FRAP, and ABTS assays and showed good results, respectively, 901.16 mg AAE/g DW, 7994.26 mg FSE/g DW and 815.66 mg TE/g DW [8]. Regarding the UAE extraction of chestnut shells, the extract (70 °C/40 min) also had good antiradical and antioxidant results in the DPPH (IC_{50}= 44.1 µg/mL), ABTS (IC_{50}= 65.4 µg/mL) and FRAP (IC_{50}= 32.0 µg/mL) assays [299]. Analyzing the results from Table 5 with the authors mentioned above, it is evident that the extraction conditions are crucial. When the plant matrix was the same, the different extraction methods used were subjected to several variations. A detailed understanding of the bioactive composition, particularly the phenolic profile, is, therefore, essential, since the spectrophotometric assays have

limitations such as the sensitivity to pH, reaction time, and presence of interferents (e.g., sugars and amino acids) [300].

2.2.3. Phenolic profile identification and quantification by HPLC-PDA

The phenolic profiles of the *C. sativa* shells extracts prepared using SWE are detailed in Table 6.

Table 6 - Identification and quantification (mg/g DW) of the phenolic compounds present in *C. sativa* shells SWE extracts by HPLC-PDA analysis. Results are expressed as mean ± SD (*n* = 3).

Phenolic compound	110˚C	120˚C	140˚C	160˚C	180˚C
	mg/g DW				
Alkaloids					
Caffeine	1.18 ± 0.06	1.14 ± 0.06	1.01 ± 0.05	0.33 ± 0.02	0.44 ± 0.02
Chalconoids					
Phloridzin	0.83 ± 0.04	0.50 ± 0.03	ND	ND	ND
Flavanols					
Catechin	4.10 ± 0.20	3.65 ± 0.18	1.60 ± 0.08	0.74 ± 0.04	0.69 ± 0.03
Epicatechin	1.68 ± 0.08	1.14 ± 0.06	0.75 ± 0.04	<LOQ	<LOQ
Flavonols					
Rutin	1.04 ± 0.05	0.64 ± 0.03	0.69 ± 0.03	0.103 ± 0.005	0.136 ± 0.007
Flavonones					
Naringin	<LOQ	<LOQ	ND	ND	ND
Phenolic acids					
3,5-di-caffeolquinic acid	<LOQ	<LOQ	<LOQ	ND	ND
4-O-caffeyolquinic acid	0.49 ± 0.02	0.46 ± 0.02	0.40 ± 0.02	0.34 ± 0.02	0.21 ± 0.01
4,5-DI-O-caffeoylquinic acid	0.126 ± 0.006	0.22 ± 0.01	0.34 ± 0.02	0.026 ± 0.001	0.28 ± 0.01
Caftaric acid	0.44 ± 0.02	0.26 ± 0.01	0.53 ± 0.03	0.16 ± 0.01	0.58 ± 0.03
Caffeic acid	0.17 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.069 ± 0.003	0.12 ± 0.01
Clorogenic acid	0.43 ± 0.02	0.27 ± 0.01	0.18 ± 0.01	0.081 ± 0.004	0.13 ± 0.01
p-Coumaric acid	<LOD	<LOD	<LOD	<LOD	<LOD
Ellagic acid	0.136 ± 0.007	0.157 ± 0.008	0.181 ± 0.009	0.064 ± 0.003	0.108 ± 0.005
Gallic acid	5.99 ± 0.30	5.08 ± 0.25	4.64 ± 0.23	3.48 ± 0.17	2.40 ± 0.12
Ferulic acid	<LOQ	<LOQ	<LOQ	0.011 ± 0.001	0.056 ± 0.003
Neochlorogenic acid	0.71 ± 0.04	0.59 ± 0.03	0.49 ± 0.02	0.21 ± 0.01	0.28 ± 0.01
Protocatechuic acid	1.68 ± 0.08	1.37 ± 0.07	1.15 ± 0.06	1.31 ± 0.07	1.59 ± 0.08
Sinapic acid	ND	ND	ND	<LOQ	<LOQ
Syringic acid	ND	ND	ND	0.019 ± 0.001	0.063 ± 0.003
Vanillic acid	1.02 ± 0.05	0.91 ± 0.05	0.55 ± 0.03	0.084 ± 0.004	0.12 ± 0.01
Quercetin-3-O-galactoside	0.115 ± 0.006	0.099 ± 0.005	0.136 ± 0.007	0.012 ± 0.001	0.120 ± 0.006
Quercetin-3-O-glucopyranoside	0.057 ± 0.003	0.069 ± 0.003	0.081± 0.004	0.043 ± 0.002	0.036 ± 0.002
Stilbenoids					
Resveratrol	<LOD	<LOD	<LOD	ND	ND
Trans-polydatin	<LOQ	<LOD	<LOD	<LOD	<LOD
Total	20.20	16.71	12.85	7.07	7.37

ND: not detected; LOD: limit of detection; LOQ: limit of quantification.

As shown in Table 6, health-promoting compounds were grouped into seven different classes. Overall, 25 phenolic compounds were identified and quantified, with phenolic acids being the main class of compounds. The extract obtained at 110 °C presented the highest amount of phenolic acids (20.20 mg phenolic acids/g DW), though higher temperatures led to a significant decrease, such as the extract prepared at 160 °C (7.07 mg phenolic acids/g DW). This may be due to the diminution of the dielectric constant and polarity of water as temperature increases, which leads to the dissolution of non-polar compounds such as polyphenols [301]. However, high temperatures have a negative effect on polyphenols above 120 °C, resulting in the breakdown of thermally unstable polyphenols [302].

As it is possible to observe, the major phenolic acid present in all extracts is gallic acid, particularly at 110 °C, 120 °C and 140 °C (5.99, 5.08 and 4.64 mg/g DW, respectively). This compound is extremely important for human health, and several studies have reported its biological and pharmacological activities, including anti-inflammatory, antioxidant, anticancer, and antimicrobial activities [303]. Moreover, the presence of protocatechuic acid may indicate the thermal degradation of catechin [8]. Flavonoids were also identified in considerable amounts, corresponding to more than 30% of the total phenolic content. Particularly, catechin ranged from 3.65 to 4.10 mg/g DW for the extracts obtained at 120 °C and 110 °C, respectively. The epicatechin amounts in the extracts obtained at 110 °C and 120 °C were, respectively, 1.68 and 1.14 mg/g DW, while for rutin, the quantities were 1.04 (110 °C) and 0.64 mg/g DW (120 °C). However, for most of the compounds mentioned, the results indicate a high temperature sensitivity that should be considered.

Oxidative stress is the main factor in the basis development of OM, which induces to the activation of the apoptotic process, leading to the clinical manifestation of mucosal damage. Pro-inflammatory cytokines are in a positive feedback mechanism, conducting to ulceration, which compromises the mucosal integrity, allowing several microorganisms to penetrate tissues and increasing the sepsis risk [265, 304]. The five phenolic compounds mentioned above were found at higher concentrations in all the extracts. Because of their capacity to control crucial cellular enzyme activities that lead to anti-inflammatory, antioxidant, antimicrobial, and anticancer activities, all of them have been associated with a wide range of beneficial properties and are expected to be potential treatments and prevention options for OM [305].

Previous studies have reported discrepancies in the phenolic composition of chestnut shells extracts, which can be explained by the use of diverse extraction techniques and also varied extraction settings. For example, Vásquez *et al*. (2012) analyzed chestnut shells extracted by organic solvents using conventional methods. The authors reported 11 compounds, with catechin/epicatechin and gallocatechin/epigallocatechin being the principal ones, while hydrolysable tannins, such as galloyl-glucoses and ellagic acid, were also present and identified [306]. Gallic acid esters of glucose were particularly present in chestnut shells extracted with water at 75 °C (conventional extraction) as well as gallocatechin and catechin [293]. In the chestnut shells extract prepared by UAE, ellagic acid (40.4 µg/mg DW), caffeic acid derivative (15.4 µg/mg DW) and epigallocatechin (15.3 µg/mg DW) were identified in high quantities [299], while by MAE the gallic acid and protocatechuic acid were also detected (117.58 and 16.8 mg/g DW, respectively) [292].

2.2.4. ROS scavenging capacity

A major global issue is the development of disorders related to oxidative stress caused by an imbalance between reactive species and antioxidants commonly present in the human body, being a key factor for the OM pathogenesis. However, its mechanism remains unclear. Nonetheless, it is known that chemo and/or radiotherapy triggers an increase in ROS generation [264]. Reactive species, such as hypochlorous acid (HOCl), superoxide ($O_2^{\bullet -}$), peroxyl radical ($ROO^{\bullet}$), and peroxynitrite ($ONOO^-$), when produced in excess in living cells are responsible, directly, or indirectly, for a number of diseases, including OM and, at last stage, oral cancer [307, 308]. One of the most significant classes of natural antioxidants that can prevent or reduce the harm caused by oxidative stress is the polyphenol family, which has a positive effect on OM [304]. Table 7 shows the ROS scavenging activity results.

The extract prepared at 140 °C (IC_{50} = 31.14 µg/mL) showed the highest activity in terms of $O_2^{\bullet -}$ scavenging capacity, whereas the extract prepared at 180 °C showed the lowest activity (IC_{50} = 73.18 µg/mL). Gallic acid was used as a positive control for this test, and it produced the best results (IC_{50} = 23.82 µg/mL). No significant differences (p = 0.123) were observed between the extracts. However, HOCl was effectively scavenged by all extracts. While the extract obtained at 160 °C had the lowest free radical scavenging capacity (IC_{50} = 22.85 °C), the extract prepared at 110 °C had the best effectiveness (IC_{50} = 4.47 °C). Significant differences were noted between extracts obtained at 110 °C and 140 °C (p = 0.026), and 160 °C and 180 °C (p = 0.027). Regarding

the ROO• quenching, the efficacy increased in the following order: gallic acid >110 °C > 140 °C > 120 °C >160 °C >180 °C. No significant difference (p = 0.379) was observed between extracts.

Table 7 - Superoxide anion radical ($O2^{\bullet-}$), hypochlorous acid (HOCl), peroxyl radical ($ROO^{\bullet}$) and peroxynitrite ($ONOO^-$) scavenging capacities of *C. sativa* shells SWE extracts. Values are expressed as mean ± standard deviations (n = 3). Different letters (a,b,c,d) in the same column indicate significant differences between extracts ($p < 0.05$).

	Reactive Oxygen Species			Reactive Nitrogen Species	
SWE extracts	$O_2^{\bullet-}$	HOCl	$ROO^{\bullet}$	$ONOO^-$ in presence of HCO_3^-	$ONOO^-$ in absence of HCO_3^-
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	(µmol TE/mg DW)	IC_{50} (µg/ml)	
110 ˚C	44.36 ± 0.34 ^{cd}	4.47 ± 0.29 ^{b}	0.73 ± 0.024 ^{b}	2.52 ± 0.19 ^{c}	2.53 ± 0.22 ^{bc}
120 ˚C	46.31 ± 1.37 ^{d}	6.49 ± 0.18 ^{b}	0.54 ± 0.016 ^{cd}	1.41 ± 0.11 ^{b}	3.32 ± 0.19 ^{c}
140 ˚C	31.14 ± 2.05 ^{b}	8.27 ± 0.77 ^{b}	0.61 ± 0.037 ^{bc}	2.77 ± 0.29 ^{c}	2.09 ± 0.21 ^{b}
160 ˚C	35.34 ± 3.14 ^{bc}	22.85 ± 1.67 ^{c}	0.50 ± 0.032 ^{cd}	2.79 ±0.26 ^{c}	1.89 ± 0.09 ^{b}
180 ˚C	73.18 ±1.86 ^{e}	20.74 ± 1.27 ^{c}	0.49 ± 0.009 ^{d}	2.93 ± 0.03 ^{c}	5.94 ± 0.30 ^{d}
Positive control					
Gallic acid	23.82 ± 0.82 ^{a}	1.68 ± 0.16 ^{a}	4.23 ± 0.25 ^{a}	0.30 ± 0.03 ^{a}	0.05 ± 0.00 ^{a}

IC_{50} = *In vitro* concentration required to decrease in 50% the reactivity of the studied reactive species in the tested media. TE= (Trolox Equivalents).

Comparing with other studies, the SWE extracts prepared in the present study showed lower ROS scavenging activity. Lameirão *et al*. (2021) related that the chestnut shells extract obtained by UAE (at 70 °C during 40 min) achieved an IC_{50} of 14.1 µg/mL, 0.7 µg/mL and 0.3 µg/mL regarding $O_2^{\bullet-}$, HOCl and $ROO^{\bullet}$, respectively [299]. Likewise, the optimal SWE extract (obtained at 220 °C) of chestnut shells prepared by Pinto *et al*. (2021) exhibited high scavenging activity regarding HOCl (IC_{50} = 0.79 µg/mL) and $O_2^{\bullet-}$ (IC_{50} = 12.92 µg/mL). However, as summarized in Table 7, the extracts in the present study had a better ability to quench $ROO^{\bullet}$, as Pinto *et al*. reported an Ssample/STrolox = 0.084 for the optimal extract [8].

Regarding RNS, the peroxynitrite ($ONOO^-$) scavenging activity was evaluated in the absence and in presence of hydrogen carbonate (HCO_3^-) to mimic the *in vivo* environment. Although the results presented in Table 3 do not attest to a temperature-

dependent correlation, a significant ability of the extracts to act as effective reducers of peroxynitrite-mediated damage can be observed. The best IC_{50} calculated was 1.41 µg/mL (for the extract prepared at 120 °C) and 1.89 µg/mL (for the extract obtained at 160 °C) in the presence and absence of HCO_3^-, respectively. However, the gallic acid achieved best results (IC_{50} = 0.30 and 0.05 µg/mL, respectively, in presence and absence of HCO_3^-). To the best of our knowledge, this is the first peroxynitrite assay performed on chestnut shells extracts. The phenolic profile, and thus the potential for compounds to degrade, as previously mentioned, could play a role in the observed discrepancies. No significant differences between the extracts were observed in the presence (p= 0.285) or absence (p = 0.357) of HCO_3^-. However, the radical scavenging capacity calculations, particularly for HOCl and $ROO^{\bullet}$, are generally consistent with the spectrophotometric results (Table 5), indicating that the extract prepared at 180 °C had the lowest potential, while the extract obtained at 110 °C had the most promising characteristics for OM treatment.

2.2.5. Antimicrobial Activity

The oral cavity contains complex microbiota compromising more than 700 different bacterial species. The oral cavity is an ideal environment for microbial growth due to its pH (6.5–7.5), temperature (37 °C), soft tissues (palate, tongue, and buccal mucosa), and hard tissues (teeth) [309]. The oral microbial population is generally balanced however, pathologies may appear when the ecological balance is disturbed. Chemotherapy-induced myelosuppression and radiation-induced xerostomia are known to cause microbiome dysbiosis, which compromise the saliva flow, a major barrier against potential pathogens [310]. Owing to its ability to affect the innate immune response, which is an activator of the mucositis pathway, the oral microbiota can aggravate or sustain oral mucositis [311]. Examples of the main species of bacteria commonly isolated from the oral cavity are *Porphyromonas*, *Lactobacillus*, *Veillonella*, *Actinobacillus*, *Streptococcus*, *Staphylo coccus, Enterococcus*, and *Escherichia* [312, 313].

The antimicrobial capacity of SWE *C. sativa* shells extracts was analyzed against several Gram-negative and Gram-positive bacteria, the majority of which are present in the oral cavity in case of OM [313, 314]. MIC and MBC were determined for each microorganism at different extract concentrations (2–64 mg/mL) using a microplate dilution assay. Table 8 summarizes the results for all strains.

Table 8 - Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *C. sativa* shells SWE extracts on the bacterial strains tested, at a range of concentrations of 2-64 mg/ml. Values are expressed as mean (n = 3).

SWE *C. sativa* extracts		Gram-negative				Gram-positive		
		E. coli ATCC 25922	*E. coli* ATCC 8739	*E. coli* CTX M2*	*P. gingivalis* ATCC 33277	*S. aureus* ATCC 25913	*S. aureus* MRSA*	*E. faecalis*
110°C	MIC	8	4	4	-	-	4	64
	MBC	64	-	-	-	-	32	-
120°C	MIC	8	4	8	8	64	8	2
	MBC	64	-	-	64	-	16	-
140°C	MIC	4	16	4	4	4	32	64
	MBC	-	-	-	32	-	64	-
160°C	MIC	4	4	4	4	4	4	8
	MBC	32	-	-	32	64	32	64
180°C	MIC	8	8	-	-	4	4	16
	MBC	16	4	-	-	64	16	64

*drug resistant bacteria; - absence of MIC/MBC

As can be observed, the chestnut shells extracts obtained at different temperatures exhibited diverse effects on the growth of microorganisms, which is probably due to the phenolic content of the extracts.

The extract prepared at 110 °C did not present MIC and MBC values for *S. aureus* (Gram-positive) at the concentrations tested. However, the extract showed a significant antimicrobial activity against two drug-resistant bacteria, *S. aureus* MRSA and *E. coli* CTX M2 (MIC of 4 mg/mL and MBC of 32 mg/mL in *S. aureus* MRSA). This extract also exhibited MIC in *E. coli* ATCC 8739, *E. coli* ATCC 25922, and *E. faecalis* (8, 4, and 64 mg/mL, respectively) and MBC of 64 mg/mL in *E. coli* ATCC 25922. *E. coli* CTX M2 was resistant to the extract obtained at 180 °C. Compared to the 110 °C extract, the extract prepared at 180 °C presented higher MIC values for the two non- resistant strains of *E. coli* (8 mg/mL), but a lower MIC for *E. faecalis* (16 mg/mL). At this temperature, the extract exhibited MBC values for all strains that had growth inhibition. As shown in Table 8, the extract prepared at 160 °C showed better antimicrobial activity, as it showed lower MIC values for most of the strains tested and displayed MBC values for all the strains, except for the drug-resistant *E. coli* CTX M2.

Phenolic compounds may affect bacterial cells by damaging their cellular membranes, binding to the cell wall, and leading to enzyme inactivation and DNA

damage [315]. Flavanols (e.g., catechin and epicatechin) are known to inhibit the *in vitro* growth of several strains, including *S. mutans* and *E. coli* [316]. Flavonols (e.g., rutin) also exhibit a significant activity against *S. aureus* (Gram-positive), possibly through an aggregation mechanism. Nevertheless, phenolic acids, such as gallic acid, also showed antibacterial activity against *S. aureus* and *E. coli*. Although these compounds present weaker efficiency in bacterial growth inhibition when compared to flavonoids, they still prove to be more efficient than standard antibiotics, such as gentamicin and streptomycin [316, 317]. Kang *et al*. (2008), studied the antimicrobial effects of gallic acid, one of the main compounds present in *Rhus chinensis* L., and reported MIC values of 8 mg/mL for *S. mutans*. The authors also reported *in vitro* inhibition of *S. mutans* biofilms, demonstrating the antimicrobial activity of gallic acid [318]. Likewise, Passos *et al*. (2021) reported the antimicrobial and anti-adherence activity of gallic acid, an isolated compound of the ethanolic extract of *L. ferrea*, which exhibited significant antimicrobial activity and inhibition of adhesion of *S. mutans* biofilms [319].

Previous research has demonstrated a correlation between phenolic compounds and the antimicrobial activity, as the quantity of phenolic compounds has a proportional inhibitory effect on the microorganism growth [320]. Though, the present results do not demonstrate a correlation as the extract prepared at 110 °C has a higher amount of phenolic compounds and the extract obtained at 160 °C showed better antimicrobial capacity. One of the possible explanations for such data is the antagonistic effect of the combination of polyphenolic compounds present in the SWE extracts, which may decrease the antimicrobial ability.

2.2.6. Cells Viability Assays

Considering the potential use of SWE *C. sativa* extracts in OM treatment, it is particularly important to assess their effects in *in vitro* models. In this study, SWE *C. sativa* shells extracts were evaluated on HSC3 and TR146 cell lines by MTT assay. Both cell lines mimic the normal human buccal epithelium and are tumorigenic, making them suitable as oral mucosal models for testing medication absorption. In particular, the TR146 cell line was established from a neck metastasis of a buccal carcinomic tumor, and the HSC3 cell line was derived from tumors of metastatic lymph nodes originating from tongue squamous cell carcinoma [321-323]. Figure 3 summarizes the obtained

results for both cell lines using various extract concentrations (125–2000 µg/mL), while Table 9 presents the IC_{50} values.

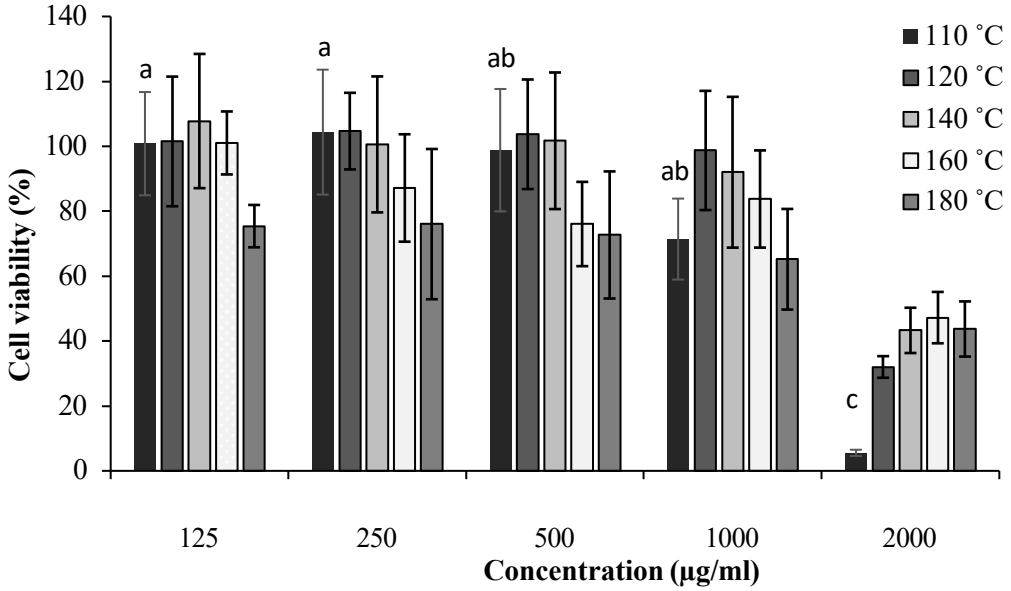


Figure 3 - Effects of C. sativa shells SWE extracts on the viability of HSC3 cells at a range of concentrations of 125-2000 µg/ml, measured by MTT assay, at 24 h. Values are expressed as mean ± standard deviation (n = 3). Different letters indicate significant differences between concentrations of the same sample (p < 0.005), according to Tukey's HSD test.

Regarding the HSC3 results, the extracts obtained at 160 °C and 180 °C presented viabilities of 87.16% and 76.03%, respectively, after exposure with the low concentrations tested (250 µg/mL). The extracts obtained at 110 °C, 120 °C, and 140 °C showed viability reductions at concentrations of 500, 1000, and 2000 µg/mL, respectively. Significant differences were noted for the different concentrations of the extracts obtained at 110 °C (500–2000 µg/mL; pn= 0.044) and 180 °C (125–250 µg/mL and 125–2000 µg/mL; p < 0.03). These results might be explained by the fact that the extract prepared at 110 °C contained the highest concentration of phenolic acids, which was in accordance with Table 5.

Table 9 - IC_{50} values of oral model cellular lines treated with *C. sativa* shells SWE extracts, measured by MTT assay. Values are expressed as mean (n = 3). IC_{50}= *In vitro* concentration required to decrease in 50% cells viability.

	Cell lines IC_{50} (µg/ml)	
SWE extracts	HSC3	TR146
110 ˚C	1325.03	468.15
120 ˚C	1730.39	496.45
140 ˚C	1862.17	663.83
160 ˚C	1923.94	553.79
180 ˚C	1707.33	572.18

As shown in Figure 4, except for the extract prepared at 110 °C, none of the extracts obtained at different temperatures resulted in a reduction in the viability of TR146 cells at a concentration of 125 µg/mL. However, with increasing concentrations, all extracts significantly decreased cellular viability, leading to viability values below 57% for all extracts at 500 µg/mL. Significant differences were observed for the extract prepared at 140 °C (125–250 µg/mL; p = 0.022) and 180 °C (25–1000 µg/mL; p = 0.016). These results proved that TR146 cells were the most sensitive to the SWE chestnut shells extracts (Table 9). Moreover, it can be concluded that the extracts exhibited antiproliferative effects for both cell lines, with the extract prepared at 110 °C achieving the lowest IC_{50} value in TR146 (468.15 µg/mL) and in HSC3 (468.15 µg/mL). This may be due to the higher content of phenolic compounds, as shown in Table 5.

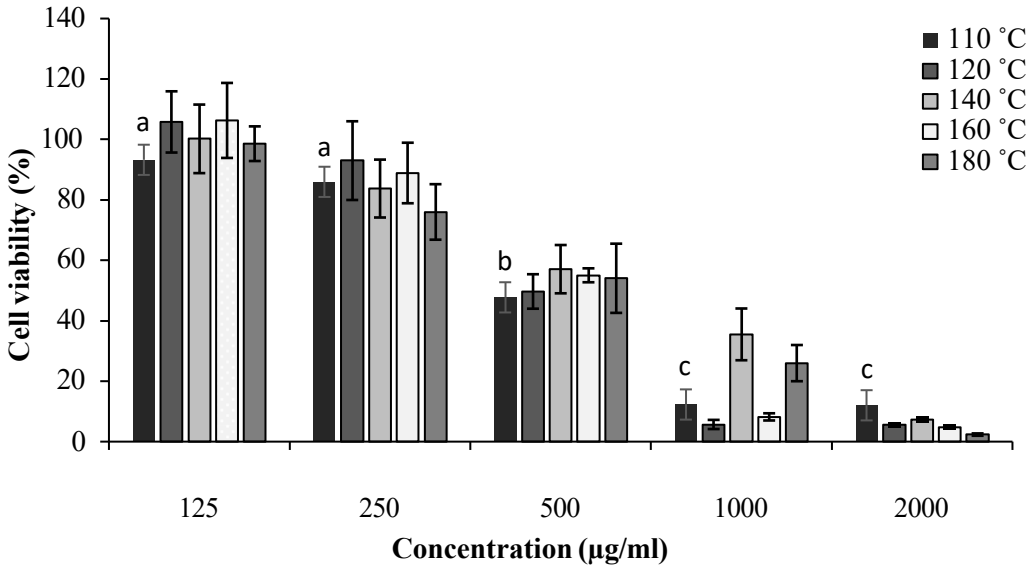


Figure 4 - Effects of *C. sativa* shells SWE extracts on the viability of TR146 cells at a range of concentrations of 125-2000 µg/ml, measured by MTT assay, at 24 h. Values are expressed as mean ± SD (n = 3). Different

letters indicate significant differences between concentrations of the same sample ($p < 0.005$), according to Tukey's HSD test.

Lambert *et al*. reported that polyphenols can inhibit different phases of carcinogenesis, including initiation, promotion, and progression of tumor cells, demonstrating anticancer properties [324]. In addition, polyphenols have been reported to prevent oral cancer, as they come into direct contact with tissues prior to being absorbed, suppressing the proliferation of oral cancer cells on the surface of epithelial cells [325]. Catechins, for example, have been demonstrated the capacity to decrease the activity of oral squamous cell carcinoma (OSCC) in OC2 cells by inhibiting the synthesis of matrix metalloproteinases (MMPs). Hence, invasion and migration of cancer cells can be regulated [326, 327]. To the best of our knowledge, this is the first study that assessed the effects of SWE chestnut shells extract on oral model cell lines. This highlights its potential as an ingredient against OM condition.

2.7. Materials and Methods

2.7.1. Chemicals

α,α′-azodiisobutyramidine dihydrochloride (AAPH), β-nicotinamide adenine dinucleotide (NADH), dihydrorhodamine 123 (DHR), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), sodium hypochlorite solution with 4% available chlorine, phenazine methosulphate (PMS), nitroblue tetrazolium chloride (NBT), fluorescein sodium salt and Trolox were obtained from Sigma-Aldrich (Steinheim, Germany). Catechin, gallic acid, Folin–Ciocalteu's reagent and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

For microbial culture, Mueller Hinton Broth (MHB) medium was purchase to Liofilmchem (Teramo, Italy), Tryptic Soy Broth (TSB), and Tryptic Soy Agar (TSA) medium was obtained from Biolife (Milan, Italy). HPLC solvents were delivered by Sigma-Aldrich (Milan, Italy). Individual's standards of phenolic compounds used for the identification and quantification in extracts were purchased from Sigma-Aldrich (Steiheim, Germany). HPLC solvents were delivered by Sigma-Aldrich (Milan, Italy). Individual's standards of phenolic compounds, namely gallic acid (≥99%), protocatechuic acid (99.63%), neochlorogenic acid (≥98%), caftaric acid (≥97%), chlorogenic acid (>95%), 4-*O*-caffeyolquinic acid (≥98%), vanillic acid (≥97%), caffeic acid (≥98%), syringic acid (≥98%), *p*-coumaric acid (≥98%), *trans*-ferulic acid (≥99%), sinapic acid (≥99%), 3,5-di-*O*-caffeyolquinic acid (≥95%), ellagic acid (≥95%), 4,5-di-*O*-caffeyolquinic acid (≥90%), (+)-catechin (≥98%), (-)-epicatechin (≥90%), naringin (≥95%), quecetin-3-

O-galactoside (≥97%), quercetin-3-*O*-glucopyranoside (≥99%), rutin (≥94%), phloridzin (99%), *trans*-polydatin (≥98%), resveratrol (≥99%) and caffeine (≥95%), used for the identification and quantification in extracts were purchased from Sigma-Aldrich (Steiheim, Germany). Dulbecco's Modified Eagle Medium (DMEM), penicillin, trypsin-EDTA and streptomycin were provided by Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). Dimethyl sulfoxide (DMSO) was purchased by AppliChem (Darmstadt, Germany).

TR146 and HSC3 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The following reference bacterial strains were obtained from the American Culture Collection (ATCC): *Escherichia coli* ATCC 25922, *E. coli* ATCC 8739, and *Staphylococcus aureus* ATCC 25913. All other strains, including resistant bacteria, were clinical isolates: *Enterococcus faecalis* and the resistant bacteria, *Escherichia coli CTX M2* and *Staphylococcus aureus MRSA*.

2.7.2. Sample

Castanea sativa shells were generously provided by Sortegel, located in Sortes, Bragança, Portugal. Dehydration of shells occurred at 41 °C for 24 h (Excalibur Food Dehydrator, Pennsylvania, USA) and then were pulverized in a miller to 1 mm particles (Ultra Centrifugal Mill ZM 200, Retsch, Germany). Prior to extraction, samples were carefully blended and kept at room temperature (20 °C), in the dark.

2.7.3. Subcritical Water Extraction (SWE) of *C. sativa* shells

Extraction of *C. sativa* shells was performed in a homemade subcritical batch-type extractor of 1.7 L [328]. For it, a built-in valve and 99.99% nitrogen (Messer) pressurization were used. The samples were then extracted for 30 min at various temperatures between 110 °C and 180 °C using 20 bars of pressure and a 1:30 sample-to-solvent ratio. The vibrating platform (3 Hz) housing the extraction vessel agitated the sample/water mixture during the extraction. The extraction vessel was then cooled using a flow-through water bath (20 ± 2 °C), and the pressure was released by opening the valve. Filtration was used to separate the extracts, and the solutions were subsequently lyophilized (Telstar, model Cryodos −80 °C, Spain) and stored at room temperature (20 °C) until further analysis. The ratio between the total weight of the lyophilized extract and the total weight of the extract obtained following SWE was used to calculate the extraction yield as follows, in Eq (1):

$$\text{Extraction yield (\%)} = \frac{\text{weight of lyophilized extract}}{\text{weight of liquid extract}} \times 100 \quad \text{Eq (1)}$$

2.7.4. Determination of total phenolic content

The Folin–Ciocalteu method was used to measure the total phenolic content (TPC) using spectrophotometry, with minor changes [8]. A calibration curve (linearity range: 5–100 µg/mL; R^2 > 0.996) was created using gallic acid solution. Results were expressed as mg of Gallic Acid Equivalents (GAE) per g of dry weight (mg GAE/g DW).

2.7.5. Antioxidant and antiradical activities

2.7.5.1. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out as described by Benzie and Strain with a few minor changes [297]. A calibration curve (linearity range: 25–500 µM; R^2 > 0.993) was established using a standard ferrous sulfate ($FeSO_4 \cdot 7H_2O$) solution at a concentration of 1 mM. Results were expressed in µmol of ferrous sulfate equivalents (FSE) per gram of DW (µmol FSE/g DW).

2.7.5.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

DPPH free radical scavenging assay was performed according to the method described by Pinto *et al*. [329]. Trolox was used as reference for the calibration curve (linearity range: 5–125 µg/mL; R^2 > 0.989). Results were presented as the radical scavenging activity's half-maximal inhibitory concentration (IC_{50}, µg/mL).

2.7.5.3. 2,2´-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity assay

Extracts were tested for ABTS radical scavenging activity according to Re *et al*. [330], with slight adjustments. The calibration curve standard (linearity range: 5–100 µg/mL; R^2 > 0.998) was ascorbic acid and the results were expressed as IC_{50} (µg/mL).

2.7.6. HPLC-PDA analysis

HPLC was used to identify and quantify polyphenols using photodiode array (PDA) detection [331]. Separation was performed at 25 °C using a Gemini C18 column (250 mm 4.6 mm, 5 m, Phenomenex, Alcobendas, Spain). Results on DW (mg/g DW)

were represented as mg of each phenolic component per gram of extract. The calibration data used for the quantification of individual phenolic compounds in the chestnut shells extracts are presented in Table S10.

2.7.7. Reactive oxygen species and reactive nitrogen species scavenging capacity

2.7.7.1. Superoxide radical scavenging assay

Superoxide radical ($O_2^{\bullet -}$) scavenging assay was performed as described by Gomes *et al*. [332]. Results were expressed as IC_{50} of the NBT reduction to diformazan.

2.7.7.2. Hypochlorous acid scavenging assay

Hypochlorous acid (HOCl) scavenging assay was performed accordingly to Gomes *et al*. [332]. Gallic acid was used as a positive control. The results were presented as an inhibition of the HOCl-induced oxidation of DHR, measured in IC_{50}.

2.7.7.3. Peroxyl radical scavenging assay

Peroxyl radical ($ROO^{\bullet}$) assay was carried out according to the method described by Ou *et al*. [333]. Trolox was used as standard control. The ratio of the slopes obtained for the extracts or positive controls to the slope of Trolox (Sample/STrolox), which represents the $ROO^{\bullet}$ induced oxidation of fluorescein, was used to express the results.

2.7.7.4. Peroxynitrite scavenging assay

The effects of the studied extracts on the of inhibition of $ONOO^-$ induced oxidation of non-fluorescent DHR to fluorescent rhodamine 123 were assessed by peroxynitrite ($ONOO^-$) scavenging activity. $ONOO^-$ was synthesized as described by Whiteman *et al*. [334]. To simulate physiological CO_2 values, parallel tests were run in the presence of 187.5 mM $NaHCO_3$, and the results were represented as IC_{50}.

2.7.8. Antimicrobial activity

2.7.8.1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC values were determined by the broth microdilution method using a 96-well plate with MHB medium with minor modifications [335]. Microorganisms were exposed to extracts (0.05 mL of dilutions + 0.05 mL of MHB medium) in the following concentration

range: 2–64 mg/mL. The extracts were dissolved in deionized water and the results were compared with those bacteria that had grown in MHB in the presence of water as a control. The MIC of each extract was determined as the lowest concentration that showed no turbidity after 24 h of incubation at 37 °C. All the 96-well plates and petri dish plates were sealed.

2.7.8.2. Growth rate (GR) studies

The GR parameter was determined using the broth microdilution method in a 96-well plate using TSB. All different strains of bacteria were exposed to extracts concentrations superior, equal, or inferior to the determined MIC, performing 1 h interval readings of the optical density (OD) at 625 nm, 37 °C, for 24 h [335]. Growth rate was determined by fitting a linear function to an exponential (log) phase curve.

2.7.8.3. Minimum bactericidal concentration (MBC) determination

MBC assays were evaluated by the broth dilution method, where MBC was established as the lowest extract concentration that inhibited 99.9% of the bacterial inocula after 24 h of incubation at 37 °C. Briefly, 5 µL was taken from the well obtained from the MIC experiment from each extract and its dilutions and added to a petri dish plate with TSA medium. All the petri dish plates were sealed. The plate was divided into six equal areas, and for all extracts, the area of the lower dilution with no visible colonies was determined as MBC [336].

2.7.9. Cell viability assay

A cell viability assay was performed using two human oral cancer cell lines, namely TR146 and HSC3. Passages 28 and 6 were used for TR146 and HSC3, respectively. The cells were cultured in a CellCulture® CO_2 Incubator (ESCO GB Ltd., Barnsley, UK) for 24 h at 37 °C and exposed to various extract concentrations (125–2000 g/mL). This assay was performed according to Pinto *et al*. [8]. Extracts were dissolved in cell culture medium or absent and then incubated (2.5×10^4 cells/mL). MTT reagent was added, and the cells were incubated for 3 h at 37 °C, the number of viable cells was estimated, and the crystals were solubilized using DMSO. The positive and negative control were DMEM and 1% (*w/v*) Triton X-100. The background absorbance (630 nm) was subtracted at the 590 nm reading. Cell viability results were expressed as percentages.

2.7.10. Statistical analysis

Results are expressed as mean ± standard deviation (n = 3). IBM SPSS Statistics 27.0 software (SPSS Inc., Chicago, IL, USA) was used to conduct statistical analysis. Tukey's HSD test was used for post hoc comparisons of the means, and one-way ANOVA was used to analyze sample differences. p values lower than 0.05 ($p < 0.05$) were recognized as significant.

2.8. Conclusions

The scientific data presented in this study support the valorization of *C. sativa* shells extracts generated by SWE, an innovative and environmentally safe extraction method that may be applied as a therapeutic agent for the treatment of OM. The extract that produced the best results in terms of antioxidant and antiradical activities as well as the ability to scavenge free radicals was that produced at 110 °C, which was the optimal temperature for extraction. This extract also had a higher variety of polyphenols (25 phenolic compounds), with a particular focus on gallic acid, protocatechuic acid, catechin, epicatechin, caffeine, and rutin. Furthermore, antimicrobial activity assays demonstrated that, in general, all extracts significantly inhibited bacterial growth against strains commonly found in OM patients. The chestnut shells extracts induced cytotoxic effects in HSC3 and TR146 cells, with a particular focus on TR146 cell line, and the extract obtained at 110 °C exhibited the lowest IC_{50} values for both cell lines. All the biological properties exhibited by the SWE *C. sativa* shells extract, particularly the extract obtained at 110 °C, may target the oxidative stress caused by anticancer treatments through the antioxidant activity and radical species scavenging capacity and, consequently, the inflammatory state. In addition, in advanced conditions (ulceration), it can manage bacterial proliferation and exert antiproliferative effects in head and neck squamous cell carcinoma. More research is required to reach a conclusion on the effects on the oral mucosa, particularly on the absorption of phenolic compounds via the buccal route. These may include *in vitro* oral epithelial permeation assays and oxidative stress experiments in cell lines. Furthermore, the sustainable use of chestnut shells will improve the applicability of food by-products, improve their economic value, and contribute to environmental sustainability.

Supplementary Material

Table S10 - Calibration data used for the quantification of individual phenolic compounds in chestnut shells extract.

Phenolic Compounds	(m ± Δm)[a]	(b ± Δm)[b]	r^2	LOD[c] (mg/L)	LOQ[d] (mg/L)
Alkaloids					
Caffeine	54054 ± 257	6136 ± 307	0.9999	0.313	1.043
Chalconoids					
Phloridzin	47726 ± 42	78 ± 8	0.9999	0.125	0.416
Flavanols					
Catechin	13826 ± 56	3021 ± 1127	0.9999	0.568	1.89
Epicatechin	15117 ± 60	1178 ± 118	0.9999	0.558	1.86
Flavonols					
Rutin	83207 ± 51	1937 ± 1034	0.9999	0.087	0.289
Quercetin-3-*O*-galactoside	23597 ± 27	466 ± 47	0.9999	0.159	0.531
Quercetin-3-*O*-glucopyranoside	48964 ± 119	17.0 ± 0.9	0.9999	0.161	0.535
Flavonones					
Naringin	36536 ± 25	1384 ± 499	0.9999	0.095	0.317
Phenolic acids					
3,5-di-*O*-caffeolquinic acid	62289 ± 829	-11003 ± 3153	0.9991	0.341	1.13
4-*O*-caffeyolquinic acid	12334 ± 202	-1032 ± 52	0.9995	0.19	0.632
4,5-di-*O*-caffeoylquinic acid	28503 ± 410	32 ± 3	0.9991	0.383	1.27
Caftaric acid	18700 ± 50	966 ± 48.3	0.9999	0.177	0.591
Caffeic acid	69190 ± 45	124 ± 12	0.9999	0.090	0.302
p-Coumaric acid	146520 ± 1176	-33763 ± 3376	0.9991	1.70	5.68
Ellagic acid	44947 ± 1040	-30767 ± 20152	0.9973	3.09	10.3
Gallic acid	54220 ± 90	57.7 ± 6	0.9999	0.120	0.400
trans-Ferulic acid	61448 ± 39	1501.6 ± 789	0.9999	0.089	0.298
Neochlorogenic acid	50502 ± 344	5751 ± 288	0.9998	0.449	1.495
Protocatechuic acid	32064 ± 24	1024 ± 477	0.9999	0.104	0.346
Sinapic acid	29742 ± 16	124 ± 12	0.9999	0.075	0.25
Syringic acid	61262 ± 72	2088 ± 209	0.9999	0.165	0.551
Vanillic acid	36254 ± 55	1215 ± 121	0.9999	0.215	0.715
Chlorogenic acid	27244 ± 57	-1445 ± 144	0.9999	0.292	0.972
Stilbenoids					
Resveratrol	181186 ± 1303	-16059 ± 803	0.9998	0.474	1.579
Trans-polydatin	64539 ± 221	4280 ± 214	0.9999	0.226	0.753

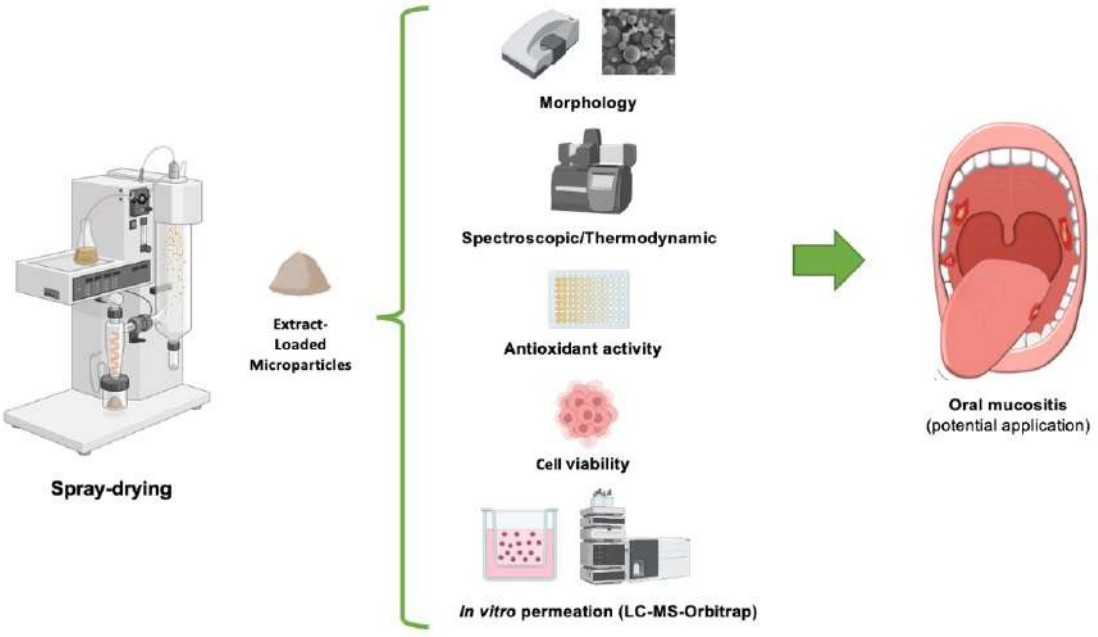
[a]m: slope ± standard deviation (*n* = 5) expressed in μV min/mg L; [b]b: intercept ± SD (*n* = 5) expressed in μV min; [c]LOD: limit of detection; [d]LOQ: limit of quantification.

Chapter 3

Incorporation of Chestnut Shells Extract into Spray-Drying Microparticles

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Statement of contribution:

The contribution of the first author, Ana Sofia Ferreira, in this work, includes the literature review, investigation, methodology and writing of the original draft.

3.1. Introduction

Oral Mucositis (OM) is a common and debilitating side effect of cancer treatments, being characterized by pain, erythema, and ulceration that can significantly impair the patient's nutritional intake and disrupt the cancer treatment regimens. Currently, there are no effective therapies to prevent or treat OM, with almost standard managements being primarily palliative [264]. (Poly)phenols, a diverse group of plant secondary metabolites, have attracted an exponential attention of the pharmaceutical industry owing to their outstanding biological properties, low toxicity, and broad range of health-promoting properties, and are promising candidates for the design and development of novel pharmacological applications, such as OM treatment [337, 338]. The antioxidant, anti-inflammatory, antitumor, and antibacterial activities of (poly)phenols have been deeply reported [339]. Nevertheless, oral administration of (poly)phenols has several drawbacks that difficult the *in vivo* therapeutic effects. Factors such as low water solubility, poor absorption, and rapid metabolism, may significantly reduce the oral bioavailability of (poly)phenols. Additionally, (poly)phenols are highly unstable and sensitive to environmental conditions, particularly temperature, light and oxygen, as well as some human body characteristics, such as acidic pH, and digestive system enzymatic activity [337, 340].

Among natural matrixes, chestnut shells (CS) (*Castanea sativa* Mill.) is an excellent source of (poly)phenols. This by-product is generated in large quantities by chestnut industry, particularly the one located in Southern Europe. The extraction of CS compounds demonstrated their richness in (poly)phenols, including phenolic acids (gallic acid and protocatechuic acid), flavonoids (epicatechin and quercetin) and ellagitannins, as well as vitamins and amino acids [341]. The most recent studies of our research team proved the *in vitro* (through *in vitro* gastrointestinal digestion coupled or not to intestinal co-culture models) and *in vivo* (using mice models) antioxidant/antiradical activities of CS extract, suggesting its potential role in mitigating the development of several diseases induced by oxidative stress, such as OM [341, 342].

Encapsulation is a highly effective strategy to delivery sensitive bioactive compounds with precision, such as CS extract (poly)phenols, allowing their protection until release at targeted locations and achieving controlled release profiles [343, 344]. This approach involves the incorporation of active ingredients into coating materials, which can be accomplished by different methodologies including spray drying [344]. Spray drying is an encapsulation technique particularly common owing to its simplicity,

fast processing, cost-effectiveness, and scalability, being reported excellent results in what concerns to (poly)phenols encapsulation [343, 345].

The main goal of this study is to produce microparticles of (poly)phenolic-rich CS extract for potential prevention or treatment of OM. This research addresses sustainability concerns by promoting waste valorization, utilizing a cost-effective raw material that aligns with circular economy principles. This approach seeks to reduce the environmental impact, while enhancing the economic sustainability of chestnut and pharmaceutical industries. To achieve this objective, the process yields, encapsulation efficiency, and particle characterization in terms of size, hydration ability, antioxidant/antiradical activities, and cytotoxicity in oral cell lines were assessed, and *in vitro* buccal permeation in a 3D model was performed.

3.2. Materials and Methods

3.2.1. Chemicals

All chemicals, solvents (HPLC-HRMS grade), and standards were provided by Sigma-Aldrich (Steinheim, Germany), Sigma Chemical Co. (St. Louis, MO, USA), Merck (Darmstadt, Germany) and Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). Eudragit® RS 30 D was kindly supplied by Evonik Operations GmbH. TR146 and HSC-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

3.2.2. *Castanea sativa* shells extract

C. sativa shells were provided by Sortegel, located in Sortes, Bragança, Portugal. After dehydration and shredding, the samples were subjected to subcritical water extraction (SWE), as outlined by Ferreira *et al*. [341]. Briefly, CS were extracted at 110 ºC using a homemade subcritical batch-type extractor (1.7 L) and pressurization with 99.99% nitrogen (Messer) pressurization. The extraction was performed during 30 min at 20 bars of pressure, with a sample-to-solvent ratio of 1:30. The extraction vessel was agitated through a vibrating platform (3 Hz) and cooled with a flow-through water bath (20 ± 2 °C) [341]. Subsequently, the liquid extract was centrifuged at 11 000 rpm for 10 min to eliminate any residual pellet.

3.2.3. Encapsulation by spray-drying

Spray-drying was carried out in a mini spray dryer BÜCHI B-290 (Flawil, Switzerland) with a liquid feed volumetric flow of 4 mL/min (15%), drying air inlet temperature of 115 °C, outlet temperature between 55-60 °C, standard 0.5 mm nozzle, aspiration rate 100% (36 m3/h) and 5-6 bar of air pressure [346]. Eudragit® RS 30 D was used as coating material (30%, w/w). A blank only consisting only of the encapsulating agent and water was also prepared. The extract loaded solution fed to the spray-dryer were prepared by mixing 4 mL of CS extract with 6.66 mL of encapsulating agent solution (3%, v/v) and 186 mL of distilled water, based on initial testing.

3.2.4. Microencapsulation yield

The yield of the processed powder was calculated considering the ratio between the total amount of powder collected from the collection vessel and the total quantity of extract and/or excipients in the feed solution [346]. The results were determined according to Eq. (2):

$$\text{Process Yield (\%)} = \frac{\text{Powder after spray-drying}}{\text{Solids introduced in the spray-drying feeding solution}} \times 100 \qquad \text{Eq (2)}$$

3.2.5. LC-ESI-LTQ-Orbitrap-HRMS analysis

The identification and quantification of the phenolic compounds present in the CS extract and CS-loaded microparticles were assessed using LC-ESI-LTQ-Orbitrap-HRMS, following the methodology used by Pinto *et al*. [347]. Quantitative analysis was performed according to a validated chromatographic method [348]. Calibration curves were prepared from a mixture of phenolic standards that were expected to be found in CS extract using six different concentrations, ranging from 0.05 to 1 ppm for chlorogenic acid, 2,5-dihydroxybenzoic acid, phloridzin, quercetin-3-*O*-galactoside, protocatechuic acid, *o*-coumaric acid, trans-polydatin, castalagin, neochlorogenic acid, 2,6-dihydroxybenzoic acid, gallic acid, apigenin, luteolin, naringin, rutin, 3-hydroxyphenylacetic acid, 3,5-di-caffeoylquinic acid, dihydroxyphenylpropionic acid 3-hydroxybenzoic acid, epicatechin, *p*-coumaric, ferulic acid, secoisolariciresinol, isorhamnetin, dihydroferulic acid, vanillin, catechin and methyl gallate. Phenolic metabolites were quantified based on the calibration curves of the parent compounds that originated them. In the absence of the parent compound, the chemically most similar standard was selected.

3.2.6. Encapsulation efficiency

The encapsulation efficiency (%) was calculated as the ratio of the mass of each bioactive compound encapsulated within the microparticles to the mass of each bioactive compound in the preparation of the feed solution for the spray-dryer, considering the process yield (Eq. (3)) [349]. The mass of each bioactive compound was analyzed by LC-ESI-LTQ-Orbitrap-HRMS (section 3.2.5).

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{mass of each encapsulated compound}}{\text{mass of each compound in the spray-drying feed solution}} \times 100 \quad \text{Eq (3)}$$

3.2.7. Microparticles characterization

3.2.7.1. Microparticles morphology

The scanning electron microscopy (SEM) was employed to provide detailed imaging of the microparticles morphology, including their shape, size, and surface features. Microparticles were analyzed using a Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M (Eindhoven, The Netherlands) [349]. The microscope was operated at 15 kV accelerating voltage and magnification of 100–50,000 X, in a JEOL JFC-100 apparatus. Samples were fixed in a brass stub using double-sided adhesive tape and then coated by a thin layer of gold.

3.2.7.2. Microparticles size and distribution

The particle size and distribution and key size metrics such as percentiles and span, which are critical for understanding uniformity and behavior of microparticles, were determined in a particle size analyzer by laser diffraction with a Mastersizer 3000 (Malvern Instruments, Worcestershire, UK) [350]. Briefly, samples were dispersed in MilliQ water (500 mL) under constant stirring (2000 rpm) using a Hydro EV dispersion unit to achieve a homogeneous suspension. Size, the percentiles 10, 50, and 90 of the equivalent spherical diameters based on volume (Dv10, 50 and 90), and the span were provided by the Mastersizer's software (version 3.63, Malvern Instruments; Worcestershire, UK) and considered for the microparticles characterization.

3.2.7.3. Moisture content and swelling capacity

The microparticles moisture content and swelling capacity were assessed by determining the water content before and after hydration, according to Campos *et al*. [350], with minor modifications. Initially, 150 mg of dry microparticles were weighed and

placed in an infrared moisture balance AD-4713 (A&D Company; Tokyo, Japan). An infrared lamp heated the microparticles at 103 °C for 60 min, and the automatically calculated the moisture percentage based on the weight differential before and after heating. Subsequently, new microparticles were hydrated for 6 h using artificial saliva (pH 6.9) and then collected by filtration using Whatman nº 1 paper to remove excess water. The increase in water mass was determined as an indirect measure of the microparticles swelling capacity.

3.2.7.4. Total phenolic content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu method [341]. Gallic acid was used as standard (linearity range: 5–100 µg/mL; R^2 > 0.998), and the results were expressed as mg of gallic acid equivalents (GAE) per gram of microparticles (mg GAE/g).

3.2.7.5. Ferric reducing antioxidant power (FRAP) Assay

The FRAP assay was performed following the protocol described by Ferreira *et al*. [341]. Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was the standard solution (linearity range: 25–500 µM; R^2 > 0.996). The results were expressed in µmol of ferrous sulphate equivalents (FSE) per gram of microparticles (mg FSE/g).

3.2.7.6. DPPH radical scavenging activity assay

DPPH free radical scavenging assay was carried out in accordance to Pinto *et al*. [8]. Trolox was used as standard (linearity range: 5–125 µg/mL; R^2 > 0.998). Results were expressed as mg of Trolox equivalents (TE) per gram of microparticles (mg TE/g).

3.2.7.7. Differential scanning calorimeter (DSC)

Differential Scanning Calorimeter (DSC) was performed to evaluate the thermal properties of the produced microparticles and on the lyophilized shells extract. This technique aimed to identify thermal transitions, such as melting points or glass transitions, which are crucial for understanding the thermal stability. These studies were conducted using a DSC 200 F3 Maia (Netzsh–Gerätebau GmbH, Germany). Samples were sealed in an aluminum pans with a perforated lid, using the following setting parameters: temperature from 0 °C to 200 °C, and heating rate of 10 °C/min. The onset temperatures were calculated through the Proteus Analysis software (Version 6.1, Netzsh-Gerätebau GmbH, Germany).

3.2.7.8. Rheological behavior

The rheological properties, such as the flow and deformation characteristics of blank and loaded microparticles was evaluated according to Sharkawy *et al*. [351], with minor modifications. A rotational rheometer with a plate-plate configuration (geometry PU20 SR4367) was used (Malvern Kinexus Lab+, Germany) at a temperature of 25 °C and with a 1 mm gap between plates, in an oscillatory mode. A small amount of sample volume was inserted on the bottom plate of the equipment, and the torque was applied to the upper plate, with an initial frequency of 0.10 Hz and a final frequency of 10.00 Hz, and a 0.25% strain (inside the linear viscoelastic region - LVER). The rSpace Kinexus Lab+ (Version 1.75) software was employed to calculate viscosity.

3.2.7.9. Fourier transform infrared spectroscopy (FTIR)

The interactions between the polymer used and the encapsulated extract, and thus their compatibility and stability, were assessed using a FTIR Nicolet 6700—diamond point (ThermoFisher Scientific, Waltham, MA, USA), and employing the potassium bromide (KBr) disc method [352]. Each of the microparticle powders were separately loaded into the sampler, and spectra analysis was conducted within a range of 4000 and 400 cm^{-1}, with 32 scans at a resolution of 4 cm^{-1}.

3.2.8. Cell viability assays

Cell viability assays were carried out utilizing passages 31-32 of TR146 and 23-24 of HSC-3 cells [341]. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed following the procedure described by Pinto *et al*. [8], and employing DMEM and Triton X-100 at 1% (*w*/*v*) as positive and negative controls, respectively. The results were expressed as percentages (%) of cell viability.

3.2.9. *In vitro* buccal permeation model

The *in vitro* permeability of the bioactive compounds present in microparticles was carried out in a co-culture buccal model composed by TR146 cells [322]. The extract and the CS-loaded microparticles were added to the apical side of the model. A sample from the basolateral side was collected at different timepoints (0, 15, 30, 45, 60, 90, 120, 150, 180, and 240 min). Samples were analyzed by LC-ESI-LTQ-Orbitrap-HRMS (section 3.2.5). The TEER of the model was evaluated before, during and at the end of the

permeability assay using an EVOM Epithelial Volthometer Instrument equipped with a chopstick electrode (World Precision Instruments, Sarasota, FL, USA).

3.2.10. Statistical analysis

Results are presented as mean ± standard deviation from a minimum of three independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's HSD tests with IBM SPSS Statistics 28.0 software (Chicago, IL, USA). Statistical significance was considered as $p < 0.05$.

3.3. Results and discussion

3.3.1. Process yield and encapsulation efficiency

The selected spray-drying conditions resulted in process yields for blank and CS-loaded microparticles, respectively 39.73% and 43.90% [349]. These results were good considering the laboratorial scale and comparing with other studies of the literature [343, 349, 353, 354]. Specifically, Ribeiro *et al*. reported process yields of elderberry extract ranging from 25 to 41% *via* spray-drying, whereas Estevinho *et al.* demonstrated that yields for various types of spray-drying microparticles varied between 26.5 and 65.5% [343, 349]. The small volume of liquid feed (200 mL) and low solid content sprayed, as well as the loss of the smallest and lightest particles during spray-drying atomization, are the main reasons for the decrease in the process yield. The use of low inlet temperatures (115 °C) also leads to higher particle deposition on the cylinder, decreasing the process yield [20]. In the present study, the incorporation of the extract increased the product yield, which could be associated with a higher interaction between the core material and the polymeric carrier, which probably occurs *via* covalent and hydrogen bonds, and electrostatic interactions. These results are in accordance with previous studies that reported similar process yields for natural matrix encapsulation [343, 349, 353, 354].

The compounds present in the CS extract and CS-loaded microparticles were identified and quantified using LC-ESI-LTQ-Orbitrap-HRMS, as well as the encapsulation efficiency (EE) of the CS-loaded microparticles (Table 11).

Table 11 - Quantification of *C. sativa* shells (CS) extract bioactive compounds incorporated in CS-loaded microparticles and their encapsulation efficiency (%). Results are expressed as mean ± SD (*n* = 3).

Compound/Metabolite	Quantification of CS extract (mg/ml)	Encapsulation efficiency of CS-loaded microparticles (%)
Succinic acid	1.78 ± 0.13	16.46 ± 8.10
Myo-Inositol	0.31 ± 0.01	24.19 ± 2.41
Pyrocatechol	0.20 ± 0.03	33.73 ± 4.25
Protocatechuic acid	0.29 ± 0.15	28.68 ± 14.36
Homovanillic acid	0.36 ± 0.01	48.16 ± 1.41
4-Hydroxybenzoic acid	1.66 ± 0.25	17.87 ± 7.68
Vanillin	0.54 ± 0.05	98.59 ± 8.32
Sebacic acid	36.02 ± 0.89	21.65 ± 1.72
Hydroxyadipic acid	0.27 ± 0.01	25.87 ± 3.68

Nine compounds were quantified, while the others were only identified because they were present in lower amounts (Supplementary Material Table S2). The EE ranged between 16.46% and 98.59% for succinic acid and vanillin, respectively, while the EE average for all analyzed compounds was 35.02%. Pyrocatechol (33.73%), protocatechuic acid (28.68%), homovanillic acid (48.16%), 4-hydroxybenzoic acid (17.87%) and vanillin (98.59%) were the main (poly)phenols incorporated in CS-loaded microparticles. These results are in accordance with Pinto *et al*. [355] that also quantified these compounds in supercritical fluid extract of chestnut shells at 60 °C.

Particularly, protocatechuic acid, 4-hydroxybenzoic acid, catechol, vanillic acid, and vanillin were the main (poly)phenols quantified, confirming the richness of CS extract and indicating that the use of 110 °C in our SWE extraction did not result in significant thermal degradation of these bioactive compounds [355]. Given that the CS extract comprises a mixture of compounds with distinct properties, the variability in EE values can be partially attributed to the nature of the metabolites and different affinities to the matrix [356]. Additionally, (poly)phenolic compounds are susceptible to the influence of temperature during spray-drying process, although for short times of exposure [357, 358]. Therefore, controlling the room temperature is essential to minimize the variability in EE [359], which was significant for succinic acid, protocatechuic acid, and 4-hydroxybenzoic acid.

Protocatechuic acid (PCA) has been studied for its potential biological activities, namely anti-inflammatory, antioxidant, and antimicrobial properties [359, 360]. The ability of PCA to mitigate the oxidative stress-related conditions is noteworthy. PCA inhibits free radicals by downregulating enzymes, such as xanthine oxidase (XOD) and NAPH

oxidase (NOX), as well as by reducing the levels of malondialdehyde (MDA), an oxidative stress marker [359]. Additionally, PCA enhances the activity of endogenous antioxidant enzymes, including glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD), thereby strengthening the body's defense mechanisms against oxidative damage, as well as the ability to promote wound healing [359-361]. Besides *in vitro* results, *in vivo* assays revealed that PCA reduce the carrageenan-induced inflammation by decreasing the levels of tumor necrosis factor-alpha (TNF-α), interleukin-beta (IL-β), and prostaglandin E2 (PGE2). Additionally, PCA may prevent the lipopolysaccharide (LPS)-induced sepsis in mice by decreasing the nitric oxide (NO) levels and suppressing the interleukin-10 (IL-10) [360]. Furthermore, the dietary administration of PCA showed protective effects at all stages of carcinogenesis in tongue cancer, a subtype of head and neck squamous carcinoma, in which 100% of the patients experience OM [264, 359].

Sebacic acid, a dicarboxylic acid, exhibits anti-inflammatory properties by inhibiting the production of nitric oxide (NO) and effectively suppressing TNF-α, thereby attenuating the inflammatory responses [362]. Moreover, it regulates the phosphorylation of ERK1/2 and p38 MAPK, crucial signaling pathways involved in cellular stress responses, while also reduce the JNK 1/2 phosphorylation [362, 363]. These mechanisms are pivotal in modulating the cellular stress signaling. Additionally, sebacic acid suppresses the transcriptional activity of key inflammatory genes, such as inducible nitric oxide synthase (iNOS), IL-10, TNF-α, and cyclooxygenase-2 (COX-2) mRNA by inhibiting the activation of NF-κB, a critical transcription factor in inflammatory pathways [362, 363]. Therefore, these bioactive compounds are crucial for the OM management.

3.3.2. Microparticles characterization

3.3.2.1. Morphology

The structure and size of the CS-loaded and blank microparticles were analyzed using SEM (Figure 5).

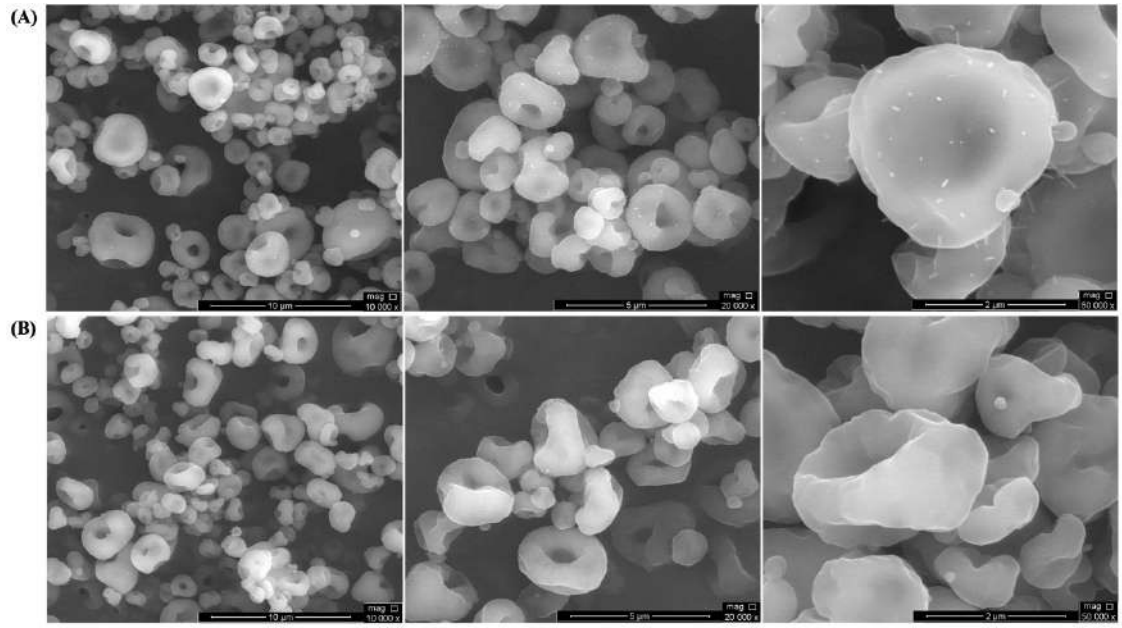


Figure 5 - Scanning electron microscopy (SEM) micrographs of blank microparticles (A) and *C. sativa* shells extract encapsulated microparticles (B) by spray-drying. Magnification of 10,000, 20,000 and 50,000x, beam intensity (HV) of 15 kV, distance between sample and lens (WD) around 10 mm.

Both formulations showed similar morphologies, presenting spherical biconcave disk forms, a heterogeneous size distribution, and no visible pores. The same shape was observed for spray-drying microparticles prepared with Eudragit L100 [364] and maltodextrin [365]. Micrographs also reveal agglomerates in both types of microparticles, which is confirmed by the bimodal volume distribution curve (Figure 2A), appearing to have the same size, which is in line with other Eudragit RS 30D microparticles [366]. These results attest that the incorporation of CS extract as core material did not affect the morphology of the microparticles produced.

3.3.2.2. Particles size and distribution

Particles size have a huge impact on the functionality and stability of final products, being a fundamental parameter according to the particle's final application [349]. Particles with sizes ranging from 1 to 1000 μm are called microparticles. Particles under this range are generally called nanoparticles. For pharmaceutical application, the particles size needs to be carefully controlled, since small ones may pass into bloodstream and penetrate cells, causing DNA damage [367]. On the other hand, larger particles can lead to the clogging of blood vessels. Particularly, particles larger than 100 nm remain at the site of administration until phagosomal clearance, which is ideal for sustained drug delivery systems for local action [367]. Size is highly influenced by spray-

drying conditions, namely the nozzle type (diameter), the spray gas flow, the feed rate, and the solid concentration of the feed solution, conditions that were kept constant during all process [349].

The size of blank and CS-loaded microparticles were evaluated by laser diffraction (Figure 6). Regarding volume distributions, the particle size was similar for blank and CS-loaded microparticles, respectively 2.90 µm and 3.23 µm, without significant differences ($p > 0.05$). Both microparticles revealed a distribution curve with two peaks (bimodal distribution), which is associated with the agglomeration phenomenon [368] and can be observed at lower magnification in the SEM micrographs. Furthermore, blank microparticles presented Dv10, Dv50, and Dv90 values of 0.31 µm, 1.94 µm and 3.71 µm, respectively, and CS-loaded microparticles achieved results of 0.38 µm, 1.95 µm and 3.22 µm, respectively, without significant differences ($p > 0.05$). The extract encapsulation did not result in an increase in particle size compared to the blank ones.

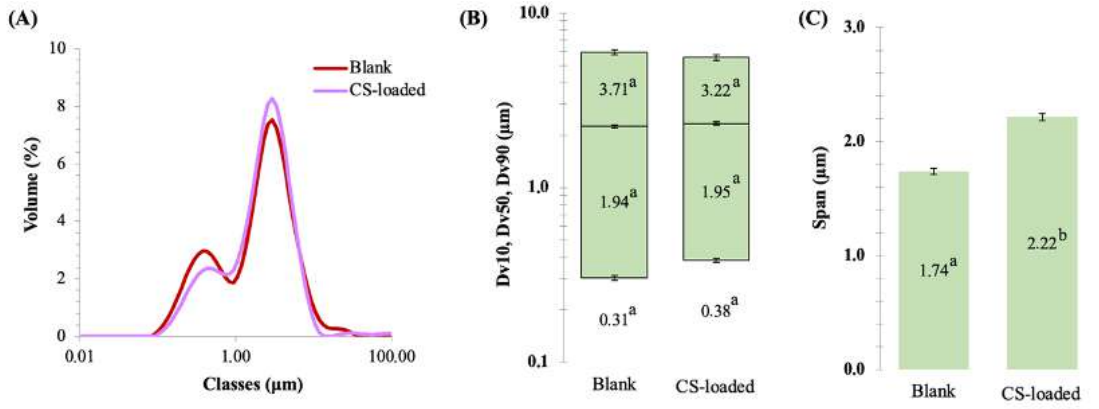


Figure 6 - Evaluation of blank and CS-loaded microparticles size distribution over volume (A), size distribution with Dv10 (bottom of the rectangles), Dv50 (middle lines), and Dv90 (top of the rectangles) (B), and span (C). Results are expressed as mean ± standard deviation ($n = 3$). Different letters (a,b) indicate statistical differences ($p < 0.05$).

Smaller span values indicate a narrow particle size distribution, demonstrating uniformity [369]. The CS-loaded microparticles showed a higher span value than the blank ones (2.22 µm vs 1.74 µm), indicating a broader size distribution and lower uniformity. This lower uniformity could potentially affect the performance of CS-loaded microparticles, such as their release profile and stability. However, it is noteworthy that the span value of our CS-loaded microparticles is considerably lower when compared to the spray-drying microparticles prepared with *Lannea macrocarpa* extract (4.75-21.09 µm) [370] or grape cane extract (6.15 µm) [369]. Further optimization of the formulation

and processing parameters may be necessary to enhance the uniformity of CS-loaded microparticles.

3.3.2.3. Moisture content and swelling capacity

Spray-drying is a drying process that uses temperature to dry the feed solution to produce solid microparticles. Nevertheless, the use of temperature can affect thermolabile bioactive compounds, reducing the encapsulation effectiveness [365]. Martinic *et al*. tested lower inlet temperatures (100-130 °C) for spray-drying plant extracts and faced challenges with a high powder moisture content, which affected the shelf life of the particles [365]. In the present study, an inlet temperature of 115 °C was employed to preserve the thermolabile (poly)phenols and prevent their conversion into other compounds [341].

Blank and CS-loaded microparticles exhibited moisture contents of 3.51% and 4.33%, respectively, without significant differences ($p > 0.05$) (Table 12). These results are in line with those obtained for the encapsulation by spray-drying of *Murraya koenigii* leaf extract with maltodextrin and gum arabic and employing an inlet temperature of 165 °C, achieving a moisture of 3.03% [371]. Moreover, the results obtained are in accordance with those reported by Navarro-Flores *et al*. for *Crotalaria longirostrata* leaf extract microencapsulation using different coating materials at 120 °C, which ranged between 3.01% and 4.31% [358]. These results indicate that the optimized conditions of the spray-drying process can allow the use of low inlet temperatures to obtain optimal moisture content.

The swelling capacity of the formulated microparticles was evaluated using artificial saliva at 6.9 pH to mimic the buccal conditions, since this property is influenced by pH variations as well as the presence of electrolytes, despite often being under-evaluated [372, 373]. Swelling capacity greatly impacts the drug release rate by increasing the volume of microparticles, enhancing the diffusion of the core material [374]. For sustained release purposes, a lower swelling capacity is preferable, while greater swelling abilities are advantageous for mucoadhesive properties and might be beneficial for local drug delivery [367, 375, 376]. CS-loaded and blank microparticles showed swelling capacities of 98.16% and 73.88%, respectively, with significant differences ($p < 0.05$), indicating that the CS extract incorporation affected the microparticles capacity to absorb water (Table 12). This significant increase can be attributed to the hydrophilic nature of the (poly)phenols present in the CS extract, which enhances the water absorption and retention. Additionally, the structural modifications

imparted by (poly)phenols may create a more porous matrix, allowing for greater swelling [373, 374].

Table 12 - Moisture content (%) and swelling capacity (%) of blank and CS-load microparticles. Results are expressed as mean ± SD (*n*= 3).

	Moisture content (%)	Swelling capacity (%)
Blank microparticles	3.51 ± 0.24	73.88 ± 12.51
CS-Loaded microparticles	4.33 ± 1.96	98.16 ± 7.05

3.3.2.4. Phenolic content and antioxidant/antiradical activities

The microencapsulation of bioactive compounds enhances their stability and enables a controlled release, improving the therapeutic efficacy, and providing protection against oxidative damage, increasing its application in pharmaceutical field [377]. The phenolic content, as well as the antioxidant and antiradical activities, are critical aspects for the effectiveness of natural extracts therapeutic delivery systems [378]. Table 13 summarizes the TPC, FRAP, and DPPH results for CS-loaded and blank microparticles.

Table 13 - Total Phenolic Content (TPC), antioxidant (FRAP) and antiradical activity (DPPH) of blank and CS-loaded microparticles. Results are expressed as mean ± SD (*n* = 3). Different letters (a,b) in the same column mean significant differences ($p < 0.05$).

	TPC (mg GAE/g)	FRAP (µmol FSE/g)	DPPH (mg TE/g)
Blank microparticles	6.54 ± 0.77^{a}	28.43 ± 3.72^{a}	2.48 ± 0.40^{a}
CS-loaded microparticles	9.73 ± 1.31^{b}	91.76 ± 5.85^{b}	7.83 ± 0.84^{b}

GAE: Gallic Acid Equivalents; FSE: Ferrous Sulphate Equivalents; TE: Trolox Equivalents

As expected, the CS-loaded microparticles exhibited higher values in all assays than the blank ones, indicating the incorporation of (poly)phenolic compounds and confirming the encapsulation effectiveness [342]. CS-loaded microparticles achieved a TPC of 9.73 mg GAE/g, a result significantly ($p = 0.043$) higher than the one observed for blank microparticles, namely 6.54 mg GAE/g. In what concerns to the FRAP assay, CS-loaded microparticles exhibited high levels of antioxidant activity, respectively 91.46 µmol FSE/g. This value was significantly greater ($p = 0.025$) compared to the blank microparticles (28.43 µmol FSE/g). Additionally, in the DPPH assay the CS-loaded microparticles demonstrated significantly ($p = 0.038$) higher antiradical capacity (7.83 mg TE/g) than blank microparticles (2.48 mg TE/g). These results are consistent with the

ones reported by Cea-Pavez *et al*. for propolis extract loaded microparticles prepared with different coating materials (inulin, pectin, sodium alginate and chitosan), which TPC ranged between 7.1 and 10.9 mg GAE/g [379]. Similarly, Gomes *et al*. determined FRAP values of 122.50 µmol FSE/g for loaded microparticles composed of strawberry aqueous extract encapsulated with inulin [380].

Ferreira *et al*. previously evaluated the lyophilized CS extract using the same assays and reported a TPC of 239.53 mg GAE/g DW, and a FRAP of 4240.38 µmol FSE/g [341]. Unlike the current study, the authors calculated the mean inhibitory concentration (IC_{50}) for the DPPH radical (IC_{50} = 426.88 µg/mL) [341]. The decreased activity observed in the loaded microparticles compared to the CS extract is indicative for the microencapsulation success. The spray-drying process effectively encapsulated bioactive compounds within the microparticles, protecting them from external factors and reducing their reactivity. Consequently, microparticles demonstrated lower activity than the CS extract [349].

3.3.2.5. Thermal and rheological behavior

Spray-drying process can be affected by various factors, such as the solvent type, temperature range, and solvent evaporation rate, which can influence stability, interactions polymer-extract and amorphous/crystalline ratio of the resulting microparticles [381]. Figure 7 shows the DSC curves of the CS extract, as well as CS-loaded and blank microparticles.

As can be observed, all curves exhibit a broad endothermic peak between 78.21 °C and 111.07 °C. These transitions are associated with water loss through evaporation (generally 2-10% of weight), related to the hydrophilic groups present in (poly)phenols and polymers [381]. Particularly, the CS extract curve shows a peak at 103.76 °C that probably corresponds to the melting point. Additionally, the blank microparticles curve, primarily composed of the coating material, aligns with the literature data, as the polymer (Eudragit RS-PO) presents a broad endothermic peak between 60 °C and 80 °C [382]. The shift of the CS-loaded microparticles to a higher temperature indicates a better thermal stability, which might be due to the interaction between CS extract and the microparticles matrix (e.g., hydrogen bonding, van der Waals forces), and to a distinct moisture content, leading to enhanced structural stability [382]. Moreover, the absence of intense peaks indicates that the sample is not pure, which was anticipated since the CS extract is a mixture of bioactive compounds, while Eudragit RS 30D is a copolymer

of ethyl acrylate, methyl methacrylate and methacrylic acid ester [382]. This polymer also has a low glass transition temperature, which was not observed in Figure 7 [383].

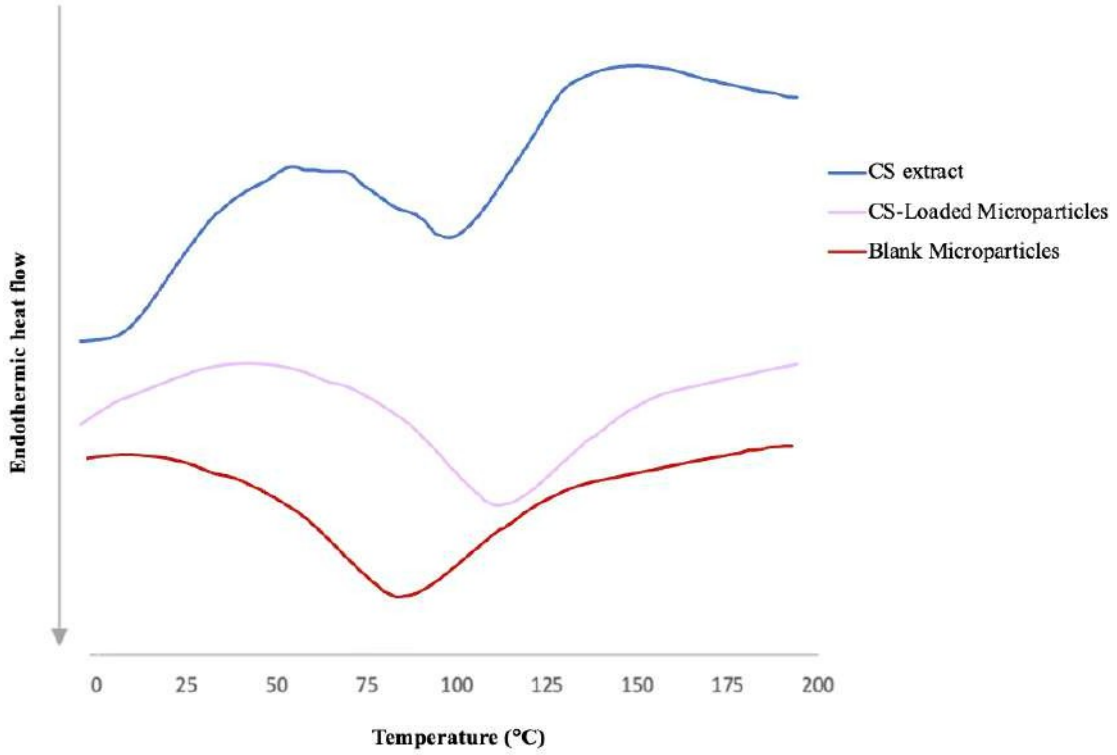


Figure 7 - Differential Scanning Calorimeter (DSC) thermograms of *C. sativa* shells (CS) extract, CS-loaded microparticles and blank microparticles.

The microparticles rheological behavior evaluation is a crucial step to obtain insights into the formulation stability, processability, performance and patient acceptability [383]. Figure 8 shows the rheological properties of CS-loaded and blank microparticles, illustrating the relationship between shear rate and viscosity.

The viscosity curves for both formulations demonstrate a non-Newtonian shear thinning behavior [384], indicating that microparticles become less viscous when subjected to higher shear forces, which is a typical behavior for many polymeric systems [384] . At low shear rates, the viscosity is relatively high for both formulations. During manufacturing or administration, this behavior ensures that microparticles can easily flow under mechanical stress. Once the stress is removed, the viscosity increases, which is advantageous for maintaining stability and facilitating controlled release [383]. The higher initial viscosity of blank microparticles compared to CS-loaded ones might be due to the absence of CS extract that could interact with the polymer matrix, altering the viscosity profile.

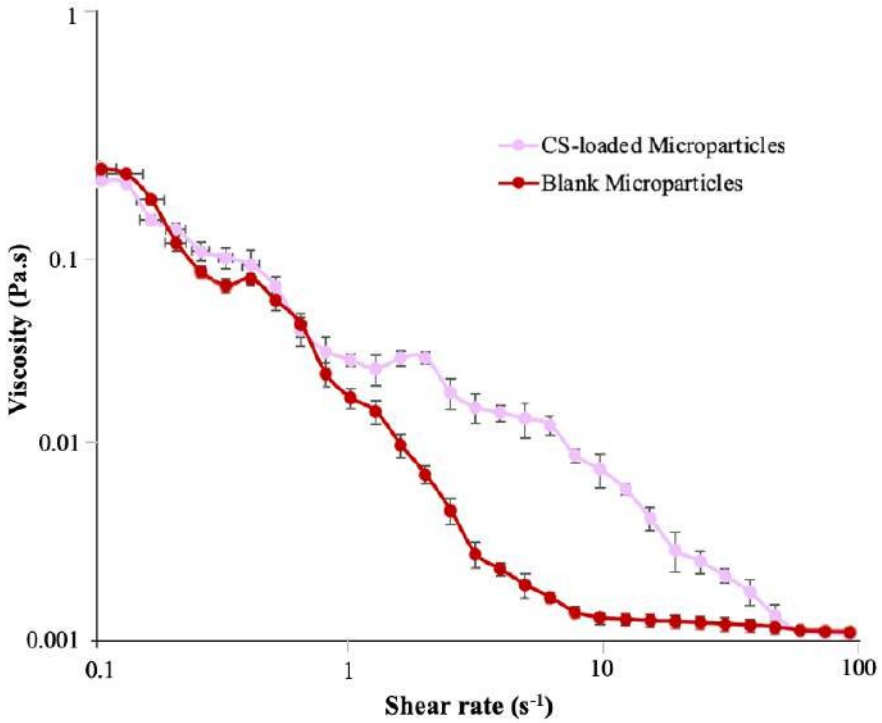


Figure 8 – Rheological behavior of blank and CS-loaded microparticles. Results are expressed as mean ± SD (*n* = 3).

3.3.2.6. Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra of CS extract, CS-loaded and blank microparticles are shown in Figure 9.

The CS extract spectrum (Figure 9A) revealed characteristic peaks at 1608 cm^{-1}, usually associated with C=O stretching vibrations, indicating the presence of carbonyl groups, possibly from phenolic acids and flavonoids, as well as peaks in the region between 1000 and 1500 cm^{-1}, that can be attributed to C-O stretching and bending vibrations, indicating the presence of alcohols, carboxylic acids, ethers, and esters [385]. The broad and intense peak at 3000-3500 cm^{-1} is typically associated with O-H and N-H stretching vibrations, demonstrating the presence of hydroxyl groups typical of (poly)phenols and other hydrophilic compounds present in the CS extract [386].

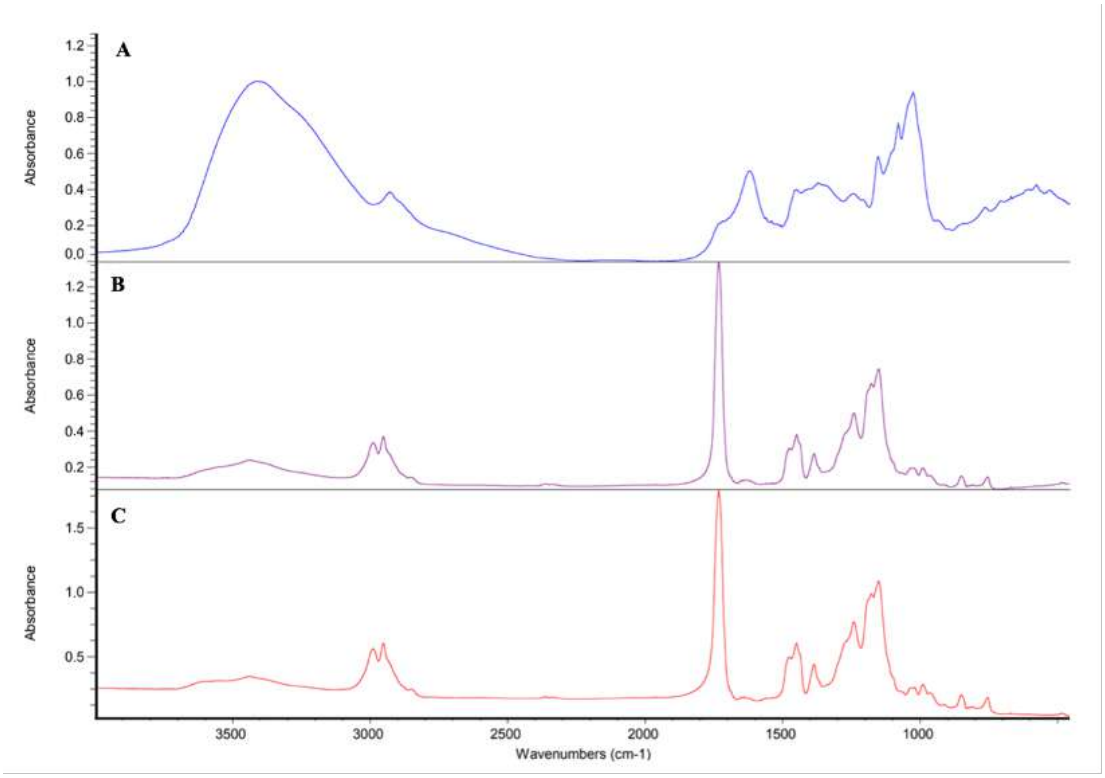


Figure 9 - Fourier-transform infrared spectroscopy (FTIR) spectrum of *C. sativa* shells (CS) extract (A), CS-loaded microparticles (B) and blank microparticles (C).

The blank microparticles spectrum (Figure 9C) showed a peak at 2953 cm^{-1}, attributed to C-H stretching, as well as peaks at 1150 cm^{-1} and 1239 cm^{-1}, corresponding to the carbonyl (C-O-C) groups in the polymeric structure, and a peak at 1728 cm^{-1}, consistent to the C=O stretching ester vibration [356]. The FTIR spectrum of the CS-loaded microparticles (Figure 5B) presents characteristic peaks closely resemble the blank microparticles. This similarity suggests that the peaks corresponding to the CS extract were likely obscured by the absorption bands of the coating material, ensuring the successful encapsulation of the CS extract within microparticles.

3.3.3. Cell viability

Figure 10 summarizes the cell viability results obtained for TR146 and HSC-3 cells after 24 h exposure to blank and CS-loaded microparticles at different concentrations.

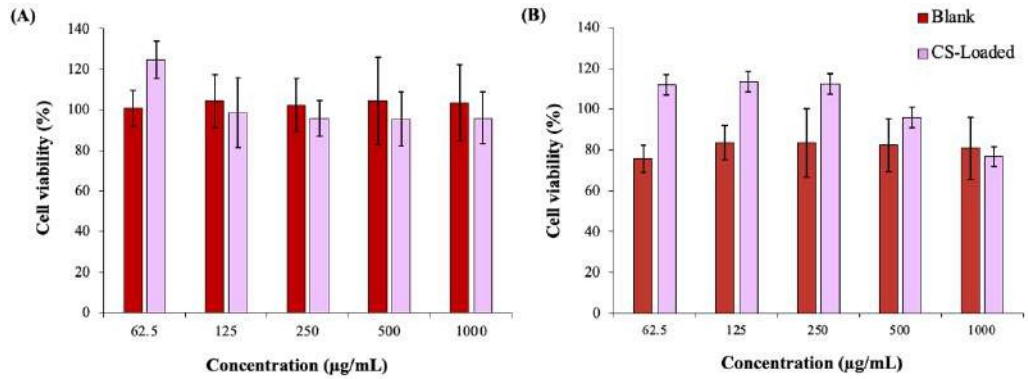


Figure 10 - Cell viability of TR146 and HSC-3 cells after exposure to blank and CS-loaded microparticles (62.5-1000 µg/mL), measured by MTT assay. Results are expressed as mean ± standard deviation (*n* = 3). **p* <0.05 means significant differences between groups at the same concentration.

The results indicated high cell viabilities across all experimental conditions, demonstrating excellent biocompatibility with both cell lines, without significant differences ($p > 0.05$). Notably, HSC-3 cells exhibited higher sensitivity when exposed to microparticles. Specifically, blank microparticles showed a mean cell viability of 83%, which did not correlate with concentration. However, the CS-loaded microparticles demonstrated a slight decrease in cell viability at the two highest concentrations when compared to the lowest ones ($p = 0.046$).

3.3.4. *In vitro* buccal model permeability

Bioactive compounds of interest must exhibit adequate permeability to reach effective concentrations in target tissues, such as the buccal mucosa [387]. The bioavailability and safety of CS-loaded microparticles was screened through an *in vitro* buccal model composed by TR146 cells. Table 14 summarizes the permeated identified compounds after the *in vitro* assay of CS-loaded microparticles at different time points, for up to 240 min.

The putative annotation of compounds in the CS extract and CS-loaded microparticles is shown in Supplementary Material Table 15, and Figure 11 shows the TEER values obtained during the *in vitro* model construction (A) and the permeation assay (B). TEER is a critical parameter for evaluating cell layer integrity in *in vitro* model experiments. In the present study, TEER values increased on the 8th day, suggesting the cellular growth and stability (171 ± 25 Ω/cm^2). The values ranged between 140.0 and 186.0 Ω/cm^2 during the permeability assay, attesting the cellular morphology preservation and the integrity of the cell monolayer.

Table 14 - Identification of (poly)phenolic compounds incorporated in CS-loaded microparticles that permeated through the *in vitro* buccal model (TR146 cells) at the different timepoints.

Rt (min)	$[M-H]^-$ (*m*/*z*)	Compound/Metabolite
0.82	863.2420	Verbascose
1.25	128.0353	Pyroglutamic acid
1.75	117.0191	Succinic acid
3.28	161.0444	Myo-Inositol
4.95	108.0483	Pyrocatechol
5.27	153.0196	Protocatechuic acid
5.40	180.0725	Homovanillic acid
5.56	137.0246	4-Hydroxybenzoic acid
5.86	151.0402	Vanillin
6.55	201.1137	Sebacic acid
13.82	161.0457	Hydroxyadipic acid

Eleven compounds were identified at the final permeation timepoint, being observed a higher abundance of (poly)phenols (46%), followed by lipids (18%), organic acids, alcohols, carbohydrates and amino acids (9% each, respectively). These results demonstrate the capability of the CS-loaded extract microparticles to operate as delivery system [387]. All the identified compounds exhibited biological properties of interest that may alleviate OM symptoms [264].

Vanillin is well recognized for its antioxidant, anti-inflammatory, and antibacterial activities [388]. Wei *et al*. demonstrated the efficiency of a vanillin hydrogel as wound healing promotor in a diabetic wound model [389], while Al Asmari *et al*. demonstrated that vanillin inhibits the pro-inflammatory state and oxidative stress by suppressing NF-κB, exhibiting anti-ulcerative effects in an *in vivo* rat model [390]. Likewise, pyrocatechol demonstrated anti-inflammatory activity by inhibiting LPS-induced activation of pro-inflammatory mediators (NF-κB) [391]. Moreover, Xie *et al*. highlighted pyrocatechol as a candidate compound against cytotoxic effects of commonly used chemotherapy drug cisplatin through *in vitro* and *in vivo* assays [392]. According to the authors, pyrocatechol effectively inhibited the oxidative stress-induced apoptosis in cisplatin-treated cells by activating glutathione peroxidase 4 and decreasing the ROS/JNK/p38 MAPK signaling pathway [392].

4-hydroxybenzoic acid also exhibits antioxidant, anti-inflammatory, anticancer, and antifungal activities that could be useful in OM management [393]. Han *et al*. revealed the positive effects of 4-hydroxybenzoic acid on an *in vivo* mice colitis model by increasing the serum levels of IL-10, an anti-inflammatory cytokine, and improving the

mucosal barrier through rising goblet cells and, consequently, the expression of mucins [394].

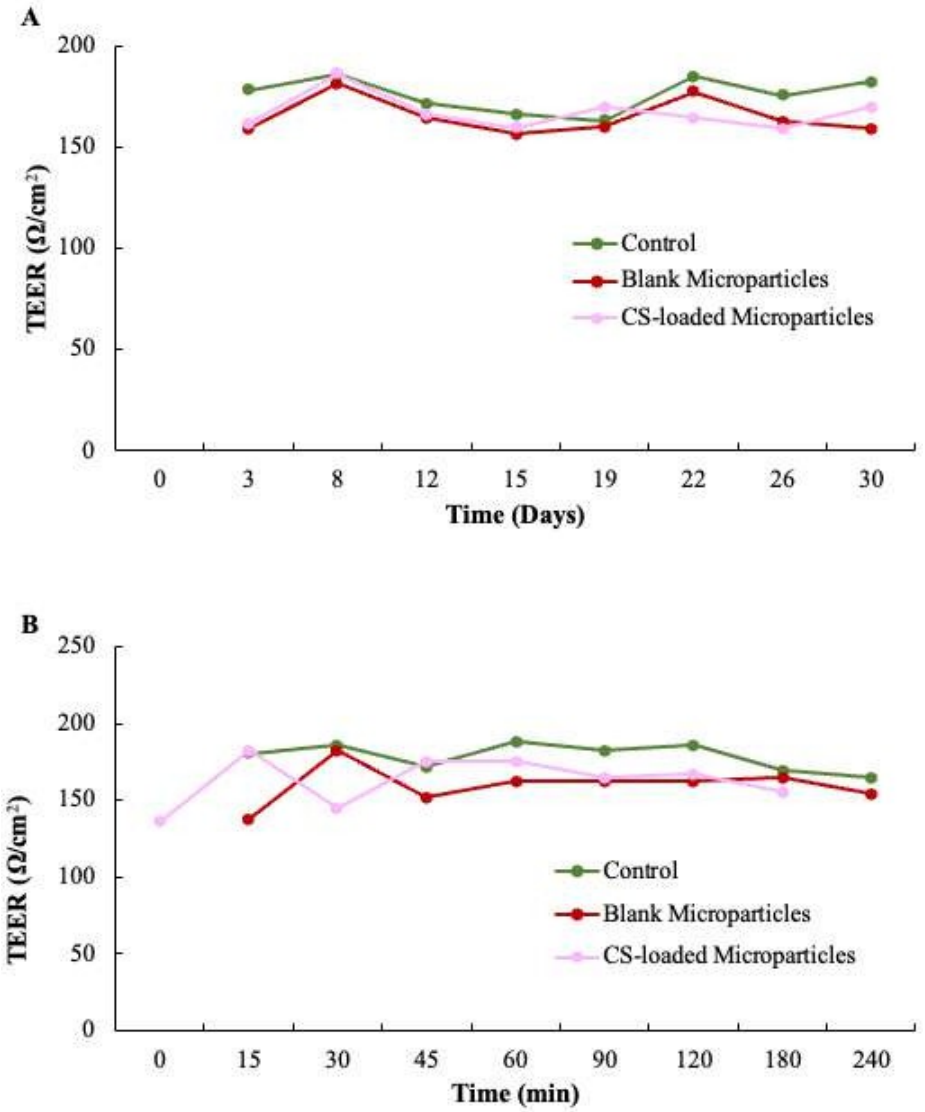


Figure 11 - Transepithelial electrical resistance (TEER) of the *in vitro* buccal model monitored during: (A) 31 days and (B) 240 min of the permeability assay.

Among other compounds, amino acids such as pyroglutamic acid have great hygroscopic properties that are important for the treatment of several skin conditions [395], and have also demonstrated antioxidant, antifungal, antibacterial and anti-inflammatory properties [396], which are key activities in OM treatment.

3.4. Conclusion

The present work provides a step forward in the valorization of CS as active ingredient for potential pharmaceutical applications in the prevention and treatment of OM. The spray-drying CS-loaded extract microparticles had a process yield of 44%,

which is considered common and excellent for this physical method at laboratory scale. The results also outlined the influence of incorporating CS extract into a heterogeneous size distribution, which is characteristic of this method. Moreover, the thermal and chemical results evidenced successful encapsulation process. Antioxidant/antiradical assays demonstrated the incorporation of CS extract (poly)phenols, which was confirmed by LC-ESI-LTQ-Orbitrap-HRMS analysis. The CS-loaded microparticles exhibited higher EE percentages for vanillin, homovanillic acid, pyrocatechol and protocatechuic acid. Furthermore, the *in vitro* viability of oral epithelial cells remained high after exposure to CS-loaded microparticles. Additionally, an *in vitro* permeability assay using a buccal model demonstrated the efficacy of these microparticles as delivery system, particularly with potential for the local treatment of OM. The next steps will be centered to enhance microparticle uniformity, stability assays, and *ex vivo* and *in vivo* permeability analyses to up-scale these results into marketed products.

Supplementary Material

Table S15 - Putative annotation of compounds detected by LC-ESI-LTQ-Orbitrap-HRMS analysis regarding untargeted metabolomic profiling using MS-dial and MS-finder platforms.

Rt (min)	Metabolite	Ontology	Neutral molecular formula	Average Mz	Theoretical mass	Error (ppm)	Total score	MS^1 isotopic spectrum
0.76	L-Malic acid	Hydroxy acid	$C_4H_6O_5$	133.0155	134.0215	-2.5373	7.23	134.01428:83116 135.01763:4074 136.02099:725
0.82	Verbascose	Carbohydrate	$C_{30}H_{52}O_{26}$	863.2420	863.2441	2.5381	6.81	863.26697:20574 864.27032:10099 865.27368:3349
1.14	Fumaric acid	Dicarboxylic acid	$C_4H_4O_4$	114.9631	115.0037	3.5164	6.67	115.00376:35380 116.00711:1547 117.01047:557
1.14	Isocitric acid	Tricarboxylic acid	$C_6H_8O_7$	191.0104	191.0202	4.1990	8.90	191.02008:1242036 192.02343:91598 193.02679:13890
1.25	Pyroglutamic acid	Amino acid	$C_5H_7NO_3$	128.0353	128.0355	2.1143	5.45	128.03522:67634 129.03857:4464 130.04193:1437
1.47	Xanthine	Purine	$C_5H_4N_4O_2$	151.0254	152.1143	3.1420	6.67	151.02547:108327 152.02882:5681 153.03218:266
1.75	Succinic acid	Dicarboxylic acid	$C_4H_6O_4$	117.0191	117.0191	3.1679	5.55	117.01947:242833 118.02282:10538 119.02618:1505
1.78	L-Tyrosine	Amino acid	$C_9H_{11}NO_3$	180.0751	181.0738	1.5371	7.43	180.91753:11073 181.92088:574 182.92424:304
1.94	Mevalonic acid	Fatty acid	$C_6H_{12}O_4$	147.0774	148.0735	3.9641	6.84	148.98022:19821 149.98357:363 150.98693:0
2.23	Gallic acid *	Benzoic acid	$C_7H_6O_5$	169.0143	169.0143	4.3446	5.18	169.01428:83201 170.01763:5937 171.02099:822
2.50	Adipic acid	Fatty acid	$C_6H_{10}O_4$	145.0583	146.0579	2.9864	6.85	146.0872:17256 147.09055:1360 148.09391:0

3.28	Myo-Inositol	Alcohols and polyols	$C_6H_{12}O_6$	161.0444	161.0457	5.6848	5.50	161.04607:715644 162.04942:42718 163.05278:8059
3.47	Quinic acid	Alcohols and polyols	$C_7H_{12}O_6$	191.0663	192.0633	3.3551	ND	191.05621:10822 192.05956:626 193.06292:0
4.13	Inosine	Carbohydrate	$C_{10}H_{12}N_4O_5$	267.0753	268.0807	4.6121	ND	268.80386:130934 269.80721:0 270.81057:344660
4.25	Shikimic acid	Alcohols and polyols	$C_7H_{10}O_5$	173.0483	174.0528	3.7482	7.84	174.08226:14201 175.08561:1232 176.08897:6558
4.37	L-Phenylalanine	Amino acid	$C_9H_{11}NO_2$	164.0751	165.0790	2.6254	8.26	165.05602:17963 166.05937:1566 167.06273:0
4.86	Panthothenic acid (Vit. B5)	Alcohols and polyols	$C_9H_{17}NO_5$	218.1034	218.1022	0.6427	5.37	218.10237:48219 219.10572:8398 220.10908:628
4.89	Vescalagin	Hydrolyzable tannin	$C_{41}H_{26}O_{26}$	466.0282	466.0290	3.5485	6.48	466.02896:63376 467.03231:11535 468.03567:412
4.95	Pyrocatechol	Benzenoid	$C_6H_6O_2$	108.0483	109.0295	0.0000	5.50	109.0295:112023 110.03285:8497 111.03621:531
5.05	Neochlorogenic acid *	Alcohols and polyols	$C_{16}H_{18}O_9$	352.0621	353.0882	-0.0272	8.42	353.08817:193141 354.09152:53331 355.09488:6245
5.15	L-Tryptophan	Amino acid	$C_{11}H_{12}N_2O_2$	203.0851	204.0899	-0.2764	ND	204.08998:27546 205.08993:5310 206.0899:0
5.27	Protocatechuic acid *	Hydroxybenzoic acid	$C_7H_6O_4$	153.0196	153.0196	0.7685	5.28	153.01956:81820 154.02291:6771 155.02627:940
5.32	4-*O*-Caffeoylquinic acid *	Hydroxycinnamic acid	$C_{16}H_{18}O_9$	353.0885	353.0885	-3.6764	8.48	353.08853:188163 354.09188:53844 355.09524:7459
5.34	Methylgallate *	Benzoic acid	$C_8H_8O_5$	182.0352	183.0306	0.0000	7.02	183.03064:819017 184.03399:100662 185.03735:6341
5.40	Acetyl-L-Leucine	Amino acid	$C_8H_{15}NO_3$	172.1515	173.1052	1.5172	8.34	173.10226:14201 174.10561:1232 175.10897:6558
5.40	Homovanillic acid	Methoxyphenol	$C_9H_{10}O_4$	180.0725	181.0512	0.0000	8.28	181.05124:447078 182.05459:63292 183.05795:4723
5.51	Epicatechin *	Flavonoid	$C_{15}H_{14}O_6$	289.0719	289.0724	-1.9624	7.77	289.07242:294892 290.07577:70716 291.07913:5734
5.54	Glycoprotein-phospho-D-hexose	Carbohydrate	$C_6H_{12}O_6$	179.0549	180.0633	2.5163	6.64	179.05487:23781 180.05822:1424 181.06158:44357

5.55	Pimelic acid	Fatty acid	$C_7H_{12}O_4$	159.0754	160.0736	1.8564	6.72	159.06566:45785 160.06901:5759 161.07237:332
5.56	3-Hydroxybenzoic acid *	Benzoic acid	$C_7H_6O_3$	137.0246	137.0246	1.7398	5.56	137.02458:87910 138.02793:7344 139.03129:668
5.61	Epigallocatechin gallate *	Flavanoid	$C_{22}H_{18}O_{11}$	457.0851	458.0849	3.4682	6.62	457.07568:34102 458.07903:12858 459.08239:2246
5.62	2,6-Dihydroxybenzoic acid *	Benzoic acid	$C_7H_6O_4$	153.0251	154.0266	1.1795	7.35	153.02629:22900 154.02964:1347 155.033:106
5.67	m-Cresol	Phenol	C_7H_8O	107.0563	108.0575	0.5316	ND	107.05032:32272 108.05367:2969 109.05703:0
5.72	Rutin *	Flavonoid glycoside	$C_{27}H_{30}O_{16}$	609.1463	609.1474	2.2707	8.19	609.1474:220069 610.15075:101117 611.15411:23019
5.80	Quercetin 3-*O*-galactoside *	Flavonoid glycoside	$C_{21}H_{20}O_{12}$	463.0891	463.0891	1.7539	8.38	463.08905:299453 464.0924:107861 465.09576:20583
5.82	Hydroxycinnamic acid isomer I	Hydroxycinnamic acid	$C_9H_8O_3$	163.0404	163.0404	0.6326	5.25	163.04042:100914 164.04377:14967 165.04713:455
5.86	Vanillin *	Methoxyphenol	$C_8H_8O_3$	151.0402	151.0402	0.8521	5.43	151.04024:55671 152.04359:4944 153.04695:342
5.87	Ellagic acid *	Benzoic acid	$C_{14}H_6O_8$	301.0051	302.0047	-2.6271	7.45	300.99747:267836 302.00082:67566 303.00418:7245
5.88	Homoveratric acid	Methoxybenzene	$C_{10}H_{12}O_4$	195.0669	195.0669	3.2409	6.19	195.06694:85437 196.07029:15019 197.07365:1141
5.93	Suberic acid	Fatty acid	$C_8H_{14}O_4$	173.0851	174.0892	1.6865	8.62	173.08127:79674 174.08462:14713 175.08798:722
5.95	Dihydroferulic acid *	Hydoxycinnamic acid	$C_{10}H_{12}O_4$	195.0762	196.0735	-1.6278	5.95	195.06584:19615 196.06919:3085 197.07255:240
6.00	Naringin *	Flavonoid glycoside	$C_{27}H_{32}O_{14}$	579.1713	579.1732	2.9924	7.42	579.17316:293758 580.17651:135313 581.17987:28635
6.01	Sinapic acid *	Hydroxycinnamic acid	$C_{11}H_{12}O_5$	223.0662	224.0684	4.2162	6.67	223.06075:25010 224.0641:5722 225.06746:349
6.14	Hydroxycinnamic acid Isomer II	Hydroxycinnamic acid	$C_9H_8O_3$	163.0462	164.0473	1.3961	5.26	163.04269:68213 164.04604:7591 165.0494:4847
6.22	Secoisolariciresinol *	Lignans	$C_{20}H_{26}O_6$	361.1655	361.1663	-1.9736	7.11	361.16629:162284 362.16964:58709 363.173:6237

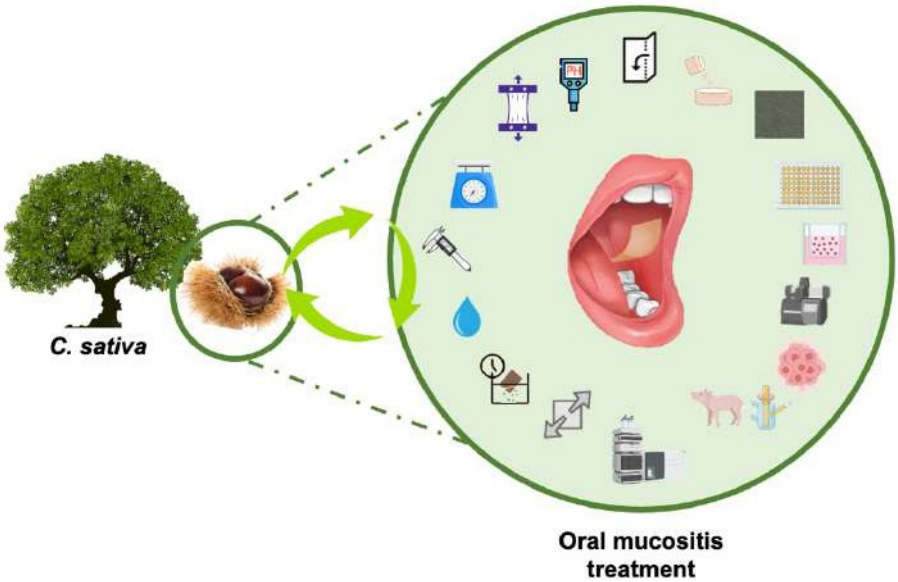
6.26	Phlorizin *	Flavonoid *O*-glycoside	$C_{21}H_{24}O_{10}$	435.1363	436.1369	4.7261	8.46	435.13037:300575 436.13372:94283 437.13708:15243
6.55	Sebacic acid	Fatty acid	$C_{10}H_{18}O_4$	201.1137	201.1137	0.0063	7.03	201.11372:104834 202.11707:15341 203.12043:1531
6.61	Luteolin *	Flavonoid	$C_{15}H_{10}O_6$	285.0461	286.0477	1.5265	ND	285.03928:136912 286.04263:38657 287.04599:2821
6.79	Sativic acid	Fatty acid	$C_{18}H_{36}O_6$	347.2439	347.2446	2.2134	6.90	347.24457:279813 348.24792:79447 349.25128:8059
6.89	Apigenin *	Flavonoid	$C_{15}H_{20}O_5$	269.0461	270.0528	3.6282	6.89	269.04443:113259 270.04778:32998 271.05114:1976
6.93	Undecanedioic acid	Fatty acid	$C_{11}H_{20}O_4$	215.1361	216.1362	-2.5168	6.93	215.12799:80355 216.13134:20670 217.1347:1032
6.94	Isorhamnetin *	Flavonoid	$C_{16}H_{12}O_7$	315.0510	315.0517	2.2463	7.38	315.0517:956562 316.05505:195939 317.05841:17997
7.36	Corchorifatty acid F	Lineolic acid	$C_{18}H_{32}O_5$	327.2291	328.2250	3.9941	5.83	327.21793:17969 328.22128:4306 329.22464:3498
7.89	Sakuranin	Flavonoid glycoside	$C_{23}H_{20}N_4O_6$	447.1361	448.1382	2.5174	7.97	447.13425:10526 448.1376:2979 449.14096:0
8.23	Crocetin	Curcuminoid	$C_{20}H_{24}O_4$	327.1641	328.1675	4.7516	5.78	327.16159:64446 328.16494:13030 329.1683:3106
8.49	Colnelenic acid	Fatty acid	$C_{18}H_{28}O_3$	291.2526	292.2038	3.6283	5.58	291.19958:49921 292.20293:6968 293.20629:2432
8.80	Herbacetin	Flavonoid glycoside	$C_{28}H_{30}O_{17}$	637.1474	638.1483	2.5179	8.80	637.14056:115806 638.14391:49807 639.14727:4868
9.39	Ajmaline	Alkaloid	$C_{20}H_{26}N_2O_2$	325.2763	326.1994	0.2774	5.37	325.18454:398558 326.18789:75607 327.19125:14636
12.07	Auraptene	Prenol lipid	$C_{16}H_{26}O_3S$	297.1583	298.1602	2.7356	6.32	297.15106:72262 298.15441:12607 299.15777:2636
13.82	Hydroxyadipic acid	Fatty acid	$C_6H_{10}O_5$	161.0457	161.0456	-1.4679	8.66	161.04562:32251 162.04897:4976 163.05233:1430

Chapter 4

Orodispersible Films with Chestnut Shells extract to prevent Oral Mucositis: *in vitro* and *ex vivo* studies

The information presented in this chapter was published in the following publication:

Ferreira, Ana Sofia; Silva, Ana Margarida.; Macedo, Catarina; Laveriano-Santos, Emily P.; Lozano-Castellón, Julián; Lamuela-Raventós, Rosa M.; Švarc-Gajić, Jaroslava; Costa, Paulo; Delerue-Matos, Cristina; Rodrigues, Francisca. Innovative orodispersible films with chestnut shells extract as active ingredient to prevent oral mucositis: *In vitro* and *ex vivo* studies. *Submitted*



Statement of contribution:

The contribution of the first author, Ana Sofia Ferreira, in this work, includes the literature review, investigation, methodology and writing of the original draft.

4.1. Introduction

Oral mucosa is highly sensitive to cytotoxic anticancer agents, such as chemo and/or radiotherapy, owing to their mechanism of action by rapidly targeting proliferative cell populations, including malignant cells [23, 397]. Nevertheless, these treatments also induce collateral effects to healthy tissues, such as the mucosal lining. The disruption of oral mucosal integrity and the generation of oxidative stress triggers an inflammatory process that result in the onset of oral mucositis (OM) [23, 62]. This condition significantly impairs the patient's quality of life by causing pain, hemorrhage, ulceration, ingestion difficulties and increased susceptibility to infections, leading to the reduction of dose treatment or cessation, thereby adversely impacting the patient prognosis [264]. OM affects almost 100% of the patients with head and neck cancer (HNC) subjected to radiotherapy, 70-90% of the patients undergoing hematopoietic stem cell transplantation, and up to 40% of those with solid tumors receiving chemotherapy [23, 61, 75]. Therefore, OM is a public health concern, particularly when 35 million new cancer cases are predicted in 2050, representing an increase of 77% when compared to 2022 [398].

The OM first line therapy is unsatisfactory as it results in a short duration of modest pain relief, being characterized by an increased need for opioid analgesics and parenteral (intravenous feeding) or enteral nutrition (tube feeding) for patients who cannot obtain a correct oral nutrition [63], being critical the exploration for new treatments. Over last years, natural agents have attracted a lot of attention due to their diverse biological properties such as anti-inflammatory, antioxidant, antibacterial, anticancer, immunomodulating, sedative or healing capacity that may represent an effective approach in preventing and treating OM [61, 264, 399, 400].

Among natural agents, *Castanea sativa* (chestnut) shells (CS), an abundant by product generated in high quantities by chestnut industry, particularly in Southern Europe, arise as an excellent option. Several authors have described the richness of CS extracts in (poly)phenols, mainly phenolic acids (gallic acid and protocatechuic acid), flavonoids (epicatechin and quercetin) and ellagitannins, as well as vitamins and amino acids (arginine and leucine) [8, 273, 294, 341, 342, 401], highlighting the associated biological activities, namely antioxidant, anti-inflammatory and antimicrobial properties, alongside with regulation of gene expression, improvement of endothelial function or inhibition of enzymes (such as matrix metalloproteinases) [8, 273, 294]. More recently, our research team attested by *in vitro* and *in vivo* assays in animals (mice) the metabolomic profile of CS extracts, supporting the pro-healthy properties ascribed and

opening new perspectives for its use as active ingredient to prevent or even treat OM [341, 401].

Oral dosage forms continue to be the prevalent approach for drug administration due to patients' compliance and convenience, cost-effectiveness, and feasibility for large-scale manufacturing, comparatively to injections and inhalers [402, 403]. Nevertheless, challenges arise for geriatric and pediatric population, as well as for dysphagic patients as consequence of OM, for example [264].

To address these limitations, orodispersible films (OFs) are gaining prominence in pharmaceutical research and development [402, 403]. OFs are composite active 108 compounds-loading films formed by a polymer matrix consisting of one or more layers, with potential to be either rapidly dispersible in mouth or mucoadhesive, leading to distinct routes of absorption [404-407]. For example, active compounds that undergo a rapid absorption via oral delivery avoid the exposure to the gastrointestinal tract, where might suffer degradation from stomach acid, bile, and first pass metabolism. Consequently, OFs have the potential to allow a local action, reduce the dose needed, and enhance the efficacy and safety profile of some active compounds [408]. This study intends to incorporate CS extract in OFs and explore their potential use to prevent OM symptoms.

To achieve this main goal, mechanical, physicochemical, antioxidant/antiradical and anticancer properties of the formulated OFs were evaluated as well as the permeation of the principal bioactive compounds through *in vitro* (buccal model) and *ex vivo* (porcine buccal mucosa) assays.

4.2. Materials and Methods

4.2.1. Chemicals

For OFs production, polyvinylpyrrolidone (PVP, Sigma-Aldrich, Germany), Carbopol® EDT 2020 (Pharma Excipients, Switzerland), Eudragit® E100, Eudragit® RS30D and Eudraguard® Protect were provided by Evonik Industries AG (Germany); HPMC K100 LV EP and HPMC E10M were a gift from Colorcon (USA); glycerol was supplied by Sigma-Aldrich, Germany. All chemicals and standards were of analytical reagent grade, all chromatographic solvents were of HPLC-HRMS grade and all chemicals were supplied by commercial sources. Acetonitrile, formic acid, water, and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Chlorogenic acid, 2,5-dihydroxybenzoic acid, phloridzin, quercetin-3-O-galactoside, protocatechuic acid, O-coumaric acid, trans-polydatin, castalagin, neochlorogenic acid, 2,6-dihydroxybenzoic

acid, gallic acid, apigenin, luteolin, naringin, rutin, 3-hydroxyphenylacetic acid, 3,5-di-caffeoylquinic acid, dihydroxyphenylpropionic acid 3-hydroxybenzoic acid, epicatechin, p-coumaric acid, ferulic acid, secoisolariciresinol, isorhamnetin, dihydroferulic acid, vanillin and catechin were supplied by Sigma-Aldrich (Steinhemin, Germany). Methyl gallate was from Phytolab (Vestenbergsgreuth, Germany). All standards were handled without exposure to light.

Human tongue squamous cell carcinoma (HSC-3), human squamous cell carcinoma (TR146), and human epidermal keratinocyte cell line (HaCaT) were obtained from the American Type Culture Collection (ATCC, USA). Cell reagents were purchased from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain).

4.2.2. *Castanea sativa* shells extract

Castanea sativa shells (CS) were provided by Sortegel, located in Sortes, Bragança, Portugal. Following dehydration and shredding, samples were subjected to subcritical water extraction (SWE), as described by Ferreira *et al*. [341]. Briefly, shells were extracted at 110 ºC using a homemade subcritical batch-type extractor (1.7 L) and a built-in valve with 99.99% nitrogen (Messer) pressurization. The extraction was performed during 30 min at 20 bars of pressure, with a sample-to-solvent ratio of 1:30. The extraction vessel was agitated through a vibrating platform (3 Hz) and cooled with a flow-through water bath (20 ± 2 °C) [341]. Afterwards, the extract in its liquid form was centrifuged at 11 000 rpm for 10 min, to remove any pellet and was incorporated in the OFs.

4.2.3. Preparation of OFs

Preliminary tests using the solvent casting method were carried out to select the OFs polymers that best fits the final application as well as its concentrations [409]. Similarly, the CS concentration was selected after preliminary tests that verified the highest concentration allowed to obtain OFs with a uniform content. After optimization, HPMC E10M was used at 1% (w/v), while glycerin was employed at 2.5% (w/v) as plasticizer. Briefly, the polymer and glycerol were dispersed in 100 mL of deionized water for the placebo OFs. For the OFs incorporating the chestnut shells extract (CS-loaded OFs), the extract in its liquid form was added to the mixture at a concentration of 25% (v/v) as solvent. Subsequently, the resulting solution was spread onto a plastic petri dish (8.5 cm) and refrigerated overnight at 4 °C, to eliminate entrapped air. The following day,

plates were placed in an oven at 60 °C for 24 h. The resulting films were covered with aluminum foil and stored in a desiccator until further analysis.

4.2.4. Characterization of OFs

4.2.4.1. Thickness and weight uniformity

Films were cut into 2 x 2 cm squares, and the thickness was determined using a digital micrometer (Powerfix Z22855, Germany) at three distinct points. The OFs weight was evaluated using an analytical weighing balance (Radwag AS 220.R2, Poland).

4.2.4.2. Mechanical properties

Films were cut into rectangles measuring 1 x 5 cm, and mechanical properties were determined using a texture analyzer (TA.XT plus Texture Analyzer, Stable Micro Systems, Cardiff, UK using Miniature Tensile Grips (Stable Micro Systems) and data were collected using Texture Exponent 32 software (version 6.1.12.0, Stable Micro Systems; Surrey, UK). Three independent film portions *(n* = 3) were vertically held with a separation of 10 mm and stretched until rupture by moving the probe at a constant speed of 0.1 mm/s. The tensile strength (N), elongation at break (%) and Young's modulus (MPa) were determined from the stress-strain curves, as in Eq (4) and (5), respectively:

$$\text{Elongation (\%)} = \frac{\text{distance at the rupture instant-initial grip distance}}{\text{initial grip distance}} \times 100 \qquad \text{Eq (4)}$$

$$\text{Young's modulus (MPa)} = \frac{\text{force at the corresponding strain}}{\text{cross-sectional area of film} \times \text{corresponding strain}} \qquad \text{Eq (5)}$$

4.2.4.3. Folding endurance

Films were cut into equal sizes (2 x 2 cm) and folded repeatedly until breakage at some point or completing a maximum of 300 folds, which is considered an excellent flexibility ability [409].

4.2.4.4. Surface pH

Films (2 x 2 cm) were immersed in a petri dish filled with artificial saliva for approximately 1 min. Artificial saliva was prepared according to Hobbs *et al*. [410] and kept at 37 ± 1 °C. A pH meter S400 (Mettler-Toledo, Ohio, USA) electrode was placed on the film surface, and pH readings were recorded.

4.2.4.5. Swelling capacity

The hydration capacity of the films (2 x 2 cm) was measured by weighing the samples over time during contact with artificial saliva. Each film was weighed (W1), placed onto a glass Petri dish containing 3 mL of artificial saliva, removed after 5 min, and reweighed (W2). The swelling index was calculated as in Eq (6):

$$\text{Swelling capacity } (\%) = \frac{W2-W1}{W1} \times 100 \qquad \text{Eq (6)}$$

4.2.4.6. Moisture content

Films (2 x cm) were placed in an infrared moisture balance AD-4713 (A&D Company, Japan) at 100 °C for 20 min. The device autonomously determined the moisture percentage by analyzing the difference in the films weight before and after heating.

4.2.4.7. Disintegration time

The disintegration time of the formulated films (2 x 2 cm) was assessed using the petri dish method [409]. Briefly, 10 mL of artificial saliva was placed in a glass petri dish, and the temperature was kept constant at 37 ± 1 °C. Afterwards, films were introduced into the petri dishes and subjected to rotation at 50 rpm using a magnetic stirrer (IKA C-MAG HS7, Carl-Roth, Germany), measuring the time taken by the films to be completely disintegrate.

4.2.4.8. Stability tests

Films (2 x 2 cm) were subjected to accelerated stability tests by storage at 40 °C with 75% relative humidity (40 °C/75% RH) and 25 °C with 65% RH (25 °C/65% RH), while wrapped in aluminum foil, both for 90 days. Subsequently, tensile strength, elongation, folding endurance, surface pH, and disintegration time were evaluated at time 0 and after 15, 30, 60, and 90 days of storage under both conditions.

4.2.4.9. Total phenolic content

Total Phenolic Content (TPC) was assessed by spectrophotometry following the Folin–Ciocalteu method, with minor changes [341]. Two square films (2 x 2 cm) were dissolved in 4 mL of artificial saliva to create a 100% film-concentration stock solution, from which serial dilutions were prepared. A calibration curve (linearity range = 5–100

µg/mL; R^2 > 0.997) was established using gallic acid as standard. Results are presented as mg of Gallic Acid Equivalents (GAE) per g of film (mg GAE/g film).

4.2.4.10. DPPH assay

The DPPH free radical-scavenging assay was performed according to the protocol described by Pinto *et al*. [8]. Trolox was used as standard for the calibration curve (linearity range, 5–125 µg/mL; R^2 > 0.996). Films (2 x 2 cm) were treated as described in Section 4.2.3.9. Results are presented as mg of Trolox Equivalents (TE) per g of film (mg TE/g film).

4.2.4.11. FRAP assay

FRAP assay was performed as described by Ferreira *et al*. [341]. A calibration curve (linearity range: 25–500 µM; R^2 > 0.998) was established using a standard ferrous sulfate ($FeSO_4.7H_2O$) solution at a concentration of 1 mM. Films (2 × 2 cm) were treated as described in Section 2.5.9. Results are expressed in µmol of ferrous sulfate equivalents (FSE) per gram of film (µmol FSE/g film).

4.2.4.12. Scanning electron microscopy/ Energy dispersive spectroscopy (SEM/EDS)

SEM/EDS analysis was performed using a High-resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis (FEI Quanta 400 FEG ESEM/EDAX Genesis X4M). Samples were coated with an Au/Pb thin film for 80 s, and with a 15 mA current by sputtering using SPI Module Sputter Coater equipment.

4.2.4.13. Fourier transform infrared spectroscopy (FTIR)

The interactions between the OFs polymeric matrix and the incorporated extract were evaluated using an FTIR Nicolet 6700 – Diamond Point (ThermoFisher Scientific, USA) and the potassium bromide (KBr) method [352]. Samples were individually placed in the sampler with spectral analysis between 4000 and 400 cm^{-1} and 32 scans at a resolution of 4 cm^{-1}.

4.2.4.15 . Thermal properties (DSC)

Differential scanning calorimetry (DSC) thermograms of the OFs and the lyophilized chestnut shells extract were obtained using a DSC 200 F3 Maia (Netzsh-Geratebau GmbH, Germany) with an empty aluminum pan as reference. Samples were placed in a sealed aluminum pan and heated from 0 to 200 °C at a ramping rate of 10 °C/min. Nitrogen was used as the purging gas at a flow rate of 20 mL/min. The onset temperatures were calculated using Proteus Analysis software (version 6.1, Netzsh-Geratebau GmbH, Germany).

4.2.4.15. Mucoadhesive strength

Porcine buccal mucosa tissue was obtained from a local slaughterhouse and served as model to evaluate the mucoadhesive properties of the prepared films. Using a texture analyzer coupled to a mucoadhesion rig (A/MUC) from Stable Micro Systems [411], 2 cm^2 of buccal tissue with 500 mm $\pm$ 100 mm in thickness was hydrated for 10 min with artificial saliva and fixed in the apparatus. Films were attached to the probe with a 10 mm diameter. Films were in contact with the mucosa tissue by applying a downward force of 0.5 N for 30 s before conducting the experiment. The probe was raised at a constant speed of 0.3 mm/s, and the force required for complete detachment (N) and work of adhesion (N/mm) was calculated with Exponent software.

4.2.5. Cytotoxicity

OFs cell viability was assessed using an MTT assay in two human cancer cell lines, namely, HSC3 and TR146, as well as in an immortalized human keratinocyte cell line, HaCaT. Passages 18, 33, and 39 were used for HSC3, TR146, and HaCaT cells, respectively. Cells were cultured and plated as described by Ferreira *et al*. [341] and exposed to various OFs concentrations. OFs were serially diluted (3-100%) in DMEM medium. Films (2 x 2 cm) were dissolved in 10 mL of DMEM as a 100% solution. Cell viability results are expressed as percentages (%).

4.2.6. *Metabolomic profiling*

4.2.6.1. *In vitro* permeation

The *in vitro* permeability of the bioactive compounds present in the OFs was carried out in a co-culture model composed by TR146 [322]. The extract and the CS-loaded OFs were added to the apical side of the model. A sample from the basolateral

side was collected at different timepoints (0, 15, 30, 45, 60, 90, 120, 150, 180, and 240 min). Samples were analyzed by LC-ESI-LTQ-Orbitrap-HRMS (section 2.8). The TEER of the model was evaluated before, during and at the end of the permeability assay using an EVOM Epithelial Volthometer Instrument equipped with a chopstick electrode (World Precision Instruments, Sarasota, FL, USA).

4.2.6.2. *Ex vivo* permeation

Porcine buccal mucosa was used to evaluate the buccal permeation of the phenolic compounds in the extract and the CS-loaded OFs. A Franz cell assembly (9 mm clear jacketed Franz cell with flat ground joint, 5 mL receptor volume and permeation area of 0.785 cm^2; PermeGear, Inc., USA) was used. The experiment followed the methodology described by Rodrigues *et al*. [412]. In the Franz apparatus, the porcine buccal mucosa was positioned with the cheek side facing to the donor chamber, which contained 500 µL (1000 µg/mL) of the chestnut shells extract or the CS-loaded OFs (2 square films (2 x 2 cm) with 500 $\pm$ 100 mm of thickness were dissolved in 4 mL of artificial saliva). The receptor chamber was filled with 5 mL of PBS, maintained at 37 °C, and stirred continuously at 150 rpm to simulate oral cavity conditions. Samples (300 µL) were collected at specific timepoints (0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420 and 480 min) and analyzed by LC-ESI-LTQ-Orbitrap-MS (section 4.2.6.3) to determine the amount of phenolic compounds that permeated the buccal mucosa.

4.2.6.3. Metabolomic profile by LC-ESI-LTQ-Orbitrap-HRMS

The identification and quantification of the phenolic compounds present in the extract and the extract incorporated in the OFs as well as the compounds that permeated from the OFs through *in vitro* and *ex vivo* assays were conducted using a LC-ESI-LTQ-Orbitrap-HRMS equipment with an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK), a photodiode array detector, a quaternary pump, and a temperature controlled autosampler coupled to an high resolution LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) with an ESI source in negative mode [347, 413]. The system was controlled using the Xcalibur v3.0 software (ThermoFisher Scientific, Hemel Hempstead, UK). The elution was performed on an AcquityTM UPLC® BEH C18 Column (2.1 x 100 mm, i.d., 1.7 µm particle size, Waters Corporation, Wexford, Ireland) kept at 30 °C. The gradient elution was performed with water (A) and acetonitrile (B), both with 0.1% formic acid, with a flow rate and an injection volume of 450 µL/min and 5 µL, respectively. The solvent gradient (v/v) of B (t (min), %B)

was set as follows: (0, 0); (2, 0); (4, 30); (8, 100); (10, 100); (11, 0); (14, 0). Samples were analyzed in full scan mode at resolving power of 30,000 and m/z 600.

Data-dependent MS/MS events were acquired at a resolving power of 15,000. Most intense ions were detected via FTMS mode-triggered data-dependent aquisition mode. Ions not sufficiently intense for a data-dependent scan were explored in MSn mode. Precursors were fragmented by collision-induced dissociation using a C-trap with a normalized collision energy (35 V) and an activation time of 10 ms. Operation parameters were as follows: source voltage, 3 kV; sheath gas, 50 units; auxiliary gas, 20 units; sweep gas, 2 units, and capillary temperature, 375 °C [347].

Compounds identified were putatively annotated using the MS-finder 299 and MS-dial software (open source version 4.25, created by Prof. Masanori Arita team (RIKEN) and Prof. Oliver Fiehn team (UC Davis)) [414-416], for data treatment, considering the high confidence provided by the fragmentation pattern, isotopic pattern (isotopic spacing and isotopic ratio) followed by exact mass and retention time alignments. A database set by combining annotations from Phenol- Explorer (http://phenol-explorer.eu/ (accessed on 27 October 2023)) and Food Database (http://foodb.ca/ (accessed on 27 October 2023)) was employed as a reference for putative annotation. Quantitative analysis was performed according to a validated chromatographic method [348].

Calibration curves were prepared from a mixture of phenolic standards that were expected to be found in the C. sativa shells extract using six different concentrations, ranging from 0.05 to 1 ppm for chlorogenic acid, 2,5-dihydroxybenzoic acid, phloridzin, quercetin-3-O-galactoside, protocatechuic acid, o-coumaric acid, trans-polydatin, castalagin, neochlorogenic acid, 2,6-dihydroxybenzoic acid, gallic acid, apigenin, luteolin, naringin, rutin, 3-hydroxyphenylacetic acid, 3,5-di-caffeoylquinic acid, dihydroxyphenylpropionic acid 3-hydroxybenzoic acid, epicatechin, p-coumaric, ferulic acid, secoisolariciresinol, isorhamnetin, dihydroferulic acid, vanillin, catechin and methyl gallate. Phenolic metabolites were quantified based on the calibration curves of the parent compounds that originated them. When there was no parent compound, the standard more similar chemically was selected. The calibration curves were as follows:

Gallic acid: y= -859.209+15446x (R^2= 0.9989)
3-Hydroxybenzoic acid: y = 2646.83+226224x (R^2= 0.9918)
2,5-Dihydroxybenzoic acid: y = -7872.05+387539x (R^2= 0.9979)
Dihydroferulic acid: y = -1597.01+39126x (R^2= 0.9953)
Epicatechin: y = -34724.6+684895x (R^2= 0.9912)

Chlorogenic acid: y = -25845.1+622416x (R^2= 0.9925)
Phlorizin: y = 1349.6+188575x (R^2= 0.9939)
Naringin: y = -424.528+572027x (R^2= 0.9975)
Rutin: y = -12789.3+365370x (R^2= 0.9945)
Protocatechuic acid: y = -9322.36+255908x (R^2 = 0.9917)

4.2.3. Statistical analysis

Results are expressed as mean ± standard deviation from at least three independent experiments. One-way ANOVA and Tukey's HSD tests were conducted for the statistical analysis using the IBM SPSS Statistics 28.0 software (Chicago, IL, USA). Results with statistical significance are denoted as $p < 0.05$.

4.3. Results and discussion

4.3.1. Preparation of OFs

In addition to resistance and stability, OFs must be manageable and flexible. Moreover, they must have adequate mucoadhesion properties and release the bioactive compounds, allowing the permeation through the buccal mucosa. The OFs polymers used in the present study were selected after conducting a literature review and after obtention of preliminary results (data not present). Based on qualitative parameters, such as flexibility and uniformity, HPMC E10M was the polymer selected. HPMC E10M is a semisynthetic polymer derived from cellulose. The application of cellulosic polymers in the production of solid extended-release dosage forms is facilitated by their exceptional film-forming properties and extensive grade selection, despite not being pH-responsive [417]. At 2% (water) and 20 ºC, HPMC E10M, a medium molecular weight HPMC, possesses a viscosity of 10,000 cPs. Due to its dispersibility in water, lower molecular weight polymers are highly correlated with higher rates of drug release [417].

With a medium molecular weight, the plasticizer glycerol increases the solution viscosity, reduces the brittleness, and fortifies the OF formulations. Furthermore, it facilitates robust intermolecular interactions between cellulose chains, thereby reducing intermolecular tension along the entire polymer chain. Glycerol films have superior properties when compared to films manufactured with sorbitol or polyethylene glycol (PEG) [418]. Glycerol is a hydrophilic substance that enhances the film flexibility, while reduces the strength when subjected to stress. Conversely, when the concentration of glycerol increases, elongation and moisture content also increase [418]. In this way, the addition of a plasticizer is mandatory to improve the mechanical properties of OFs. The

thin, transparent, homogeneous, flexible, mucoadhesive coatings produced by the HPMC E10M formulation exhibited no visible fissures or air bubbles. CS-loaded OFs presented a brown color, despite maintaining transparency (Figure 12).

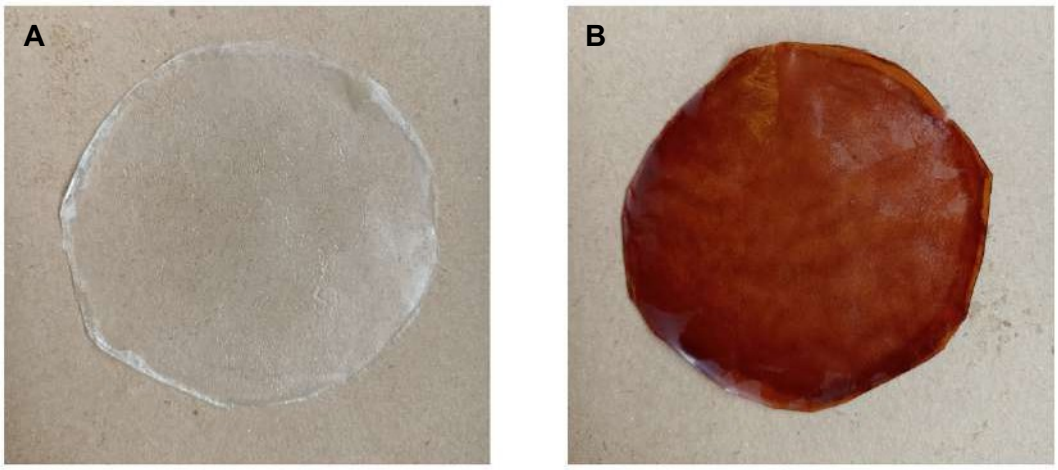


Figure 12 - Formulated Oral Films after detachment from petri dishes: (A) placebo film; (B) Loaded film with *C. sativa* shells extract.

4.3.2. Characterization of OFs

4.3.2.1. Mechanical properties

The mechanical features of an optimal OF are determined by a variety of factors, including the microstructural network and constituents, the interplay between matrix additives and preparation conditions, the plasticizer, and preexisting intermolecular forces [419-421]. Table 16 summarizes the obtained results for the formulated OFs.

Table 16 - Mechanical properties of formulated Oral Films (OFs), placebo and loaded with *C. sativa* shells (CS) extract (n = 3). Results are expressed as mean ± SD. Different letters in the same column means significant differences between samples ($p < 0.05$).

Formulation	Thickness (µm)	Rupture tensile strength (MPa)	Elongation (%)	Young's modulus (MPa)	Folding endurance
Placebo OFs	136 ± 8[a]	47.76 ± 1.11[a]	60.18 ± 7.43[a]	1245.28 ± 26.43[a]	>300
CS-loaded OFs	125 ± 9[a]	43.05 ± 1.29[a]	75.28 ± 7.27[a]	1141.74 ± 39.71[b]	>300

In addition to affect mucoadhesion and oral comfort, film thickness is an essential factor related to the accuracy of drug dosage. The barrier characteristics of the buccal mucosa and the rate of dissolution and disintegration may also be disturbed by thickness. Placebo films and CS-loaded ones (136 and 125 µm, respectively) presented thickness

with no significant differences (p = 0.23) among them. These values suggest that the formulations were relatively thin, which is advantageous since may contribute to a pleasant sensation when applied to the buccal mucosa. As reported, the ideal thickness of oral thin films should be between 50 and 1000 μm [409].

OFs must possess a substantial tensile strength to endure the stress associated with production, packaging, transportation, and routine handling, since an insufficient tensile strength will result in a rapid drug release from the matrix. As shown in Table 16, no significant differences (p = 0.35) were observed between the placebo and the CS-loaded OFs (47.76 and 43.05 MPa, respectively). Tedesco *et al*. studied the production of an OF with HPMC E15 with incorporated peanut skin extract and reported a maximum tensile strength of 26.63 MPa [254]. Borges *et al*. evaluated the tensile strength of marketed OFs reporting values between 1.47 and 33.91 MPa [257]. Therefore, the tensile strength of the OFs developed were better than reported in these studies.

Elongation is an indicator of flexibility, in which the concentration of polymer, plasticizer and extract are key aspects. CS-loaded OFs revealed a similar elongation percentage (75.28%) to placebo OFs (60.18%), with no significant differences (p = 0.42) among them. According to Kola *et al*., the ethanolic extract from pomegranate seeds reduced the cellulose film tensile strength and increased the elongation at break [422]. This effect could be attributed to the presence of (poly)phenols, indicating that the extract probably acted as a plasticizer, increasing the molecular attraction and forming new hydrogen bonds between cellulose chains and extract [418, 422].

The results for tensile strength and elongation corroborate the Young's modulus values, indicating resistance to deformation. As tensile strength decreased and elongation percentage increased, the Young's modulus decreased. The Young's modulus of the placebo OFs was greater (1245.28 MPa) when compared to the CS-loaded OFs (1141.74 MPa), being significantly different (p = 0.04). Notably, the range values of both films align with those of commercial ones that varied between 51.25 MPa and 1824 MPa [423].

After being folded more than 300 times in the same place, the OFs were still unbroken, without degradation signs and showing a high level of flexibility, probably due to the presence of glycerol, denoting high mechanical strength. These results are in line with previous studies that attested the great mechanical performance of HPMC polymers [424].

4.3.2.2. Physicochemical properties

The physicochemical properties of OFs, such as weight, moisture content, swelling capacity, disintegration time and surface pH, are crucial for patients' compliance. As reported in Table 16, the placebo and the CS-loaded OFs weighed 72 and 76 mg, respectively, without significant differences ($p > 0.05$). These results are not directly proportional to the thickness, as the CS-loaded OFs are heavier but thinner than the placebo ones. A possible explanation might be the higher density of the extract when compared to water. OFs' mucoadhesion (related with comfort application) and stability (specifically microbial contamination) are highly influenced by the moisture content.

A suitable water content not only mitigates the fragility of the OFs, but also acts as a powerful plasticizer [406]. According to Nair *et al*. [402], the ideal moisture content of OFs should be lesser than 5%. The results obtained for placebo and CS-loaded OFs were approximately 4%, without significant difference ($p > 0.05$) (Table 17). It was not surprising that adding the CS extract to the polymeric matrix had no effect on the OFs humidity.

Table 17 - Physicochemical properties of formulated Orodispersible Films (OFs), placebo and loaded with *C. sativa* shells (CS) extract ($n = 3$). Results are expressed as mean ± SD. Different letters in the same column means significant differences between samples ($p < 0.05$).

Formulation	Weight (mg)	Moisture content (%)	Swelling capacity (%)	Disintegration time (min:s)	Surface pH
Placebo OFs	72 ± 5^{a}	4.36 ± 0.37^{a}	9.13 ± 0.19^{a}	$18:35 \pm 1:17^{a}$	6.98 ± 0.09^{a}
CS-loaded OFs	76 ± 1^{a}	4.25 ± 0.35^{a}	7.31 ± 0.89^{b}	$20:43 \pm 1:38^{a}$	6.94 ± 0.19^{a}

The rate of drug release and the mucoadhesive properties of the OFs, which are affected by the structure and composition of the polymeric matrix, can be determined through swelling evaluation [425]. Upon application to the oral mucosa, water molecules permeate the OFs membrane, supplying hydration to the polymer matrix. The increase in OFs volume induced by hydration facilitates the drug diffusion, in this study the CS extract. The placebo OFs exhibited higher swelling capacity (9.13%) than the CS-loaded OFs (7.31%), due to the hydrophilic feature of HPMC. It is known that the inclusion of hydroxyl groups in the HPMC polymer guarantees the stability of the hydrophilic polymer matrix. The adhesion that occurs when swelling begins leads to the formation of a weak bond. A rise in the hydration level corresponds to an increase in the mucoadhesive

strength. On the other hand, the strength swiftly decreases when the polymer becomes overhydrated, and the interface becomes disentangled from the tissue [426].

Disintegration time indicates the onset of drug action. A low disintegration time leads to a faster release and absorption of the loaded drug through the oral mucosa. Disintegration time was evaluated in artificial saliva (pH 6.8), at 37 °C, to mimic the oral conditions. Placebo OFs disintegrated in 18 min and the CS-loaded OFs in more than 20 min, without significant differences ($p > 0.05$). These values correlate with the OFs swelling capacity, as higher swelling capacity leads to a faster disintegration [257].

The surface pH is an important attribute of OFs intended to be applied to the mucous membrane since it can be related to eventual local damage or irritation, causing discomfort to patients. The human saliva pH range between 6.2 and 7.6, with a specific value of 6.3 for the buccal mucosa [427]. The OFs presented a pH between 6.5 and 6.98, being considered suitable for oral application, without risk of irritation or inflammation of the buccal mucosa.

4.3.2.3. Stability

Stability assays are mandatory when developing new drug delivery systems. Additionally, these tests should be performed on OFs to determine the behavior of their components and to identify any potential degradations or interactions. Products must be stable after manufacturing to comply with the standards set by the International Council of Harmonization (ICH), with packaging providing mechanical protection and acting as a crucial barrier to light, moisture, and oxygen [409, 428]. Therefore, CS-loaded OFs were evaluated for short term and accelerated stability studies (Table 18).

As can be observed, OFs folding endurance remained in the acceptable range of more than 300- fold even after 90 days of storage at 40 °C/75% RH. Similarly, no significant differences were observed in terms of tensile strength, elongation, physical appearance, disintegration time or surface pH. The absence of significant differences for all parameters at room temperature (25 °C/65% RH) or under accelerated conditions (40 °C/ 75% RH) indicates the good physicochemical stability of OFs developed. To the best of our knowledge, this is the first study that assessed the stability of OFs with natural extracts as active ingredients.

Table 18 - Effects of storage at room temperature and accelerated conditions on the properties of the *C. sativa* shells (CS) extract loaded Oral Films (OFs) (n = 3). Results are expressed as mean ± SD. Different letters (a,b,c,d,e) in the same line means significant differences ($p < 0.05$).

CS-loaded OFs						
Storage conditions (25 °C/65% RH)						
Time (days)	**Rupture tensile strength (MPa)**	**Elongation (%)**	**Folding endurance**	**Disintegration time (min:s)**	**Surface pH**	**Physical appearance**
0	43.05 ± 1.29[a]	75.28 ± 7.27[a]	>300	20:43 ± 01:38[a]	6.94 ± 0.19[a]	-
15	42.78 ± 1.45[a]	76.31 ± 5.79[a]	>300	19:33 ± 02:55[a]	6.89 ± 0.24[a]	No change
30	43.20 ± 1.34[a]	77.02 ± 5.54[a]	>300	20:13 ± 01:36[a]	6.91 ± 0.13[a]	No change
60	44.05 ± 2.03[a]	77.89 ± 3.41[a]	>300	21:01 ± 00:59[a]	6.92 ± 0.09[a]	No change
90	41.05 ± 1.79[a]	76.12 ± 4.76[a]	>300	20:22 ± 01:41[a]	6.95 ± 0.21[a]	No change
Accelerated conditions (40 °C/75% RH)						
Time (days)	**Rupture tensile strength (MPa)**	**Elongation (%)**	**Folding endurance**	**Disintegration time (min:s)**	**Surface pH**	**Physical appearance**
0	41.27 ± 1.35[a]	76.82 ± 5.86[a]	>300	19:27 ± 01:13[a]	6.92 ± 0.15[a]	-
15	42.35 ± 2.64[a]	75.87 ± 6.15[a]	>300	20:14 ± 02:41[a]	6.87 ± 0.18[a]	No change
30	41.63 ± 1.67[a]	74.23 ± 4.69[a]	>300	21:53 ± 02:05[a]	6.94 ± 0.26[a]	No change
60	40.97 ± 2.53[a]	74.75 ± 2.71[a]	>300	22:05 ± 01:52[a]	6.83 ± 0.21[a]	No change
90	39.05 ± 1.39[a]	73.87 ± 3.67[a]	>300	22:43 ± 00:43[a]	6.74 ± 0.18[a]	No change

4.3.2.4. Phenolic content and antioxidant/antiradical activities

One of the main triggers in the development of OM is the oxidative stress that can be minimized with the use of natural antioxidants, particularly using delivery systems. In the current study, the TPC and the antioxidant/antiradical activities of OFs were evaluated (Table 19).

Table 19 - Total Phenolic Content (TPC) and antioxidant activity (FRAP and DPPH) of formulated Oral Films (OFs), placebo and loaded with *C. sativa* shells (CS) extract (n = 3). Results are expressed as mean ± SD.

Formulation	TPC (mg GAE/g film)	DPPH (mg TE/g film)	FRAP (µmol FSE/g film)
Placebo OFs	ND	ND	ND
CS-loaded OFs	37.05 ± 1.20	143.42 ± 3.41	0.142 ± 0.01

ND – Not Determined.

As expected, the placebo OFs did not show results for these assays. In a previous study, our team evaluated the phenolic content of the dry CS extract, reporting a TPC value of 239.53 mg GAE/g DW [294]. Considering that the extract was used as solvent at 25% (*v*/*v*) for the CS-loaded OFs, the obtained TPC values closely aligned with this ratio of solvent (59.88 mg GAE/g of film) [294]. The same ratio was not observed for the antioxidant/antiradical assays results. However, due to the potential interference of the polymer or plasticizer in these assays, it is expected that the OFs would yield lower results when compared to the dry extracts. The CS-loaded OFs achieved a result of 143.43 mg TE/g film (dry extract: 426.88 mg TE/g DW) and 0.142 µmol FSE/g film (dry extract: 4092.98 µmol FSE/g DW) for the DPPH and the FRAP assays, respectively. A potential reason may be the low ability of the natural antioxidants present in the extract to scavenge the DPPH free radicals or to bind to the FRAP ions, as they are entrapped into the polymeric matrix, particularly when the polymer was selected for its sustained release profile [49].

4.3.2.5. SEM/EDS

For an ideal buccal application, the OFs morphology should be homogeneous to ensure a uniform distribution of the bioactive content through the polymeric mixture. Additionally, interactions between drugs, polymers and plasticizers may result in a rough OFs surface [23]. The OFs surface observed by SEM are shown in Figure 13.

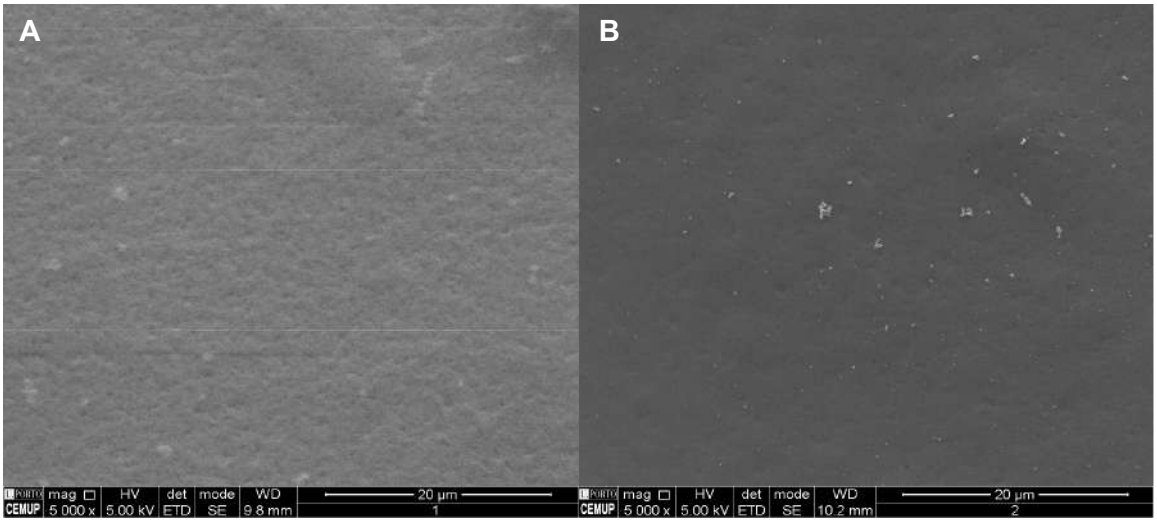


Figure 13 - Scanning Electron Microscopy (SEM) micrographs of produced Oral Films: (A) placebo film; (B) film loaded with *C. sativa* shells extract.

The microscopic appearance of the placebo OFs (A) presented a homogeneous, continuous, and smooth surface, despite being noticed the presence of white dots in

both OFs that were more noticeable in the CS-loaded OFs (B), although without irregularities. These results are in line with Porfírio *et al*. who also observed small white dots in HPMC films while adding zidovudine and lamivudine, suggesting the precipitation of the polymer involved [429].

4.3.2.6. Polymer and extract interactions

FTIR was employed to assess the CS extract incorporation impact on the intermolecular forces within HPMC E10M OFs (Figure 14).

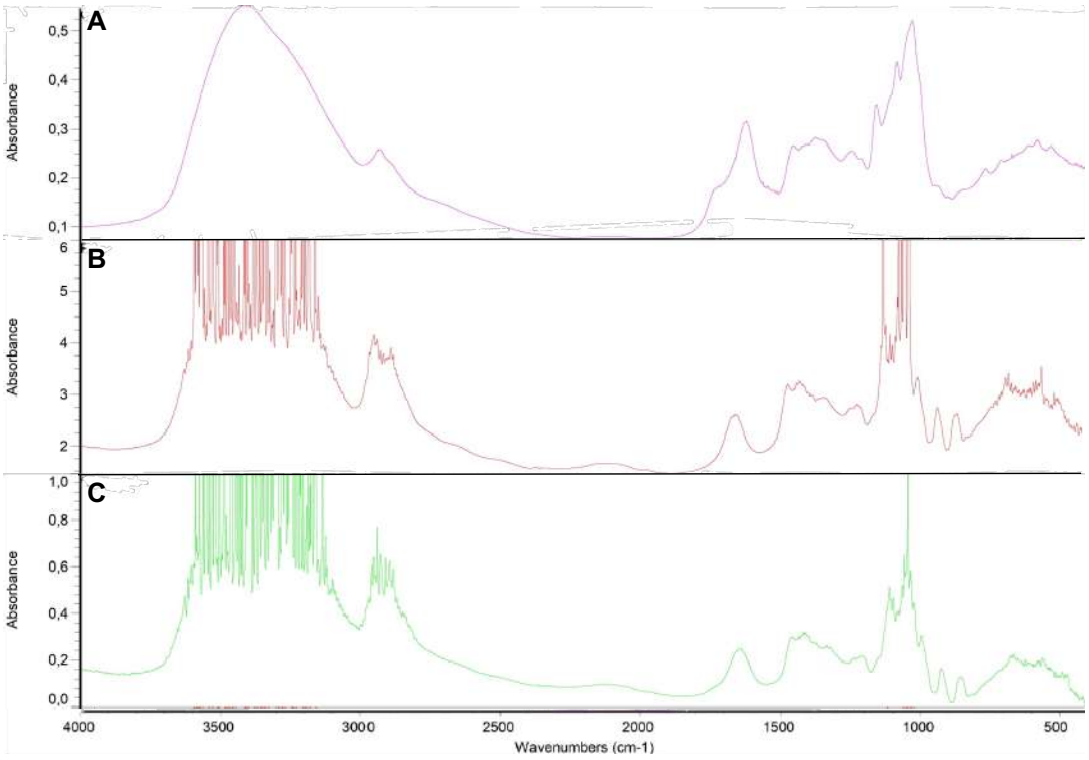


Figure 14 - FTIR spectrum: (A) *C. sativa* shells (CS) extract; (B) placebo film; (C) CS-loaded film.

The extract introduction resulted in the emergence of new peaks in the OFs, suggesting potential modifications in the chemical structure of the HPMC matrix. Notable differences were observed in the 3600-3100 cm^{-1} region, associated with the stretching vibrations of O-H bonds (hydroxyl groups) and N-H bonds (amines and amides) [430, 431]. These modifications may signify a change in the hydrogen bonding patterns or the introduction of new functional groups containing O-H or N-H bonds [430]. Furthermore, the presence of hydroxyl groups in compounds such as catechin and epicatechin may contribute to modifications in this region [432, 433]. The alterations observed in the 2950-

2850 cm^{-1} region, mainly linked to stretching vibrations of C-H, may result from the presence of alkaloids, flavonoids, phenolic acids and stilbens [430]. The 1150-1000 cm^{-1} region that is characteristic of HPMC and associated with C-O stretching, displayed differences, suggesting modifications in the cellulose backbone or the introduction of functional groups from the extract [430, 434], with compounds such as catechin and rutin with distinct C-O stretching characteristics, contributing to these modifications [432, 435].

4.3.2.7. DSC

The DSC thermograms, represented in Figure 15, showed a broad endothermic peak for the lyophilized CS extract at 103.76 °C, which may correspond to its melting point.

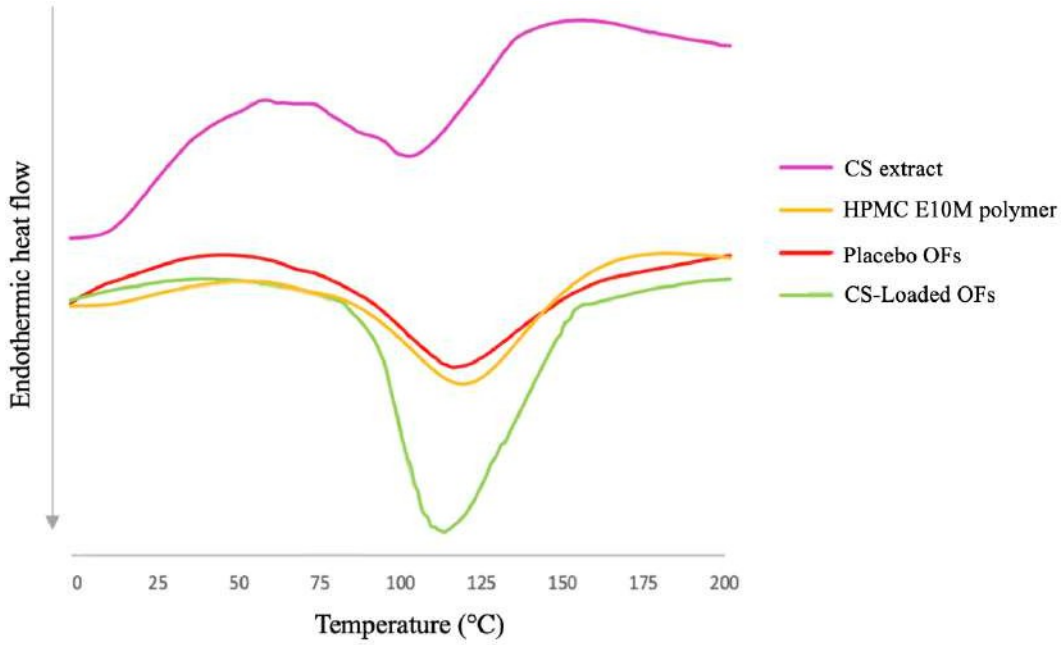


Figure 15 - DSC thermograms of *C. sativa* shells (CS) extract, pure HPMC polymer and produced Oral Films (OFs) (placebo and CS-loaded).

Since there is a mixture of (poly)phenols in the matrix that can interact with each other, thermal degradation may occur, with the conversion of some compounds into different phenolic acids [341]. However, to the best of our knowledge, there is a lack of information regarding DSC analyses of CS extract. The thermograms of pure HPMC E10M polymer and placebo films exhibit shallow, broad endothermic peaks at 119.54 °C, and 115.32 °C, respectively. Conversely, the CS-loaded OFs displayed a more pronounced peak at 118.21 °C, indicating a greater degree of heat fusion. These results

suggest that the inclusion of (poly)phenols demands a greater amount of energy to disrupt the interactions between (poly)phenols and the film matrix [436]. Based on the thermograms obtained for the mixture, no significant interactions between the extract and excipients were observed. HPMC E10M and glycerin were compatible with CS extract.

4.3.2.8. *Ex vivo* mucoadhesive strength

Mucoadhesion is influenced by various physicochemical characteristics of the polymers, such as charge, concentration, functional groups, and environmental factors. Low molecular weight polymers are more effective at permeating mucous, whereas high molecular weight ones may become entangled with mucin more frequently. HPMC and other polymers in the cellulose derivative class have hydroxyl and carboxyl groups substituted for their natural cellulose backbones. Carboxyl groups attract water, resulting in significant diffusion and enlargement of the polymer chains. Pharmacokinetics are enhanced via ionic and hydrogen bonds with mucin oligosaccharides, because of the extended residence time of these carboxylic groups at the site of application.

Furthermore, the ability of the polymeric matrix to be flexible facilitates the formation of a greater number of hydrogen bonds [419]. The mucoadhesive strength of the OFs was evaluated using porcine buccal mucosa using a texture analyzer, and the detachment force and work of adhesion were measured (Table 20).

Table 20 - Mucoadhesive strength of formulated Orodispersible Films (OFs), placebo and loaded with *C. sativa* shells (CS) extract (n = 3). Results are expressed as mean ± SD.

Formulation	Detachment Force (N)	Work of adhesion (N/mm)
Placebo OFs	1.89 ± 0.41	0.26 ± 0.01
CS-loaded OFs	1.77 ± 0.23	0.19 ± 0.02

As can be observed, the detachment force was much lower than that reported in other studies [437, 438], and no significant differences were detected between the CS-loaded OFs and the placebo ones (p > 0.05). For example, Al-Dhubiab *et al*., using polymer blends of HPMC, Eudragit and Carbopol, reported detachment force values of 7.89 to 8.34 N for different polymer ratios [437]. However, Mady *et al*. obtained detachment force values between 0.306 and 0.416 N for a polymeric mixture composed of carboxymethyl cellulose and polyvinylpyrrolidone with propylene glycol and Tween [438]. It is likely that the hydrophilicity associated with the thickness of the HPMC OFs

leads to rapid disintegration, reducing the mucoadhesive characteristics. The high solubility of polymers in water can be a positive aspect since after hydration, the OFs start to disintegrate due to dissolution.

4.3.3. Cytotoxicity

Beyond mechanical and biodegradable properties, the polymer biocompatibility is a critical factor for its use in pharmaceutical applications. The MTT assay was performed in TR146, HSC-3, and HaCaT cells after 24 h of exposure to OFs, as illustrated in Figure 16.

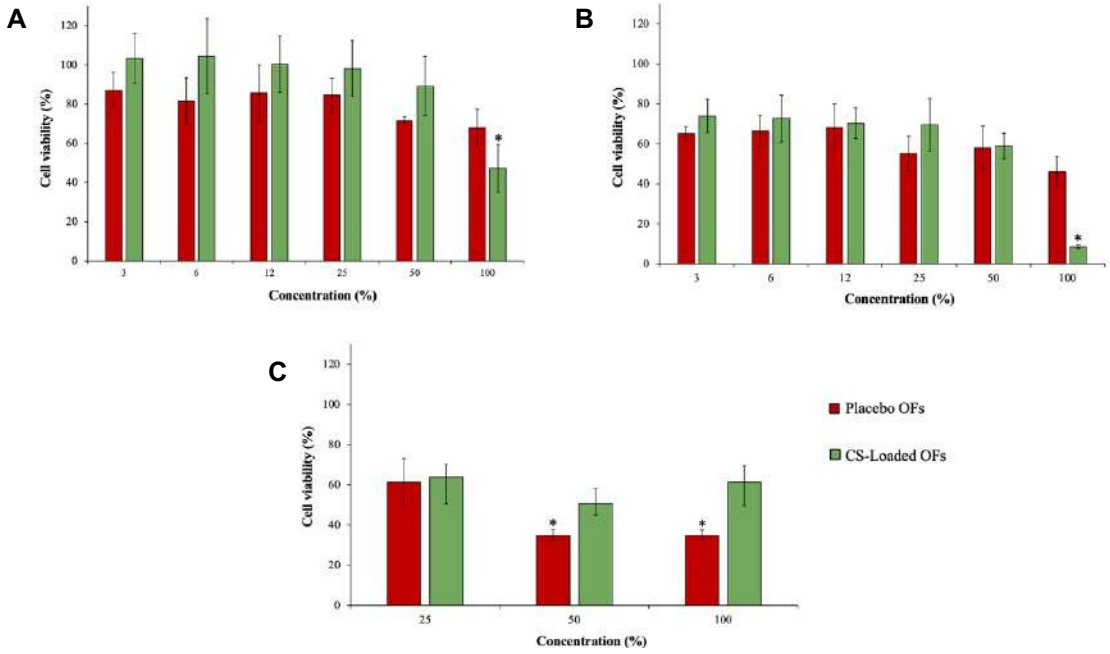


Figure 16 - Cell viability when in contact with placebo and C. sativa (CS) shells extract loaded oral films (OFs), determined by the MTT assay after 24 h of contact, of TR146 (A), HSC-3 (B) and HaCaT (C) cells. Results are expressed in percentage (%), as mean ± SD. *p <0.05 between groups at the same concentration.

TR146 and HSC-3 are human tumorigenic oral cell lines, commonly used as cellular models for studies on the oral cavity, while HaCaT is a human immortalized keratinocyte line derived from normal skin that has similarities with epidermis and epithelial tissue of the oral mucosa [439, 440]. The following criteria were used to classify the cellular viability: viability > 100%, indicating no toxicity (class 0); viability = 0%, revealing the highest toxicity (class 5); and 75–99%, 50–74%, 24–49% and 1–25% viability were categorized as classes 1, 2, 3 and 4, respectively [441]. The toxicity of CS-

loaded OFs was higher than the placebo ones at the highest tested concentration (100% - no dilution), with viabilities of 47.22% and 67.91%, respectively. Similarly, as the placebo OFs led to a viability of 46.00% in the HSC-3 cell line, the exposure to the CS-loaded OFs resulted in a viability of 8.62%. This evidence confirmed the cytotoxic effects of the CS extract on both tumorigenic cell lines at the highest tested concentration. However, for the diluted concentrations of OFs, this effect was not observed. Regarding HaCaT cells, a higher viability was observed after exposure to the highest concentration of the CS-loaded OFs (61.2%) than after exposure to the placebo OFs (35.06 %), being categorized as classes 2 and 3, respectively. These results confirm the antitumoral effect of the CS extract due to the selectivity of its cytotoxic effects. Moreover, the reduced cellular viability may be due to the high viscosity of the polymer, which prevents the proper cellular division in the 2D cellular model.

4.3.4. Metabolomic profile

4.3.4.1. *In vitro* permeability

The permeation of natural compounds from the prepared OFs must be evaluated to assess their absorption across the buccal epithelium. In this study, the safety and bioavailability of the developed OFs were evaluated using an *in vitro* buccal model composed by TR146 cells. Tables 21 and 22 present the permeated compounds identified and quantified after the *in vitro* permeation assay of CS extract and CS-loaded OFs at different time points for up to 240 min. Supplementary Table S4 summarizes the putative annotation of compounds in CS extract and CS-loaded OFs. As expected, *in vitro* and *ex vivo* assays revealed no bioactive compound permeation in placebo films assays.

Table 21 - Permeation of *C. sativa* shells extract compounds through the *in vitro* buccal model (TR146 cells) at different time points (n = 3). Results are expressed as mean ± SD. Different letters (a,b,c,d,e) in the same line means significant differences ($p < 0.05$). ND – Not Determined

Class	Subclass	Compound/Metabolite	Permeation (%)							
			Time (min)							
			15	30	45	60	90	120	180	240
Benzene	Benzoic acids	Methyl gallate	37.24 ± 0.04^{a}	ND	ND	ND	ND	ND	ND	ND
Carboxylic acids	Amino acids, peptides	Pyroglutamic acid	13.92 ± 0.12^{a}	15.39 ± 1.05^{a}	18.22 ± 1.41^{b}	23.34 ± 0.88^{b}	22.87 ± 1.52^{b}	25.66 ± 1.84^{b}	21.35 ± 0.97^{b}	28.47 ± 2.52^{b}
	Dicarboxylic acids	Succinic acid	20.51 ± 1.62^{b}	14.86 ± 2.71^{a}	16.09 ± 1.85^{a}	14.96 ± 0.46^{a}	19.54 ± 0.94^{b}	20.71 ± 1.67^{b}	21.62 ± 0.61^{b}	23.76 ± 1.68^{b}
	Tricarboxylic acid	Isocitric acid	0.04 ± 0.01^{a}	0.12 ± 0.03^{a}	0.15 ± 0.01^{a}	0.15 ± 0.01^{a}	0.24 ± 0.03^{a}	0.33 ± 0.02^{ab}	0.54 ± 0.04^{ab}	0.70 ± 0.01^{b}
Dibenzylbutane lignans	Dibenzylbutanediol lignans	Secoisolariciresinol	ND	ND	ND	ND	ND	ND	22.32 ± 0.12^{a}	ND
Fatty Acyls	Fatty acids	Hydroxyadipic acid	25.51 ± 1.51^{a}	54.36 ± 0.58^{b}	24.25 ± 0.84^{a}	24.30 ± 0.10^{a}	25.06 ± 0.79^{a}	34.93 ± 2.05^{a}	29.87 ± 1.69^{a}	62.63 ± 2.33^{b}
		Sativic acid	1.85 ± 0.01^{a}	3.02 ± 0.05^{a}	3.77 ± 0.02^{a}	4.46 ± 0.03^{a}	6.17 ± 0.08^{ab}	7.52 ± 1.13^{ab}	10.80 ± 0.16^{ab}	12.34 ± 0.09^{b}
		Sebacic acid	121.43 ± 3.045^{a}	108.30 ± 1.07^{a}	117.63 ± 2.87^{a}	122.27 ± 3.63^{a}	120.02 ± 4.05^{a}	113.74 ± 2.24^{a}	117.88 ± 1.74^{a}	101.09 ± 3.73^{a}
Flavonoids	Flavans	Epicatechin	97.57 ± 1.07^{a}	96.86 ± 2.25^{a}	97.35± 1.74^{a}	98.05 ± 3.81^{a}	97.75 ± 5.73^{a}	97.75 ± 6.01^{a}	97.36 ± 2.57^{a}	96.86 ± 1.86^{a}
	Flavonoid glycosides	Quercetin 3-*O*-galactoside	ND	ND	ND	49.94 ± 0.98^{a}	ND	ND	ND	ND
	Flavones	Isorhamnetin	99.26 ± 2.34^{a}	99.73 ± 1.83^{a}	98.36 ± 3.74^{a}	99.10 ± 2.96^{a}	99.00 ± 4.70^{a}	98.36 ± 1.07^{a}	99.08 ± 2.69^{a}	98.36 ± 1.91^{a}
Organooxygen	Alcohols and polyols	Caffeoyl quinic acid	ND	ND	29.74 ± 0.73^{a}	ND	ND	60.35 ± 1.67^{b}	29.76 ± 0.92^{a}	30.86 ± 0.63^{a}
		Myo-Inositol	39.51 ± 0.87^{ab}	79.23 ± 1.43c	12.98 ± 0.01^{a}	33.80 ± 0.08^{ab}	30.46 ± 0.05^{ab}	41.34 ± 1.01^{ab}	25.02 ± 0.09^{ab}	56.50 ± 0.37^{c}
		Neochlorogenic acid	ND	ND	ND	ND	ND	ND	14.58 ± 0.08^{a}	ND

Table 22 - Permeation of compounds present in formulated Oral Films with *C. sativa* shells extract incorporated through the *in vitro* buccal model (TR146 cells) at different time points (n = 3). Results are expressed as mean ± SD. Different letters (a,b,c,d,e) in the same line means significant differences ($p < 0.05$).

Class	Subclass	Compound/Metabolite	Permeation (%)							
			Time (min)							
			15	30	45	60	90	120	180	240
Carboxylic acids	Amino acids, peptides	Pyroglutamic acid	17.03 ± 0.23^{a}	39.63 ± 0.24^{ab}	43.32 ± 1.12^{ab}	46.32 ± 2.65^{ab}	65.40 ± 0.82^{b}	66.95 ± 3.71^{b}	103.24 ± 2.71^{c}	111.34 ± 1.62^{c}
	Dicarboxylic acids	Succinic acid	44.20 ± 2.19^{a}	64.44 ± 1.03^{c}	56.70 ± 1.23^{b}	57.45 ± 0.78^{b}	79.12 ± 4.41^{c}	88.23 ± 1.26^{c}	103.25 ± 1.42^{d}	100.86 ± 1.75^{d}
	Tricarboxylic acid	Isocitric acid	0.15 ± 0.001^{a}	0.29 ± 0.05^{a}	0.50 ± 0.06^{a}	0.62 ± 0.02^{a}	1.04 ± 0.01^{b}	1.40 ± 0.04^{b}	2.77 ± 0.01^{b}	3.38 ± 0.06^{b}
Fatty Acyls	Fatty acids	Sativic acid	7.64 ± 0.45^{a}	13.72 ± 0.58^{b}	19.16 ± 0.87^{b}	21.17 ± 2.41^{b}	34.01 ± 1.38^{c}	43.66 ± 3.51^{c}	58.08 ± 0.63^{d}	71.88 ± 1.47^{d}
Flavonoids	Flavones	Isorhamnetin	105.62 ± 3.71^{a}	105.51 ± 3.25^{a}	105.05 ± 2.24^{a}	101.34 ± 2.95^{a}	98.29 ± 2.14^{a}	99.38 ± 1.47^{a}	98.29 ± 1.94^{a}	100.20 ± 3.56^{a}
Organooxygen	Alcohols and polyols	Caffeoylquinic acid	ND	ND	47.99 ± 1.41	ND	ND	95.85 ± 2.72	39.15 ± 0.84	69.64 ± 2.61
	Carbohydrates	Verbascose	2.14 ± 0.01^{a}	4.25 ± 0.15^{a}	4.45 ± 0.98^{a}	4.86 ± 0.41^{a}	7.90 ± 0.51^{b}	11.25 ± 0.13^{b}	16.17 ± 0.44^{ab}	20.82 ± 0.97^{ab}
Phenolic acids	Hydroxybenzoic acids	Protocatechuic acid	89.42 ± 3.08^{b}	ND	85.88 ± 2.01^{b}	94.08 ± 3.56^{b}	96.88 ± 1.43^{b}	106.06 ± 0.78^{b}	84.76 ± 2.51^{b}	75.80 ± 0.91^{a}
Phenols	Benzenoids	Pyrocatechol	99.63 ± 2.51^{c}	107.69 ± 3.06^{c}	54.19 ± 1.35^{a}	ND	57.35 ± 0.85^{a}	ND	58.10 ± 1.73^{a}	68.19 ± 2.65^{b}
	Methoxyphenols	Homovanillic acid	106.01 ± 1.46^{a}	104.89 ± 1.08^{a}	105.41 ± 0.99^{a}	103.62 ± 2.72^{a}	105.25 ± 2.83^{a}	109.09 ± 1.97^{a}	106.07 ± 2.67^{a}	110.05 ± 3.61^{a}

ND – Not Determined.

Regarding the CS extract, a total of 20 compounds were presumptively annotated in the *in vitro* buccal model permeation, with a higher abundance of (poly)phenols (7 compounds, representing 35%) and organic acids (5 compounds, representing 25%), followed by lipids (3 compounds, representing 15%), alcohols or polyols (3 compounds, representing 15%), carbohydrates (1 compound, representing 5%), and amino acids and derivatives (1 compound, representing 5%). From the CS extract, 16 compounds permeated through the *in vitro* buccal model after up to 4 h of assay. Sebacic acid achieved the highest permeation in most of the timepoints, reaching a permeability of 101.09% after 240 min, followed by isorhamnetin (98.36%), epicatechin (96.86%) and homovanillic acid (87.20%).

Reactive oxygen species (ROS) play a central role in the OM pathogenesis since epithelial cell damage triggers apoptosis and upregulates several transcription factors [341]. This cascade culminates in the induction of a pro-inflammatory state characterized by the production of cytokines, including tumor necrosis factor-α (TNF-α), interleukin- and -6 (IL-β and IL-6) [341]. Particularly, an increase in salivary IL-6 levels during the third week of treatment in head and neck cancer patients is associated with the development of severe OM [442]. Sebacic acid, a fatty acid also present in royal jelly, has been associated with anti-inflammatory properties by reducing the expression of IL-6 induced by lipopolysaccharide (LPS) in human macrophage cells [364]. Besides, isorhamnetin has shown protective capacity against H_2O_2-induced endothelial cell injury through antioxidative, anti-inflammatory and anti-mitochondria-dependent apoptosis [443]. Shin *et al*. demonstrated that epicatechin protects *in vitro* HaCaT cells from radiation-induced damage by inhibiting the ROS generation, preserving the mitochondrial integrity, and suppressing the MAPKs activation. Moreover, in a rat model, epicatechin improved wound healing after radiation exposure in the oral cavity [444], while homovanillic acid has potent antioxidant properties in rats after olive oil consumption [445].

Regarding the CS-loaded OFs permeated samples, the results demonstrated a lower abundance of the annotated compounds, probably due to the concentration of CS extract used in the OFs (25%, *v*/*v*) as well as the evaporation process that occurs during the OFs production. Zelbiene *et al*. tested different types of polyacrylic acid gels with horse chestnut seed extract and demonstrated that the permeability of (poly)phenolic compounds was dependent on the polymer used, highlighting the importance of the polymeric matrix for release purposes. This problem may be solved by the incorporation of a permeation enhancer [446]. The concentration, structure, and molecular size, as well as hydrophilicity and permeation time may also impact the compounds permeation

through the oral mucosa [409, 447]. Nonetheless, it should be highlighted that this cellular model has drawbacks, including the influence of oncogenesis on the permeability barrier [448]. Pyroglutamic acid (PGA) achieved the highest permeation percentage in CS-loaded OFs, reaching a permeability of 111.34% at the timepoint 240 min, followed by homovanillic acid (110.05%), succinic acid (100.86%), isorhamnetin (100.20%), protocatechuic acid (75.80%) and caffeoylquinic acid (69.64%). PGA is a low molecular weight carboxylic acid well known for its great hygroscopic properties, alongside demonstrated antitumoral and antimicrobial activities [449]. Additionally, PGA has been associated with a decrease concentration of inflammatory cytokines within the stratum corneum, suggesting potential anti-inflammatory effects. Moreover, the synergy between PGA and antioxidants may offer enhanced restoration of physiological skin functions. In this context, the CS-loaded OFs may represent a promising strategy to mitigate the OM ulceration.

Protocatechuic acid (PCA) and caffeyolquinic acid are bioactive compounds with diverse health benefits, due to its anti-inflammatory, antioxidant, antitumoral and antibacterial activities [450, 451]. Moreover, *in vitro* and *in vivo* studies attested the PCA antiulcer activity and analgesic effect [450]. During the OM process, the skin epithelial barrier is compromised, exposing patients to potential pathogenic bacteria invasion, such as *Staphylococus aureus* and Escherichia coli, increasing the risk of sepsis. Caffeoylquinic acid has demonstrated inhibitory effects against these bacteria, suggesting its capacity to mitigate secondary infections [451]. It should be noted that these compounds showed higher permeations in the CS-loaded OFs than the CS extract, supporting that the formulated delivery system is a good vehicle for these natural compounds.

During *in vitro* models' assays, TEER is a decisive parameter to evaluate the integrity of cell layers. In the present study, TEER was monitored for 31 days. As shown in Figure 17, the values increased on the 8th day, indicating that TR146 cells grew and remained stable (165 ± 20 Ω/cm^2). During the permeability assay, the values ranged between 166.0 and 210.0 Ω/cm^2, indicating the viability of the cell culture. Mazzinelli *et al*. reported that TEER values between 150 and 200 Ω/cm^2 are greater than those of healthy epithelium, which is due to their derivation from a carcinoma [452]. On the other hand, these values are lower than 646 the ones obtained for models with Caco-2 cells due to the absence of tight junctions [452]. Therefore, the TR146 cell layers maintained the cellular morphology as well as the integrity of the cell monolayer during the experiments.

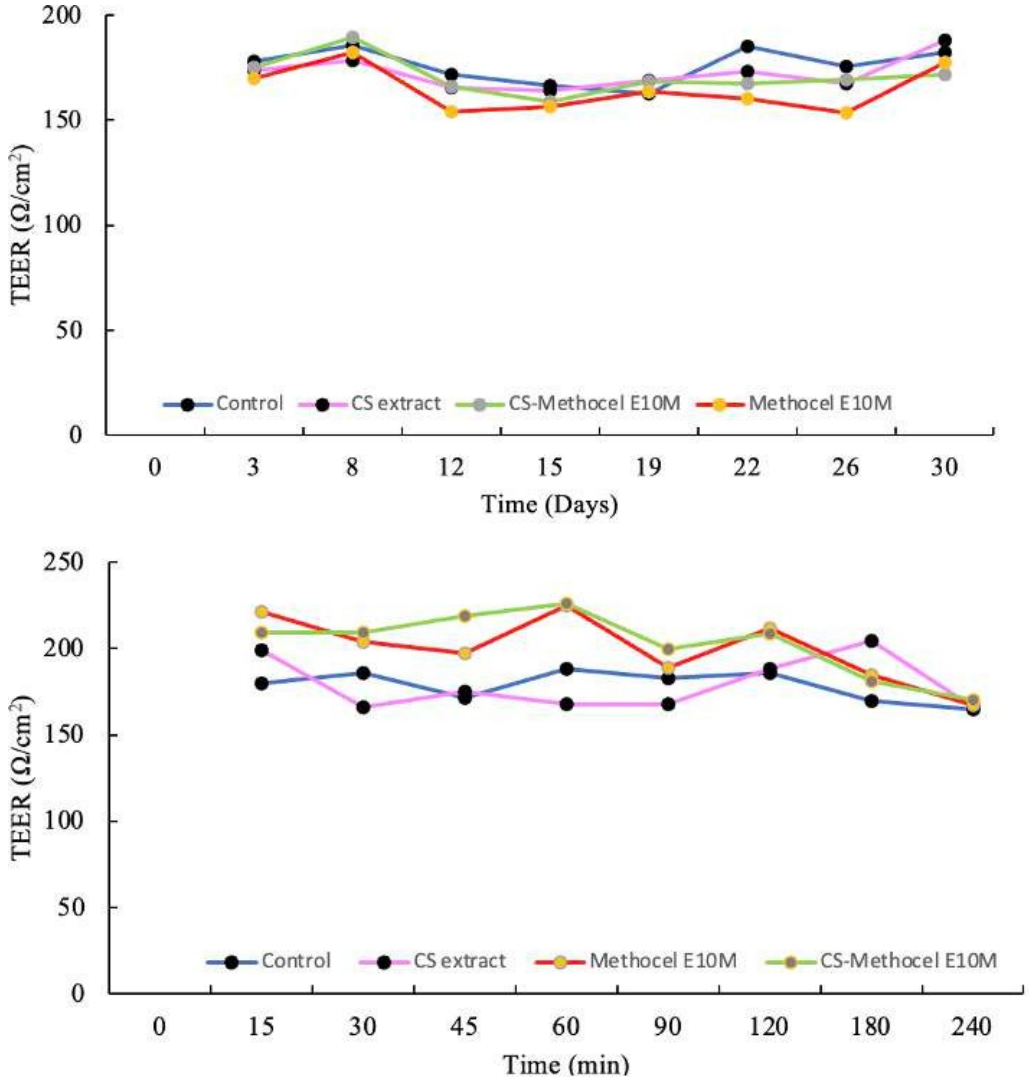


Figure 17 - Transepithelial electrical resistance (TEER) measurements of the 3D buccal model (TR146 cells) monitored during: (A) 31 days and (B) 240 min of the permeability assay.

4.3.4.2. *Ex vivo* permeability

Ex vivo studies are used as screening permeation tools and offer the advantage of reducing labor and experimental costs when compared to *in vivo* animal studies. Porcine buccal mucosa is the standard animal model for oral permeability studies, due to its close resemblance to the human buccal mucosa, as it is a nonkeratinized tissue and has a similar enzymatic composition [388]. In fact, the oral mucosa shows intermediate permeability properties between skin epidermis and gut, acting as a barrier to compounds permeation [452]. It is also worth noting that in buccal administration, the mucosa barrier properties rely on the structural and physicochemical features of the oral tissue and the active ingredients [452]. Therefore, an *ex vivo* permeation study was

performed using a vertical Franz type diffusion cell apparatus coupled to porcine buccal mucosa.

Table 23 outlines the permeated compounds identified and quantified after the *ex vivo* permeation assay of CS extract and CS-loaded OFs at different time points, up to 30 min under conditions that mimic the human oral environment, once the OFs disintegrate within 20 min.

Table 23 - Permeation of compounds present in *C. sativa* shells extract (CS) and CS-loaded Orodispersible Films (OFs) in porcine buccal mucosa (Franz cells) up to 30 minutes. Results are expressed as mean ± SD. Different letters (a,b,c,d,e) in the same line means significant differences ($p < 0.05$).

			Permeation (%)			
			CS extract		CS-loaded OFs	
			Time (min)			
Class	Subclass	Compound/Metabolite	15	30	15	30
Carboxylic acids	Amino acids, peptides	L-Phenylalanine	8.67 ± 0.03[a]	8.57 ± 1.47[a]	ND	ND
		L-Tyrosine	7.39 ± 0.79[a]	7.26 ± 0.06[a]	74.20 ± 1.98[b]	76.52 ± 2.73[b]
		Acetyl-L-leucine	63.81 ± 2.85[a]	66.76 ± 0.28[a]	104.42 ± 3.78[b]	135.84 ± 5.25[b]
	Dicarboxylic acids	Fumaric acid	15.02 ± 0.04[a]	16.78 ± 0.19[a]	ND	ND
		Succinic acid	60.67 ± 0.21[b]	70.72 ± 3.61[b]	14.00 ± 0.63[a]	16.11 ± 0.72[a]
	Indoyl carboxylic acids	L-Tryptophan	37.21 ± 0.01[a]	27.24 ± 0.23[a]	ND	ND
	Tricarboxylic acid	Isocitric acid	15.13 ± 0.29[a]	13.91 ± 1.51[a]	19.05 ± 1.12[a]	19.02 ± 0.90[a]
Cinnamic acids	Hydroxycinnamic acids	Hydroxycinnamic acid Isomer II	67.14 ± 2.35[a]	64.79 ± 2.04[a]	ND	ND
Fatty Acyls	Fatty acids	Adipic acid	11.41 ± 0.03[a]	9.47 ± 0.07[a]	98.93 ± 2.05[b]	97.59 ± 2.36[b]
		Mevalonic acid	1.26 ± 0.01[a]	1.35 ± 0.01[a]	ND	ND
		Pimelic acid	65.10 ± 1.87[a]	67.06 ± 2.58[a]	76.61 ± 4.85[a]	ND
		Sebacic acid	77.63 ± 2.51[a]	76.42 ± 1.23[a]	ND	ND
		Undecanedioic acid	85.64 ± 4.12[a]	86.86 ± 3.52[a]	ND	ND
Flavonoids	Lineolic acids	Corchorifatty acid F	4.29 ± 0.13[a]	3.79 ± 0.09[a]	ND	ND
	Flavonoid glycosides	Phlorizin	18.69 ± 0.04[a]	17.60 ± 0.30[a]	ND	ND
Organooxygen	Alcohols and polyols	Xanthine	6.94 ± 0.02[a]	6.48 ± 0.02[a]	ND	ND
		Panthothenic acid (Vit. B5)	8.70 ± 1.03[a]	10.65 ± 1.22[a]	ND	ND
		Quinic acid	29.36 ± 1.68[a]	28.33 ± 1.16[a]	70.71 ± 1.52[b]	74.31 ± 1.78[b]
		Shikimic acid	24.23 ± 0.07[a]	23.97 ± 0.92[a]	ND	ND
	Carbohydrates	Glycoprotein-phospho-D-hexose	2.42 ± 0.02[a]	2.60 ± 0.04[a]	73.62 ± 0.83[b]	89.40 ± 2.62[b]
Phenolic acids	Hydroxybenzoic acids	3-Hydroxybenzoic acid	5.91 ± 0.06[a]	4.76 ± 0.02[a]	45.86 ± 0.03[b]	105.28 ± 3.37[b]
		Protocatechuic acid	19.05 ± 1.63[a]	19.06 ± 0.61[a]	ND	ND
Phenols	Methoxyphenols	Homovanillic acid	99.88 ± 2.79[a]	100.53 ± 3.72[a]	ND	ND
Tannins	Hydrolyzable tannis	Ellagic acid	10.61 ± 0.08[a]	6.26 ± 0.75[a]	35.46 ± 0.32[b]	60.75 ± 1.62[c]

ND – Not Determined.

Regarding the CS extract, a total of 24 compounds were presumptively annotated, with a greater abundance of lipids (6 compounds, representing 25%) and amino acids (5 compounds, representing 21 %), followed by (poly)phenols (5 compounds, representing 21%), organic acids (4 compounds, representing 17%), alcohols and polyols (3 compounds, representing 12%), and carbohydrates (1 compound, representing 4%). Similarly to the *in vitro* study, compounds of interest permeated in lesser extent for CS-loaded OFs when compared to the CS extract. A total of 11 compounds were annotated for the CS-loaded OFs, with amino acids being present in greater amounts (3 compounds, representing 27%), followed by (poly)phenols (2 compounds, representing 18%), lipids (2 compounds, representing 18%), organic acids (2 compounds, representing 18%), carbohydrates (1 compound, representing 9%), and alcohols and polyols (1 compound, representing 9%).

The amino acid L-tyrosine permeated the porcine buccal mucosa up to 66.76% and 135.84% at 30 min, for CS extract and CS-loaded OFs, respectively. This amino acid can be converted from phenylalanine, also identified in CS extract, through hydroxylation and it is recognized for its antioxidant properties that help to protect skin from oxidative stress induced by UV radiation, supporting the skin's natural repair mechanism and promoting tissue regeneration [453]. L-tyrosine is also involved in the synthesis of neurotransmitters, such as dopamine and adrenaline, which play roles in modulating inflammatory responses and promoting wound healing [453]. Acetyl-L-leucine was another amino acid identified that permeated both samples. It is one of the main amino acids present in a commercialized formulation (Elental®) for the treatment of 5- fluorouracil-induced mucositis [454]. Notably, the cytotoxic effects of the chemotherapeutic agents extend to salivary tissues, leading to diminished saliva volume and alterations in salivary protein composition. These changes significantly contribute to the intensification of OM [454].

Ellagic acid was also identified, permeating 6.26% and 60.75%, respectively, for CS extract and CS-loaded OFs. This phenolic acid is well recognized by its antioxidant activity in lipid peroxidation and metal chelation, safeguarding cells against oxidative harm. It also shows chemopreventive, antimicrobial, antiapoptotic, anti-inflammatory and antimutagenic activities [455]. The *in vitro* and *ex vivo* models, as well as the CS-loaded OFs and the CS extract, presented similar permeated (poly)phenols profiles, with 35% and 20%, respectively, in the *in vitro* study (free extract and CS-loaded OFs, respectively), and 20% and 18%, respectively, in the *ex vivo* permeation assay (free extract and CS-loaded OFs, respectively). Therefore, it is possible to conclude that the bioactive compounds present in the CS-loaded OFs can permeate across oral

epithelium. To the best of our knowledge, this study provides, for the first time, an evaluation of the potential of CS administrated through OFs as active ingredient for OM prevention/treatment using an *in vitro* and *ex vivo* models.

4.4. Conclusion

In the present study, a CS extract was used as active ingredient in OFs to prevent OM. The HPMC E10M OFs showed potential for application in the buccal mucosa, as its thickness may contribute to a comfortable sensation for the patient, while simultaneously presented outstanding mechanical properties to withstand the production, packaging, transportation, and handling constraints. Stability studies indicated that OFs remains stable at room and accelerated conditions for up to 3 months, with the FTIR and DSC results supporting this statement. Cytotoxicity studies showed a selective effect of the CS-loaded OFs against tumorigenic cell lines, confirming the antitumoral effects of the CS extract, probably related with its phenolic composition. Considering that the CS-loaded OFs disintegrates within 20 min and a detachment force of 1.77 N may be sufficient to manage the tension of tongue movement, this formulation can be considered as a buccal film. The metabolic profile of the CS extract and CS-loaded OFs permeation assay revealed that (poly)phenolic compounds represented 20% of the annotated compounds, with protocatechuic acid, isorhamnetin, homovanillic acid, 4-hydroxybenzoic acid and ellagic acids achieving the best results.

This study provides, for the first time, insights in the phenolic-rich extracts potentialities to be absorbed through oral mucosa and exert pro-healthy effects against OM. Nonetheless, to determine the bioavailability of drugs administered via buccal mucosa, *in vivo* studies are crucial future steps. Moreover, the exploration of encapsulation tools or micronization to enhance bioavailability should also be considered.

Supplementary Material

Table S24 - Putative annotation of compounds detected by LC-ESI-LTQ-Orbitrap-HRMS analysis regarding untargeted metabolomic profiling using MS-dial and MS-finder platforms.

Rt (min)	Metabolite	Ontology	Neutral molecular formula	Average Mz	Theoretical mass	Error (ppm)	Total score	MS' isotopic spectrum
0.76	L-Malic acid	Hydroxy acid	$C_4H_6O_5$	133.0155	134.0215	-2.5373	7.23	134.01428:83116 135.01763:4074 136.02099:725
0.82	Verbascose	Carbohydrate	$C_{30}H_{52}O_{26}$	863.2420	863.2441	2.5381	6.81	863.26697:20574 864.27032:10099 865.27368:3349
1.14	Fumaric acid	Dicarboxylic acid	$C_4H_4O_4$	114.9631	115.0037	3.5164	6.67	115.00376:35380 116.00711:1547 117.01047:557
1.14	Isocitric acid	Tricarboxylic acid	$C_6H_8O_7$	191.0104	191.0202	4.1990	8.90	191.02008:1242036 192.02343:91598 193.02679:13890
1.25	Pyroglutamic acid	Amino acid	$C_5H_7NO_3$	128.0353	128.0355	2.1143	5.45	128.03522:67634 129.03857:4464 130.04193:1437
1.47	Xanthine	Purine	$C_5H_4N_4O_2$	151.0254	152.1143	3.1420	6.67	151.02547:108327 152.02882:5681 153.03218:266
1.75	Succinic acid	Dicarboxylic acid	$C_4H_6O_4$	117.0191	117.0191	3.1679	5.55	117.01947:242833 118.02282:10538 119.02618:1505
1.78	L-Tyrosine	Amino acid	$C_9H_{11}NO_3$	180.0751	181.0738	1.5371	7.43	180.91753:11073 181.92088:574 182.92424:304
1.94	Mevalonic acid	Fatty acid	$C_6H_{12}O_4$	147.0774	148.0735	3.9641	6.84	148.98022:19821 149.98357:363 150.98693:0
2.23	Gallic acid *	Benzoic acid	$C_7H_6O_5$	169.0143	169.0143	4.3446	5.18	169.01428:83201 170.01763:5937 171.02099:822
2.50	Adipic acid	Fatty acid	$C_6H_{10}O_4$	145.0583	146.0579	2.9864	6.85	146.0872:17256 147.09055:1360 148.09391:0
3.28	Myo-Inositol	Alcohols and polyols	$C_6H_{12}O_6$	161.0444	161.0457	5.6848	5.50	161.04607:715644 162.04942:42718 163.05278:8059
3.47	Quinic acid	Alcohols and polyols	$C_7H_{12}O_6$	191.0663	192.0633	3.3551	ND	191.05621:10822 192.05956:626 193.06292:0
4.13	Inosine	Carbohydrate	$C_{10}H_{12}N_4O_5$	267.0753	268.0807	4.6121	ND	268.80386:130934 269.80721:0 270.81057:344660
4.25	Shikimic acid	Alcohols and polyols	$C_7H_{10}O_5$	173.0483	174.0528	3.7482	7.84	174.08226:14201 175.08561:1232 176.08897:6558
4.37	L-Phenylalanine	Amino acid	$C_9H_{11}NO_2$	164.0751	165.0790	2.6254	8.26	165.05602:17963 166.05937:1566 167.06273:0
4.86	Panthothenic acid (Vit. B5)	Alcohols and polyols	$C_9H_{17}NO_5$	218.1034	218.1022	0.6427	5.37	218.10237:48219 219.10572:8398 220.10908:628

4.89	Vescalagin	Hydrolyzable tannin	$C_{41}H_{26}O_{26}$	466.0282	466.0290	3.5485	6.48	466.02896:63376 467.03231:11535 468.03567:412
4.95	Pyrocatechol	Benzenoid	$C_6H_6O_2$	108.0483	109.0295	0.0000	5.50	109.0295:112023 110.03285:8497 111.03621:531
5.05	Neochlorogenic acid *	Alcohols and polyols	$C_{16}H_{18}O_9$	352.0621	353.0882	-0.0272	8.42	353.08817:193141 354.09152:53331 355.09488:6245
5.15	L-Tryptophan	Amino acid	$C_{11}H_{12}N_2O_2$	203.0851	204.0899	-0.2764	ND	204.08998:27546 205.08993:5310 206.0899:0
5.27	Protocatechuic acid *	Hydroxybenzoic acid	$C_7H_6O_4$	153.0196	153.0196	0.7685	5.28	153.01956:81820 154.02291:6771 155.02627:940
5.32	4-*O*-Caffeoylquinic acid *	Hydroxycinnamic acid	$C_{16}H_{18}O_9$	353.0885	353.0885	-3.6764	8.48	353.08853:188163 354.09188:53844 355.09524:7459
5.34	Methylgallate *	Benzoic acid	$C_8H_8O_5$	182.0352	183.0306	0.0000	7.02	183.03064:819017 184.03399:100662 185.03735:6341
5.40	Acetyl-L-Leucine	Amino acid	$C_8H_{15}NO_3$	172.1515	173.1052	1.5172	8.34	173.10226:14201 174.10561:1232 175.10897:6558
5.40	Homovanillic acid	Methoxyphenol	$C_9H_{10}O_4$	180.0725	181.0512	0.0000	8.28	181.05124:447078 182.05459:63292 183.05795:4723
5.51	Epicatechin *	Flavonoid	$C_{15}H_{14}O_6$	289.0719	289.0724	-1.9624	7.77	289.07242:294892 290.07577:70716 291.07913:5734
5.54	Glycoprotein-phospho-D-hexose	Carbohydrate	$C_6H_{12}O_6$	179.0549	180.0633	2.5163	6.64	179.05487:23781 180.05822:1424 181.06158:44357
5.55	Pimelic acid	Fatty acid	$C_7H_{12}O_4$	159.0754	160.0736	1.8564	6.72	159.06566:45785 160.06901:5759 161.07237:332
5.56	3-Hydroxybenzoic acid *	Benzoic acid	$C_7H_6O_3$	137.0246	137.0246	1.7398	5.56	137.02458:87910 138.02793:7344 139.03129:668
5.61	Epigallocatechin gallate *	Flavanoid	$C_{22}H_{18}O_{11}$	457.0851	458.0849	3.4682	6.62	457.07568:34102 458.07903:12858 459.08239:2246
5.62	2,6-Dihydroxybenzoic acid *	Benzoic acid	$C_7H_6O_4$	153.0251	154.0266	1.1795	7.35	153.02629:22900 154.02964:1347 155.033:106
5.67	m-Cresol	Phenol	C_7H_8O	107.0563	108.0575	0.5316	ND	107.05032:32272 108.05367:2969 109.05703:0
5.72	Rutin *	Flavonoid glycoside	$C_{27}H_{30}O_{16}$	609.1463	609.1474	2.2707	8.19	609.1474:220069 610.15075:101117 611.15411:23019
5.80	Quercetin 3-*O*-galactoside *	Flavonoid glycoside	$C_{21}H_{20}O_{12}$	463.0891	463.0891	1.7539	8.38	463.08905:299453 464.0924:107861 465.09576:20583
5.82	Hydroxycinnamic acid isomer I	Hydroxycinnamic acid	$C_9H_8O_3$	163.0404	163.0404	0.6326	5.25	163.04042:100914 164.04377:14967 165.04713:455
5.86	Vanillin *	Methoxyphenol	$C_8H_8O_3$	151.0402	151.0402	0.8521	5.43	151.04024:55671 152.04359:4944 153.04695:342
5.87	Ellagic acid *	Benzoic acid	$C_{14}H_6O_8$	301.0051	302.0047	-2.6271	7.45	300.99747:267836 302.00082:67566 303.00418:7245

5.88	Homoveratric acid	Methoxybenzene	$C_{10}H_{12}O_4$	195.0669	195.0669	3.2409	6.19	195.06694:85437 196.07029:15019 197.07365:1141
5.93	Suberic acid	Fatty acid	$C_8H_{14}O_4$	173.0851	174.0892	1.6865	8.62	173.08127:79674 174.08462:14713 175.08798:722
5.95	Dihydroferulic acid *	Hydoxycinnamic acid	$C_{10}H_{12}O_4$	195.0762	196.0735	-1.6278	5.95	195.06584:19615 196.06919:3085 197.07255:240
6.00	Naringin *	Flavonoid glycoside	$C_{27}H_{32}O_{14}$	579.1713	579.1732	2.9924	7.42	579.17316:293758 580.17651:135313 581.17987:28635
6.01	Sinapic acid *	Hydroxycinnamic acid	$C_{11}H_{12}O_5$	223.0662	224.0684	4.2162	6.67	223.06075:25010 224.0641:5722 225.06746:349
6.14	Hydroxycinnamic acid Isomer II	Hydroxycinnamic acid	$C_9H_8O_3$	163.0462	164.0473	1.3961	5.26	163.04269:68213 164.04604:7591 165.0494:4847
6.22	Secoisolariciresinol *	Lignans	$C_{20}H_{26}O_6$	361.1655	361.1663	-1.9736	7.11	361.16629:162284 362.16964:58709 363.173:6237
6.26	Phlorizin *	Flavonoid *O*-glycoside	$C_{21}H_{24}O_{10}$	435.1363	436.1369	4.7261	8.46	435.13037:300575 436.13372:94283 437.13708:15243
6.55	Sebacic acid	Fatty acid	$C_{10}H_{18}O_4$	201.1137	201.1137	0.0063	7.03	201.11372:104834 202.11707:15341 203.12043:1531
6.61	Luteolin *	Flavonoid	$C_{15}H_{10}O_6$	285.0461	286.0477	1.5265	ND	285.03928:136912 286.04263:38657 287.04599:2821
6.79	Sativic acid	Fatty acid	$C_{18}H_{36}O_6$	347.2439	347.2446	2.2134	6.90	347.24457:279813 348.24792:79447 349.25128:8059
6.89	Apigenin *	Flavonoid	$C_{15}H_{20}O_5$	269.0461	270.0528	3.6282	6.89	269.04443:113259 270.04778:32998 271.05114:1976
6.93	Undecanedioic acid	Fatty acid	$C_{11}H_{20}O_4$	215.1361	216.1362	-2.5168	6.93	215.12799:80355 216.13134:20670 217.1347:1032
6.94	Isorhamnetin *	Flavonoid	$C_{16}H_{12}O_7$	315.0510	315.0517	2.2463	7.38	315.0517:956562 316.05505:195939 317.05841:17997
7.36	Corchorifatty acid F	Lineolic acid	$C_{18}H_{32}O_5$	327.2291	328.2250	3.9941	5.83	327.21793:17969 328.22128:4306 329.22464:3498
7.89	Sakuranin	Flavonoid glycoside	$C_{23}H_{20}N_4O_6$	447.1361	448.1382	2.5174	7.97	447.13425:10526 448.1376:2979 449.14096:0
8.23	Crocetin	Curcuminoid	$C_{20}H_{24}O_4$	327.1641	328.1675	4.7516	5.78	327.16159:64446 328.16494:13030 329.1683:3106
8.49	Colnelenic acid	Fatty acid	$C_{18}H_{28}O_3$	291.2526	292.2038	3.6283	5.58	291.19958:49921 292.20293:6968 293.20629:2432

8.80	Herbacetin	Flavonoid glycoside	$C_{28}H_{30}O_{17}$	637.1474	638.1483	2.5179	8.80	637.14056:115806 638.14391:49807 639.14727:4868
9.39	Aimaline	Alkaloid	$C_{20}H_{26}N_2O_2$	325.2763	326.1994	0.2774	5.37	325.18454:398558 326.18789:75607 327.19125:14636
12.07	Auraptene	Prenol lipid	$C_{16}H_{26}O_3S$	297.1583	298.1602	2.7356	6.32	297.15106:72262 298.15441:12607 299.15777:2636
13.82	Hydroxyadipic acid	Fatty acid	$C_6H_{10}O_5$	161.0457	161.0456	-1.4679	8.66	161.04562:32251 162.04897:4976 163.05233:1430

Table S25 - Quantification of permeated compounds of *C. sativa* shells (CS) extract and Oral Films (OFs) incorporated with CS extract through the *in vitro* buccal model (TR146 cells). Results are expressed as mean ± SD (n = 3). Different letters in the same line means significant differences between samples ($p < 0.05$).

Rt (min)	$[m-H]^-$ (m/z)	Compound/Metabolite	Quantification	
			CS extract (mg/ml)	CS-loaded OFs (mg/g film)
0.82	863.2420	Verbascose	36.03 ± 1.04^a	80.50 ± 3.96^b
1.14	191.0104	Isocitric acid	222.23 ± 6.57^a	306.36 ± 13.65^b
1.25	128.0353	Pyroglutamic acid	41.32 ± 2.63^b	28.93 ± 1.88^a
1.75	117.0191	Succinic acid	1.60 ± 0.03^a	0.82 ± 0.01^a
3.28	161.0444	Myo-Inositol	0.54 ± 0.01^a	0.42 ± 0.01^a
4.95	108.0483	Pyrocatechol	0.46 ± 0.01^a	0.36 ± 0.02^a
5.05	352.0621	Neochlorogenic acid	0.48 ± 0.05^a	0.36 ± 0.02^a
5.27	153.0196	Protocatechuic acid	0.53 ± 0.03^a	0.41 ± 0.02^a
5.32	353.0885	Caffeoyl quinic acid	0.40 ± 0.02^a	0.38 ± 0.03^a
5.34	182.0352	Methyl gallate	0.37 ± 0.01^a	0.37 ± 0.03^a
5.40	180.0725	Homovanillic acid	0.58 ± 0.03^a	0.41 ± 0.01^a
5.51	289.0719	Epicatechin	0.41 ± 0.03^a	0.36 ± 0.02^a
5.56	137.0246	4-Hydroxybenzoic acid	1.52 ± 0.02^a	0.36 ± 0.03^a
5.80	463.0891	Quercetin 3-galactoside	0.36 ± 0.01^a	0.36 ± 0.01^a
5.86	151.0402	Vanillin	0.70 ± 0.02^a	0.44 ± 0.05^a
6.22	361.1655	Secoisolariciresinol	0.43 ± 0.02^a	0.37 ± 0.01^a
6.55	201.1137	Sebacic acid	26.39 ± 1.68^b	10.60 ± 0.96^a
6.79	347.2439	Sativic acid	66.13 ± 3.84^b	39.85 ± 2.53^a
6.94	315.0510	Isorhamnetin	0.38 ± 0.04^a	0.38 ± 0.01^a
13.82	161.0457	2-Hydroxyadipic acid	0.51 ± 0.02^a	0.43 ± 0.03^a

Table S26 - Quantification of permeated compounds of *C. sativa* shells (CS) extract and Oral Films (OFs) incorporated with CS extract through porcine buccal mucosa (Franz cells). Results are expressed as mean ± SD (n = 3). Different letters in the same line means significant differences between samples ($p < 0.05$).

Rt (min)	$[m-H]^-$ (m/z)	Compound/Metabolite	Quantification	
			CS extract (mg/ml)	CS-loaded OFs (mg/g film)
1.14	114.9631	Fumaric acid	4.47 ± 0.98^b	0.40 ± 0.01^a
1.14	191.0104	Isocitric acid	2.20 ± 0.05^a	2.21 ± 0.16^a
1.47	151.0254	Xanthine	25.47 ± 0.13^b	1.16 ± 0.02^a
1.75	117.0191	Succinic acid	1.26 ± 0.01^b	0.38 ± 0.01^a
1.78	180.0751	L-Tyrosine	9.02 ± 0.38^b	0.99 ± 0.03^a
1.94	147.0774	Mevalonic acid	94.49 ± 4.52^b	7.60 ± 1.00^a
2.50	145.0583	Adipic acid	93.48 ± 2.39^b	11.84 ± 2.11^a
3.47	191.0663	Quinic acid	1.02 ± 0.02^b	0.39 ± 0.01^a
4.25	173.0483	Shikimic acid	1.59 ± 0.01^b	0.27 ± 0.02^a
4.37	164.0751	L-Phenylalanine	5.52 ± 0.24^b	0.42 ± 0.01^a
4.86	218.1034	Panthothenic acid (Vit. B5)	20.13 ± 1.64^b	0.89 ± 0.05^a
5.15	203.0851	L-Tryptophan	4.18 ± 0.26^a	1.18 ± 0.09^a
5.27	153.0196	Protocatechuic acid	0.97 ± 0.03^b	0.13 ± 0.01^a
5.40	172.1515	N-Acetyl-L-leucine	9.12 ± 0.17^b	0.99 ± 0.01^a
5.40	180.0725	Homovanillic acid	2.03 ± 0.03^a	1.67 ± 0.03^a
5.54	179.0549	Glycoprotein-phospho-D-mannose	22.71 ± 1.95^b	3.11 ± 0.05^a
5.55	159.0754	Pimelic acid	26.17 ± 2.25^a	30.41 ± 0.01^a
5.56	137.0246	4-Hydroxybenzoic acid	1.18 ± 0.1^b	0.09 ± 0.02^a
5.87	301.0051	Ellagic acid	7.15 ± 2.50^b	1.27 ± 0.05^a
6.14	163.0462	2-Hydroxycinnamic acid	0.49 ± 1.28^a	0.13 ± 0.02^a
6.26	435.1363	Phlorizin	1.93± 0.06^a	0.87 ± 0.06^a
6.55	201.1137	Sebacic acid	38.01± 3.41^a	32.46 ± 3.54^a
6.93	215.1361	Undecanedioic acid	23.93 ± 1.12^a	23.67 ± 5.00^a
7.36	327.2291	Corchorifatty acid F	32.51 ± 0.74^b	0.85 ± 0.01^a

Chapter 5

Conclusion and Final Remarks

This thesis explored the potential valorization of *Castanea sativa* Mill. (European chestnut) shells, a significant by-product of chestnut processing, as a source of bioactive compounds with considerable therapeutic potentiality. This work demonstrated that CS, which constitute approximately 20% of the fruit's weight, are predominantly enriched with (poly)phenols, lignans, vitamins and amino acids, which possess a broad range of beneficial properties, including antioxidant, anti-inflammatory, antimicrobial and anticancer properties, making them valuable for the OM condition.

It is particularly noteworthy that as OM is the most prevalent secondary effect of anticancer treatments, and the worldwide burden of cancer is constantly increasing, OM constitutes a great socioeconomic concern, with high impact on national health services as well as the patient's life's quality. OM is characterized by the inflammation and ulceration of the mucous membranes lining of the mouth, which induces severe pain, reduces oral intake, and can lead to weight loss. Additionally, the presence of ulcers increases the susceptibility to infections, posing a particular risk to immunocompromised individuals. Despite existing OM treatment guidelines, these interventions often provide only short-term, modest relief from pain, resulting in increased reliance on opioid analgesics and the need for parenteral or enteral nutrition. Given these challenges, natural products are garnering interest as a therapeutic alternative, due to their safety profiles and minimal or negligible toxicity.

The first step was the optimization of the SWE to maximize the yield of the bioactive compounds present in the CS. The optimal extraction temperature was 110 ºC, resulting in an extract with high phenolic content, with a particular focus on gallic acid, protocatechuic acid, catechin, epicatechin, rutin and vanillic acid. Furthermore, antimicrobial assays showed that the 110 ºC extract inhibits the growth of five out of six bacterial strains tested, particularly, two drug-resistant bacteria. The antioxidant/ antiradical activities and cytotoxic effects on TR146 and HSC-3 of this extract may be due to its higher phenolic content.

This extract was subsequently processed into spray-drying Eudragit RS 30D microparticles, overcoming issues related to the instability and poor water solubility of (poly)phenols. The encapsulation process success was evidenced by thermal and chemical results, as well by antioxidant assays, which were confirmed by LC-ESI-LTQ-Orbitrap-HRMS analysis. The microparticles were also found to be non-toxic to oral epithelial cell lines and demonstrated effective permeation of bioactive compounds in *in vitro* buccal models.

The core innovation of this work lies in the development of OFs incorporating CS extract. The resulted films exhibited excellent mechanical properties, uniform content,

and stability under standard and accelerated conditions, as confirmed by FTIR and DSC analyses. These films demonstrated selective cytotoxic effects against tumorigenic cell lines, which can be attributed to the phenolic composition of the CS extract. Metabolomic profiling from both *in vitro* and *ex vivo* permeation assays revealed that 20% of permeated compounds were (poly)phenols, including protocatechuic acid, isorhamnetin, homovanillic acid, 4-hydroxybenzoic acid and ellagic acid.

In summary, this research not only demonstrated the effective valorization of chestnut shell by-products for developing innovative therapeutic formulations but also sets a precedent for future studies to explore similar sustainable practices, thereby promoting a circular economy within the food industry. The development of OFs have the potential to offer a novel approach for the prevention and treatment of OM, as it has the ability to deliver bioactive compounds directly to the oral mucosa, enhancing treatment efficacy and patient comfort.

Further research should focus on comprehensive *in vivo* studies and clinical trials to validate the clinical efficacy, safety, and patient acceptability of the OFs for managing OM. These studies must include diverse patient populations to ensure a broad applicability and to identify any variations in treatment response. Long-term studies are essential to understand the sustained effects and potential side effects of OFs, which will be critical in determining their suitability as a standard treatment option. Additionally, further refinement of the formulation process is needed to optimize the concentration and release profile of the bioactive compounds, ensuring consistent therapeutic effects.

Finally, scaling production processes and assessing the economic feasibility of commercializing these OFs are crucial steps towards their broader adoption. Evaluating cost-effective manufacturing methods and ensuring regulatory compliance will facilitate the transition from research to market-ready products.

By addressing these areas, future research can significantly enhance the development of OFs, establishing them as a novel and effective solution for managing OM and potentially other health conditions, while contributing to sustainable advancements in pharmaceutical technology.

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