Master in Bioengineering

Formulation of a gel with antioxidant and antibacterial properties based on grape pomace extracts for topical treatment of chronic wound infections

Dissertation for Master's degree in Biological Engineering

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July 2022

U.PORTO
FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

ALiCE Lepabe Laboratory for Process Engineering, Environment, Biotechnology and Energy
Biofilm Engineering Lab
IJUP EMPRESAS
SOGRAPE ORIGINAL LEGACY WINES
FCT Fundação para a Ciência e a Tecnologia
REPÚBLICA PORTUGUESA
COMPETE 2020
PORTUGAL 2020
UNIÃO EUROPEIA Fundo Europeu de Desenvolvimento Regional

Acknowledgments

Firstly, I would like to show my tremendous gratitude to Dr. Anabela Borges, for her relentless availability, dedication, and guidance. Her knowledge, passion, and trust in me really helped me grow throughout these past few months. I would also like to thank Professor Manuel Simões for his advice and support in the elaboration of this project.

Thank you to the technicians Carla, Joana, and Sílvia from Laboratory E-103 for all the help provided, as well as the researchers from the from Laboratory E007.

Additionally, I would like to thank my lab partners, Andriy, Carolina, Igor, Mariana, Francisca and Luís, for enabling a great work environment and always being there to help. They made this project even more enjoyable. I will never forget all the great times we shared. Also, thank you to the Ph.D. students Ariana and Miguel for all the help and immense kindness.

A special thanks to Marcelo for his endless support and encouragement throughout these years. Thank you for believing in me even when I didn't and helping me through the good and the bad days.

Lastly, I would like to immensely thank my parents, for all the sacrifices, the love and the help they always have given me. Thank you for always encouraging me. I am extremely grateful to both of you.

This work was financially supported by: LA/P/0045/2020 (ALiCE), UIDB/00511/2020 and Programmatic - UIDP/00511/2020 Funding of the Laboratory for Process Engineering, Environment, Biotechnology, and Energy – LEPABE - funded by national funds through the FCT/MCTES (PIDDAC; Lisbon, Portugal). Project Biocide_for_Biofilm-PTDC/BII-BTI/30219/2017-POCI-01-0145-FEDER-030219, funded by FEDER funds through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI; Lisbon, Portugal) and by national funds (PIDDAC) through FCT/MCTES. Project POMACE4Wounds-Valorization of grape pomace by-products as antibiotic resistance modifiers to counteract biofilms-associated chronic wound infections (IJUP2021-SOGRAPE-23) funded by SOGRAPE VINHOS, Portugal through the IJUP-Companies 2021 Edition, an Initiation Research Programme (IJUP Multidisciplinary Projects Programme) of the University of Porto (U. Porto, Porto, Portugal).

Thesis outputs

The results presented in this thesis are partially presented in "IJUP'22 – 15º Encontro de Investigação Jovem da Universidade do Porto":

Teixeira, L., Simões, M., Borges, A., (2022). Antioxidant properties of pomace extracts directed for chronic wound healing. Rectory of Universidade do Porto, Porto-Portugal, 4th-6th May.

Abstract

The development of chronic wounds is increasing and affects millions of people around the world, causing great psychological, social, and economic impacts. Nonetheless, nowadays, there is still a lack of treatments that can effectively promote wound healing. Hydrogels, namely natural hydrogels, are ideal candidates for the development of advanced wound dressings that tackle current dressings limitations. This is due to their capacity to absorb exudates and inherent biological activity that promotes wound healing.

The winemaking industry generates a vast number of residues, such as grape pomace (GP), which are usually undervalued and discarded into the environment. In this regard, there is an urgent need to implement more ecological and sustainable winemaking as well as to seek innovative applications for their residues, adding value to the wine production chain and thus promoting a circular economy. Considering GP is rich in phytochemicals that are bioactive compounds with antioxidant and antibacterial activity, these can be of great value for the development of new alternatives for wound treatment, with the integration of such extracts in a hydrogel formulation to promote wound healing.

In this study, bioactive compounds were extracted from GP originating from a mixture of varieties of red (*Touriga Nacional*, *Touriga Franca*, *Tinta Roriz*, and *Tinta Barroca*) and white (*Malvasia Fina*, *Viosinho*, *Rabigato*, *Códega do Larinho* and *Gouveio*) grapes, composed by grape stalks or a mixture of skin and seeds. The solid-liquid extraction by a modified maceration process (under magnetic agitation) was performed with water, ethanol, and acetone in two water ratios (50:50% v/v and 70:30% v/v). The obtained GP extracts were evaluated for their antioxidant capacity (ABTS and DPPH assays), as well as for the richness of polyphenols (total phenolic content - TPC assay) and flavonoids (total flavonoid content – TFC assay).

The highest extraction yields, and antioxidant activity was obtained for the white GP (WGP) extracts, however, the TPC and TFC were higher for the red GP (RGP) extracts. The crude ethanolic (70:30% v/v) WGP extract from the mixture (skin and seeds) fraction was selected for incorporation in a hydrogel designed for topical wound care application.

A chitosan-alginate hydrogel cross-linked with glutaraldehyde and calcium chloride and incorporated with WGP extracts, as previously mentioned, was fabricated. It demonstrated high swelling, being potentially suitable for exudates absorption, however, almost no degradation nor release of polyphenols (TPC assay) was detected. Also, the

incorporation of WGP extracts did not enhance the antioxidant activity of the hydrogels (assessed by the ABTS and DPPH assays), with the hydrogel and its control presenting low values.

Nonetheless, the hydrogel presented antibacterial activity toward the bacterial pathogen *Staphylococcus aureus*. Using a modified hydrogel disk diffusion assay, it was possible to observe an increase in the inhibition zone diameter when compared to the negative control (Chit/Alg hydrogel, the hydrogel without any compound incorporated). The bacterial cell growth was also reduced when in contact with the hydrogel for 24h. Besides, 100% of the bacterial cells, both cell suspensions and cells adhered to hydrogel presented disrupted cytoplasmic membranes (Live/Dead assay). Lastly, the cells were not able to be cultured in solid medium after 24h of incubation with the hydrogel (total log (CFU/mL) reduction), except the washed Chit/Alg hydrogel. Actually, it was detected that glutaraldehyde can slightly influence the observed antibacterial effects of the hydrogels since, when an additional step of hydrogel washing was included, a decrease in the antibacterial capacity was denoted. The irradiation of the hydrogel with a UV light of 254 nm did not influence the antibacterial potential.

With this study, it was possible to confirm the antioxidant potential of GP extracts rich in polyphenolic compounds and flavonoids. The incorporation of these extracts in a hydrogel resulted in a biomaterial with antibacterial activity and high swelling capacity, that can potentially promote wound healing by exudate absorption and combating infection.

Keywords: Antibacterial Activity; Antioxidant Activity; Chronic Wounds; Circular Economy; Degradation; Grape Pomace Extracts; Hydrogels; Release; Swelling.

Resumo

O desenvolvimento de feridas crónicas é um problema que afeta milhões de pessoas em todo o mundo, causando danos psicológicos, sociais e económicos. No entanto, atualmente ainda existe uma carência de tratamentos que consigam promover a cicatrização deste tipo de feridas eficientemente. Os hidrogéis, nomeadamente os hidrogéis naturais, são candidatos ideais para o desenvolvimento de curativos avançados que lidem com as atuais limitações dos curativos, uma vez que estes têm a capacidade de absorver exsudados e atividade biológica inerente que promove a cicatrização de feridas.

A indústria vinícola gera um vasto número de resíduos, tais como bagaço de uva (GP), que geralmente são desvalorizados e descartados no ambiente. Neste sentido, há uma urgente necessidade de promover um processo de vinificação mais ecológico e sustentável, bem como procurar aplicações inovadoras para os estes resíduos, acrescentando valor à cadeia de produção do vinho e promovendo uma economia circular. Considerando que o GP é rico em fitoquímicos que são compostos bioativos com atividade antioxidante e antibacteriana, estes podem ser interessantes para o desenvolvimento de novas alternativas para o tratamento de feridas, através da integração destes extratos num hidrogel.

Neste estudo, foram extraídos compostos bioativos de GP originário de uma mistura de variedades de uvas tintas (*Touriga Nacional*, *Touriga Franca*, *Tinta Roriz* e *Tinta Barroca*) e brancas (*Malvasia Fina*, *Viosinho*, *Rabigato*, *Códega do Larinho* e *Gouveio*), compostas por engaço de uva ou uma mistura de pele e sementes. A extração sólido-líquido foi realizada através de um processo de maceração modificado (sob agitação magnética) com água, etanol e acetona em duas proporções de água diferentes (50:50% v/v e 70:30% v/v). Os extratos de GP obtidos foram avaliados em relação à sua capacidade antioxidante (ensaios ABTS e DPPH), bem como o conteúdo de polifenóis (conteúdo fenólico total - ensaio TPC) e flavonóides (conteúdo flavonóide total - ensaio TFC).

Os rendimentos de extração e atividade antioxidante mais elevados foram obtidos para os extratos de GP de uva branca (WGP), contudo, o TPC e o TFC foram mais elevados para os extratos de GP de uva tinta (RGP). O extrato etanólico bruto de WGP (70:30% v/v) da mistura (pele e sementes) foi o extrato selecionado para incorporação num hidrogel concebido para aplicação tópica no tratamento de feridas.

Assim, fabricou-se um hidrogel de quitosano e alginato reticulado com glutaraldeído e cloreto de cálcio e incorporado com extratos de WGP. Este demonstrou elevados valores de *swelling*, sendo potencialmente adequado para absorção de exsudados, no entanto, demonstrou degradação muito baixa e a libertação de polifenóis (ensaio de TPC) não foi detetada. Além disso, a incorporação de extratos de WGP não aumentou a atividade antioxidante do hidrogel (avaliada pelos ensaios ABTS e DPPH).

No entanto, o hidrogel apresentou atividade antibacteriana para a bactéria patogénica *Staphylococcus aureus*. Utilizando um ensaio de difusão em disco de hidrogel modificado, foi possível observar um aumento no diâmetro da zona de inibição quando comparado com o controlo negativo (hidrogel Chit/Alg, hidrogel sem qualquer composto incorporado). O crescimento das células bacterianas também foi reduzido quando em contacto com o hidrogel durante 24h. Além disso, tanto nas suspensões celulares como nas células aderidas ao hidrogel, todas as células bacterianas apresentaram integridade das membranas citoplasmáticas comprometidas (ensaio Live/Dead). Por último, as células bacterianas não se apresentaram cultiváveis em meio sólido após 24h de incubação com o hidrogel (redução total de log (CFU/mL)), exceto para o hidrogel Chit/Alg lavado. Na verdade, foi incluída uma etapa adicional de lavagem do hidrogel sem nenhum composto incorporado e foi constatado que a reticulação com glutaraldeído pode influenciar ligeiramente os efeitos antibacterianos dos hidrogéis. A irradiação dos hidrogéis com uma luz UV de 254 nm não influenciou o seu potencial antibacteriano.

Com este estudo foi possível confirmar o potencial antioxidante de extratos de GP ricos em compostos polifenólicos e flavonóides. Estes extratos foram incorporados num hidrogel com atividade antibacteriana e alta capacidade de *swelling*, que pode potencialmente promover a cicatrização de feridas por absorção de exsudato e combate de infeções.

Palavras-chave: Atividade Antibacteriana; Atividade Antioxidante; Degradação; Economia Circular; Extratos de Bagaço de Uva; Feridas Crónicas; Hidrogéis; Libertação; *Swelling*.

Declaration

I hereby declare, under word of honor, that this work is original and that all non-original contributions are indicated, and due reference is given to the author and source.

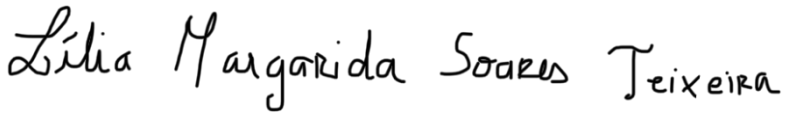


Table of Contents

Chapter 1: Work outline **1**
1.1. Background and project presentation 1
1.2. Research objectives 1
1.3. Thesis organization 2

Chapter 2: Literature review **4**
2.1. Wounds 4
2.1.1. The skin 4
2.1.2. Wound Types and Healing Process 5
2.1.3. Chronic wounds 7
2.1.4. Wound care strategies 9
2.2. Hydrogels 10
2.2.1. Hydrogel types and classifications 10
2.2.2. Hydrogels for Chronic Wound Healing 11
2.3. Grape Pomace Extracts for Hydrogel Incorporation 13

Chapter 3: Grape Pomace Extracts Preparation and Characterization **17**
3.1. Introduction 17
3.2. Materials and Methods 18
3.2.1. GP source, collection, transport and storage 18
3.2.2. Solid-liquid Extraction (SLE) 18
3.2.3. Extract Stock-solution Preparation 19
3.2.4. Total Phenolic Content Assay 19
3.2.5. Total Flavonoids Content Assay 20
3.2.6. ABTS Assay 20
3.2.7. DPPH Assay 21
3.2.8. Statistical analysis 21
3.3. Results and Discussion 21
3.3.1. Extraction Yield 21
3.3.2. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) 22
3.3.3. Antioxidant Activity of the GP Extracts by ABTS and DPPH Assay 25
3.4. Conclusions 28

Chapter 4: Hydrogel Fabrication and Characterization **30**
4.1. Introduction 30
4.2. Materials and Methods 31
4.2.1. Hydrogel Fabrication 31
4.2.2. Hydrogel Characterization 31
4.2.3. Hydrogel Antioxidant Activity 32
4.2.4. Statistical analysis 32
4.3. Results and Discussion 32
4.3.1. Hydrogel Characterization 32
4.3.2. Hydrogel Antioxidant Activity 36
4.4. Conclusions 37

Chapter 5: Hydrogel Antibacterial Activity **39**

5.1. Introduction 39
5.2. Materials and Methods 40
5.2.1. Bacterial Strains and Culture Conditions 40
5.2.2. Modified Hydrogel Disk Diffusion Assay 40
5.2.3. Bacterial Transfer from Hydrogel Disks Assay 40
5.2.4. Bacterial Growth, Membrane Integrity, and Culturability Assays 41
5.2.5. Statistical analysis 42
5.3. Results and Discussion 42
5.3.1. Modified Hydrogel Disk and Agar Well Diffusion Assays 42
5.3.2. Hydrogel Bacterial Transfer 44
5.3.3. Bacterial growth reduction 45
5.3.4. Membrane Integrity Disruption 46
5.3.5. Bacterial culturability 48
5.4. Conclusions 49

Chapter 6: Concluding remarks and future research perspective 50
6.1. General conclusions 50
6.2. Future research perspective 51

References 52

Annexes ..... a

List of Figures

Figure 1. Schematic representation of the skin...……..……..4

Figure 2. Schematic representation of wound healing process with cells involved in each phase……..……..6

Figure 3. Chemical structure of chitosan...……..……...……..12

Figure 4. Chemical structure of alginate with repeating units G (L-guluronic acid) and M (D-mannuronic acid)……..……..……..13

Figure 5. Chemical structure of flavonoids, with A and B illustrating an aromatic rings and C a heterocyclic……..……16

Figure 6. Schematic representation of the samples used (GP types, fractions and extraction solvents used).....19

Figure 7. Extraction yields of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%) RGP and WGP extracts obtained from mixture and stalks fractions……..……...22

Figure 8. Total phenolic content (TPC) of crude aqueous, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%) RGP and WGP extracts obtained from mixture and stalks fractions……..……23

Figure 9. Total flavonoid content (TFC) of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%) RGP and WGP extracts obtained from mixture and stalks fractions……..……...24

Figure 10. Antioxidant activity obtained by ABTS assay, expressed in mg of TE/g of extract, of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%.) RGP and WGP extracts obtained from mixture and stalks fractions……..……...25

Figure 11. Antioxidant activity obtained by DPPH assay, expressed in mg of TE/g of extract, of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%.) RGP and WGP extracts obtained from mixture and stalks fractions……..……...26

Figure 12. Swelling percentage (%) of Chit/Alg, Chit/Alg/GP and Chit/Alg/Trolox Hydrogel through time (0, 0.5, 1, 2, 4, 8 and 24 hours)……..……32

Figure 13. Degradation percentage (%) of Chit/Alg, Chit/Alg/GP and Chit/Alg/Trolox Hydrogel through time (0, 0.5, 1, 2, 4, 8 and 24 hours)……..……34

Figure 14. Antioxidant activity by the ABTS assay represented in µM of TE a) and scavenging activity (%) b)……..……....35

Figure 15. Antioxidant activity by the ABTS assay represented in µM of TE a) and scavenging activity (%) b)……...……36
Figure 16. Representative pictures of the bacterial transfer assay, where both conditions present *S. aureus* transfer after hydrogel removal with no growth observed in condition a) (24h incubation at 37ºC) and growth in condition b). a) is Chit/Alg/GP Non-irradiated hydrogel and b) is the Washed Chit/Alg Non-irradiated hydrogel……..…….43
Figure 17. Percentage (%) of cells with disrupted membrane and cells with preserved membrane integrity in a) bacterial suspensions in contact with the hydrogels after 24h of exposure (Chit/Alg/GP, Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg and washed Chit/Alg hydrogel) and b) suspensions with the cells previously adhered in the hydrogels surface (Chit/Alg/GP, Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg and washed Chit/Alg hydrogel) after 24 hours of exposure……45

List of Schemes

Scheme 1. Global overview of the thesis organization……3

List of Tables

Table 1. Proximate composition of GP based on dry weight…….14

Table 2. Hydrogels (Chit/Alg/GP, Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg and washed Chit/Alg hydrogel) disk diffusion inhibition zone dimeter (IZD) in mm, obtained by the subtraction of the hydrogel's diameter (8 mm) to the diameter of the halo……....40

Table 3. Agar well diffusion inhibition zone diameter (IZD) in mm, obtained by the subtraction of the well's diameter (6 mm) to the diameter of the halo……..41

Table 4. O.D. reduction (%) at 600nm of *S. aureus* growth when in contact with the hydrogels (Chit/Alg/GP, Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg and washed Chit/Alg hydrogel) in comparison with *S. aureus* cells grown without hydrogel…….……43

Table 5. Effect of the contact of *S. aureus* (CECT 978) with hydrogels (Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg/GP, Chit/Alg and washed Chit/Alg hydrogels) on their culturability in PCA culture medium, expressed in Log (CFU/mL) reduction…….…….46

Table 6. Effect of the adherence of *S. aureus* (CECT 978) with hydrogels (Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg/GP, Chit/Alg and washed Chit/Alg hydrogels) on their culturability in PCA culture medium, expressed in Log (CFU/mL) reduction……..46

Nomenclature

ABTS	2,2-azinobis (3-ethyl-benzothiazolina-6-sulfonic acid)
CE	Catechin Equivalents
CFU	Colony-Forming Unit
DPPH	2,2-diphenyl-1-picrylhydrazyl
EMC	Extracellular Matrix
FA	Fusidic Acid
FC	Folin-Ciocalteu
FTIR	Fourier-Transform Infrared Spectroscopy
SEM	Scanning-Electron Microscopy
GAE	Gallic Acid Equivalents
GP	Grape Pomace
HPLC	High-Performance Liquid Chromatography
IZD	Inhibition Zone Diameter
LCMS	Liquid Chromatography–Mass Spectrometry
MHA	Müller-Hinton agar
MHB	Müller-Hinton broth
O.D.	Optical Density
PBS	Phosphate-Buffered Saline Solution
PCA	Plate Count Agar
RGP	Red Grape Pomace
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SD	Standard Deviation
SLE	Solid-Liquid Extraction
TE	Trolox Equivalents
TFC	Total Flavonoids Content
TPC	Total Phenolic Content
WGP	White Grape Pomace

Chapter 1: Work outline

1.1. Background and project presentation

Chronic wounds are a global economic problem that continuously increases the patients´ morbidity and mortality[1]. Wound care strategies for the treatment of chronic wounds exist in great quantity, however the availability of effective treatment strategies remains limited[2,3]. The interest in the development of advanced wound dressings based on natural biopolymers, such as hydrogels, has been growing[4]. Furthermore, the incorporation of bioactive compounds in hydrogels formulation is highly beneficial since it provides healing properties to the dressings due to their antioxidant and antibacterial activities[5,6].

Grape pomace (GP) are residues of the wine production process, rich in phytochemicals that provide the extracts with attractive properties for biomedical application[7]. The extraction of bioactive compounds by sustainable methods for further incorporation in natural-based hydrogel wound dressings promotes the valorization of the winemaking industry's by-products while stimulating a circular economy.

1.2. Research objectives

This thesis was developed on the scope of POMACE4Wounds project, financed by the IJUP-Companies 2021 Edition program and developed in collaboration with *Sograpе Vinhos S.A.* (Portugal) (IJUP2021-SOGRAPE-23). This project aims to develop new bioactive mixtures based on the combination of grape pomace extracts and useless topical antibiotics, to safely achieve a more robust antibiofilm/therapeutic effect.

In particular, the aim of this study was to extract bioactive compounds from white grape pomace (WGP) and red grape pomace (RGP) obtained from grape stalks or a mixture of seeds and skin. The phenolic and flavonoid content, as well as antioxidant activity was evaluated to understand the best extraction solvent that enhances the extracts bioactivity. After selection of the best extract, a hydrogel was fabricated for its incorporation.

The characterization of the hydrogel's properties, such as water uptake capacity, degradation, and release, was assessed to evaluate the fabricated hydrogel's capacity to absorb exudates and deliver the incorporated compounds to the wound site. The

antioxidant activity of the hydrogel was studied to understand if the bioactive compounds maintained their activity after incorporation, and their potential to promote wound healing by fighting reactive species, mainly reactive oxygen species (ROS), but also reactive nitrogen species (RNS). The antibacterial activity was also examined to evaluate the hydrogel's potential to fight infections, by using pathogen *Staphylococcus aureus*, a bacterium commonly present in chronic wound infections[8–10].

Lastly, the valorization of winemaking process byproducts and promotion of circular economy, as well as the development of green sustainable alternatives for the extraction of bioactive compounds and the production of a wound directed hydrogel is a very important goal that this study goes towards.

1.3. Thesis organization

This dissertation is divided in six chapters (Scheme 1). Chapter 1 presents the context, motivation, objectives, and a guideline of the work described in the following chapters.

Chapter 2 provides a brief literature review. The characterization of wounds and their healing mechanisms are discussed, with focus on chronic wounds and the limitations of current wound care strategies. An introduction to hydrogels is also provided, where the types of hydrogels are described, and their application as wound dressings is discussed. Also in this chapter, GP and its composition is exploited, enhancing their bioactive properties and relevance as potential incorporated compounds.

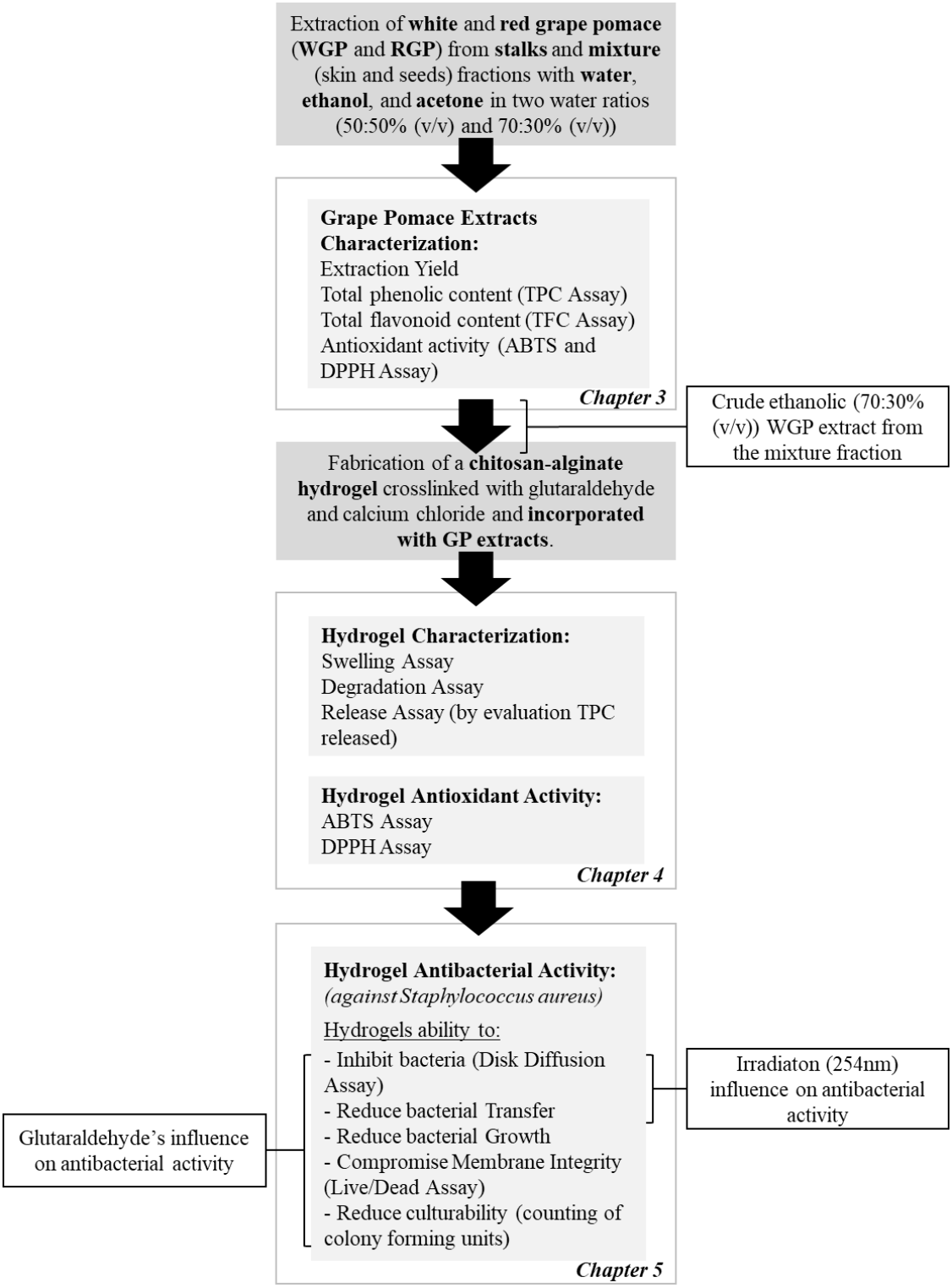
Chapter 3 focuses on obtaining bioactive extracts from WGP and RGP derived from stalks or a mixture of skin and seeds fractions, and with different extraction solvents. The richness of flavonoids and polyphenols of the GP extracts was evaluated for each extract as well as their antioxidant capacity. This led to the selection of the "best" extract, most bioactive, for further incorporation on a hydrogel.

Chapter 4 is based on the characterization of the fabricated hydrogel incorporated with GP extracts by evaluating its swelling, degradation, and release capacities. These enable a better understating on the important properties of hydrogels, water uptake and drug delivery. The antioxidant activity of the hydrogel was also explored.

Chapter 5 explores the antibacterial activity of the fabricated hydrogel by assessing its diffusion capacity and inhibition of *S. aureus*, the possibility of transferring the bacteria, promoting bacterial growth reduction, and the effect on cell membrane integrity

and cell culturability. The photosensitivity of the hydrogel and its effect on antibacterial activity is also studied by irradiation with a UV light, as well as the influence of the utilization of glutaraldehyde as the hydrogel cross-linker on the antibacterial effects.

The main conclusions and perspectives for further research were summarized in Chapter 6, giving an overview of the developed work.



Scheme 1. Global overview of the thesis organization.

Chapter 2: Literature review

2.1. Wounds

2.1.1. The skin

The skin is the largest organ of the human body which corresponds to about 15% of its weight. It has important functions such as thermal regulation, avoiding desiccation of the organism, initialization of vitamin D synthesis, excretion, and protection from the environment[11,12].

The skin has a multilayer structure constituted by epidermis, dermis, and a subcutaneous layer as can be seen in Figure 1. The outer barrier of the skin is called the epidermis and is composed mainly of keratinocytes. The epidermis's high impermeability enables the control of water loss and it serves as the main barrier against external harmful stimulation, such as radiation, microbial agents, and mechanical effects[11,13].

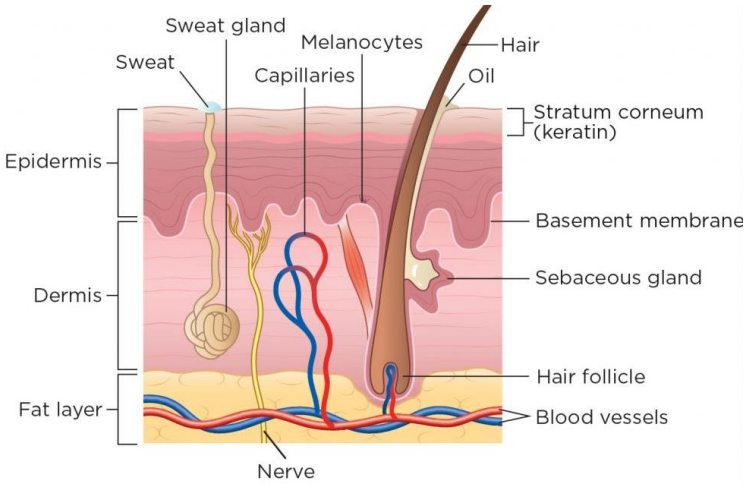


Figure 1. Schematic representation of the skin. From Lawton[14].

The dermis is located beneath the epidermis and consists of a connective tissue rich in collagen, with an extracellular matrix (EMC), fibroblasts (that release elastin and collagen), glycosaminoglycan, living cells, nerve endings, and lymphatic and blood vessels. The dermis is responsible for providing nutrition and energy to the epidermis, as well as flexibility and support for the skin. The subcutaneous layer or hypodermis, on the

other hand, is the deepest layer of the skin, rich in adipose tissue with vascularization that provides thermal isolation, regulation, and mechanical properties [11,13].

2.1.2. Wound Types and Healing Process

Any damage, defect, breakage, or disorder that affects the structure and function of the skin are called wounds. These can be caused by thermal and physical trauma, like injuries or surgical interventions, or by the presence of medical or physiological conditions, that lead to instability of the area and can jeopardize normal body processes [11,13]. Generally, wounds can be classified by open or close wounds regarding the nature of the damage on the skin, or acute and chronic wounds depending on the time of healing and the factors that induced determined injuries[11].

Open wounds are a type of injury that causes external or internal breakage in the body's tissue, which, if left untreated, can potentially expose the body to pathogens, risking infection. These consist of lacerations, cutting-pricking tool and surgical wounds, insect bites, stings, radionecrosis, or vascular, neurological, and metabolic wounds[15,16]. Close wounds, however, consist of contusions, hematomas, and abrasions such as damage of soft tissues, small blood vessels, or deep tissue layers[11].

On the other hand, an acute wound is characterized by their spontaneous healing of about 8 to 12 weeks and is caused by external damage to the skin, such as radiation, extreme temperature changes, or contact with chemicals. This type of wound includes surgical wounds, bites, burns, minor cuts, and abrasions. Treatment might be required, varying according to the location, and depth of the wound. When it comes to chronic wounds, these have a very slow healing process, and some never heal due to prolonged inflammation[11,13,16]. These are caused by endogenous mechanisms associated with a predisposing condition that potentially compromises the dermal and epidermal tissue. Pathophysiological abnormalities can lead to chronic wounds like leg and foot ulcers, and pressure sores, due to compromised arterial supply, venous drainage, and metabolic diseases like diabetes. Other factors, like advanced age, obesity, smoking, malnutrition, and immunosuppression can aggravate chronic ulceration[16].

Contrarily to the continuous skin cell regeneration process, which is a process where the epithelial cells of the skin are eliminated and replaced regularly, the wound healing process is one of the most complex dynamic biological processes in the organism. It results from the cooperation of various biological molecules and stem cells that promote

wound repair[13]. Separate parts of the same wound can be at different stages in a moment in time, and the timing and interactions can vary with the type of wound. However, independently of the type, the main phases are maintained, which are the hemostasis, inflammatory, proliferation, and remodeling phase[17], as subsequently illustrated in Figure 2.

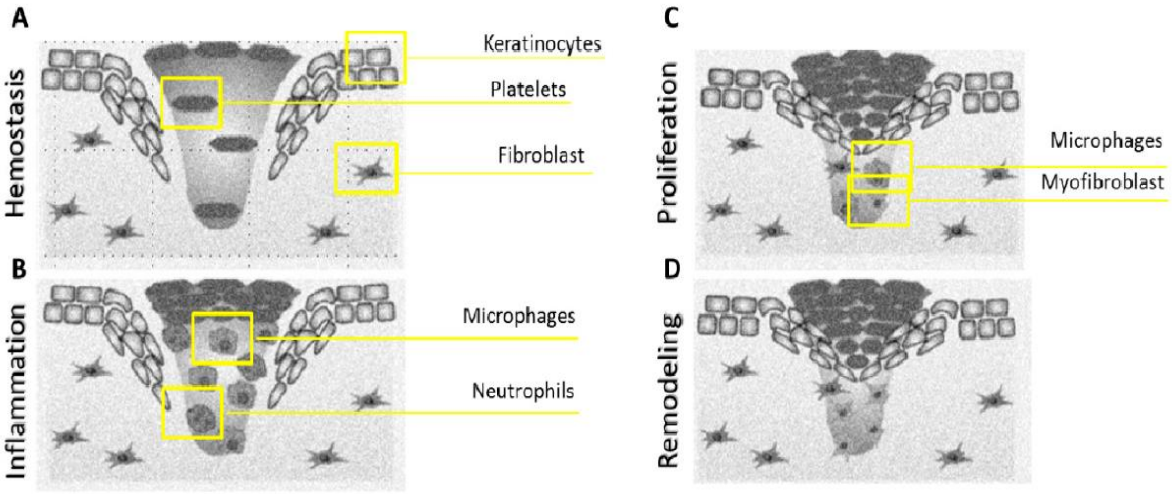


Figure 2. Schematic representation of wound healing process with cells involved in each phase. From Tottoli *et al.* [12].

The first stage is the hemostasis phase that occurs immediately after the injury. In this stage, the blood and fluid loss in the wound site is prevented by a coagulation cascade. It starts by the production of a platelet clot and then the formation of a fibrin matrix that facilitates cell infiltration[11,12]. This provides a matrix for the cells that intervene in the following steps, which is an important goal of this phase[17]. The prevention of infections and the removal of necrotic or devitalized tissue from the wound is facilitated by the inflammatory responses and the immune system involvement[11,12].

Consequently, the second stage of the healing process is the inflammatory phase. This phase can last from 24h to 6 days[11], and it aims to convey an immune barrier against potential pathogens that might populate the wound[17]. It starts by the release of immune cells with proteolytic enzymes, ROS, and pro-inflammatory cytokines to the wound region. In this phase neutrophils and macrophages remove particles and tissue debris from the wound bed, preventing infections[18,19]. Firstly, neutrophils actuate by phagocytizing bacteria, foreign particles, and damaged tissue. After the removal of contaminating bacteria, their activity changes, and they are eliminated by apoptotic bodies, which are subsequently phagocytized by macrophages[17]. Macrophages coordinate all events evolved in response to damage[12], act as regulatory cells and provide growth factors to the

tissue. Lymphocytes act about 72h after injury being the last cells to act on the wound site in this phase. They have an important role in collagenase regulation that is needed in the following phases, namely for collagen remodeling, development of the ECM, and degradation[17].

The third stage is the proliferation phase, which starts about 3 days after injury and can last up to 2 weeks[17]. Here, stimulated by the release of cytokines and enzymes, fibroblasts and myofibroblasts deposit in the new ECM, acting as a substitute for fibrin and fibronectin[11,17]. Granulation tissue is typically formed in the edges of the wound, where keratinocytes tend to migrate and proliferate[12]. By the end of the first week, EMC accumulates, promoting the support for cell migration. Then fibroblasts change to their myofibroblast phenotype, which helps attach the fibronectin and collagen to the ECM. This is important for the following phases since it provides integrity and strength to the tissues, playing a relevant role in the proliferative and remodeling phases of repair. Furthermore, angiogenesis, the process of formation of blood vessels, occurs mainly in this phase, as well as epithelization, the migration of epithelial cells or the surrounding tissue, that starts a few hours after wounding by a formation of a single layer of cells over the injury[17].

Finally, the last stage of the healing process is remodeling. Here new epithelium and final scar tissue is formed. It can last from 1 to 2 years[17]. During this phase the various processes that occurred in the previous phases stop and macrophages, isolated endothelial cells, and myofibroblasts suffer apoptosis or leave the wound site. This way the region becomes rich in collagen and the deposition of ECM proteins occurs, leading to an increase in the new tissue tensile strength. The existing interactions between the epidermis and dermis allow the continuous regulation of skin integrity and homeostasis [11,12].

2.1.3. Chronic wounds

Chronic wounds are characterized by their persistent inflammatory phase. This benefits microorganisms which form biofilms and release platelet-derived factors, which can lead to damage in ECM proteins and premature cellular senescence. Chronic injuries are also known by their inability to effectively respond to wound healing signals[12].

The main physical manifestations of chronic wounds are represented by exudation**,** persistent infection, and necrosis. The wound exudation is a great indicator of the state of an injury or the effectiveness of a wound treatment since it reflects the micro-environment

of the wound bed. In addition, exudates can possibly be an indicator for systemic complications, helping with the selection of the most appropriate treatment approach. However, it must be tackled promptly since the consequences of chronic injuries can be aggravated by the exudate components that cause continuous ECM degradation[12].

Infection is also an indication of chronic wounds. In response to infection, the skin stimulates an inflammatory response that produces exudate, as well as activates the production of antimicrobial peptides (AMPs). The access of bacteria to the wound bed is an inevitable occurrence that in extreme cases can lead to severe complications[12].

Healthy skin microbiota plays an important role in protecting against pathogens, regulating the immune system, as well as providing a barrier. In the case of injury, the bacteria can move to the wound region and cause infections by colonizing and proliferating[12,20]. In fact, the exposed tissue is favorable for microorganism colonization and microbial growth especially if the tissue is devitalized and the immune response is compromised. The bacteria may originate from the external environment, such as *S. aureus*, the surrounding skin, such as *Staphylococcus epidermidis*, or from endogenous sources. Most wound infections involve a mixed population of both aerobic microorganisms, such as *Pseudomonas aeruginosas* and *Escherichia coli*, and anaerobic microorganisms, such as *Prevotella bivia* and *Clostridium clostridioforme* [16].

An additional bacterial risk is represented by the formation of biofilms, which are complex communities of microbial cells embedded in a self-produced matrix with the ability to adhere to the wound bed. This protects bacteria and enhances their proliferation [12,20]. The biofilm matrix facilitates the bacteria's survival in difficult conditions and confers resistance to antibacterial treatments. In addition, biofilms cause various chronic diseases, such as chronic wounds, and the emerging antibiotic resistance in bacteria can difficult its treatment[12].

Lastly, devitalization or/and necrosis is another manifestation of chronic wounds, and it occurs when there is unresolved infection, or irreversible damage of the tissues. This is a problem defined as cells or tissue death for pathological reasons[12].

Something that also greatly influences the wound healing process is the antioxidant activity on the wound site. However, chronic wounds sustained inflammatory response can lead to the accumulation of ROS to an extent that exceeds the antioxidant capacity of host cells and prevents the transition to the proliferative phase[11].

The organisms' production of proteolytic enzymes and ROS occurs during the inflammatory stage, where the main goal is to remove the debris, damaged tissue, and

bacteria[19]. The production of ROS acts as a defense mechanism[21]. At low levels of concentration, ROS are beneficial to normal wound healing promoting homeostasis, stimulating cell migration and angiogenesis[22]. Yet, high concentrations of ROS can interfere with the integrity of tissues and the ECM, sometimes to a severe extent[23], decreasing the healing process by damaging cellular membranes, proteins, DNA, and lipids. Fibroblasts can be killed and skin lipids flexibility is decreased by excessive ROS [19,24]. Antioxidants, on the other hand, counter the excess of proteases and ROS, and protect protease inhibitors from oxidative damage [25].

Hence, the role of antioxidant and antibacterial agents is extremely important for the treatment and management of chronic wounds, since they help combat adverse effects on the wounds by removing products of inflammation and fighting bacterial infection.

2.1.4. Wound care strategies

When it comes to wound care strategies, most of the approaches are directed to the wound bed itself. However, as previously mentioned, the skin surrounding the wound, which is called periwound, has also a very important role in the healing process. It provides the proper environment to facilitate healing as the source of epithelial cells needed for wound closure[26].

The periwound skin area extends 4 cm around the wound edge, but the damage can reach beyond this zone[27,28]. Normally the wound is cleaned by a normal sterile saline solution. However, contamination of the periwound can be observed even though the wound bed was cleaned[29]. This area faces several challenges during the healing process that include: intrinsic factors, such maceration, which is the excess of humidity in the wound; excoriation, a simple lesion in the superficial layer of the skin; dry skin; and hyperkeratosis, which is the increasing of keratin and the thickening of the outer layer of the epidermis[26].

The exudate is one of the main reasons for excessive moisture in the skin, which impairs the epidermal barrier function, increasing the permeability of the stratum corneum[26]. Other body secretions can lead to periwound inflammation, such as urine, saliva, mucus, sweat, and stool[27]. This forestalls the wound from healing, possibly resulting in chronic wounds and expansion of the damaged area. Currently, the available treatment options do not take into consideration the periwound and can even represent a risk for its cicatrisation[26].

The wound assessment and treatment is based on four main concepts: tissue evaluation and debridement of the wound bed, infection assessment and treatment by systemic or topical antibiotics, moisture balance evaluation and management of exudates, and lastly, evaluation of epithelial advancement of the surrounding skin[12].

Current wound dressings adhere to the wound surface which can cause mechanical debridement during the replacement of dressings, traumatizing the new epithelial tissue and periwound, delaying healing, and causing suffering to the patient[30]. Besides, they mostly act as passive advanced dressings since they provide protection from the environment but do not have an active role in stimulating healing. Besides, it is required the addition of external factors as antibiotics to combat infection[12].

Considering all that was previously mentioned, new alternative wound healing strategies must be developed. Some characteristics should be met to obtain an ideal wound dressing, which are described as follows. Firstly, they should stop bleeding, relieve pain, provide mechanical protection, absorb excess exudate, and keep the wound moist. Also, they should attach to the skin, but do not adhere with new granulation tissue, in fact it should promote and accelerate its formation. They should enhance healing and the re-epithelialization rate, have good water vapor, and gas permeability and reduce the risk of infection. Lastly, they should be biocompatible, and nontoxic. Keeping this in mind, several wound dressings based on synthetic and natural polymers have been developed, with hydrogels being considered ideal candidates[4].

2.2. Hydrogels

2.2.1. Hydrogel types and classifications

Hydrogels are three-dimensional cross-linked networks of natural or synthetic polymers that have the ability to absorb or retain large amounts of water or biological fluids[31,32]. They are characterized by having at least 10% water in their constitution, which gives them a similar flexibility as natural tissues[33]. They have high absorbing capacities while presenting resistance to dissolution. This is due to the hydrophilic groups present in their polymeric chains and the cross-linking extent between chains[34].

The action of cross-linking agents is very important in the synthesis of any hydrogel, since the resulting structure highly influences the materials' characteristics. The forces that enable the cross-linking of a polymer are mainly covalent, but other forces also occur such as electrostatic, hydrophobic, dipole-dipole interactions, or hydrogen bonds[35].

Hydrogels can be classified according to the source of the polymers as natural and synthetic. Natural hydrogels are biocompatible, biodegradable, and contain biologically recognizable moieties. However, oftentimes these polymers lack the required mechanical properties for application and can trigger an inflammatory or immunological response in the host. On the other hand, synthetic hydrogels can be designed to have the desired properties, which is an advantage, but do not have any inherent bioactivity[36].

Another classification method is based on their preparation, being homopolymers when only one type of monomer is part of the polymeric chain, copolymers when two or more monomers compose the chain, or interpenetrating networks (IPN) when two different types of polymers are cross-linked with each other[36].

When it comes to the types of cross-linking connections of the hydrogels, they can be physical or chemical. Physical if the cross-linked networks are temporary due to chain entanglements, ionic interactions, hydrogen bonds or hydrophobic interactions; and chemical if the cross-linked networks are permanent[34].

Other classifications for this type of material can be related to the ionic charges of the polymer (cationic, anionic, nonionic, zwitterionic, and ampholytic) or according to their configuration, being amorphous if they have a random network structure, crystalline if they have an organized polymer network, and semi-crystalline if they present a mixture of both[34,36].

Hydrogels can react to stimuli that lead to increases or decreases in their volume by absorption of water, swelling or de-swelling. These changes can be due to physical stimuli, like temperature, electro-magnetic fields, irradiation, and pressure, or chemical stimuli, such as pH, ions, or specific chemical compositions. In most of the cases the transitions are reversible, returning to the initial state when the trigger is removed[33].

Nowadays, some of the most frequent types of hydrogels used are light-responsive, pH, temperature, and electro sensitive hydrogels. These have various applications such as drug delivery, dyes and heavy metal ions removal, scaffolds in tissue engineering, biosensors, and others[33,35].

2.2.2. Hydrogels for Chronic Wound Healing

Hydrogels are considered to be ideal dressing candidates for wound healing, because of their 3D structure, good permeability, excellent biocompatibility and ability to provide a moist environment to the wound. Also they enable the degradation of wound

debridement, absorb the wound exudates, and have low adherence to the wound, which overcomes the limitations of traditional dressings[13,37].

Natural polysaccharides, such as chitosan and alginate, are frequently used for biomedical applications, namely chronic wound healing, due to their biodegradability, biocompatibility and drug loading capacity[38,39].

Chitosan is a copolymer constituted by β-(1→4)-linked 2-acetamido-2-deoxy-d-glucopyranose and 2-amino-2-deoxy-d-glucopyranose units. It is derived from chitin, the main component of the exoskeleton of crustaceans, and obtained by alkaline deacetylation of this compound. The degree of deacetylation as well as chitosan's molecular weight highly influences its properties[40]. Chitosan is commonly used in hydrogels formulation due to its biodegradability, non-toxicity, antibacterial effect, and biocompatibility. Also, it presents beneficial characteristics for wound healing since it stimulates homeostasis and accelerates tissue regeneration[39]. Chitosan and alginate present oppositely charged molecules, therefore their complexation is an effective approach to improve chitosan properties for controlled drug delivery applications, and also to promote the mechanical properties of the hydrogel[4,41,42].

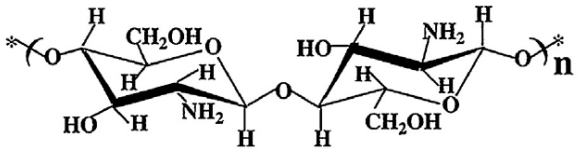


Figure 3. Chemical structure of chitosan. From Baysal *et al.* [39].

Alginate is a linear anionic polysaccharide derived from brown algae or bacteria, that consists of β-1,4-linked D-mannuronic acid (M) and L-guluronic acid (G) repeating units in varying ratios, illustrated in Figure 4[43]. A common conformation of alginate is the egg-box conformation, where G monomers of antiparallel chains form dimers[44]. Its hydrophilicity, excellent biocompatibility, biodegradability, moisture permeability and liquid absorbing capacity of up to 300 times of its own weight make alginate an attractive material for wound dressings[45,46]. Most importantly, alginate hydrogels do not adhere to the wound tissues[47], and their removal will not cause injuries to the wound when replaced, namely to the periwound[4]. Alginate can also accelerate chronic wound healing by stimulating macrophages, monocytes, fibroblasts, endothelial cells and tumor necrosis factors[46].

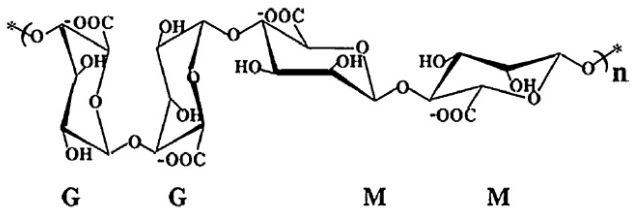


Figure 4. Chemical structure of alginate with repeating units G (L-guluronic acid) and M (D-mannuronic acid). From Baysal *et al.*[39].

Alginate-based wound dressings can be applied in the form of hydrogels, films, foams, nanofibers, membranes, and sponges. The film/gel option is used as a primary waterproof dressing due to its conformable and adhesive properties, with the transparency of the formulation facilitating wound inspection[48]. Foams are solid porous matrices that absorb exudate and are also conformable when applied to body surfaces[49], however, these are usually non-adhesive and require the use of secondary dressings[4]. Nanofibers are highly absorbent and can form gels when they come into contact with fluid, however they should not be used in dry wounds as they can produce fibrous residues[48,50].

Besides the inherent activity of some natural polymers, the hydrogels can be incorporated with functional substances, such as vitamins, minerals, oils, proteins, and biologically active natural products. With the aim to accelerate wound healing, many hydrogel dressings with antioxidant and antibacterial functions have emerged in literature. In these cases, bioactive compounds are delivered to the wounds in a controlled way imposed by the absorption and release strategies of the hydrogels[37]. Hereby more favorable conditions are created for the treatment of wounds, providing alternatives for the lacking existing solutions for wound care in the current days[13].

2.3. Grape Pomace Extracts for Hydrogel Incorporation

To minimize the undesirable effects of synthetic drugs, natural extracts have been investigated as an alternative approach. Due to their high phytochemical content their incorporation in hydrogels has been studied to promote hydrogels' bioactivity. Actually, the utilization of natural compounds might reduce side effects and promote a green and sustainable alternative for the current treatment strategies[51].

Grapes are one of the most produced crops worldwide with a production estimate of about 79 million tons in 2020, according to the Food and Agriculture Organization (United Nations)[52]. Approximately 75% of produced grapes are intended for wine production, out of which grape pomace (GP) represents around 20–30%, being classified

as waste products[53,54]. GP results from the pressing of whole grape bunches, and it consists of skins, remaining pulp, seeds, and stalks from the plant[54,55].

Currently, the fate of these by-products is waste disposal, alcohol production, or as fertilizers and animal ration. However, these options are insufficient and often environmentally unsustainable. Therefore, the search for alternatives for the valorization of these by-products is imperative[56].

GP is a complex substrate composed mainly of water content (55-75%). Its chemical composition includes dietary fiber (43-75%), proteins (6–15%), lipids, sugars, and a wide diversity of phenolic compounds[57], as can be seen in Table 1.

Table 1. Proximate composition of GP based on the dry weight. From Antonić *et al* [56].

Compounds	Quantity g/100g	Compounds	Quantity mg/100g
Ash	1.73-9.10	Na	87-244
Protein	3.57-14.17	K	1184-2718
Fat	1.14-13.90	Mg	92-644
Total Dietary Fiber	17.28-88.70	Ca	91-961
Insoluble Fiber	16.44-63.70	Mn	6-1356
Soluble Fiber	0.72-12.78	Fe	5-5468
Carbohydrates	12.20-40.53	Zn	2-2254
TPC	0.28-8.70	Cu	39-130
Frutose	0.38-8.91	P	4-3157
Glucose	0.21-26.34		

Dietary fibers are predominant compounds in RGP though in WGP their content is significantly reduced. The main group of dietary fibers are insoluble fibers like cellulose and hemicelluloses. Some fiber compounds can bond with phenolic substances and create antioxidant dietary fibers, giving the pomace stronger radical scavenging potential. Since fibers from grape skin mainly consist of lignin, cellulose and hemicellulose, these constituents can be used as a source of supporting materials[56].

The content of the soluble sugars, like glucose and fructose, is usually low in RGP. This is because normally RGP is obtained after red wine fermentation has already started, leading to the consumption of these sugars by yeasts. On the other hand, higher glucose and fructose contents are found in WGP, since it is mainly obtained before the fermentation process[56,57].

The main representatives of phenolic compounds are anthocyanins (only in RGP), catechins, flavonol glycosides, phenolic acids, and alcohols[53]. Together with dietary

fibers, phenolic compounds are the most valuable compounds of GP with health beneficial properties, such as the prevention of intestinal and chronic diseases, and cancer[19,53]. The polyphenols in the winemaking process mainly remain in GP after the process (about 70%) due to incomplete extraction[56]. In the case of RGP, the phenolic content represents about 9 kg per tonne of GP. Nonetheless, this type of compound is present in both RGP and WGP[54].

Phenolic compounds are phytochemicals found in most plant tissues[57]. They are known to have several effects on human health due to their bioactive properties, such as, antimutagenic, anticancer, antitumor, anti-inflammatory, antioxidant, and antimicrobial activities[58]. Also, they are the most abundant secondary metabolites in plants and possess a chemical structure that has an aromatic ring with one or more hydroxyl groups and can be divided into several classes. The main groups of phenolic compounds include flavonoids, phenolic acids, tannins, coumarins, stilbenes, quinones, curcuminoids, and lignans[59,60].

Phenolic compounds can be also categorized by the way they occur. In nature, they mainly exist in either a soluble or bound form. The intracellular endoplasmic reticulum synthesizes the majority of soluble phenolic compounds, which are then stored in the plant's vacuoles. While bound phenolic compounds are formed by the interaction of soluble phenolic compounds with the cell wall macromolecules, contributing to cell wall formation[60].

As mentioned before, flavonoids are phenolic compounds that form a large group of natural products. To date, more than 8000 different flavonoids have been documented[61]. They are plant pigments that function as chemical deterrents, UV-protectants and pollination enhancers, that provide defense mechanisms against microorganisms and other non biotic stress[62]. They also exhibit attractive pharmacological properties as described for phenolic compounds in general[63].

Flavonoids are polyhydroxy compounds composed of two aromatic (A and B) and a heterocyclic rings (C), that are produced by various biosynthetic pathways[64]. Their chemical structure is dependent on a basic skeleton of C6-C3-C6[61] (see Figure 5). These types of phenolic compounds are structurally distinct while containing a conserved core, the phenyl benzodihydropyran (flavan) where R groups are commonly hydroxy, methoxy, prenyl, or glycosyl groups[63]. Flavonoids can be divided into six main subclasses according to their chemical structure: flavones, isoflavones, flavanones, flavonols, flavan-3-ols (flavanols), and anthocyanins[64].

Anthocyanins and flavonols are the most abundant polyphenols in GP[57]. However, in WGP anthocyanins are reduced. Up to 65% of the total grape flavanols content is found in seeds, while for the skins the total flavanol content decreases to 21%[56]. Grape stems contain phenolic acids, flavonols, and flavanol monomers, condensed tannins, with catechin being the major flavanol monomer, and epicatechin being present in trace amounts [65].

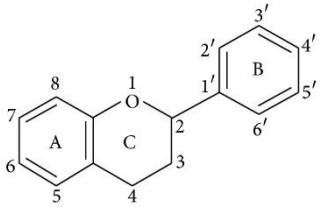


Figure 5. Chemical structure of flavonoids, with A and B illustrating aromatic rings and C a heterocyclic ring. From Kumar *et al.* [66].

The antioxidant activity of polyphenols highly depends on the structure of the molecules, the initiation conditions, and its microenvironment[67]. Considering the amount and diversity of phenolic content in GP, it is natural that GP possesses a high antioxidant capacity, and therefore it can be a valuable source of natural antioxidants[54].

Polyphenols also have antibacterial activity, being able to deal with drug-resistant bacteria. Flavonoids especially, act directly by killing bacteria, activating antibiotics and attenuating bacterial pathogenicity[68].

Therefore, incorporation of GP bioactive extracts and/or extracted phytochemicals like phenolic compounds in hydrogels can potentially be used as wound care alternative strategies, while promoting the valorization of the winemaking industry's by-products and fomenting circular economy.

Chapter 3: Grape Pomace Extracts Preparation and Characterization

3.1. Introduction

GP is the resultant residue of the winemaking industry, that consists of remaining pulp, skins, seeds, and stalks, and amounts to 20 to 30% of the weight of the grapes processed[56]. Currently, GP is used to produce distilled drinks, animal feed and fertilizers, however alternative applications to valorize this residue have arisen in the food, cosmetic and pharmaceutical industry[69].

GP is described to be rich in bioactive phenolic compounds, such as flavonoids, that provide antioxidant and antibacterial properties[70]. Anthocyanins and flavonols are the most prevalent polyphenols in GP, being anthocyanins, the pigment responsible for red color, mainly present in RGP, while flavonols are more abundant in WGP[70]. Also, RGP and WGP exhibit different compositions in terms of sugar content, since RGP undergoes alcoholic fermentation before being removed contrarily to WGP[69].

With the aim to take advantage of GP properties the recovery of bioactive compounds has gained interest by various researchers throughout the years[71]. Solid-liquid extraction (SLE) is a technique used to extract the compounds from the interior of a solid material, that can be performed by various methods such as maceration, percolation, supercritical fluid extraction, and accelerated solvent extraction[72]. This is the most reported process for the recovery of phenolic content in GP[71]. The extraction yield of bioactive compounds can be affected by many factors such as the composition of the sample (type of phytochemicals), the extraction method, polarity of the solvents, the size of the samples (if it's a powder or not), the ratio between solid and solvent, and the physical conditions of the extraction. Amongst of them, the selection of the solvent is very important since it is the parameter that weighs more when the samples are treated under the same extraction time and temperature[73,74].

In this study a modification of the maceration extraction technique using 3 solvents of different polarities (ethanol, acetone, and water) at different ratios of water (50:50% v/v and 70:30% v/v) were applied to obtain bioactive GP extracts. Additionally, the total phenolic and flavonoid content of the extracts was assessed, as well as their antioxidant

activity. This way a better insight was provided for the selection of the most suitable GP extract for incorporation in a new topical hydrogel for chronic wound treatment.

3.2. Materials and Methods

3.2.1. GP source, collection, transport and storage

The GP was harvested at *Sogrape Vinhos SA.*, Vila Real, Portugal, on the 20th of September 2021 in different parts of the winemaking process. The RGP was constituted by the *Touriga Nacional*, *Touriga Franca*, *Tinta Roriz* and *Tinta Barroca* varieties, and the WPG was constituted by *Malvasia Fina*, *Viosinho*, *Rabigato*, *Códega do Larinho* and *Gouveio* varieties. In the case of the WPG, the samples were collected after grape stalks removal and the pressing stage. However, for RGP the harvesting was carried out after the initiation of the fermentation process.

Then, the samples were immediately taken into the laboratory, and RGP and WGP were divided into two types of fractions based on their composition. One was composed of stalks only, while the other was a mixture of grape seeds and skins. After, the GP were separated in bags of 500 g and frozen at -20ºC, followed by lyophilization at -80ºC and 1.3 Pa (SP Scientific, New York, USA) until complete dehydration. The dehydrated GP was then powdered in the blender and conserved at 4ºC until extracts preparation.

3.2.2. Solid-liquid Extraction (SLE)

A SLE was carried out by a modified maceration process (under magnetic agitation) for each fraction type (stalks and mixture of seeds and skins) with 3 different solvents at different water ratios. The solvents used were water, ethanol 50% (ethanol:water, 50:50 v/v), ethanol 70% (ethanol:water, 70:50 v/v), acetone 50% (acetone:water, 50:50 v/v), and acetone 70% (acetone:water, 70:50 v/v) (VWR International).

The powdered GP was mixed with the solvent 1:10 (w/v) and left reacting in agitation for 24h in the dark. Then, the mixture was centrifuged at 4000 rpm for 15 minutes (Eppendorf centrifuge 5810R; Eppendorf AG, Germany), and the supernatant was collected. Figure 6 schematizes the 20 different extracts obtained from both WGP and RGP. Then the solvent was evaporated in the rotary evaporator (BÜCHI Heating Bath B-490) at 60ºC for ethanol and 30ºC for acetone at 235 mbar. The extracts were frozen once again at -80ºC and lyophilized until they were completely dry.

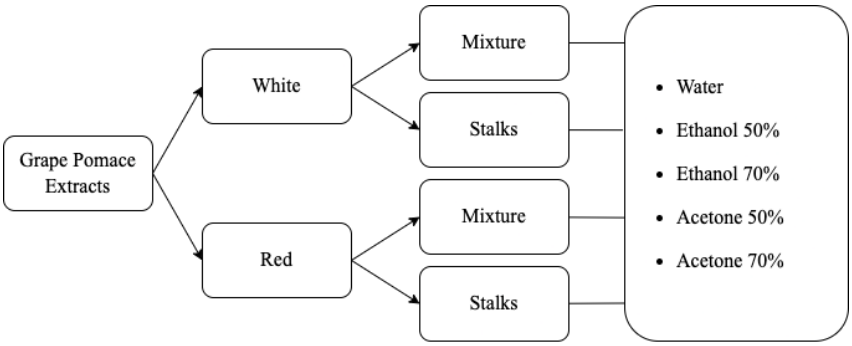


Figure 6. Schematic representation of the samples used (GP types, fractions and extraction solvents used).

The yield of this extraction was calculated by comparing the weight of the dried sample before solvent extraction, and the dry weight of each sample after the lyophilization process, as can be seen in equation 1.

$$Extraction\ yield\ (\%) = \frac{mass_{final}\ (g)}{mass_{initial}(g)} \times 100\% \qquad (1)$$

3.2.3. Extract Stock-solution Preparation

Previously lyophilized extracts of RGP and WGP were dissolved in ethanol or methanol (when the extracts were unable to be weighed due to adherence to the flasks) with the help of an ultrasound bath to obtain stock solutions. The concentrations were then normalized to 0.04 mg/mL by dilution of the samples with the corresponding solvent. All stock solutions were kept in the dark, at -20ºC, until further necessity.

3.2.4. Total Phenolic Content Assay

The quantification of the total phenolic content (TPC) was estimated by the method of Folin-Ciocalteu (FC) according to the protocols proposed in Lamuela-Raventós *et al.*, (2017)[75], and H. Abramovič *et al.*, (2017)[76]. The GP extracts stock solutions were firstly diluted by 1:19 and then 96 µL were mixed with 736 µL of ultrapure water (Milli-Q) and 48 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, Portugal). After 5 minutes of reaction 120 µL of a solution of sodium carbonate (20%) was added. The mixture was left 1 hour reacting in the dark and the absorbance was read in a microplate reader (SPECTROstar Nano, BMG Labtech) at 765 nm.

The calibration curve was obtained by using gallic acid solutions (Sigma-Aldrich, Portugal) with concentrations ranging from 14.1 µM to 338.6 µM. The TPC was expressed in mg of gallic acid equivalents per gram of extract (mg GAE/g extract) and

all experiments were carried out in triplicate. Two independent assays were conducted, on different days, to validate the obtained results.

3.2.5. Total Flavonoids Content Assay

The quantification of the total flavonoid content (TFC) was estimated by the protocol proposed in Derakhshan *et al.*, (2018)[77], and El-Sayed *et al.*, (2018)[78]. The GP extracts stock solutions were diluted by 1:19 and 100 µL was mixed with 30 µL of $NaNO_2$ and 400 µL of ultrapure water. After 6 minutes, 30 µL of $AlCl_3$ (10%) was added, followed by 400 µL of NaOH (4%) and 40 µL of ultrapure water after another 6 minutes. The absorbance was read in a microplate reader (SPECTROstar Nano, BMG Labtech) at 510 nm after 15 minutes of reaction.

The catechin (Sigma-Aldrich, Portugal) calibration curve was obtained by using concentrations ranging from 10 µM to 1000 µM. The TFC was expressed as mg of catechin equivalents per gram of extract (mg CE/g extract) and all experiments were carried out in triplicate. Two independent assays were conducted, on different days, to validate the obtained results.

3.2.6. ABTS Assay

The antioxidant capacity of GP extracts was estimated by the evaluation of ABTS (2,2-azinobis (3-ethyl-benzothiazolina-6-sulfonic acid)) radical scavenging activity, adapted from the methods proposed by Xiao *et al.*, (2020)[79]. ABTS (7 mM) and potassium persulfate (2.45 mM) (both Sigma-Aldrich, Portugal) were mixed 1:1 v/v and allowed to react for 12-16 hours in the dark at room temperature. After, the solution was diluted with acetate buffer (50 mM) at pH 4.6, 1:40 v/v. The absorbance of the ABTS solution was set to 0.700±0.02 in a spectrophotometer (GENESYS 10UV, Thermo Fisher Scientific) at a wavelength of 734 nm. A Trolox (Sigma-Aldrich, Portugal) calibration curve was made by dissolving Trolox in glacial ethanol with concentrations ranging from 0.25 mM and 0.0125 mM.

In a 96 wells microplate, the samples and Trolox were mixed with reactive ABTS 1:10 v/v in triplicates. The absorbance was read in the microplate reader after 15 minutes at a wavelength of 734 nm. The control was made with the diluted ABTS solution. The antioxidant activity was expressed in mg of Trolox equivalents (TE) per gram extract (mg TE /g extract) and scavenging activity (%) by using equation 2. The absorbance of the

control corresponds to the absorbance of the ABTS reactive solution with the same dilution of the samples. Two independent assays were conducted, on different days, to validate the obtained results.

$$Scavenging\ Activity\ (\%) = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100\% \quad (2)$$

3.2.7. DPPH Assay

The antioxidant capacity of GP extracts was determined by a DPPH (2,2-diphenyl-1-picrylhydrazyl) protocol adapted from Magalhães *et al.*, (2011) and (2012); Xiao *et al.*, (2020), and Marinova *et al.*, (2011)[79–83]. DPPH (0.06 mM) was dissolved in glacial ethanol and set to an absorbance of 0.700±0.02 at a wavelength of 517 nm. The Trolox calibration curve was made with concentrations ranging from 0.25 mM and 0.0025 mM.

In a 96 wells microplate, the samples and Trolox were mixed with DPPH 1:10 v/v in quadruplicates. The absorbance was read, in the microplate reader, after 30 minutes at a wavelength of 517 nm. The control was made with the diluted DPPH. The antioxidant activity was expressed and calculated as in section 2.2.6.. Three independent assays were conducted, on different days, to validate the obtained results.

3.2.8. Statistical analysis

The statistical analysis was performed by ordinary one-way ANOVA analysis and mean differences were evaluated by Tukey's multiple comparisons test, for a confidence level of $\geq 95\%$, where $p < 0.05$ was considered statistically significant. Results are presented as mean ± standard deviation (SD).

3.3. Results and Discussion

3.3.1. Extraction Yield

Solvent extraction is a simple and inexpensive way to obtain bioactive extracts and/or compounds present in the GP's matrix[84]. The effect of the solvent on the efficiency SLE of bioactive GP extracts was studied. The samples lyophilization is important since drying leads to greater chemical stability, and phytochemicals are subjected to various chemical reactions when in aqueous solutions[85]. Through this method it was possible to determine the most adequate extraction solvents for each type of GP sample (stalks or mixture) in terms of dry mass recovered from the initial mass of the GP samples.

As can be seen in Figure 7, for the RGP mixture and stalk extracts, the solvent that provided a higher extraction yield was acetone 70%, with extraction yields of 13% and 17% respectively. Meanwhile, for WGP mixture extracts, water achieved an extraction yield of 41%, which is to the highest value obtained for the various extraction yields for the different conditions and fractions. For WGP stalk extracts the optimal solvent was acetone 50% that reached an extraction yield of 39%. The weighed masses (before/after extraction) and the extraction yields are presented for further consultation in Annex 1.

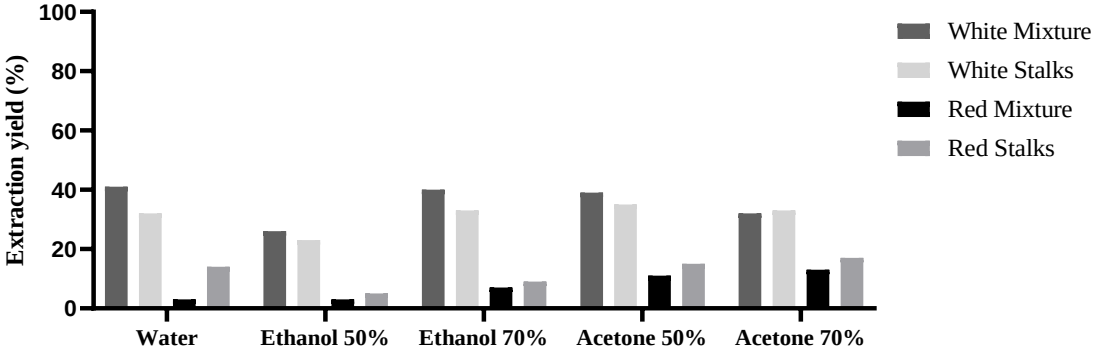


Figure 7. Extraction yields of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%) RGP and WGP extracts obtained from mixture and stalks fractions.

The obtained RGP yields were similar to Xu *et al.*[86], since it reported extraction yields for RGP ranging from 5.3% to 12.4%, and in this study 3 to 17% of extraction was achieved. In the case of WGP the results are in a range of 23 to 41%, while according to the same author they ranged from 20.6 – 24.9%.

3.3.2. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The FC method is a colorimetric method based on unspecific oxidation of the FC reagent by polyphenols, that is commonly used to quantify the total phenolic content in samples[87]. The gallic acid calibration curves can be consulted in Annex 2. The TPC of each sample was illustrated in Figure 8, and the discrete values in mg GAE/g extract can be consulted in Annex 3.

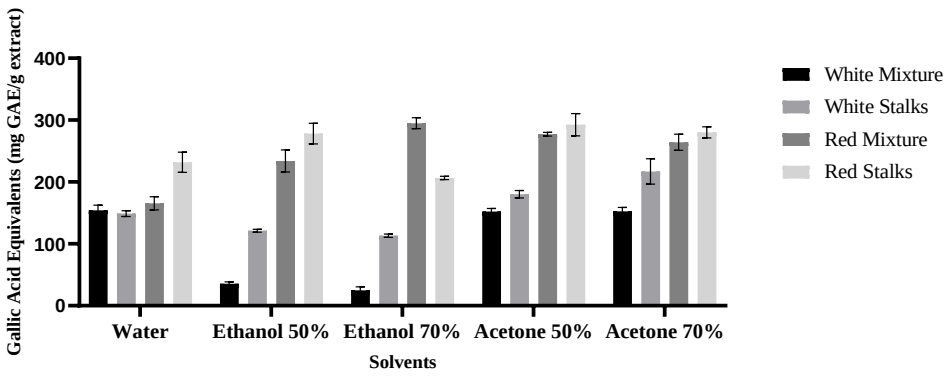


Figure 8. Total phenolic content (TPC) of crude aqueous, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%) RGP and WGP extracts obtained from mixture and stalks fractions.

Overall, the TPC results are mostly statistically similar ($p<0.05$). Thus, the best results of polyphenols' extraction for RGP mixture were obtained by crude ethanolic (ethanol 70%) and acetonic (acetone 50%) extracts ranging from 277.2±2.9 to 294.9±8.9 mg GAE/g extract. For RGP stalks the most promising TPC results were obtained by the crude acetonic (acetone 50% and acetone 70%) and ethanolic (ethanol 50%) extracts with values ranging from 278.1±16.8 to 292.5±17.9 mg GAE/g of extract. On the other hand, for WGP mixture the best TPC values were obtained by the crude aqueous and acetonic extracts with values ranging from 152.1±5.1 to 154.2±8.2 mg GAE/g extract. For WGP stalks the crude acetonic extract (acetone 70%) was the highest ($p>0.05$) with 216.9±20.4 mg of GAE/g extract. Lastly, the samples with lower TPC were the crude aqueous extracts for RGP mixture and the crude ethanolic extracts (ethanol 70%) for WGP.

According to the literature the obtained TPC values for the RGP are higher than expected for the same grape varietal types used in this work. For instances, Tournour *et al*[88]., reported 104.1±5.5 mg GAE/g extract for a mix of varieties (*Touriga Roriz*, *Touriga Franca* and *Touriga Nacional*)[88]. However, more similar values were obtained by Spigno *et al*.[89], and Amendola *et al*.[90], reported values of 441 mg GAE/g extract and 269.0 mg GAE/g extract, and Monagas *et al*.[91], a range of 78.5 to 563 mg GAE/g extract for commercial grape seeds extracts.

The present results suggest that higher extraction yields do not translate into higher TPC values as WGP extracts presented higher extraction yields, but RGP extracts presented higher TPC values. This can possibly be explained by the fact that WGP extracts have a higher content of soluble sugars than RGP extracts. This is because, RGP undergoes a period of fermentation before collection, enabling the consumption of sugars

by yeasts in the fermentation process[56]. On the other hand, WGP extracts were collected before alcoholic fermentation and thus the sugars remained unconsumed[57].

The aluminum chloride colorimetric method was used for the quantification of flavonoid content in the samples. This assay is based on the capacity of aluminum chloride to form stable complexes with the keto and hydroxyl groups, and the A and B rings of flavonoids, providing them color[92]. This way, by using spectrophotometry, a catechin calibration curve was determined (Annex 4). The TFC of each sample is illustrated in Figure 9 and the corresponding values discriminated in Annex 5.

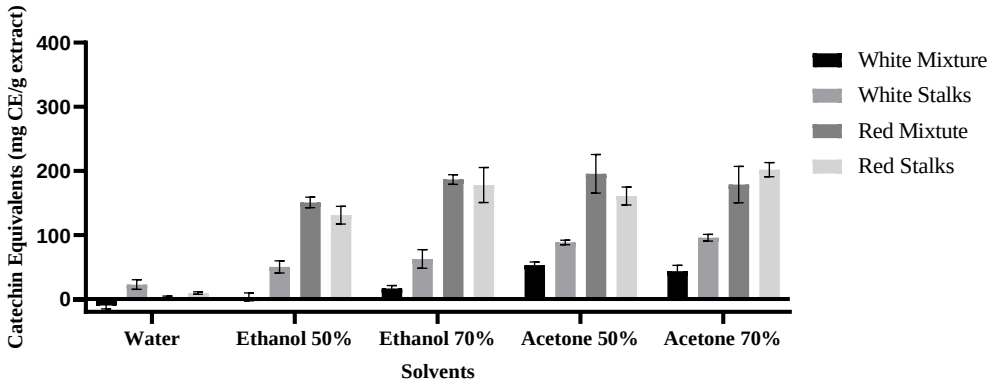


Figure 9. Total flavonoid content (TFC) of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%) RGP and WGP extracts obtained from mixture and stalks fractions.

The crude acetonic extracts (acetone 50% and 70%) mainly obtained the highest values of TFC for each extract. The best results for extraction of TFC in the RGP mixture were the crude acetonic (acetone 50% and 70%) and ethanolic (ethanol 70%) extracts ($p>0.05$) which ranged from 178.7±28.4 to 195.4±30.1 mg CE/g extract. For RGP stalks the highest TFC values were obtained by crude acetonic (acetone 70%) and ethanolic (ethanol 70%) extracts ($p>0.05$) ranging from 177.9±27.4 to 201.8±11.1 mg CE/g extract. For the WGP mixture and stalks the highest TFC results were obtained for crude acetonic extracts (acetone 50% and 70%) ($p>0.05$), ranging from 43.6±9.1 to 52.8±5.2 mg CE/g extract, and 88.3±3.6 to 95.7±5.3 mg CE/g extract, respectively. However, the crude aqueous extracts displayed lower values of extracted TFC for every type of GP samples.

According to the literature, the obtained TFC results presented higher values for RGP than common, while the WGP was in the expected range[93,94]. Xu *et al.*[86] described for *Chambourcin* and *Cabernet Franc* (RGP varieties) a range of 38.9–91.7 mg CE/g extract

and for the *Vidal Blanc* and *Viognier* (WGP varieties) a range of 32.8–75.0 mg CE/g extract. Other authors also reported similar values[95].

According to Xia *et al*[96], flavonoids are the main polyphenols in grapes, especially grape's skin and seeds, and are mostly present in red grapes with high abundance of anthocyanins[96]. This comes in agreement with the obtained results that indicate that most of the phenolic content in the samples are flavonoids. In fact, higher TPC seems to indicate a higher TFC for all types of extracts, except for the crude aqueous extracts that present very low quantities of flavonoids.

The WGP extracts presented higher TFC values for stalks extracts than for mixture, while in the RGP extracts nothing could be concluded due to the statistical similarity between results ($p>0.05$). According to Zainol *et al*.[97], flavonoid content can be affected by the thermal treatment of the samples. The loss of flavonoid was found to be around 31 to 73% in samples treated by lyophilization. The remaining flavonoids are dependent on their stability, with catechins presenting higher thermal stability[97,98]. Catechins are one of the major types of flavonoids present in stalks, which can explain the obtained results[99].

3.3.3. Antioxidant Activity of the GP Extracts by ABTS and DPPH Assay

The solvents' capacity of extracting antioxidant compounds from GP was evaluated by analyzing the antioxidant capacity by means of ABTS and DPPH assays. The results were calculated through Trolox calibration curves (Annex 6), a water-soluble analog of vitamin E known to have antioxidant activity, which reacts with ABTS and DPPH reagents. The results in mg TE/g extract are shown in Figure 10 for the ABTS assay.

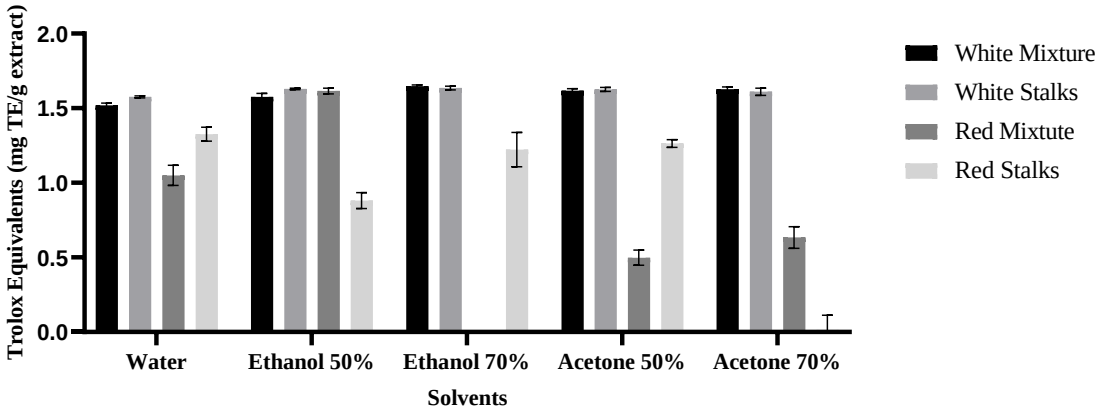


Figure 10. Antioxidant activity obtained by ABTS assay, expressed in mg of TE/g of extract, of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%.) RGP and WGP extracts obtained from mixture and stalks fractions.

The results for the DPPH assay are described in Figure 11. The values for both assays are discriminated in Annex 7. The scavenging activity was also evaluated, resulting in similar graphics (Annex 8).

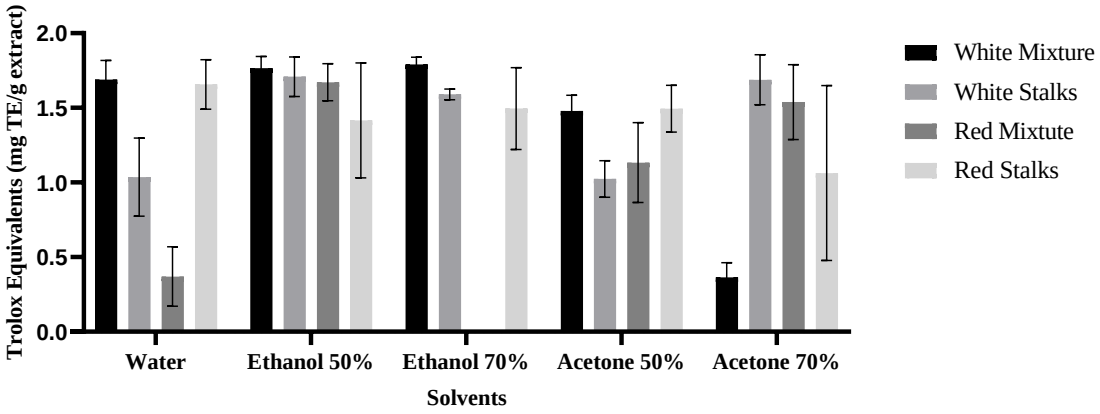


Figure 11. Antioxidant activity obtained by DPPH assay, expressed in mg of TE/g of extract, of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%.) RGP and WGP extracts obtained from mixture and stalks fractions.

In a more general way, it is possible to observe that in the ABTS assay the WGP extracts have a higher antioxidant activity compared with the RGP extracts, which comes in disagreement with current literature. In fact, Yang *et al.*[100], reported a positive correlation between the antioxidant capacity and the TPC and anthocyanins and flavonols content[100]. Usually, red grapes present high antioxidant activity due to their high content of anthocyanins, while white grapes lack this compound. Yet, the same author reported that the varietal difference of the GP has a bigger influence in the antioxidant activity than the grape color itself [100]. Also, it should be noted that a higher maturity of the grapes seems to affect the TPC, thus if the WGP derived from more mature grapes than RGP it could influence their antioxidant activity[101]. Regarding the DPPH assay, the variations between the types of extracts are more evident than in the ABTS assay leading to greater difficulty in comparing them. In this case, the WGP extracts present a favorable antioxidant activity when extracted with ethanol 50% and 70%.

In the ABTS assay the extracts with higher antioxidant capacity for WGP, both in mixture and stalks fractions, are difficult to identify since all the extracts presented statistically similar results ($p>0.05$), except for the crude aqueous extract in WGP mixture that presented a lower result ($p<0.05$). This way the highest WGP mixture antioxidant activity ranged from 1.576±0.023 to 1.648±0.08 mg TE/g extract (86.4±1.3 to 90.4±0.4% of scavenging activity), while for WGP stalks ranged from 1.575±0.007 to 1.635±0.012

mg TE/g extract (86.4±0.4 to 89.7±0.7% of scavenging activity), being the highest values attained for the crude ethanolic extract (ethanol 70%).

For the RGP extracts, the mixture and stalks fractions highest values of antioxidant activity were 1.327±0.047 mg TE/g extract (72.6±2.6% of scavenging activity) for crude aqueous stalks extract, and 1.615±0.018 mg TE/g extract (88.6±1.0% of scavenging activity) for the crude ethanolic mixture (ethanol 50%) extract ($p<0.05$). The lowest value for the stalks fractions was 0.0137±0.098 mg TE/g extract (0.0457±5.4% of scavenging activity) for the crude acetonic extract (acetone 70%). For the mixture fraction the lowest value was obtained for a crude ethanolic extract (ethanol 70%), however the absorbance measured at 734 nm was higher than the ABTS absorbance itself (0.952±0.061 > 0.625±0.015) resulting in a negative scavenging percentage (-52.4±3.3%). So, no antioxidant activity was considered since there was no apparent consumption of the reactive ABTS. Some interference occurred since the absorbance increased.

In the DPPH assay, the statistical similarity between results was higher than the ABTS assay. For the WGP mixture extracts, all the results were statistically similar ($p>0.05$) except for the lowest value, obtained for the crude acetonic extract (acetone 70%). So, the highest values ranged from 1.479±0.105 to 1.792±0.047 mg TE/g extract (78.8±5.5 to 95.3±2.5% of scavenging activity), with the maximum value corresponding to crude ethanolic extract (ethanol 70%). For the WGP stalks extracts every result was statistically similar ($p>0.05$) ranging from 1.023±0.122 to 1.751±0.073 mg TE/g extract (54.7±6.4 to 93.2±3.9% of scavenging activity), with the crude acetonic extract (acetone 70%) having the highest value.

Regarding RGP mixture extracts the highest antioxidant values were obtained for crude ethanolic (ethanol 50%) and acetonic (acetone 50% and 70%) extracts, with statistically similar results ($p>0.05$) ranging from 1.333±0.268 to 1.673±0.124 mg TE/g extract (60.5±14.2 to 89.0±6.6% of scavenging activity), with the crude ethanolic extract (ethanol 50%) presenting the highest value. Lastly for the RGP stalks extracts every result was statistically similar ($p>0.05$) ranging from 1.062±0.586 to 1.657±0.166 mg TE/g extract (56.8±31.0 to 88.2% of scavenging activity), with the crude aqueous extract presenting the highest value.

The comparison with literature, in terms of antioxidant activity, can be difficult due to the lack of a standardized format for results presentation. Thus, in Annex 9 a conversion of the antioxidant activity units from mg TE/g extract to μmol TE/g extract can be consulted. The obtained results range from 0–6.584 μmol TE/g extract for ABTS

and 0–7.158 μmol TE/g extract for DPPH, which are comparable to the ones obtained by Rocha & Noreña[102], where the ABTS and DPPH results for lyophilized samples range from 6.29–8.32 μmol TE/g extract and 7.99–10.92 μmol TE/g extract, respectively. However the overall obtained results are relatively lower than the ones presented in the literature[102]. This can be due to the fact that phenolic compounds present unsaturated bonds, which are sensitive to light and heat[103]. Bearing in mind that the samples were frequently manipulated to perform several tests, being exposed to light (although limited) and removed from the cold, their activity may have been compromised.

WGP extracts present higher values of antioxidant activity. Since these extracts demonstrated higher extraction yields and lower TPC and TFC, it is possible that the solvents used can also potentiate the extraction of other bioactive compounds that also provide the samples with antioxidant capacity such as vitamins and carotenoids[104], or has mentioned before the manipulation inactivated the compounds.

In the present work, different solvents did not seem to influence the antioxidant activity neither did the aqueous ratio. Nonetheless, the selected solvent for extraction of antioxidant bioactive compounds was ethanol for various reasons. Nayak *et al.*, stated that aqueous alcoholic mixtures are considered good extraction solvents[105]. In fact, other authors also described ethanol to be the best solvent among water and acetone, with optimal composition of 40 to 70% of water content[106–109]. Ethanol is affordable and is classified as safe, green, and sustainable since it can be acquired by renewable sources[109]. Also, the highest antioxidant activity was obtained for a crude ethanolic extract. Therefore, the extract that should be incorporated in a hydrogel to promote topical wound healing is the crude ethanolic (70:30% v/v) WGP extract from the mixture fraction.

3.4. Conclusions

WGP extracts generally demonstrated a higher antioxidant activity than RGP extracts. The extraction did not seem to be directly related with the amount of TPC and TFC extracted, since the high amount of TPC and TFC of the RGP extracts did not culminate in higher antioxidant activity. In terms of the difference between stalks and mixture types of extracts nothing could be concluded. However, it was verified by both ABTS and DPPH assays that the higher antioxidant activity was obtained for the crude ethanolic (70:30% v/v) WGP extract from the mixture fraction. Ethanol is considered a green solvent that consequently favors the concept of circular economy and promotes the valorization of the winemaking industry's by-products, which is the focus by which this

project aims towards. Therefore, the extract with best results and that should be incorporated in a hydrogel directed for chronic wound treatment is the crude ethanolic (70:30% v/v) WGP extract from the mixture fraction.

Chapter 4: Hydrogel Fabrication and Characterization

4.1. Introduction

Hydrogels are 3D polymer cross-linked networks with the ability to absorb and retain a large amount of water. They are considered to be a good wound dressing alternative, due to their good permeability and biocompatibility, anti-inflammatory, antioxidant, and antibacterial effect, as well as the ability to provide a moist environment for wound repair [13,110].

Hydrogels can be obtained by the cross-linking of natural polymers such as chitosan and alginate, with glutaraldehyde and calcium chloride[39]. Their hydrophilic nature provides them with swelling capacity, which is their ability to absorb water content[34]. This represents a major benefit to hydrogels as chronic wound dressings since they are able to absorb the exudates from wounds. Natural hydrogels are often associated with inherent bioactivity, however the incorporation of bioactive compounds, as GP extracts in hydrogels' polymeric network potentially enhance their activity, providing them with important biomedical potential[36].

Strong mechanical properties to enable manipulation and durability, but also degradation properties to potentiate the release of the bioactive compounds incorporated, is a compromise that should be attained. Therefore, the cross-linking level between polymeric chains, the pH of the media, the solubility of the compounds, and the porosity of the matrix, are all factors that influence the efficiency of the hydrogel [111].

In this chapter, the swelling behaviour of the fabricated hydrogel, as well as its capability of degradation and release of bioactive compounds like polyphenols from the incorporated crude ethanolic (70:30% v/v) WGP extract from the mixture fraction as previously selected, will be studied. The antioxidant activity will also be assessed by the ABTS and DPPH method to determine if the incorporation of extracts favors the hydrogel with similar properties.

4.2. Materials and Methods

4.2.1. Hydrogel Fabrication

A hydrogel formulation protocol for the incorporation of crude ethanolic (70:30% v/v) WGP extract from the mixture fraction was adapted from Ehterami *et al.*[112], and K. Baysal *et al.*[39]. Firstly, sodium alginate and chitosan were dissolved in autoclaved ultrapure water to obtain 2 stock solutions (20 mg/mL each). In the chitosan stock solution acetic acid was added (0.0286 v/v). The stock solutions were agitated for 24h. Then they were mixed in a ratio of 1:2 chitosan/alginate and the extract was added leading to a final concentration in the hydrogel of 4 mg/mL. The mixture was agitated for 24h in the dark, until a homogenous solution was obtained. Next, the hydrogel was cross-linked with 1:5 $CaCl_2$ (50 mM)/hydrogel and glutaraldehyde in a molar proportion of 1:20 chitosan/Glutaraldehyde. NaOH at 1M was also added in a proportion of 2:25 NaOH/ hydrogel. The Chit/Alg/WGP hydrogel was obtained (Annex 10).

4.2.2. Hydrogel Characterization

4.2.2.1. Swelling Assay

The swelling protocol was adapted from Ehterami *et al.*[112], and Baysal *et al.*[39]. Briefly, the Chit/Alg/WGP hydrogel was cut into 618 mm^3 disks and frozen at -80ºC for lyophilization. Then each disk was immersed in 5 mL of phosphate-buffered saline solution (PBS) (pH= .4) at 37ºC. The disks were removed from the solution, wiped gently with tissue paper, and weighed quickly. The samples were weighed before being placed in PBS (m_0), and at various times of contact with the solution (0.5h, 1h, 2h, 4h, 8h, 24h) (m_1). The swelling percentage was calculated using the following formula:

$$Swelling\ (\%) = \frac{m_1 - m_0}{m_0} \times 100 \qquad (3)$$

Two other hydrogels were also tested, a positive control with incorporated Trolox (25 μM) (Chit/Alg/Trolox hydrogel) and a negative control with the hydrogel alone (Chit/Alg hydrogel). Two independent assays were conducted, on different days.

4.2.2.2. Degradation Assay

The degradation protocol was adapted from Ehterami *et al.*[112]. The Chit/Alg/WGP hydrogel was cut into 618 mm^3 disks, frozen at -80ºC for lyophilization, and immersed in 5 mL of PBS (pH = 7.4) at 37ºC. The hydrogel disks were removed from PBS at various

times (0.5h, 1h, 2h, 4h, 8h, 24h), and dried at 60ºC for 72h. The dried hydrogel disks were weighed before being placed in PBS (w_0), and after being dried (w_1). The tested hydrogels and independent assays were as in section 4.2.2.1. The degradation percentage was calculated using the following formula:

$$Degradation\ (\%) = \frac{w_0 - w_1}{w_1} \times 100 \qquad (4)$$

4.2.2.3.Release Assay

The protocol for the TPC released by the hydrogel was adapted from Ehterami *et al.*[112], Lamuela-Raventós *et al.*[75], and H. Abramovič *et al.*[76]. The Chit/Alg/WGP hydrogel was cut into 618 mm^3 disks and frozen at -80ºC for further lyophilization. Then, the samples were immersed in PBS (pH = 7.4) at 37ºC for various times (0.5h, 1h, 2h, 4h, 8h, 24h). After, 1 mL of the PBS solution was analyzed by the FC method described in 3.2.5. The hydrogels and independent assays were the same as in section 4.2.2.1.

4.2.3. Hydrogel Antioxidant Activity

The hydrogel antioxidant capacity was determined by adapting the protocols proposed by Zhao *et al.*[30] and Thongchai *et al.*[113]. The Chit/Alg/WGP hydrogel was cut into 618 mm^3 disks and frozen at -80ºC for lyophilization. Then they were immersed in 2 mL of methanol and agitated until the hydrogel was completely dissolved. The antioxidant activity of the samples was assessed by the protocols described in 3.2.7 and 3.2.8. and presented as µM TE. The tested hydrogels and independent assays were the same as in section 4.2.2.1.

4.2.4. Statistical analysis

The statistical analysis was performed by ordinary one-way ANOVA analysis and mean differences were evaluated by Tukey's multiple comparisons test, for a confidence level of $\geq 95\%$, where $p < 0.05$ was considered statistically significant. Results are presented as mean ± SD.

4.3. Results and Discussion

4.3.1. Hydrogel Characterization

4.3.1.1. Swelling, degradation, and release assessment

Polymer 3D networks have the capacity to absorb water molecules leading to the swelling of the hydrogels. This is verified in hydrogels similar to the fabricated hydrogel since both chitosan and sodium alginate contain several hydrophilic functional groups[114]. The swelling percentage enables the characterization of the water uptake capacity and the stability of the hydrogel[115]. To characterize the "in house" fabricated hydrogel incorporated with WGP extracts (Chit/Alg/WGP hydrogel), a negative and positive control were also evaluated (Chit/Alg and Chit/Alg/Trolox hydrogel). The swelling percentage of the hydrogels is illustrated in Figure 12 and described in Annex 11.

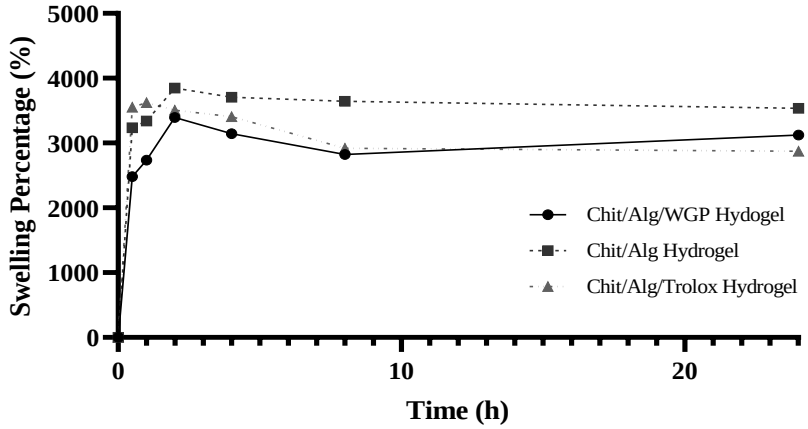


Figure 12. Swelling percentage (%) of Chit/Alg, Chit/Alg/WGP and Chit/Alg/Trolox Hydrogel through time (0, 0.5, 1, 2, 4, 8 and 24 hours).

The swelling percentage of the studied hydrogels seems to lead to a burst growth in the first hours and then slightly decreases and stabilizes in the following time. For the Chit/Alg and Chit/Alg/WGP hydrogels the swelling percentage peaked after 2h of contact with the PBS solution, reaching values of 3848% and 3392% respectively, and then decreased obtaining 3535% and 3121% of swelling after 24h. For the Chit/Alg/Trolox hydrogel, the maximum value is reached sooner with 3623% of swelling after 1 hour of contact, followed by a decrease to a value of 2873% after 24h.

The displayed results, demonstrate a high swelling percentage that is in accordance to most of the literature for various hydrogels[46,112,114,116–120]. However they come particularly in line with the reports from Baysal *et al.* that studied the swelling capacity of Chit/Alg hydrogels with different proportions of the same polymers, and described around 3000% of swelling for Chit/Alg 1:2 (w/w) hydrogel[39]. Swelling is an important attribute of hydrogels developed for wound treatment since it enables the absorption of

excessive exudates from wounds, which in turn prevents a delayed healing process, the maceration of periwound skin and leakage[121].

By comparing the hydrogels, it could be observed that the negative control obtained higher values of swelling then the rest of the hydrogels. This could be related to the difference in the chemical structure or size of the incorporated compounds that can affect the dislocation and the mobility of the polymeric chains as reported in Khan *et al.*[122]. In fact, if the incorporated compounds affect the porosity of the hydrogel by decreasing it, a decrease of the swelling capacity can be expected since the pores promote the exposition of the polymeric chains' hydrophilic groups to the water molecules[114].

The Chit/Alg/Trolox hydrogel mostly presents a slightly higher swelling percentage than the Chit/Alg/WGP hydrogel. This could be due to the solubility nature of the compounds present in the WGP extract, since hydrophobicity has been proved to influence the swelling of the hydrogels. If less polar compounds were extracted by ethanol, these can decrease the swelling capacity of the hydrogel when incorporated by decreasing the amount of water molecules capable of binding to the hydrogel matrix[122].

The degradation of the hydrogels is closely related with their swelling capacity. The penetration of the water molecules in the hydrogel matrix causes dissociation of hydrogen bonds leading to the loss of structural integrity of the polymeric chains[122]. This is evaluated by determining the dry weight loss of the studied hydrogels. The degradation percentage can be observed in Figure 13, and the respective values are discriminated in Annex 12.

Since the hydrogel demonstrated high values of swelling, it was expected that it also showed higher degradation percentages. In fact, after the swelling reached its peak, the slight decrease that can be observed in Figure 13 normally would signify some degradation of the hydrogels. However, the degradation capacity of the hydrogels was mostly negative for the three tested hydrogels, with the Chit/Alg/WGP hydrogel showing some degradation at 0.5 and 1 hours (5.6 and 3.7%) and then again after 8 hours (4.2%) of contact with the PBS solution. Negative degradation values seem to indicate that not all the water content absorbed was possible of being evaporated during the drying process. Possibly, to obtain the original weight a lyophilization of the hydrogels should be performed, as done initially, instead of dehydration in the stove.

The incapacity of the hydrogels to degrade is reported to be related to the cross-linking level of the matrix. A possible explanation is that the presence of the incorporated WGP extracts can interfere structurally or chemically in the cross-linking capacity of the

polymeric chains leading to a reduction in the stability of the hydrogel matrix, causing degradation[122].

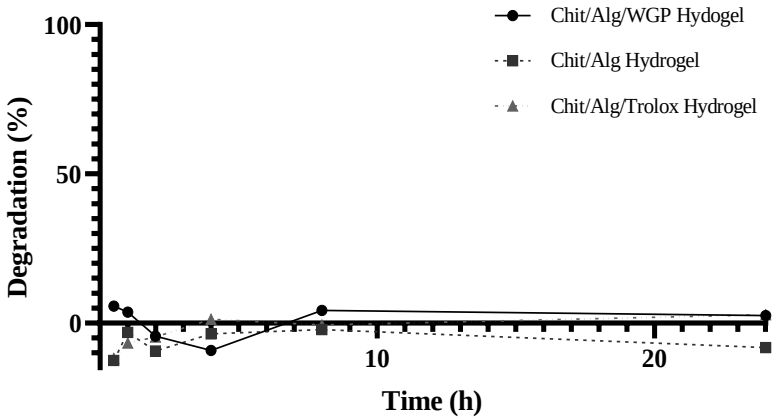


Figure 13. Degradation percentage (%) of Chit/Alg, Chit/Alg/WGP and Chit/Alg/Trolox Hydrogel through time (0, 0.5, 1, 2, 4, 8 and 24 hours).

The release of the incorporated compounds from the hydrogel comprises an important part of its function. Hydrogels are described as capable of providing spatial and temporal control over drug release[123]. Thus, a release assay was performed by determining the TPC of the PBS solution where the hydrogels were immersed for various time stamps in a range of 24h. The obtained absorbances of the supernatant were below the spectrophotometer limit detection, so the calibration curve could not be diluted. Additionally, the hydrogel concentration could not be increased since there was a limited amount of extract available for all the following experiments. Actually, the extraction process already provided a low concentration/amount of extracts, and the volume of PBS used could not be lower otherwise the hydrogels would not be totally immersed.

Considering the TPC values obtained in section 3.3.2. for crude ethanolic (70:30% v/v) WGP extract from the mixture fraction, the calculated dilution of the hydrogel in the PBS solution would lead to values that could not be evaluated by the calibration curve, as they were below the intersection of the curve with the y axis. Thus, the concentration could be so low that no release was determined even though it might have occurred.

On the other hand, the degradation rate has been reported as being linked to the release capability of a hydrogel, as it enables the discharge of the compounds from the polymeric matrix[112]. Considering the degradation assay data, almost no degradation was verified. This could have led to the entrapment of incorporated compounds and therefore, no release.

4.3.2. Hydrogel Antioxidant Activity

The antioxidant activity of the hydrogels was evaluated by ABTS and DPPH assays, to understand if the incorporation of WGP extracts containing bioactive compounds in the hydrogel, affects its activity. The obtained results are illustrated in Figure 14 a) and b) and the numerical results and calibration curves are discriminated in Annex 13.

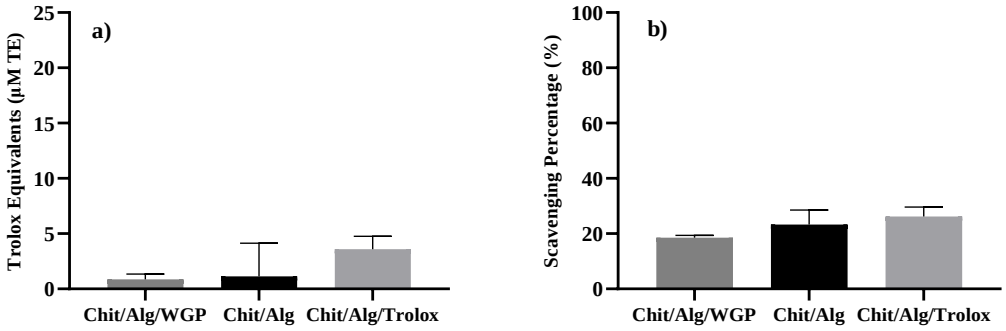


Figure 14. Antioxidant activity by the ABTS assay represented in µM of TE a) and scavenging activity (%) b).

The highest antioxidant activity was obtained for the Chit/Alg/Trolox hydrogel with 3.602±1.151 µM TE (26.2±3.4% of scavenging activity), which is statistically different from the Chit/Alg/WGP hydrogel that obtained 1.016±0.288 µM TE (18.5±0.9% of scavenging activity) ($p>0.05$). The WGP extracts incorporated in the hydrogel are at the same concentration as the extracts analysed in section 3.3.2. In fact, the data reported in this section, indicates values of 26.33±0.129 µM TE for the crude ethanolic (70:30% v/v) WGP extract from the mixture fraction which is similar to the Trolox concentration incorporated in Chit/Alg/Trolox hydrogel (25 µM). Considering the amount of TE calculated and the dilution of the hydrogel in PBS solution, the expected values of TE should be about 7.7 µM. However, the obtained results for the three hydrogels are around half to seven times lower than the incorporated values. This implies that the hydrogel is limiting the antioxidant activity of the incorporated compounds (WGP extracts and Trolox). As mentioned earlier the natural antioxidants' effectiveness depends on the preservation of their stability. Since they are sensitive to light, oxygen, and heat, the manipulation of the hydrogel could induce loss of activity[124]. Also, if the dissolution of the hydrogels was not complete, the compounds could still be structurally linked to hydrogel components, hindering their activity.

Both Chit/Alg/Trolox and Chit/Alg/WGP hydrogel antioxidant activities are statistically similar to the Chit/Alg hydrogel antioxidant activity ($p<0.05$), with values of 2.630±1.757 µM TE (23.3±5.2% of scavenging activity). It has been reported that chitosan, among many other beneficial characteristics, has antioxidant properties[125]. This possibly indicates that the antioxidant activity detected by the ABTS method was mostly due to the intrinsic antioxidant activity of the hydrogel constituents alone.

A DPPH assay was also performed however some type of interference seems to have occurred, since the absorbance mostly increased with the addition of the reactive solution instead of decreasing. No conclusion was attained from these results. Anyhow, Figure 15 illustrates the obtained antioxidant activity, and the values can be consulted in Annex 14.

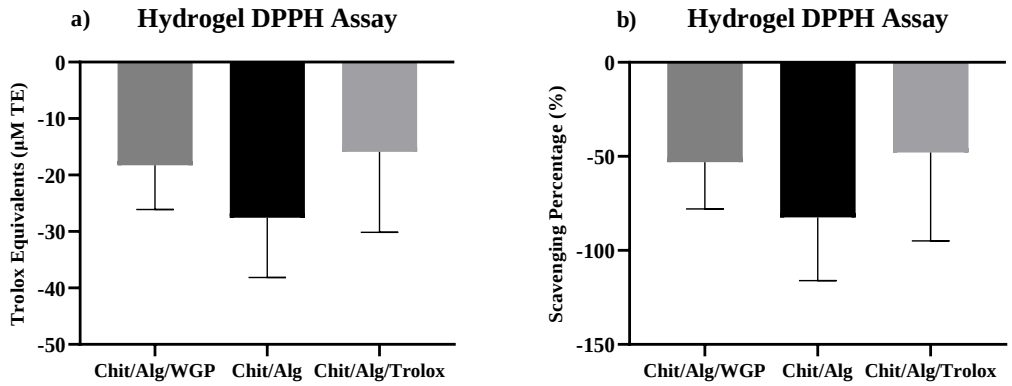


Figure 15. Antioxidant activity by the ABTS assay represented in µM of TE a) and scavenging activity (%) b).

4.4. Conclusions

The swelling percentages obtained were very favorable, with the maximum values ranging from 3392 to 3848%. Even though the controls presented higher values than the Chit/Alg/WGP hydrogel the swelling percentage obtained was still high and can possibly provide the hydrogel with exudate absorption capacity and thus promote wound healing. However, only the hydrogel with incorporated WGP extracts presented degradation, with low levels after 0.5, 1 and 8h (5.6, 3.7 and 4.2%). The release rate was not detected either due to the low concentration of the incorporated compounds in the PBS solution or due to the lack of degradation. Also, the antioxidant activity of the hydrogels ranged from 1.016±0.288 to 3.602±1.151 µM TE (23.3±5.2 to 26.2±3.4% of scavenging activity), with the potential antioxidant activity of compounds being limited

by incorporation in the hydrogel. The antioxidant activity detected seems to be mostly due to the chitosan bioactive properties.

Chapter 5: Hydrogel Antibacterial Activity

5.1. Introduction

Treatment of chronic wounds requires various steps where cleansing and debridement are performed followed by antibiotics administration and exudate management[12]. This is due to the humidity, warm and nutritious environment of the wound that can promote the growth of microorganisms, which in turn can cause risk of infection. Therefore, the development of alternative wound dressings with antibacterial properties are a priority for wound healing[11].

The incorporation of bioactive compounds in the hydrogel, such as WGP, might be beneficial since GP has been reported to have antibacterial activity due to their high TPC[126], as well as photosensitive compounds, such as resveratrol, which might affect the bioactivity of the extracts by photoactivation or inactivation[127].

Hydrogels' cross-linking is an important process to enhance mechanical properties, as well as swelling and degradation capacity of the hydrogels. Glutaraldehyde is one of the most common cross-linkers used for chitosan hydrogels, where glutaraldehyde's aldehyde groups link with chitosan's amine groups. Several hydrogels have been developed with this cross-linker which demonstrate biocompatibility, biodegradability, and low cytotoxicity[40]. However, glutaraldehyde has been reported as toxic, being commonly used in disinfectants since it can cross-link the proteins in the cells[128].

The fabricated chitosan-alginate hydrogel aims to manage the risk of infection by incorporation of WGP extracts, and the usage of natural polymers such as chitosan, that present antibacterial properties. Irradiation of the hydrogel with a UV-light at 254 nm is also evaluated in this chapter, as well as the influence of glutaraldehyde in the antibacterial properties of the hydrogel.

Therefore, a hydrogel disk diffusion assay, bacterial transfer, growth, membrane integrity and culturability assays were performed for the hydrogel with incorporated WGP extracts (Chit/Alg/WGP). Additionally, controls were also tested that include hydrogels incorporated with Trolox and fusidic acid (FA), a commonly used topical antibiotic, as well as the washed and unwashed hydrogel without incorporated compounds. For the appointed antibacterial assays, the Gram-positive bacterium *S. aureus* was used due to its relevance in wound infections recurrence and persistence/chronicity[9,129].

5.2. Materials and Methods

5.2.1. Bacterial Strains and Culture Conditions

The microorganisms used in this study were the Gram-positive bacterium *S. aureus* CECT976, obtained from the Spanish Type Culture Collection. The bacteria preserved at -80ºC were transferred onto Mueller–Hinton agar (MHA, Merck) at 37ºC for 24h before testing. Then the bacterial cells were grown overnight (16-18h) in MHA at 37ºC (for 5.2.2,) and Mueller–Hinton broth (MHB, Merck) at 37ºC and 160 rpm (for 5.2.3 and 5.2.4). Then, the culture turbidity was adjusted to an optical density (O.D.) of 0.132 at 600 nm (corresponding to 0.5 McFarland standard) by resuspending some colonies in saline solution (NaCl, at 85%) (for 5.2.2.) or MHB (for 5.2.3 and 5.2.4).

5.2.2. Modified Hydrogel Disk Diffusion Assay

A modification of the disk diffusion assay was used to attest for the antibacterial properties of the fabricated hydrogels, which is an adaptation of the method proposed by Saberian *et al.*[130]. The bacterial suspension was cultured by a sterile swap on MHA medium Petri dishes. Chit/Alg/WGP hydrogel disks (8mm diameter) were prepared aseptically and rapidly placed on the Petri dishes. The effect of irradiation on the bacterial growth inhibition capacity of the hydrogels was assessed by exposing the disks 15 min, before incubating, to a lamp (λ=254 nm). After a 24h incubation period at 37ºC the bacterial growth around the disks was inspected and the inhibition zone diameter (IZD) measured in mm. The hydrogel control disks were the same as in section 4.2.2.1., with the addition of the hydrogel incorporated with FA(10μg/mL) (Chit/Alg/FA hydrogel) and the hydrogel alone washed with autoclaved ultrapure water (washed Chit/Alg hydrogel).

A comparison between the IZD values of the hydrogel disk diffusion assay with the ones of the same "components" (WGP extract, Trolox and FA) without being incorporated in the hydrogel (using compounds at the same concentration of the hydrogels but in a well-made in the culture media - agar well diffusion method), was also made. Two independent assays with duplicates were performed.

5.2.3. Bacterial Transfer from Hydrogel Disks Assay

To evaluate the capability to transfer bacteria of the hydrogel disks to MHA medium an adaptation of the method proposed by Rippon *et al.*[131]; Singh *et al.*[132] was followed.

Firstly, 40 µL drop of bacterial suspension was placed in an MHA plate and incubated for 24h at 37ºC. Chit/Alg/EWP hydrogel disks with 11 mm diameter were placed on the drop and were either directly incubated at 37ºC for 15 min or exposed to a lamp (λ=254 nm) to study the effect of irradiation on the inhibition of bacterial transference by the hydrogels. Then the disks were moved to a new MHA plate and incubated for 24h at 37ºC. The disks were then removed, and the Petri dishes without the hydrogels were once again incubated for 24h at 37ºC. The assay was evaluated for the same hydrogels as in section 5.2.1. Two independent assays with duplicates were performed.

5.2.4. Bacterial Growth, Membrane Integrity, and Culturability Assays

The assessment of the fabricated hydrogels ability to preclude bacterial cells growth, membrane integrity and culturability was evaluated following the protocol of Singh *et al.* [132], and Lee *et al.*[121]. Firstly, 11 mm hydrogel disks were submerged in 1mL of bacterial suspension (1) in a 48 well microplate with a well with a bacterial suspension (2) that was not in contact with hydrogel and incubated 24h at 37ºC and 160 rpm. After the incubation time the turbidity was read in a microplate reader (SPECTROstar Nano, BMG Labtech) at 600 nm for evaluation of the bacterial growth.

$$Bacterial\ growth\ (\%) = \frac{O.D._{.2} - O.D._{.1}}{O.D._{.2}} \qquad (5)$$

The hydrogel in the suspension was transferred to 4 mL of 85% saline solution and vortex for 1 minute. Both, the bacterial suspension, and the resulting suspension from the vortexed hydrogel, were diluted 10 times and then 1:3 v/v of dye solution was added (Live/Dead BacLight kit; Invitrogen) composed by SYTO9 and propidium iodide (PI) in a proportion of 5:1 v/v. After 7 min of reaction, 900 µL of the solution were filtered through a filter (Cyclepore Track Cytiva Whatman Etched Membrane, 0.2 µm pores size, 100PK, 25 mm diameter). Images were acquired by using a Leica Microscope and the amount of green and red cells were counted for evaluation of cells' membrane integrity, the following equation was used.

$$N = \frac{n \times A}{B \times V} \times D \qquad (6)$$

Where N is the sample's number of cells per mL, n is the average number of cells per microscopic field, A is the membrane area (cm^2), B is the microscopic field area (cm^2), V is the volume of the filtered sample (mL), and D is the dilution factor. The results were presented as a ratio percentage of cells stained with SYTO9 and PI.

Additionally, from the bacterial and hydrogel suspension, 10 µL of the original and diluted solutions were cultured in plate count agar (PCA) Petri dishes. After 24h of incubation at 37ºC the number of colony-forming units (CFU) were counted for each condition to evaluate the culturability of the bacteria that was in contact or adhered to the hydrogel. The following equation was used.

$$\frac{CFU}{mL} = \frac{N}{V \times D} \quad (7)$$

Where N is the number of CFU in the PCA plates and S is the sample volume in mL and D is the dilution. The assays were evaluated for the same hydrogels as section 5.2.1., as well as *S. aureus* growth alone. Two independent assays were performed.

5.2.5. Statistical analysis

The statistical analysis was performed by ordinary one-way ANOVA analysis and mean differences were evaluated by Tukey's multiple comparisons test, for a confidence level of $\geq 95\%$, where $p < 0.05$ was considered statistically significant. Results are presented as mean ± SD.

5.3. Results and Discussion

5.3.1. Modified Hydrogel Disk and Agar Well Diffusion Assays

The disk diffusion assay is commonly used to assess antibacterial activity of an extract or a pure compound, as well as the agar well diffusion assay. These are two of the most known and basic methods, advantageous by their simplicity, low cost, applicability to many microorganisms and antimicrobial agents, and the ease to interpret the results provided[133]. The obtained results are reported in Table 2.

Table 2. Hydrogels (Chit/Alg/WGP, Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg and washed Chit/Alg hydrogel) disk diffusion inhibition zone dimeter (IZD) in mm, obtained by the subtraction of the hydrogel's diameter (8 mm) to the diameter of the halo.

	Chit/Alg/Trolox	Chit/Alg/FA	Chit/Alg/WGP	Chit/Alg	Washed Chit/Alg
Irradiated	3.75±0.50	13.75±0.50	5.75±0.50	3.25±0.50	2.25±0.50
Non-Irradiated	2.75±0.50	11.75±1.50	5.50±1.00	3.25±0.50	1.25±0.50

Table 3 presents the results for the agar well diffusion. Representative images are displayed in Annex 15 for further consultation.

Table 3. Agar well diffusion inhibition zone diameter (IZD) in mm, obtained by the subtraction of the well's diameter (6 mm) to the diameter of the halo.

	Trolox	FA	WGP extract
Irradiated	2.75±1.26	23.00±0.50	0.25±0.50
Non-Irradiated	3.25±0.50	22.75±1.50	0.25±0.50

All the hydrogel formulations presented inhibition towards *S. aureus*, as can be seen in Table 2 and 3, since all demonstrated an IZD. The hydrogels incorporated with FA correspond to the highest IZD values of the hydrogels. However, compared to its control, a well of FA at the same concentration, the incorporation in the hydrogel reduced its diffusion in the medium. This indicates that even though no degradation or release was previously observed, the hydrogel can diffuse the incorporated antibiotic to some extent, developing an IZD illustrative of antibacterial effect. However, the observed decrease could indicate that compared to the control there is some type of restriction in the release capacity of the hydrogel.

Chit/Alg/Trolox hydrogel presented similar values of inhibition as the Trolox well, and the Chit/Alg hydrogel alone ($p<0.05$), leading to the conclusion that when Trolox is incorporated it does not promote any inhibition. In this case, the IZD must be attributed to the antibacterial properties of the hydrogel formulation alone. The incorporation of WGP extracts seems to potentiate the IZD values, as the complexation of the compounds promotes an enhancement of the antibacterial activity compared to the negative control (Chit/Alg hydrogel) ($p>0.05$). Also, by comparing to the inhibition capacity of the WGP extract in the agar well, it is possible to verify that the incorporation of the WGP extract in the hydrogel promotes a synergetic effect improving its antibacterial activity (higher IZD values). The lack of antibacterial activity of the WGP extracts alone was not expected since the literature describes these extracts and their compounds (namely phenolic content) as antibacterial agents against *S. aureus*, however the low concentration of the extracts in the hydrogel can explain the low activity displayed[126].

The Chit/Alg hydrogel was washed by ultrapure water since it has been reported that glutaraldehyde has toxicity, and the washing of the hydrogels can liberate the glutaraldehyde that might have remained unlinked in the formulation[134,135]. By comparing

the washed Chit/Alg hydrogel and the control (Chit/Alg hydrogel without the washing step) no difference was observed ($p<0.05$). This indicates that the cross-linking with glutaraldehyde is not affecting inhibition capacity of the hydrogel. Perhaps the glutaraldehyde was fully cross-linked, or the amount of free glutaraldehyde is very reduced not showing toxicity towards *S. aureus*. The IZD for both these hydrogels can be due to the polymers' bioactivity, namely chitosan, since has been reported by Moeini *et al.*, that this polymer has antibacterial capacity[11].

By irradiating *S. aureus* with the UV-lamp it was concluded that the irradiation had no influence in the bacterial growth. The difference between irradiated and non-irradiated hydrogel disks is not statistically significant ($p<0.05$). Therefore, it can be assumed that the compounds in the WGP extract were not photosensitive to the UV light in a macroscopical way when incorporated.

5.3.2. Hydrogel Bacterial Transfer

The hydrogels' ability to transfer bacteria (*S. aureus* (CECT 978)) onto agar plates was studied to understand the capacity of the material to absorb the bacteria, avoiding its permanence in contact with wound surface[132]. This was accomplished by placing the hydrogels in contact with the bacteria for 15 minutes with UV irradiation or not, and then transferring the hydrogel to a clean plate for 24h at 37ºC. After removal of the hydrogel, it was assessed macroscopically if the bacteria remained in the agar. After 24h of incubation of the agar plate, it was also assessed if the bacteria were able to grow.

All the hydrogels presented a transfer of bacteria from the petri dish with *S. aureus* to the clean petri dish with MHA medium, as can be seen in the representative Figure 16. After the hydrogel was removed and the 24h incubation period of the petri dishes at 37ºC was finished, no growth of the bacteria was observed in all the hydrogels, except for the washed Chit/Alg hydrogel, as can be seen in the representative Figure 16 b). This indicates that the hydrogels did not absorb most of bacteria, maintaining the cells in contact with the wound bed, but mostly their growing capacity was halted. This could be due to a possible high cross-linking level of the hydrogels, that reduces the porosity and does not enable bacterial absorption[136].

The presence of glutaraldehyde seems to influence the hydrogels' ability to hinder bacteria's growth even after the hydrogel is no longer in contact with the bacteria, while the irradiation of the hydrogels does not seem to have any influence in the bacterial transfer and growth. Since growth was observed only when the glutaraldehyde was

washed, possibly the limitation of bacterial growth in most hydrogels can be due to the interaction of the cells with the glutaraldehyde present on the surface that is removed when the hydrogel is washed. However, to state such conclusions further research is required to evaluate the washing efficiency in removing free glutaraldehyde.

No difference was observed between the incorporation of the extract in the hydrogel (Chit/Alg/WGP hydrogel) and the rest of the unwashed conditions.

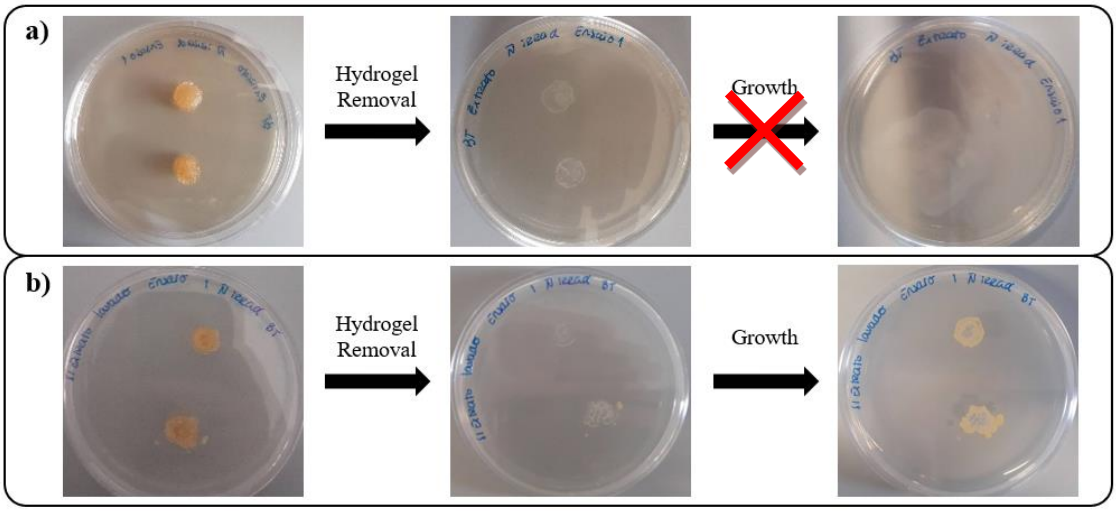


Figure 16. Representative pictures of the bacterial transfer assay, where both conditions present *S. aureus* transfer after hydrogel removal with no growth observed in condition a) (24h incubation at 37ºC) and growth in condition b). a) is Chit/Alg/WGP Non-irradiated hydrogel and b) is the Washed Chit/Alg Non-irradiated hydrogel.

5.3.3. Bacterial growth reduction

The ability of the bacteria to grow when in contact with the hydrogel was evaluated by determining the turbidity of the bacterial suspension in MHB culture medium. The results are described in the following Table 4.

Table 4. O.D. reduction (%) at 600nm of *S. aureus* growth when in contact with the hydrogels (Chit/Alg/WGP, Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg and washed Chit/Alg hydrogel) in comparison with *S. aureus* cells grown without hydrogel.

Hydrogel	**Chit/Alg/Trolox**	**Chit/Alg/FA**	**Chit/Alg/WGP**	**Chit/Alg**	**Washed Chit/Alg**
Reduction (%)	96.59±0.65	96.99±2.03	97.39±1.59	95.89±0.42	98.96±0.18

The growth of *S. aureus* when in contact with the hydrogel was reduced by more than 95% for all the conditions. The highest value was obtained for the washed Chit/Alg hydrogel with 98.96±0.18%, being statistically different from the other results ($p>0.05$). This was not expected since the goal of washing was to remove glutaraldehyde that potentially could be enhancing the antibacterial capacity of the hydrogel, and therefore, halting bacterial growth. Since glutaraldehyde was theoretically removed, the reduction of bacterial growth should be inferior. In fact, even if the washing was inefficient in removing the toxic compound, the reduction value should be similar to the unwashed control (Chit/Alg hydrogel).

By comparing the Chit/Alg with the Chit/Alg/WGP hydrogel, the hydrogel containing WGP extract had a higher reduction of bacterial growth (95.89±0.42% < 97.39±1.75%) ($p>0.05$). This indicates that the incorporation of WGP extracts in the hydrogel incapacitates the bacteria exposed to the hydrogel to grow. The other conditions (Chit/Alg/Trolox and Chit/Alg/FA) presented statistically similar values compared to the negative control ($p>0.05$). It was expected that Chit/Alg hydrogel presented the lowest values as observed, however it was not expected that the Chit/Alg/FA hydrogel presented similar results, since, as seen before, the incorporation of FA increases the IZD.

With attention to the disk diffusion assay, the Chit/Alg/WGP hydrogel promotes inhibition and reduces growth compared with the negative control. The positive control, however, presented higher inhibition capacity than the other conditions but no relevant growth reduction when compared to the others.

5.3.4. Membrane Integrity Disruption

The integrity of cells' membranes can be assessed by the Live/Dead assay. This methodology is based on the evaluation of PI's ability to penetrate the cytoplasmic membrane of bacterial cells, since it only penetrates cells with a damaged membrane. Bacterial suspensions in contact with the hydrogels and suspensions with the cells previously adhered to the hydrogels' surface after removal from the culture medium were tested to evaluate cell membrane compromise. The results can be observed in Figure 17, and Annex 16 shows some representative images of the Live/Dead assay.

After a 24h of incubation of the bacteria at 37ºC, 93.6±8.2% of the bacterial cells grown without exposure to the hydrogel presented membrane integrity, while 6.4±8.2% presented a disrupted membrane. Most of the results display mainly non-viable cells except for cells adhered to the washed Chit/Alg hydrogel. These presented 71.3±13.7%

of cells with preserved membrane integrity and 28.7±13.7% of cells with a disrupted membrane. For the suspension of cells in contact with the washed Chit/Alg hydrogel, 5.9±16.7% of cells presented preserved membrane integrity and 94.1±16.7% of cells had a disrupted membrane. This can further explain the previous bacterial transfer results (section 5.3.2.), where the washed Chit/Alg hydrogel presented growth after removal of the hydrogel. Since the membrane integrity of the cells is maintained after contact with the hydrogel then the cells might be able to grow, contrarily to the other conditions.

Once again glutaraldehyde seems to influence the antibacterial activity of the hydrogels, as reported in 5.3.2., which states that the interaction of glutaraldehyde at the cell's surface can produce a significant effect on membrane integrity[137]. The adhesion of cells to the washed hydrogel seems to affect at a lesser extent the cells membrane integrity. Possibly, if free glutaraldehyde is mainly present on the surface of the hydrogel, it is removed when washed, leading to less damage in the cells' membrane.

The high values of cells with disrupted membrane comes in accordance with the high values of growth reduction of bacterial cells in section 5.3.3., however, still does not explain the high values of reduction for the washed Chit/Alg hydrogel.

The cells membrane integrity seems to be affected mainly by the hydrogel formulation without much regard to the incorporated compounds, especially in the cells adhered to the hydrogel. In the suspension, the Chit/Alg hydrogel did preserve in a small extent the integrity of bacterial cells; however, the antibacterial activity of the formulation of the hydrogel alone once again is verified.

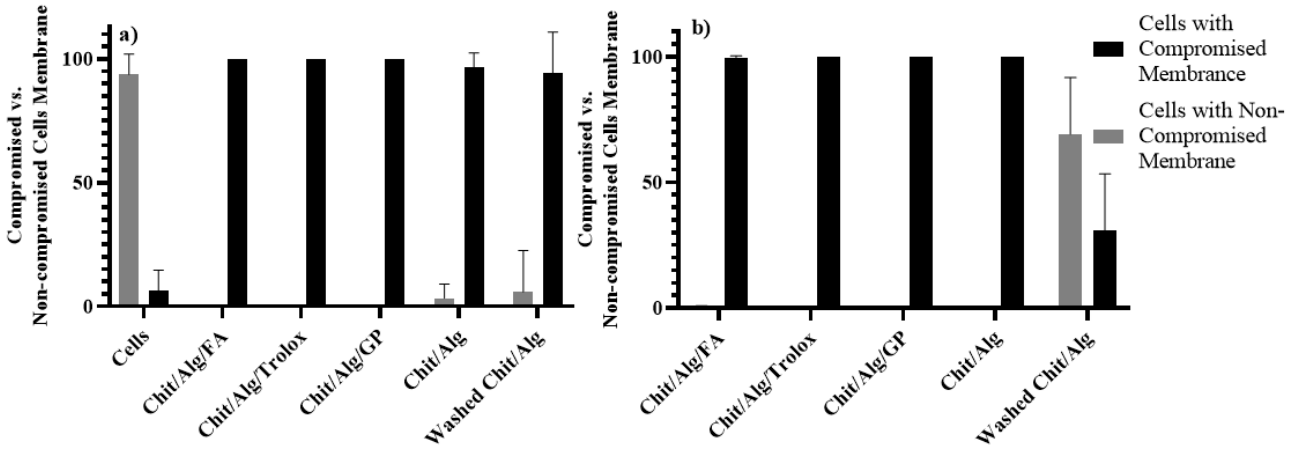


Figure 17. Percentage (%) of cells with disrupted membrane and cells with preserved membrane integrity in a) bacterial suspensions in contact with the hydrogels after 24h of exposure (Chit/Alg/WGP, Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg and washed Chit/Alg hydrogel) and b) suspensions with the cells previously adhered in the hydrogels surface (Chit/Alg/WGP,

Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg and washed Chit/Alg hydrogel) after 24 hours of exposure.

5.3.5. Bacterial culturability

The culturability of the bacteria grown in a suspension with hydrogel and the adhered hydrogel bacteria was evaluated by their capacity to form CFUs in solid medium after hydrogel exposition. Table 5 represents the total Log (CFU/mL) reduction for the bacterial suspension.

Table 5. Effect of the contact of *S. aureus* (CECT 978) with hydrogels (Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg/WGP, Chit/Alg and washed Chit/Alg hydrogels) on their culturability in PCA culture medium, expressed in Log (CFU/mL) reduction.

Suspension	Chit/Alg/Trolox	Chit/Alg/FA	Chit/Alg/WGP	Chit/Alg	Washed Chit/Alg
Log Reduction	9.62±0.00	9.62±0.00	9.62±0.00	9.62±0.00	5.49±0.25

Table 6, on the other hand, represents the total Log (CFU/mL) reduction for the cells adhered to the hydrogel.

Table 6. Effect of the adherence of *S. aureus* (CECT 978) with hydrogels (Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg/WGP, Chit/Alg and washed Chit/Alg hydrogels) on their culturability in PCA culture medium, expressed in Log (CFU/mL) reduction.

Hydrogel	Chit/Alg/Trolox	Chit/Alg/FA	Chit/Alg/WGP	Chit/Alg	Washed Chit/Alg
Log Reduction	9.62±0.00	9.62±0.00	9.62±0.00	9.62±0.00	5.33±0.12

All the conditions exhibited a change on the ability of bacterial cells to proliferate on solid culture medium, since total Log (CFU/mL) reduction (9.62±0.00 CFU/mL reduction) was observed. In both cases only the washed Chit/Alg hydrogel conditions were less affected in terms of culturability leading to reduction values of Log (CFU/mL) reduction of 5.49±0.25 for the suspension and of 5.33±0.12 for the bacteria adhered to the hydrogel. This indicates that the washing of the hydrogel enhances the bacterial cell's culturability compared to the remaining conditions, suggesting a possible influence of glutaraldehyde's presence, as stated in the previous sections. No conclusion could be assessed in terms of the incorporation of the WGP extracts in the hydrogel compared to

the other unwashed hydrogels. However, the antibacterial activity was high for all cases even the washed hydrogel. These results seem to indicate that a higher percentage of cells with damaged membrane leads to a lower culturability capacity of the bacterial cells, being an indication of the loss of cell viability (cell dead).

5.4. Conclusions

The incorporation of WGP extracts in the hydrogel formulation led to an increase in antibacterial activity, according to the modified disk diffusion assay and the bacterial growth reduction assay. In fact, in the disk diffusion assay a synergetic effect was observed, since an increase of the IZD of the hydrogel in comparison with the IZD of the WGP extracts was verified. The other methodologies did not display a difference of the integration of the WGP extracts from the other formulations. However, maximum values of cells with disrupted membrane obtained for the unwashed hydrogels (Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg/WGP). Chit/Alg hydrogel also obtained high percentages of cells with a disrupted membrane. Also, no culturability was observed for all the unwashed hydrogels, which indicates that the membrane disruption jeopardized the viability of the cells. Nonetheless the overall hydrogel formulations exhibited high antibacterial activity.

Furthermore, most of the assays denoted a decrease in the antibacterial capacity when the washed Chit/Alg hydrogel was used. The IZD was lower, the bacterial transfer and growth post contact increased and the membrane integrity of the bacterial cells and their culturability also obtained higher values. Therefore, the fabrication of the hydrogels with glutaraldehyde seems to affect the hydrogels antibacterial activity according to the premise that the free unlinked glutaraldehyde molecules are removed or significantly reduced by the washing of the hydrogels.

Lastly, by assessing through the bacterial transfer and disk diffusion assay the potential capacity of the WGP extract to be photoactivated or inactivated and affect antibacterial activity of the hydrogel due to its photosensitivity, it was verified that the irradiation of a UV light with 254nm did not influence the antibacterial activity.

Chapter 6: Concluding remarks and future research perspective

6.1. General conclusions

WGP extracts demonstrated higher antioxidant activity and extraction yields than RGP extracts. However, this did not signify a higher amount of TPC and TFC extracted. In terms of the difference between stalks and mixture types of extracts, nothing could be concluded. All extracts presented high antioxidant activity, nonetheless the higher antioxidant activity was obtained for the mixture sample of WGP extracts treated with ethanol 70%. Ethanol is considered a green solvent, which promotes sustainability, circular economy, and valorization of the winemaking industry's by-products. Therefore, the extract incorporated in a hydrogel directed for chronic wound treatment was the crude ethanolic (70:30% v/v) WGP extract from the mixture fraction.

The swelling percentages obtained for Chit/Alg/WGP hydrogel were high, potentially providing a high exudate absorption capacity to the hydrogel. However, the degradation values were very low, and the release rate was unable to be calculated. Also, the antioxidant activity of the hydrogels was very low compared to the capacity of the WGP extracts, indicating that the potential antioxidant activity of the compounds is being limited by incorporation in the hydrogel. Possibly, the antioxidant activity detected is mostly due to hydrogel formulation intrinsic properties.

On the other hand, the incorporation of WGP extracts in the hydrogel formulation led to an increase in antibacterial activity, being observed an increase of the IZD when the extracts are incorporated in the hydrogel. The *S. aureus* growth was also reduced when in contact with the Chit/Alg/WGP hydrogel. The other methodologies did not display a difference of the integration of the WGP extracts from the other formulations, since all the unwashed hydrogel (Chit/Alg/Trolox, Chit/Alg/FA, Chit/Alg/WGP) obtained maximum values of cells with disrupted membrane, with Chit/Alg also obtaining very low values of viability. Also, no culturability was observed for all the unwashed hydrogels. This indicates that low membrane integrity and low culturability lead to a reduced viability of the cells and that the overall hydrogel formulation exhibited high antibacterial activity.

In terms of glutaraldehyde influence on the antibacterial activity of the hydrogels, most of the assays denoted a decrease in the antibacterial capacity when the washed

Chit/Alg hydrogel was use. The IZD was lower, the *S. aureus* bacterial transfer and growth post contact with the hydrogel increased, and the viability of the bacterial cells and their culturability also obtained higher values. To conclude, the bacterial transfer and disk diffusion assay indicated the irradiation of a UV light with 254nm did not influence the antibacterial activity.

6.2. Future research perspective

Although this work presented some answers on GP extracts bioactivity and the formulation of a hydrogel with high swelling capacity and antioxidant activity, some questions and limitations are still not addressed. This way, suggestions for future work are presented as follows.

In terms of extraction, a higher amount of GP should be used for extraction in order to obtain a higher mass of extracted GP and consequently stock solutions with higher concentrations. This could result in beneficial implications in the release, antioxidant, and antibacterial assays, as the concentration of GP extracts in the hydrogel can be increased.

In terms of characterization of the constituents of GP extracts, other methods besides the TPC and TFC assay could be performed for the detection of polyphenols and flavonoids, since the TPC method is known to have some limitations[138]. For this HPLC (High-performance liquid chromatography) could be a good alternative. Also, LCMS (Liquid chromatography–mass spectrometry) could be used to identify monomeric phenolic content. For antioxidant activity, the hydrogen peroxide scavenging assay can be an also interesting assay, since it does not rely on synthetic radicals contrarily to the ABTS and DPPH method.

When it comes to the hydrogel fabrication, different concentrations of cross-linkers should also be analyzed in terms of swelling, degradation, and release capacity. To determine if the glutaraldehyde was being washed, the Fehling Test could be employed. Other purification methods may also be used to verify if the effect of glutaraldehyde is accentuated, such as washing with sodium borohydride after washing with ultrapure water[139]. Utilization of a different cross-linker for chitosan could also be an option, with genipin being a good alternative[40].

Also, to detect porosity and the cross-linking levels of the hydrogels a Fourier-Transform Infrared Spectroscopy (FTIR) could be performed. The surface hydrophobicity of the hydrogels could also be determined by contact angle measurement. Lastly, *in vitro* biocompatibility assays would also be important.

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Annexes

Annex 1

Table A1. RGP and WGP mixture and stalks samples extraction yields (%) for different extraction solvents (water, ethanol 50%, ethanol 70%, acetone 50%, acetone 70%).

RGP	***Mixture***			***Stalks***		
Extraction Solvent	Initial mass (g)	Final mass (g)	Extraction Yield (%)	Initial mass (g)	Final mass (g)	Extraction Yield (%)
Water	3	0.1	3	3	0.33	11
Ethanol 50%		0.1	3		0.36	12
Ethanol 70%		0.21	7		0.32	11
Acetone 50%		0.33	11		0.5	17
Acetone 70%		0.4	13		0.46	15
WGP	***Mixture***			***Stalks***		
Water	3	1.22	41	3	0.96	32
Ethanol 50%		0.79	26		0.68	23
Ethanol 70%		1.2	40		0.99	33
Acetone 50%		1.17	39		1.04	35
Acetone 70%		0.95	32		1.0	33

Annex 2

Table A2.1. Concentrations (µM) used for the gallic acid calibration curve and the respective absorbances measured at a wavelength of 765nm for Assay 1.

Concentrations (mg/L)	**338.6**	**282.2**	**225.7**	**169.3**	**112.9**	**56.4**	**28.2**	**14.1**
Abs at 734 nm	4.651	3.666	2.921	2.300	1.449	0.695	0.358	0.155

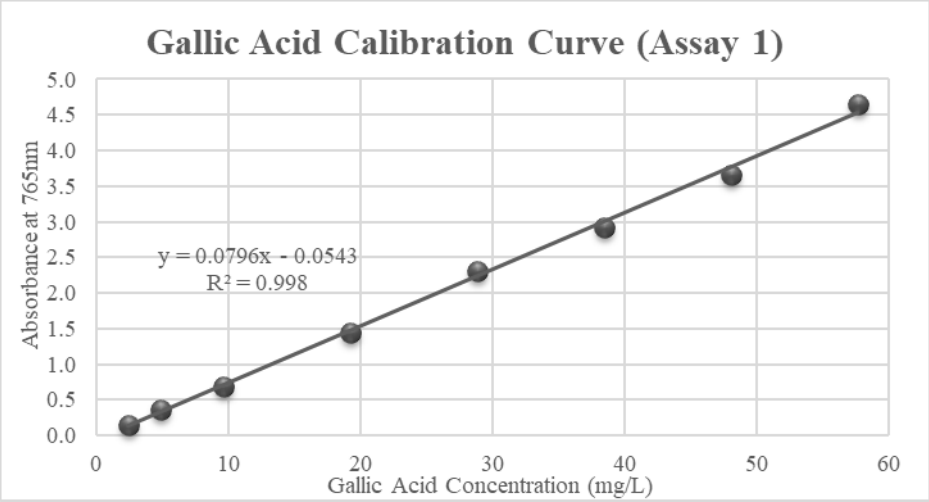


Figure A2.1. Gallic Acid calibration curve at a wavelength of 765nm for Assay 1.

Table A2.2. Concentrations (µM) used for the gallic acid calibration curve and the respective absorbances measured at wavelength of 765nm for Assay 2.

Concentrations (mg/L)	**338.6**	**282.2**	**225.7**	**169.3**	**112.9**	**56.4**	**28.2**	**14.1**
Abs at 734 nm	4.651	3.863	3.136	2.459	1.558	0.752	0.386	0.175

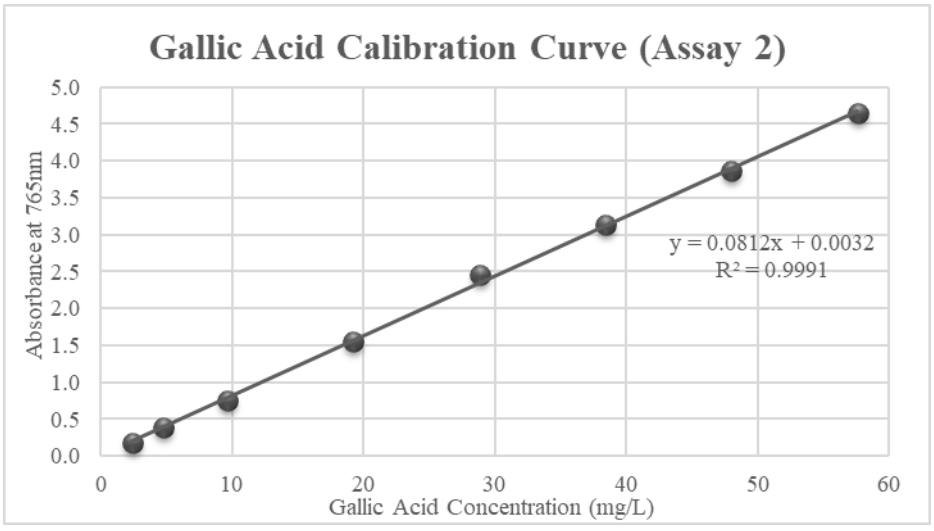


Figure A2.2 Gallic Acid calibration curve at wavelength of 756nm for Assay 2.

Annex 3

Table A3. Total phenolic content (TPC) in mg GAE/g extract of crude aqueous, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%) RGP and WGP extracts obtained from mixture and stalks.

Solvents	**TPC (mg GAE/g extract)**			
	RGP Mix	**RGP stalks**	**WGP Mix**	**WGP stalks**
Water	165.5±10.7	231.8±16.4	154.2±8.2	150.0±4.7
Ethanol 50%	234.0±17.9	278.2±16.8	35.6±2.9	121.0±2.4
Ethanol 70%	294.9±8.9	206.4±2.6	24.9±5.8	113.2±2.5
Acetone 50%	277.2±2.9	292.5±17.9	152.1±5.1	180.3±6.0
Acetone 70%	264.1±13.1	280.0±9.0	152.7±6.3	216.9±20.4

Annex 4

Table A4.1. Concentrations (µM) used for the catechin calibration curve and the respective absorbances measured at a wavelength of 510nm for Assay 1.

Concentrations (µM)	**800**	**400**	**200**	**100**	**75**	**50**	**25**	**10**
Abs at 734 nm	4.031	2.315	1.216	0.693	0.549	0.400	0.251	0.168

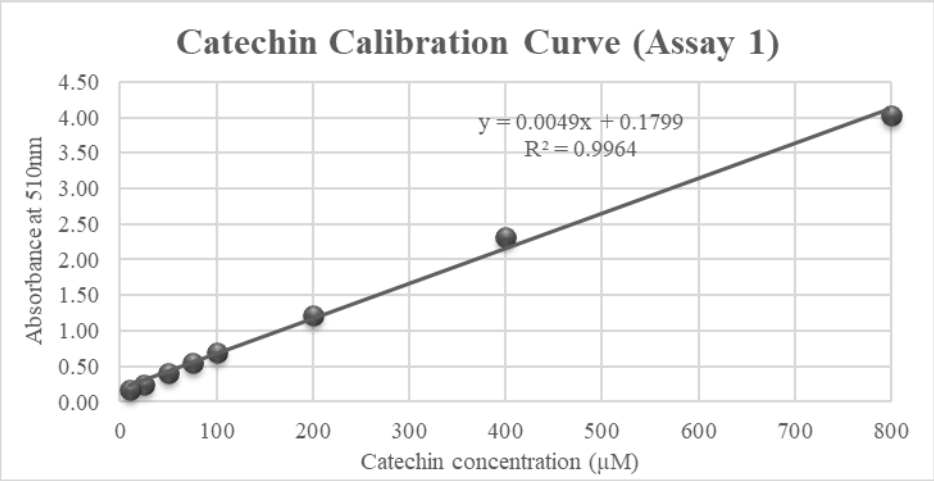


Figure A4.1. Catechin calibration curve at a wavelength of 510nm for Assay 1.

Table A4.2. Concentrations (µM) used for the catechin calibration curve and the respective absorbances measured at a wavelength of 510nm for Assay 2.

Concentrations (µM)	**1000**	**800**	**600**	**400**	**200**	**100**	**75**	**50**	**25**	**10**
Abs at 734 nm	6.414	4.520	3.261	2.809	1.685	0.632	0.528	0.490	0.293	0.161

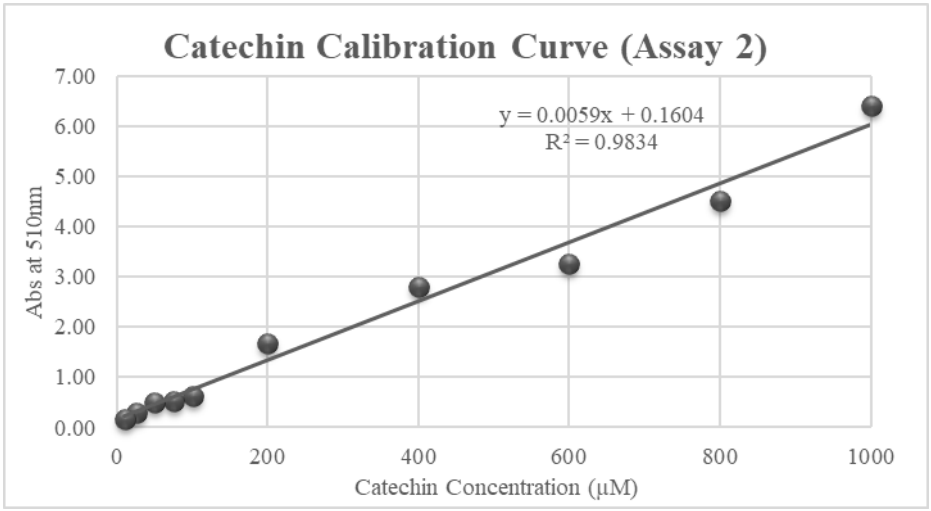


Figure A4.2. Catechin calibration curve at a wavelength of 510nm for Assay 2.

Annex 5

Table A5. Total flavonoid content (TFC) in mg GAE/g extract of crude aqueous, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%) RGP and WGP extracts obtained from mixture and stalks.

Solvents	**TPC (mg GAE/g extract)**			
	RGP Mix	**RGP stalks**	**WGP Mix**	**WGP stalks**
Water	3.2±1.3	9.3±1.9	-10.1±5.2	22.6±7.45
Ethanol 50%	150.8±8.3	130.9±13.7	9.0±2.1	50.4±9.5
Ethanol 70%	186.5±7.5	177.9±27.4	16.4±4.6	62.6±14.4
Acetone 50%	195.4±30.1	160.9±14.1	52.8±5.2	88.3±3.6
Acetone 70%	178.7.1±28.4	201.8±11.1	43.6±9.1	95.7±5.3

Annex 6

Table A6.1. Concentrations (µM) used for the ABTS assay's Trolox calibration curve and the respective absorbances measured at a wavelength of 734nm for Assay 1.

Concentrations (µM)	**25**	**20**	**15**	**10**	**5**	**2.5**	**1.25**
Abs at 734 nm	0.061	0.223	0.315	0.421	0.521	0.578	0.585

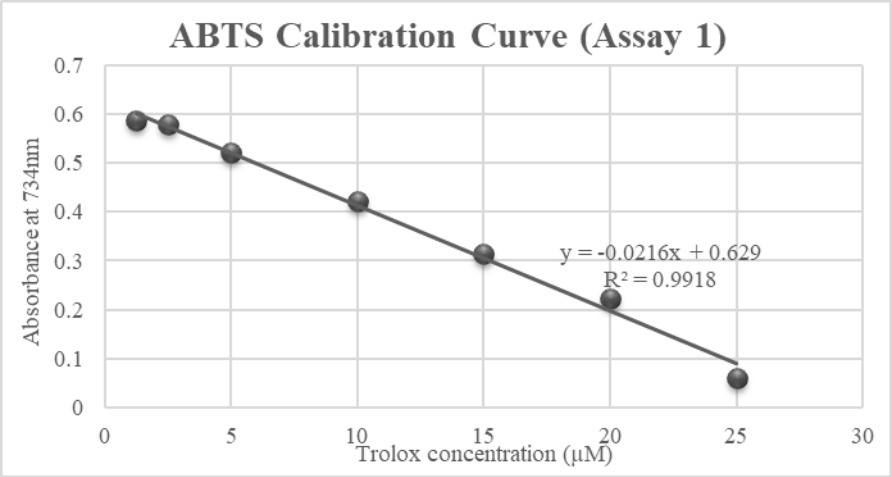


Figure A6.1. Trolox calibration curve at a wavelenght of 734nm for Assay 1.

Table A6.2. Concentrations (µM) used for the ABTS assay's Trolox calibration curve and the respective absorbances measured at a wavelength of 734nm for Assay 2.

Concentrations (µM)	**25**	**20**	**15**	**10**	**5**	**2.5**	**1.25**
Abs at 734 nm	0.065	0.183	0.288	0.404	0.516	0.565	0.596

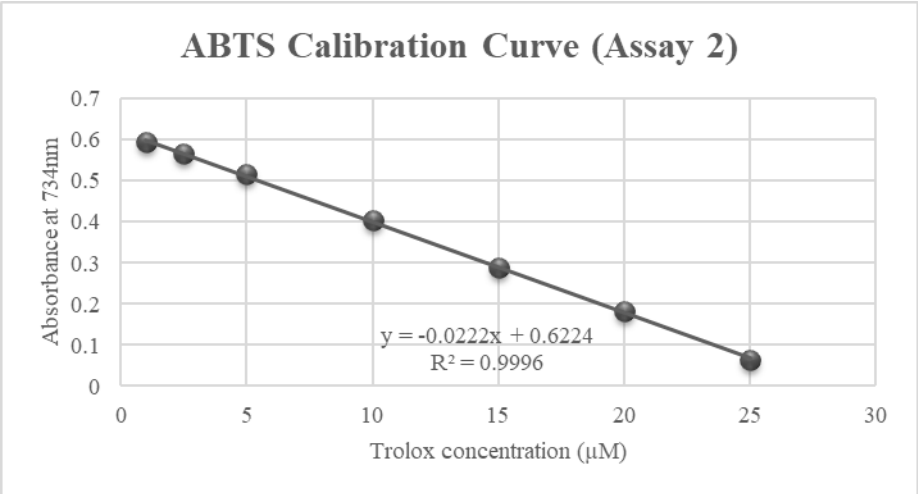


Figure A6.1. Trolox calibration curve at a wavelength of 734nm for Assay 2.

Table A6.3. Concentrations (µM) used for the DPPH assay's Trolox calibration curve and the respective absorbances measured at a wavelength of 517nm for Assay 1.

Concentrations (µM)	**25.00**	**20.00**	**15.00**	**10.00**	**5.00**	**2.50**	**1**
Abs at 734 nm	0.130	0.165	0.304	0.425	0.520	0.558	0.592

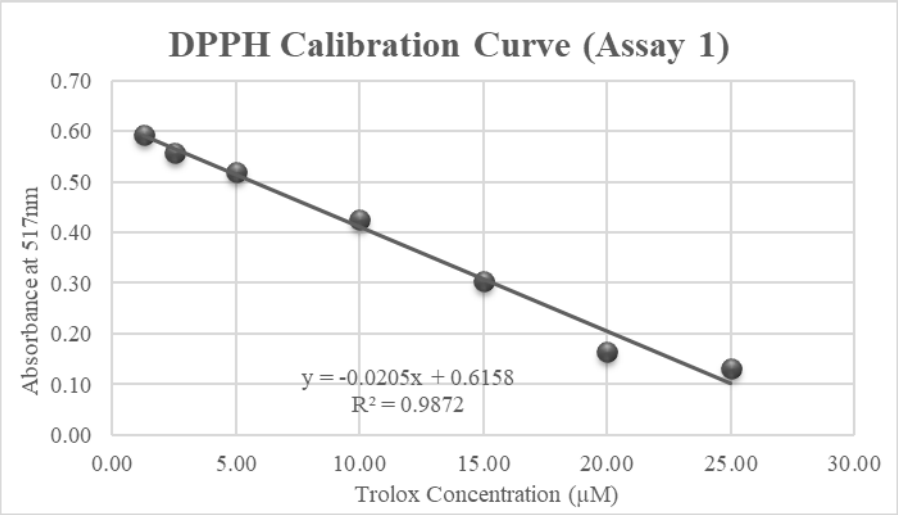


Figure A6.3. Trolox calibration curve at a wavelength of 734nm for Assay 1.

Table A6.4. Concentrations (µM) used for the DPPH assay's Trolox calibration curve and the respective absorbances measured at a wavelength of 517nm for Assay 2.

Concentrations (µM)	**25.00**	**20.00**	**15.00**	**10.00**	**5.00**	**2.50**	**1.25**
Abs at 734 nm	0.127	0.159	0.298	0.352	0.466	0.528	0.538

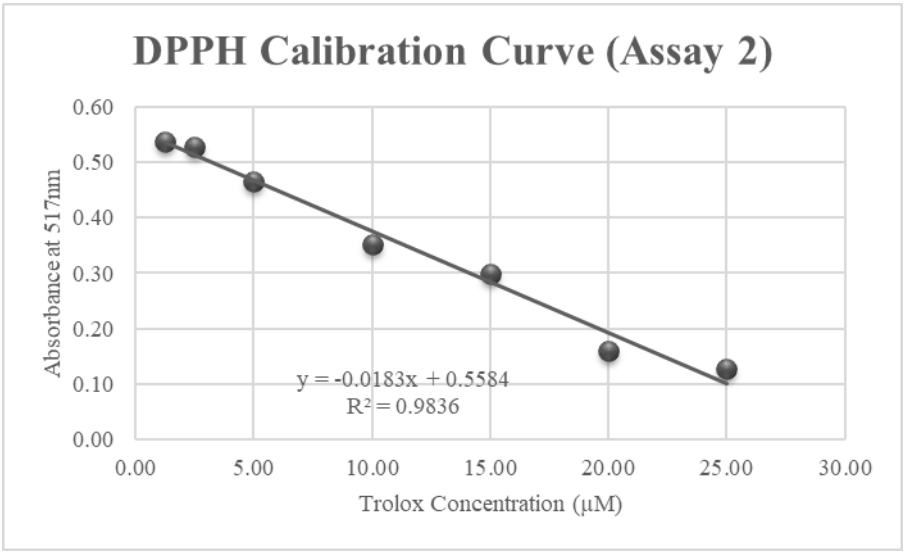


Figure A6.3. Trolox calibration curve at a wavelength of 734nm for Assay 2.

Annex 7

Table A7.1. Trolox Equivalents (TE) of the ABTS assay in (mg of TE/g of extract) for mixture and stalks samples of RGP and WGP extracted by water, ethanol 50%, ethanol 70%, acetone 50% and acetone 70% and respective standard deviation.

Solvents	ABTS TE (mg TE/g extract)			
	RGP Mix	RGP stalks	WGP Mix	WGP stalks
Water	1.050±0.070	3.701±1.326	1.519±0.015	1.575±0.007
Ethanol 50%	1.615±0.019	1.035±0.881	1.576±0.023	1.629±0.005
Ethanol 70%	-0.936±0.061	1.222±0.115	1.648±0.008	1.635±0.012
Acetone 50%	0.498±0.051	1.264±0.026	1.618±0.013	1.626±0.013
Acetone 70%	0.633±0.072	0.014±0.098	1.627±0.015	1.610±0.024

Table A7.2. Trolox Equivalents (TE) of the DPPH assay in (mg of TE/g of extract) for mixture and stalks samples of RGP and WGP extracted by water, ethanol 50%, ethanol 70%, acetone 50% and acetone 70% and respective standard deviation.

Solvents	DPPH TE (mg TE/g extract)			
	RGP Mix	RGP stalks	WGP Mix	WGP stalks
Water	0.369±0.199	1.657±0.166	1.689±0.128	1.037±0.265
Ethanol 50%	1.671±0.125	1.416±0.385	1.766±0.078	1.709±0.132
Ethanol 70%	*	1.496±0.274	1.792±0.047	1.590±0.035
Acetone 50%	1.133±0.268	1.494±0.157	1.479±0.105	1.023±0.122
Acetone 70%	1.539±0.251	1.063±0.586	0.364±5.542	1.751±0.073

*does not fall under the calibration curve

Annex 8

Table A8.1. Scavenging Activity (%) of the ABTS assay for mixture and stalks samples of RGP and WGP extracted by water, ethanol 50%, ethanol 70%, acetone 50% and acetone 70% and respective standard deviation.

Solvents	ABTS Scavenging Activity (%)			
	RGP Mix	RGP stalks	WGP Mix	WGP stalks
Water	57.315±3.701	72.568±2.619	83.253±0.015	86.359±0.365
Ethanol 50%	88.568±1.035	147.996±2.940	86.391±0.023	89.318±0.257
Ethanol 70%	-52.425±3.347	66.857±6.382	90.367±0.008	89.662±0.659
Acetone 50%	26.807±2.823	69.145±1.425	88.696±0.013	89.181±0.733
Acetone 70%	34.302±4.000	0.046±5.438	89.209±0.015	88.289±1.344

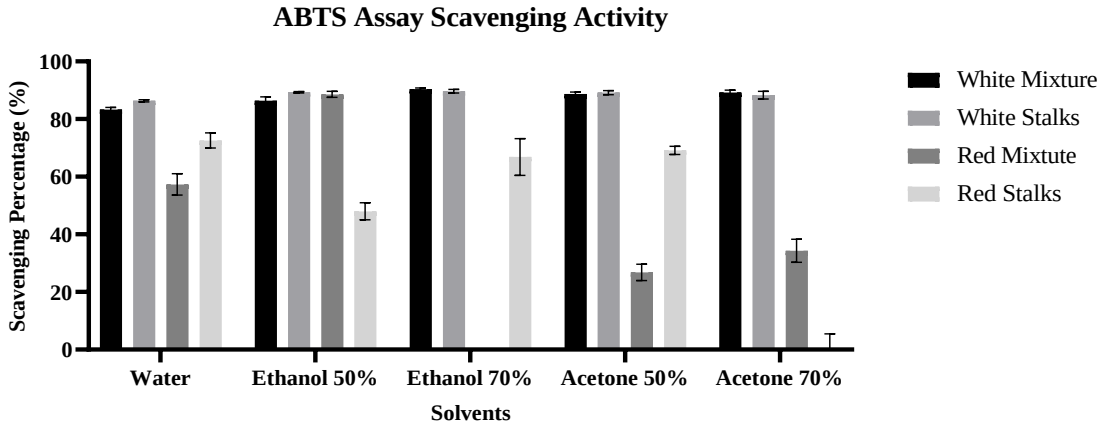


Figure A8.1. Scavenging Activity (%) of the ABTS assay for mixture and stalks samples of RGP and WGP extracted by water, ethanol 50%, ethanol 70%, acetone 50% and acetone 70% and respective standard deviation.

Table A8.2. Scavenging Activity (%) of the DPPH assay for mixture and stalks samples of RGP and WGP extracted by water, ethanol 50%, ethanol 70%, acetone 50% and acetone 70% and respective standard deviation.

Solvents	DPPH Scavenging Activity (%)			
	RGP Mix	RGP stalks	WGP Mix	WGP stalks
Water	20.169±10.544	88.237±8.778	89.942±6.781	55.385±8.178
Ethanol 50%	88.988±6.580	75.474±20.354	94.003±4.117	90.964±6.997
Ethanol 70%	223.633±43.634	79.696±12.427	95.348±2.489	84.698±1.859
Acetone 50%	60.549±14.174	79.589±8.289	78.809±5.542	54.740±6.444
Acetone 70%	81.982±13.264	056.811±30.970	19.887±5.236	93.191±3.875

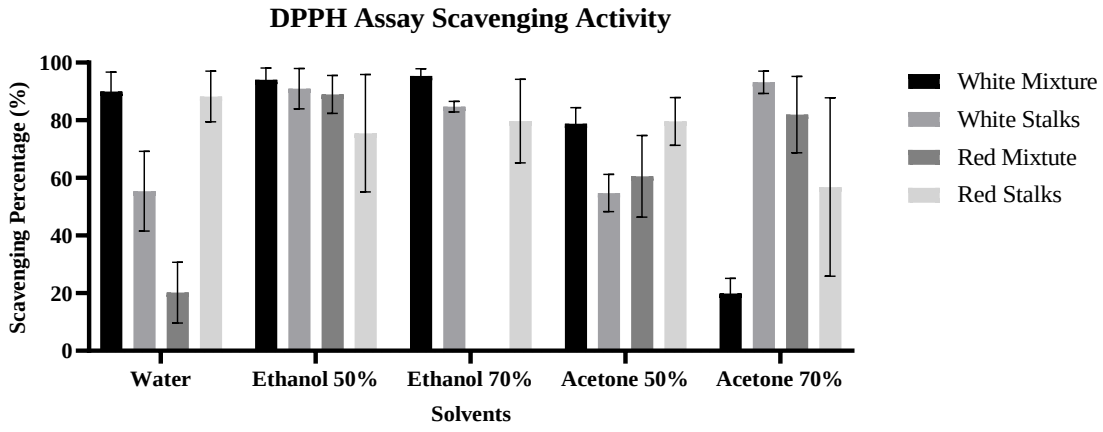


Figure A8.2. Scavenging Activity (%) of the DPPH assay for mixture and stalks samples of RGP and WGP extracted by water, ethanol 50%, ethanol 70%, acetone 50% and acetone 70% and respective standard deviation.

Annex 9

Table A9. Conversion of Trolox Equivalents (TE) of the ABTS assay in (mg of TE/g of extract) to µmol of TE /g of extract of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%.) RGP and WGP extracts obtained from mixture and stalks.

RGP	***Mixture***		***Stalks***	
Extraction Solvent	mg TE /g extract	µmol/g	mg TE /g extract	µmol /g
Water	1.050	4.194	1.326	5.297
Ethanol 50%	1.615	6.454	0.881	3.521
Ethanol 70%	-0.936	>0	1.222	4.884
Acetone 50%	0.498	1.989	1.264	5.050
Acetone 70%	0.633	2.531	0.014	0.055
WGP	***Mixture***		***Stalks***	
Water	1.519	6.069	1.575	6.294
Ethanol 50%	1.576	6.296	1.629	6.508
Ethanol 70%	1.648	6.584	1.635	6.533
Acetone 50%	1.618	6.463	1.626	6.498
Acetone 70%	1.627	6.500	1.610	6.484

Table A9.2. Conversion of Trolox Equivalents (TE) of the DPPH assay in (mg of TE/g of extract) to µmol of TE /g of extract of crude aqueouse, ethanolic (ethanol 50%, ethanol 70%) and acetonic (acetone 50%, and acetone 70%.) RGP and WGP extracts obtained from mixture and stalks.

RGP	***Mixture***		***Stalks***	
Extraction Solvent	mg TE /g extract	µmol/g	mg TE /g extract	µmol /g
Water	0.369	1.476	1.657	6.657
Ethanol 50%	1.671	6.677	1.416	5.656
Ethanol 70%	<0	<0	1.496	5.975
Acetone 50%	1.133	4.528	1.494	5.967
Acetone 70%	1.539	6.148	4.246	1.063
WGP	***Mixture***		***Stalks***	
Water	1.689	6.750	1.036	4.138
Ethanol 50%	1.766	7.057	1.709	6.827
Ethanol 70%	1.791	7.158	1.590	6.353
Acetone 50%	1.479	5.908	1.023	4.089
Acetone 70%	0.364	1.455	1.751	6.995

Annex 10

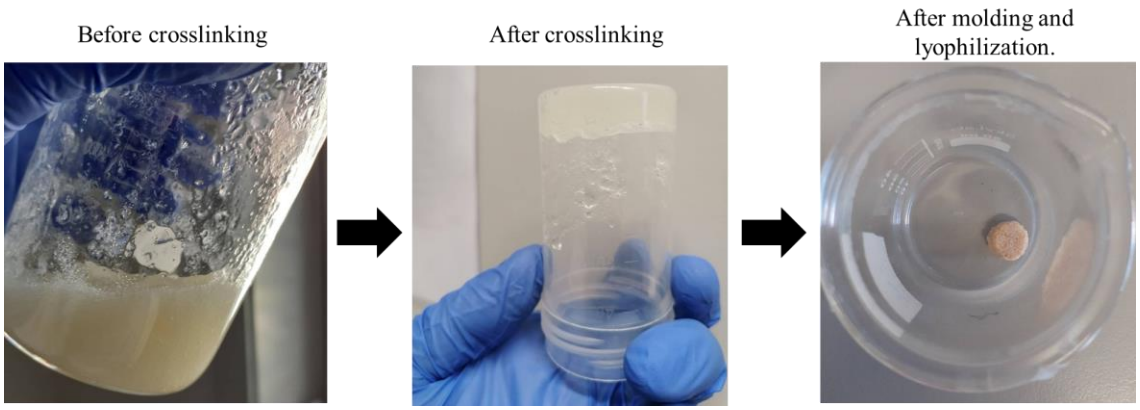


Figure A10. Chitosan-alginate hydrogel cross-linked with glutaraldehyde and calcium chloride.

Annex 11

Table A11.1. Swelling percentage (%) of Chit/Alg, Chit/Alg/GP and Chit/Alg/Trolox Hydrogel through time (0, 0.5, 1, 2, 4, 8 and 24 hours).

	Swelling (%)					
Time (h)	**0.5**	**1**	**2**	**4**	**8**	**24**
Chit/Alg/GP	2479.89	2734.32	3391.93	3143.44	2821.27	3697.67
Chit/Alg	3234.25	3338.42	3848.48	3705.29	3821.69	3535.12
Chit/Alg/Trolox	3551.70	3274.99	3376.83	3407.29	2395.16	2694.35

	Degradation (%)						
Time (h)	**0.5**	**1**	**3**	**4**	**6**	**8**	**24**
Sample	5.647	3.661	-4.388	-9.178	4.198	2.488	5.648
Control	-12.597	-3.139	-9.366	-3.655	-2.163	-8.245	-12.597
Trolox	-11.839	-6.737	-4.701	1.221	-0.654	2.761	-11.839

Table A11.2. Degradation percentage (%) of Chit/Alg, Chit/Alg/GP and Chit/Alg/Trolox Hydrogel through time (0, 0.5, 1, 2, 4, 8 and 24 hours).

Annex 12

Table A12.1. Trolox Equivalents (TE) and Scavenging Capacity of Chit/Alg/GP, Chit/Alg (negative control) and Chit/Alg/Trolox (positive control).

	ABTS Assay		DPPH Assay	
Hydrogels	**mg TE/g extract**	**Scavenging Capacity (%)**	**mg TE/g extract**	**Scavenging Capacity (%)**
Chit/Alg/GP	0.508±0.144	18.513±0.878	-9.144±4.036	-53.091±25.560
Chit/Alg	1.317±0.868	23.327±5.178	-13.784±5.389	-82.477±34.132
Chit/Alg/Trolox	1.802±0.576	26.228±3.433	-7.946±7.134	-47.907±47.079

TableA12.2. Concentrations (µM) used for the ABTS assay's Trolox calibration curve and the respective absorbances measured at a wavelength of 517nm for Assay 1.

Concentrations (µM)	**25**	**20**	**15**	**10**	**5**	**2.5**	**1.25**
Abs at 734 nm	0.089	0.150	0.277	0.385	0.478	0.548	0.089

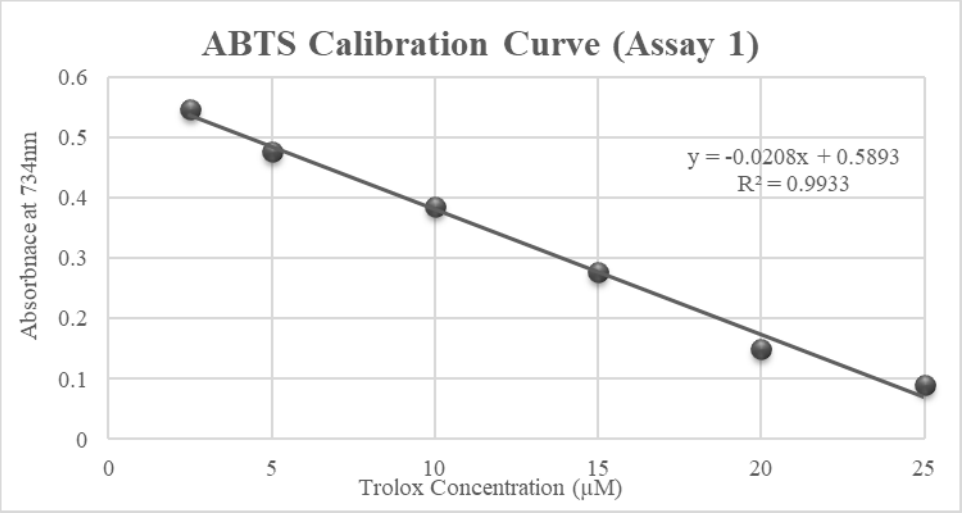


Figure A12.1 ABTS Trolox calibration curve at a wavelength of 734nm for Assay 1.

Table A12.3. Concentrations (µM) used for the ABTS assay's Trolox calibration curve and the respective absorbances measured at a wavelength of 517nm for Assay 2.

Concentrations (µM)	**25**	**20**	**15**	**10**	**5**	**2.5**	**1.25**
Abs at 734 nm	0.061	0.223	0.315	0.421	0.521	0.578	0.585

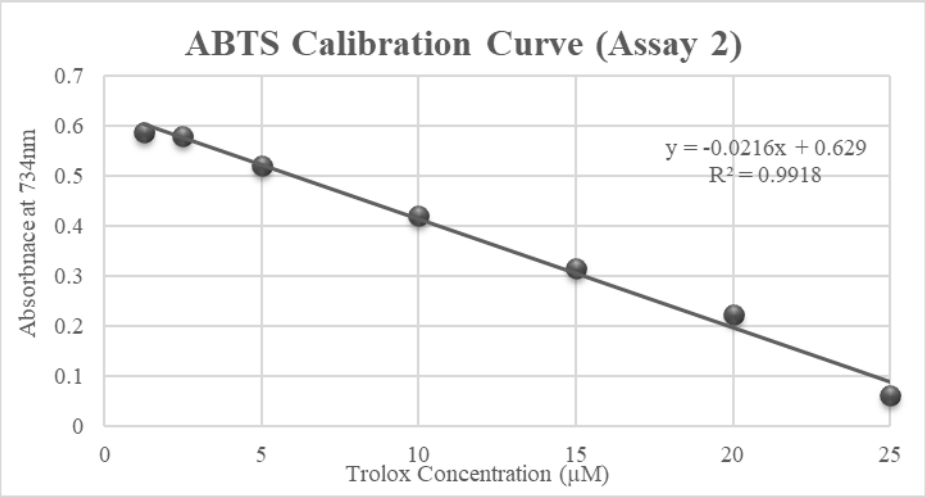


Figure A12.2. ABTS Trolox calibration curve at an absorbance at 734nm for Assay 2.

Table A12.4. Concentrations (µM) used for the DPPH assay's Trolox calibration curve and the respective absorbances measured at a wavelength of 517nm for Assay 1.

Concentrations (µM)	**25**	**20**	**15**	**10**	**5**	**2.5**	**1.25**
Abs at 734 nm	0.130	0.165	0.304	0.425	0.520	0.558	0.592

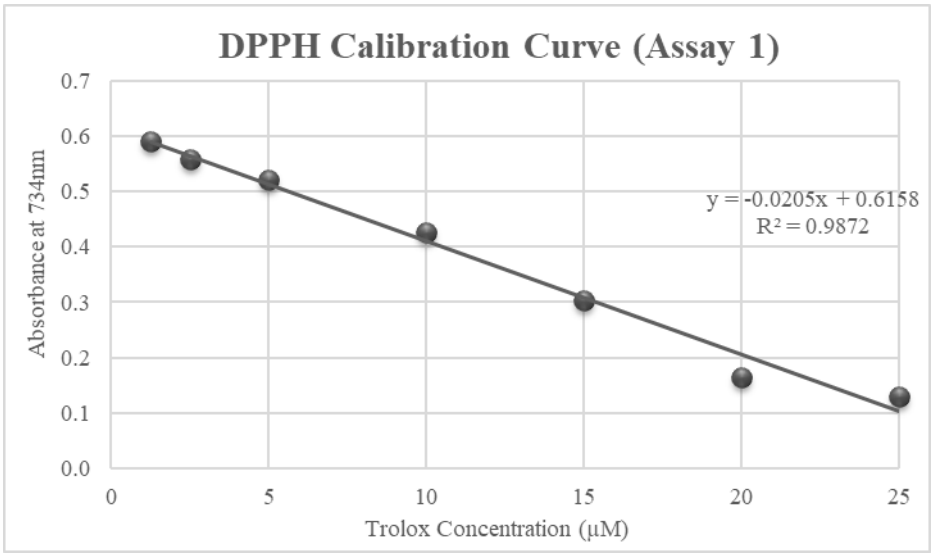
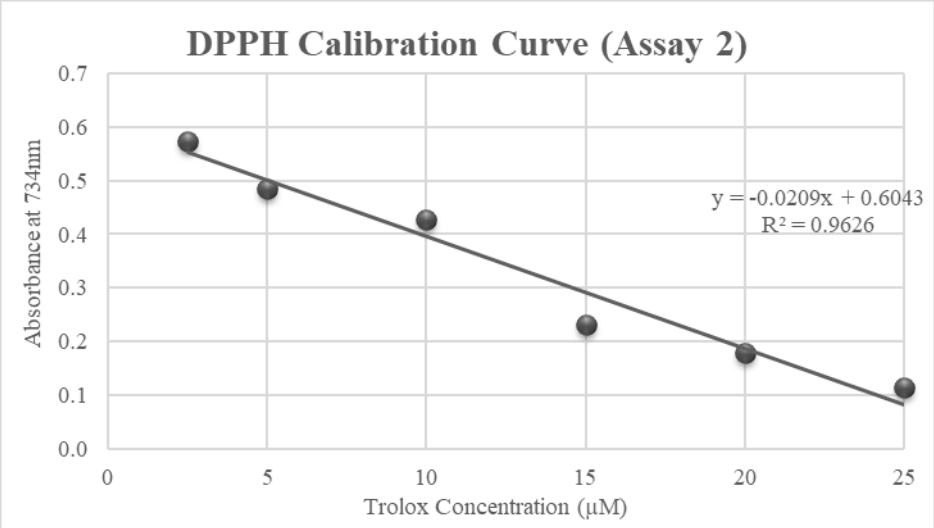


Figure A12.3. DPPH Trolox calibration curve at a wavelength of 734nm for Assay 2.

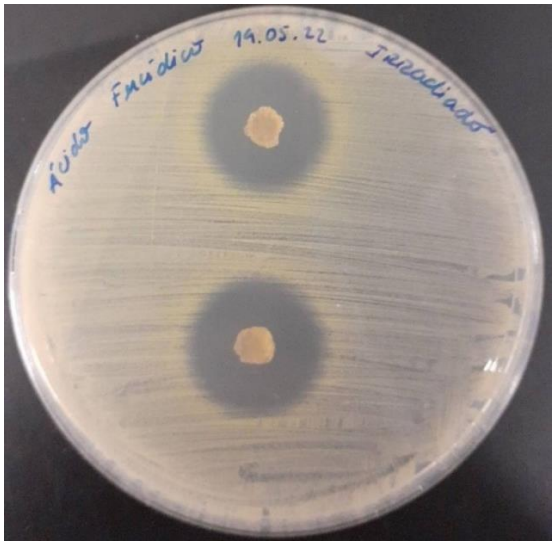
TableA12.5. Concentrations (µM) used for the DPPH assay's Trolox calibration curve and the respective absorbances measured at a wavelength of 517nm for Assay 2.

Concentrations (µM)	**25**	**20**	**15**	**10**	**5**	**2.5**	**1.25**
Abs at 734 nm	0.1138	0.1783	0.2303	0.4263	0.4843	0.5743	0.1138



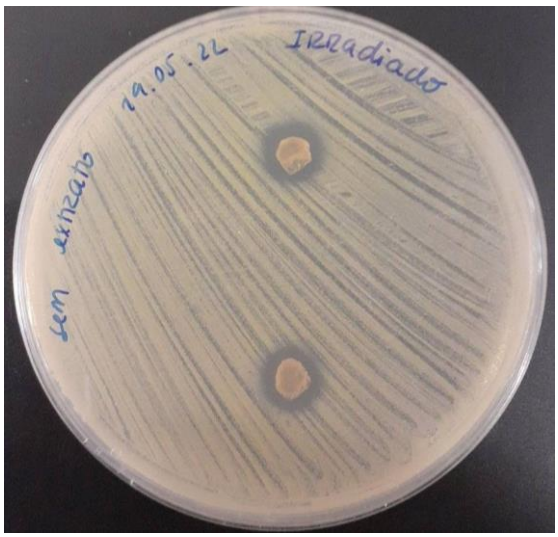
FigureA12.4. DPPH Trolox calibration curve at a wavelength of 734nm for Assay 2.

Annex 13

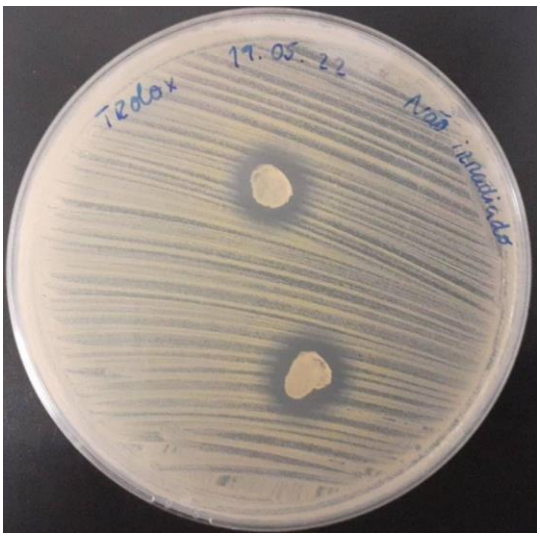


FigureA13.1. Chit/Alg/FA representative image.

FigureA13.2. Chit/Alg/GP representative image.

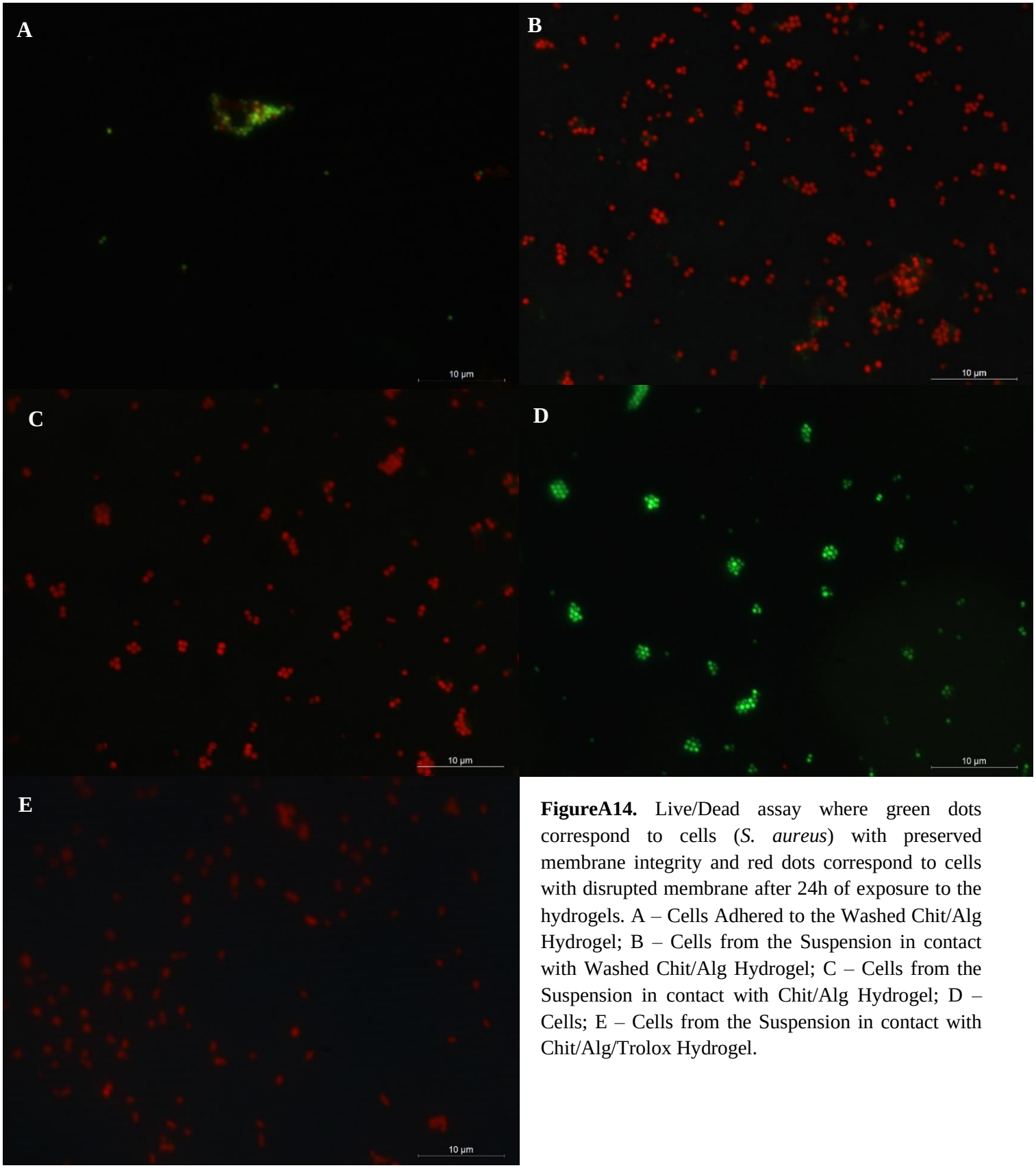


FigureA13.3. Chit/Alg representative image.



FigureA13.4. Chit/Alg/Trolox representative image.

Annex 14



FigureA14. Live/Dead assay where green dots correspond to cells (*S. aureus*) with preserved membrane integrity and red dots correspond to cells with disrupted membrane after 24h of exposure to the hydrogels. A – Cells Adhered to the Washed Chit/Alg Hydrogel; B – Cells from the Suspension in contact with Washed Chit/Alg Hydrogel; C – Cells from the Suspension in contact with Chit/Alg Hydrogel; D – Cells; E – Cells from the Suspension in contact with Chit/Alg/Trolox Hydrogel.