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Response surface evaluation of Subcritical Water Extraction conditions of *Castanea sativa* shells bioactive compounds: A cosmetic application

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Abstract

Castanea sativa is the chestnut species most abundant in south Europe, being an added value resource in producing countries due to its nutritional composition and health-promoting properties. In the last two decades, chestnut production has increased worldwide. During chestnut processing, huge amounts of by-products are generated, mainly shells. Chestnut shells are excellent sources of polyphenols, fibers and vitamin E. The richness in bioactive compounds has aroused the interest in the reuse of this by-product for cosmetic purposes, particularly polyphenol-enriched extracts that could be effective as cosmetic ingredients.

The main aim of this study was to optimize the extraction conditions of antioxidant compounds and polyphenols from chestnut shells using Subcritical Water Extraction (SWE) through Response Surface Methodology (RSM). The optimum conditions that maximized the antioxidant activity (through DPPH, ABTS and FRAP assays) and the polyphenol content occurred at 220 °C during 30 minutes. The optimized extract was evaluated for free radical scavenging activity, phytochemical profile as well as cell viability. A hydrogel containing the optimized *C. sativa* shell extract was developed and its technological properties were evaluated. A stability study of the hydrogel was also performed at different times (1, 7 and 30 days) and temperatures (25 and 40 °C).

The response surface profiles of ABTS, FRAP and TPC were similar, reaching the optimal conditions at 220 °C/30 min. The lowest responses were obtained at 150 °C probably due to the degradation of tannins. Due to the similarity of results, the phenolic composition of the extracts prepared at 80 °C/10 min and 220 °C/30 min was analysed by LC-DAD/ESI-MS. The extract prepared at 80 °C/10 min was richer in tannins, while the extract obtained at 220 ºC/30 min had higher amounts of pyrogallol and protocatechuic acid. The optimized extract did not lead to a decrease on fibroblasts and keratinocytes viability up to a concentration of 1000 and 100 µg/mL, respectively. Considering the RSM results and the bioactive composition, the extract prepared at 220 ºC/30 min was incorporated in a hydrogel. The pH of the hydrogel was compatible with the skin pH and its stability was retained when stored at 25 °C. However, the hydrogel stored at 40 °C during 30 days showed significant changes in colour, texture and viscosity. Overall, the results support the bioactivity and safeness of *C. sativa* shell extract as well as its possible use as cosmetic ingredient. The valorization of this by-product represents a new challenge for cosmetic industry to develop new sustainable and environmental friendly products.

Keywords: *Castanea sativa* shells; Subcritical Water Extraction; bioactivity; cosmetic.

Resumo

Castanea sativa é a espécie de castanha mais abundante no sul da Europa, sendo um recurso de valor acrescentado nos países produtores devido à sua composição nutricional e propriedade benéficas para a saúde. Nas últimas décadas, a produção mundial de castanha tem aumentado. Durante o processamento da castanha são geradas elevadas quantidades de subprodutos, sobretudo cascas, os quais são excelentes fontes de polifenóis, fibras e vitamina E. A riqueza em compostos bioativos tem despertado o interesse na reutilização deste subproduto para fins cosméticos.

O principal objetivo deste estudo foi otimizar as condições de extração de compostos antioxidantes e polifenóis das cascas de castanha com recurso à Extração Subcrítica com Água (ESA), através da Metodologia de Superfície de Resposta (MSR). As condições ótimas que maximizaram a atividade antioxidante e o teor de polifenóis ocorreram a 220 °C durante 30 minutos. O extrato otimizado foi avaliado quanto à capacidade de captação de radicais livres, perfil fitoquímico e viabilidade celular e incorporado num hidrogel. As propriedades tecnológicas desta formulação foram avaliadas a diferentes tempos (1, 7 e 30 dias) e temperaturas (25 e 40 °C).

Os perfis de resposta dos ensaios ABTS, FRAP e teor de polifenóis foram semelhantes, atingindo as condições ótimas a 220 °C/30 min. As respostas mais baixas foram obtidas a 150 °C provavelmente devido à degradação de taninos. Devido à semelhança de resultados, a composição fenólica dos extratos preparados a 80 °C/10 min e 220 °C/30 min foi analisada por LC-DAD/ESI-MS. O extrato preparado a 80 °C/10 min mostrou ser mais rico em taninos, enquanto o extrato obtido a 220 ºC/30 min apresentou maiores quantidades de pirogalol e ácido protocatecuico. O extrato otimizado não conduziu a uma diminuição da viabilidade celular em fibroblastos e queratinócitos à uma concentração de 1000 e 100 µg/mL. Considerando os resultados da MSR e a composição em antioxidantes e fenólicos, o extrato a 220 ºC/30 min foi selecionado para incorporação num hidrogel. O pH do hidrogel foi compatível com o pH cutâneo e a sua estabilidade foi mantida a 25 °C durante 30 dias. No entanto, o hidrogel armazenado a 40°C durante 30 dias apresentou alterações significativas na cor, textura e viscosidade. Com efeito, os resultados sustentam a bioatividade e segurança do extrato de cascas de *C. sativa*. A valorização deste subproduto constituiu um novo desafio para a indústria cosmética a fim de desenvolver novos produtos cosméticos sustentáveis e amigos do meio ambiente.

Palavras-chave: cascas de *Castanea sativa*; Extração Subcrítica com Água; bioatividade; cosmético.

List of publications and communications

1. **Diana Pinto**, Nair Braga, Ana Margarida Silva, Paulo Costa, Cristina Delerue-Matos, Francisca Rodrigues, Chapter 6 - Chestnut, “Valorization of fruit processing by-products”, Ed. Charis M. Galanakis, Academic Press, 2019, pp: 127 – 144 (Paperback ISBN: 9780128171066).

2. **Diana Pinto**, Andreia F. Peixoto, Cristina Freire, Cristina Delerue-Matos, Francisca Rodrigues, Bioactivity and radical scavenging capacity of *Castanea sativa* shell extract obtained by subcritical water extraction, 4th Meeting of Medicinal Biotechnology, 17th May 2019, Porto, Portugal

Index

1 Introduction 1
1.1 *Castanea sativa* Mill 2
1.2 *Castanea sativa* shell 7
1.3 Bioactive compounds from *C. sativa* shells as potential ingredients for cosmetics 13
1.4 Green extraction techniques for recovery of bioactive compounds 22
1.4.1 Subcritical water extraction 25
2 Objectives 30
3 Materials and Methods 32
3.1 Chemicals 33
3.2 Sample 33
3.3 Experimental design and optimization of extraction conditions 34
3.4 Subcritical water extraction of bioactive compounds from *C. sativa* shells 35
3.5 Quantification of bioactive compounds 36
3.5.1 Determination of Total Polyphenols Content 36
3.6 *In vitro* antioxidant activity 36
3.6.1 DPPH free radical scavenging assay 36
3.6.2 Ferric reducing antioxidant power (FRAP) assay 37
3.6.3 ABTS free radical scavenging assay 37
3.7 Reactive oxygen species scavenging capacity assays 38
3.7.1 Superoxide radical scavenging assay 38
3.7.2 Hypochlorous acid scavenging assay 39
3.7.3 Peroxyl radical scavenging assay 39
3.8 Phytochemical profile of *C. sativa* shell extract 40
3.9 Cell viability assays 40
3.9.1 Cell lines, primary cell isolation and culture conditions 40
3.9.2 MTT assay 41
3.10 Preparation of *C. sativa* shell hydrogel 41
3.11 Characterization of *C. sativa* shell hydrogel 43
3.11.1 Colour evaluation 43
3.11.2 Determination of pH 43

3.11.3 Texture analysis 44
3.11.4 Rheological behaviour analysis 45
3.12 Stability Study 45
3.13 Statistical analysis 46
4 Results and Discussion 47
4.1 Validation of the experimental design 48
4.2 Analysis of response surface 51
4.3 Phytochemical profile of *C. sativa* shell extracts 55
4.4 Reactive oxygen species scavenging capacity assays 60
4.5 Effects of *C. sativa* shell extract towards fibroblasts and keratinocytes 62
4.6 Characterization of *C. sativa* shell hydrogel 64
4.6.1 Analysis of organoleptic properties 64
4.6.2 Colour evaluation 66
4.6.3 Determination of pH 67
4.6.4 Texture analysis 68
4.6.5 Rheological behaviour analysis 69
5 Future perspectives 74
6 Conclusion 77
7 References 80
8 Annexes 88

Index of Figures

Figure 1. World production of chestnut, in tonnes, for a 20 year-period (from 1997 to 2017) (3). 2
Figure 2. Ranking of the ten countries with the largest volume of chestnut output in 2017 (3). 3
Figure 3. Composition of chestnut (*Castanea sativa* Mill.). 4
Figure 4. Chestnut by-products. 6
Figure 5. Concept of circular economy. 7
Figure 6. Representation of the three pillars of the sustainability. 8
Figure 7. Schematic representation of the skin damage and its consequences induced by UV-mediated oxidative *stress*. 15
Figure 8. Schematic representation about the six principles of green extraction. 22
Figure 9. The four steps of a complete extraction process. 23
Figure 10. Schematic representation of an SWE equipment. 27
Figure 11. *C. sativa* shells: a) dried chestnut shells; b) powdered dried chestnut shells. . 34
Figure 12. Equipment used for SWE. 35
Figure 13. *C. sativa* shell extract: a) liquid extract obtained by SWE after filtration and centrifugation; b) freeze-dried extract. 35
Figure 14. Preparation of hydrogels containing *C. sativa* shell extract: a) heating and dispersion of Carbopol® in the aqueous mixture containing the extract; b) gels prepared and packed in glass jars. 42
Figure 15. Colorimeter used to analyse the colour of the hydrogels. 43
Figure 16. pHmeter used to analyse the pH of the hydrogels. 43
Figure 17. Texturometer used to analyse the texture of the hydrogels. 44
Figure 18. Rheometer used to analyse the rheological behaviour of the hydrogels. 45
Figure 19. Response surface 3D plots for the interaction effects of independent variables of time (min) and temperature (°C) on dependent variables of ABTS (A), FRAP (B), DPPH (C) and TPC (D) and on the desirability index for combined responses (E) of *C. sativa* shell extract obtained through SWE. In the response surfaces were represented the optimal points. 52
Figure 20. LC-DAD/ESI-MS chromatogram of *C. sativa* shell extracts obtained at 280 nm: a) extract prepared by SWE at 80 ºC/10 min; b) extract prepared by SWE at 220 ºC/30 min. 57
Figure 21. Graphic that represents the variation of shear viscosity (Pa.s) of the hydrogel containing *C. sativa* shell extract with the shear rate, at different times and temperatures (mean ± standard deviation, *n* =3). 69
Figure 22. Graphics that represent the variation of shear viscosity (Pa.s) of hydrogel containing *C. sativa* shell extract, at different times and temperatures: a) at shear rate of 1 s^{-1}; b) at shear rate of 10 s^{-1}; c) at shear rate of 100 s^{-1} (mean ± standard deviation, *n* = 3). 71
Figure 23. Graphic that represents the variation of the shear viscosity (Pa.s) with time of deformation (s) of the hydrogel containing *C. sativa* shell extract, at a deformation velocity from 0.1 to 100 s^{-1}. 72

Index of Tables

Table 1. Examples of different antioxidant defense systems, including natural (enzymatic and nonenzymatic) and synthetic antioxidants. 14
Table 2. Some commercial skin care products rich in polyphenols obtained from natural extracts available on the market. 16
Table 3. Advantages and disadvantages of subcritical water extraction (SWE) (77). 27
Table 4. Composition of the hydrogel. 42
Table 5. Experimental and predicted values of ABTS (mg AAE/g dw), FRAP (mg FSE/g dw), DPPH (mg TE/g dw) and TPC (mg GAE/g dw) of *C. sativa* shell extracts obtained by CCD. 49
Table 6. Model summary and analysis of variance (ANOVA) of ABTS (mg AAE/G dw), FRAP (mg FSE/g dw), DPPH (mg TE/g dw) and TPC (mg GAE/g dw) of *C. sativa* shell extracts. 50
Table 7. TPC and antioxidant activity evaluated by ABTS, FRAP and DPPH assays of the optimized extract of *C. sativa* shells (220 °C/30 min). 53
Table 8. Identification and quantification of the phenolic compounds present in *C. sativa* shell extracts prepared at 80 °C/10 min and 220 °C/30 min by LC-DAD/ESI-MS analysis. 55
Table 9. Superoxide anion radical ($O_2^{\bullet -}$), hypochlorous acid (HOCl) and peroxyl radical ($ROO^{\bullet}$) scavenging capacities of freeze-dried *C. sativa* shell extract. Values are expressed as mean ± standard deviation (n = 4). 61
Table 10. Effects of *C. sativa* shell extract prepared at 220 °C/30 min by SWE on the cell viability of fibroblasts and keratinocytes at a range of concentrations between 0.1 and 1000 µg/mL (mean ± standard deviation, n = 6). * indicate significant differences (p<0.05). 63
Table 11. Evaluation of organoleptic properties of the hydrogel containing *C. sativa* shell extract as active ingredient after 1, 7 and 30 days of preparation. 65
Table 12. Colour variation (L*, a* and b*) of the hydrogel containing *C. sativa* shell extract evaluated at different times and temperatures (mean ± standard deviation, n = 3). 66
Table 13. pH of the hydrogel containing *C. sativa* shell extract evaluated at different times and temperatures (mean ± standard deviation, n = 3). 67
Table 14. Firmness and adhesiveness of the hydrogel containing *C. sativa* shell extract evaluated at different times and temperatures (mean ± standard deviation, n = 3). 68
Table 15. Yield *stress* of the hydrogel containing *C. sativa* shell extract determined at T1 (mean ± standard deviation, n = 3). 73

Index of Annexes

Annex 1. Graphic force (N) *versus* distance (mm) plotted from the results obtained in the texture analysis. 89
Annex 2. Graphic force (N) *versus* time (sec) plotted from the results obtained in the texture analysis. 89

Abbreviations

AAE	Ascorbic acid equivalents
AAPH	α,α'-azodiisobutyramidine dihydrochloride
AP	Activator protein
ATCC	American Type Culture Collection
AUC	Area under curve
BHT	Butylated hydroxytoluene
CE	Catechin equivalents
COX	Cyclooxygenase
DAF-2	4,5-diaminofluorescein
DHR	Dihydrorhodamine 123
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dw	Dry weight
EC	European Commission
EC_{50}	Half Maximal Effective Concentration
FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal Bovine Serum
FRAP	Ferric reducing antioxidant power
FSE	Ferrous sulphate equivalents
FTIR	Fourier-transform infrared
fw	Fresh weight
GAE	Gallic acid equivalents
GRAS	Generally recognized as safe
HaCaT	Keratinocytes cell line
HBSS	Hank's Balanced Salt Solution
HOCl	Hypochlorous acid
IC_{50}	Half Maximal Inhibitory Concentration
ICS	Inner chestnut shells
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IOCS	Inner and outer chestnut shells
LPO	Lipoperoxidation
MAE	Microwave-assisted extraction

MAPKs	Mitogen-activated protein kinases
MMP	Metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	β-nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium chloride
NF-κB	Nuclear factor-κB
NMF	Natural moisturizing factor
NO	Nitric oxide
NOC-5	3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene
$O_2^{\bullet-}$	Superoxide anion radical
ORAC	Oxygen Radical Absorbance Capacity
PDO	Protected Designation of Origin
PLE	Pressurized liquid extraction
PMS	Phenazine methosulphate
PUFA	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RSM	Response surface methodology
SFA	Saturated fatty acids
SFE	Supercritical fluid extraction
SWE	Subcritical water extraction
TNF-α	Tumor necrosis factor
TFC	Total flavonoids content
TPC	Total polyphenols content
UAE	Ultrasound-assisted extraction
USFA	Unsaturated fatty acids
UV	Ultraviolet

1 Introduction

1.1 *Castanea sativa* Mill.

Castanea sativa Mill., commonly known as European or sweet chestnut, is a species of the Fagaceae family abundant in south Europe. The fruit, chestnut, is a seasonal nut (autumn) in the Mediterranean countries, being highly appreciated all over the world (1). Nuts are mainly produced for human and animal feed and used in typical dishes and desserts. Indeed, chestnut tree has a key role in the economy and environment of mountainous areas, being an invaluable historical and cultural heritage in these regions (2).

According to Food and Agriculture Organization of the United Nations (FAO) statistics, the worldwide production of chestnut has been increasing since 2001, from 691,279 K in 2001 to 2,313,934 K in 2017 (3). The major chestnut producer is Asia (89.7%), followed by Europe (6.5%) and America (3.8%). Figure 1 represents the world production of chestnut for a 20-year period (3).

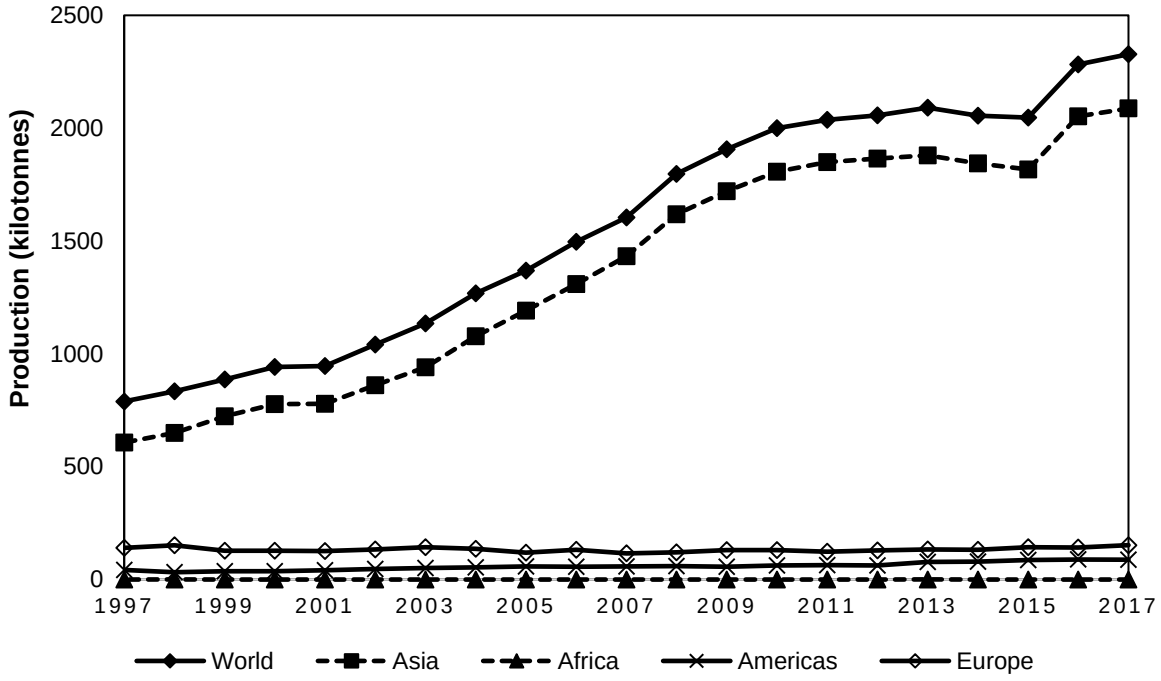

Figure 1. World production of chestnut, in tonnes, for a 20 year-period (from 1997 to 2017) (3).

China was the country with the highest chestnut output (1939 thousand tonnes) in 2017, accounting for more than 83% of global production, followed by Bolivia (3.7%), Republic of Korea (2.8%), Turkey (2.7%) and Italy (2.3%) (3). Greece, Portugal, Japan, Spain and France are also in the top 10 producers, with the Portuguese production

contributing with 29,875 tonnes and a total area of 36,759 ha. The ranking of the ten countries with the highest chestnut production in 2017 is represented in Figure 2 (3).

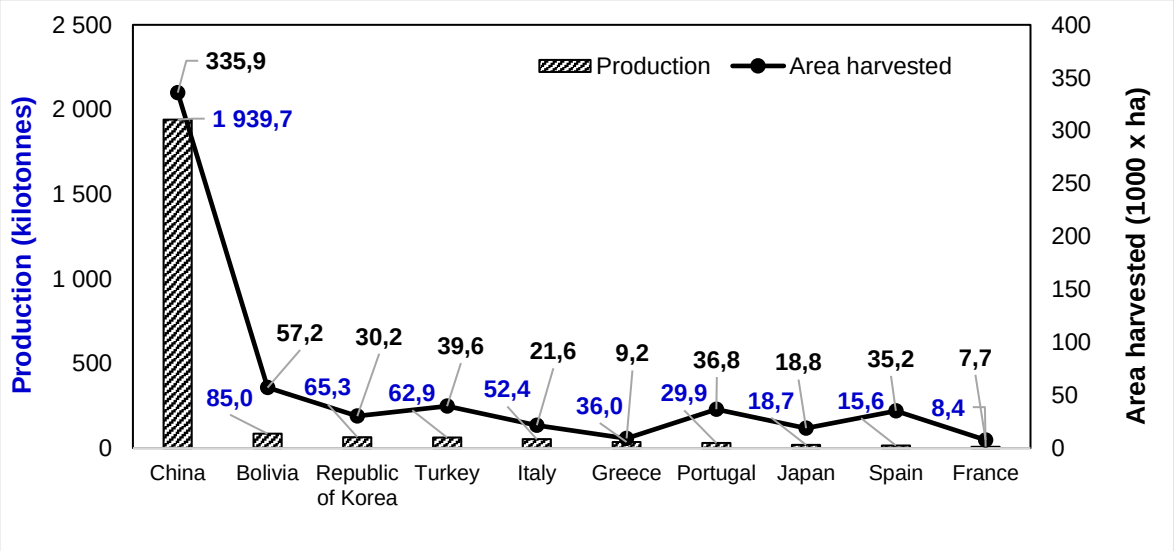

Figure 2. Ranking of the ten countries with the largest volume of chestnut output in 2017 (3).

Beyond *C. sativa*, there are other chestnut species with high commercial importance spread around the world. In Japan, *Castanea crenata* Sieb. and Zucc. is the predominant chestnut species, while *Castanea dentata* Borkh is widely disseminated in North America and *Castanea mollissima* Bl. is mostly present in China and Korea (2).

Regarding the national chestnut production, the north of Portugal (mostly Trás-os-Montes and Alto Douro) is the main producer region with 25,652 tonnes, representing more than 85% of the total production. A huge part of the nuts produced (20,768 tonnes) are exported, generating an economic input of about 53 million euros (4). In Portugal, there are four regions distinguished as Protected Designation of Origin (PDO) by their excellent chestnut quality: Marvão, Padrela, Soutos da Lapa and Terra Fria. This distinction supports the preservation of genetic heritage (2).

The chestnut is composed by the fruit, the pericarp (outer shell), the integument (inner shell), and the bur that surrounds the edible nuts. The composition of *C. sativa* is represented in Figure 3 (2).

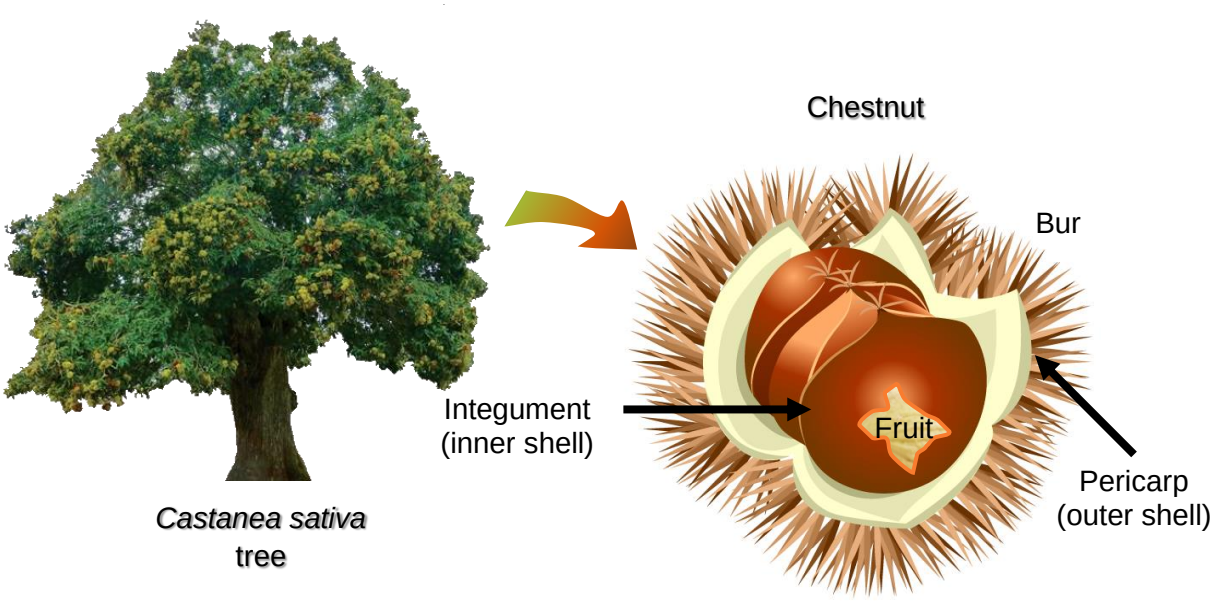

Figure 3. Composition of chestnut (*Castanea sativa* Mill.).

From a nutritional point of view, *C. sativa* fruits have high moisture (~50%) and starch (43 g/100 g of dry weight (dw)) contents, considerable amounts of fiber (3% dw) and low fat content (3% dw) (5). Chestnut fruits are a good dietary source of vitamin C (400 – 693 mg/kg dw) and amino acids (6-9 g/100 g dw). *L*-aspartic acid is the predominant amino acid, closely followed by *L*-glutamic acid, leucine, *L*-alanine and arginine (5, 6). Moreover, significant levels of minerals, particularly potassium (~750 mg/100 g dw), phosphorous (~120 mg/100 g dw) and magnesium (~75 mg/100 g dw), and vitamins, such as vitamins E, B1, B2, B3, pantothenic acid, pyridoxine and folate, were also found in chestnuts (5). Concerning to the fatty acids profile, low amounts of saturated fatty acids (SFA) (17%) and high levels of unsaturated fatty acids (USFA) (83%) were reported (7). Linoleic, oleic and palmitic acids were the main fatty acids, accounting for more than 85% of the total amount (7). Interestingly, high levels of polyunsaturated fatty acids (PUFA) have been associated to anticancer activity and low risk of cardiovascular diseases and neurological function disorders. In addition, chestnut fruits are excellent sources of polyphenols (9.6-19.4 mg of gallic acid equivalents (GAE)/g) and displayed remarkable antioxidant properties (ABTS: EC_{50} = 5.2 – 14.1 mg/g; DPPH: EC_{50} = 7.3 – 33.5 mg/g; ferric reducing antioxidant power (FRAP): EC_{50} = 6.6 – 14.6 mg/g; hemolysis inhibition: EC_{50} = 0.63 – 1.31 mg/g) (8). Gallic acid and ellagic acid were the most predominant polyphenols (8). Tannins are also abundant in European chestnuts, providing astringent bitter taste, reducing the palatability and contributing to the antimicrobial activity (9). Chestnut is considered a high added value nut due to its sensorial, nutritional and pro-healthy properties, as well as interesting phytochemical profile. A positive correlation between polyphenols content and beneficial health effects, such as anticancer, anti-inflammatory and antioxidant activities and low risk

of cardiovascular diseases, has been reported in chestnuts (10). Moreover, these fruits did not present gluten and cholesterol in the composition (7, 10).

C. sativa fruits are mostly consumed raw or after cooking, being roasting, boiling or frying the most common cooking procedures. Some authors have reported considerable changes on the sensorial and nutritional properties of chestnuts submitted to heat treatments (1). A significant reduction of the total amount of organic acids was determined after roasting, boiling and frying procedures (11). Boiled chestnuts exhibited higher amounts of gallic and ellagic acids, while roasted chestnuts showed higher protein and dietary fiber contents and lower fat levels (12). A significant decrease in vitamin C levels was found in boiled (25-54%) and roasted chestnuts (2-77%) (6). Therefore, cooked chestnuts have been associated to positive health impacts based on the significant organic acids and polyphenols contents and low amount of fat (12). During cooking, gallic acid is probably transferred from the peels to the fruits, contributing to the antioxidant activity (6). Wani *et al.* suggested that pan and microwave roasting procedures can be industrialized for the production of chestnuts with improved nutritional and aromatic properties (9).

New applications for chestnuts are being explored. Although chestnuts are highly perishable and seasonal fruits, they are also available on the market as frozen and boiled frozen fruits. Another chestnut-derived products that can be easily found on the market are marrons glacé sweet and chestnut flour (10). Chestnut flour has been used to develop gluten-free products (e.g. breads) suitable for celiac disease patients, non-celiac gluten sensitivity people and consumers who avoid gluten for lifestyle reasons, due to its gluten-free properties, ameliorating the response of the body's immune system to gluten proteins (6). Chestnut flour could be prepared by milling of dried fruits after removal of pericarp and endocarp, using small chestnuts or chestnuts with double embryos. Therefore, this product might be used as a confectionary paste in desserts and jams (10). Thus, chestnut flour has a great potential for commercialization being a safe and sustainable ingredient, easy to use and having a high nutritional value (10). Chestnut fruits might also be used as prebiotic due to the presence of non-digestible components (6).

The largely spread commercialization of chestnut has demanded for novel and suitable preservation techniques. Irradiation has been recommended as a promising option to chemical fumigation (such as methyl bromide that was legally banished in 2010 by the European Union because of its high toxicity and harmful effects on health and environment). Indeed, Carocho *et al.* reported high antioxidant activity and preservation of the total polyphenols content for irradiated chestnuts (13).

During the chestnut fruit processing, different by-products are generated, such as leaves, shells and burs. Nevertheless, these crop wastes are valuable sources of bioactive compounds (14). In addition, the flowers could also be considered a by-product in some cases. Figure 4 summarizes the by-products generated during chestnut production.

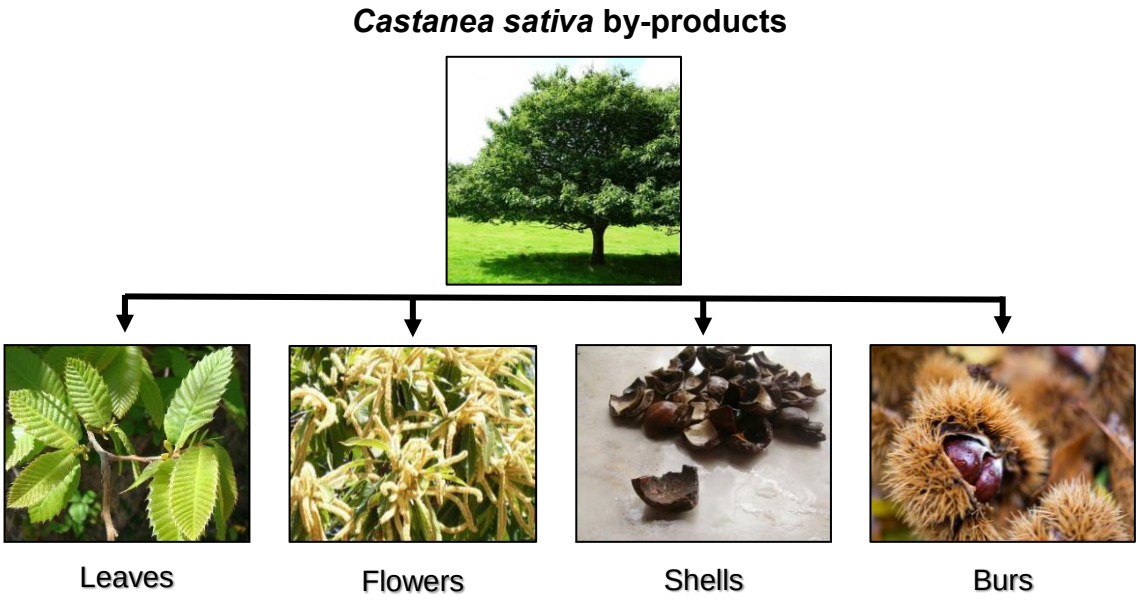

Figure 4. Chestnut by-products.

Noteworthy, these bioactive compounds are associated with health-promoting properties, namely anticarcinogenic, antioxidant and cardioprotective activities (2, 15, 16). In a sustainable point of view, *C. sativa* by-products might afford a new way to recycle for chestnut processing industries, developing cost-efficient processing methods, decreasing the negative impacts of these wastes on the environment, and, simultaneously, providing other economic advantages. The three main goals established by the European Commission (EC): “more value – less impact – better alternatives” strongly support the reutilization impact of these wastes as valuable products, contributing with benefits for the environment, pollution costs, and economy at a world level, and following the principles of sustainability and circular economy (17). Over the last decade, these two concepts, sustainability and circular economy have gained more attention, particularly from food industries, governments and consumers. Taking into consideration the new policies developed by the EC, the main goal for sustainable development is to achieve “*a continuous long-term improvement of quality of life through the creation of sustainable communities able to manage and use resources efficiently, (…) to tap the ecological and social innovation potential of the economy and (…) to ensure prosperity, environmental protection and social cohesion*” (18). In this way, the chestnut industry can be part of the sustainable and circular

economy. In Figure 5 are cited the different steps for a manufacturing process that follows the principles of circular economy.

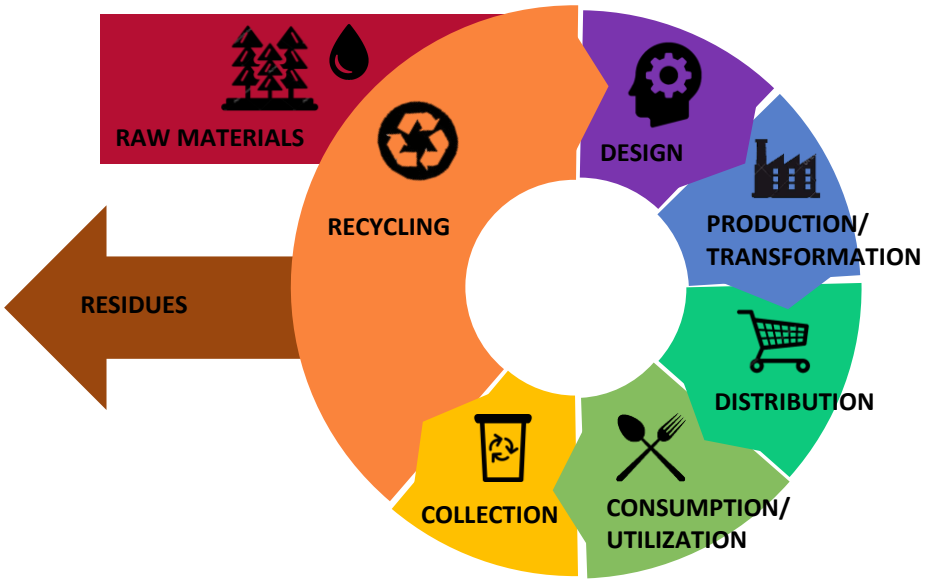

Figure 5. Concept of circular economy.

According to the Sustainable Development Goals of 2030 Agenda, the valorization of chestnut and its by-products perfectly fit in almost all of these new goals (19). Furthermore, since chestnut is a product largely distributed all over the world, it is expected a huge impact on different economies in the future.

1.2 *Castanea sativa* shell

The chestnut production might contribute to the conservation and management of natural resources (fruit, wood and by-products) with health and social benefits for the population and economic advantages for several industries that directly or indirectly are part of the process. In this way, the valorization of these agro-industrial residues is one of the main aims of current researches in order to be used by other industries, such as pharmaceutical, food and cosmetic, improving social, economic and environmental sustainability, reducing pollution costs and, simultaneously, generating more profits. Figure 6 summarizes the three pillars of the sustainability concept.

Figure 6. Representation of the three pillars of the sustainability.

Chestnut processing industry generates huge amounts of by-products as inner shell (6.3–10.1%) and outer shell (8.9–13.5%) (15). Some studies have been performed with this agro-residue in order to improve its value and find novel applications in different fields (15, 20-24). Several authors described the richness of *C. sativa* shells extracts in polyphenols, mostly phenolic acids and tannins (condensed and hydrolysable) (15, 22, 24). Tannins were used to prepare some medicines and play an important role in the flavouring of wine (e.g. tannins from the barrels are released into the wine during storage) (15). Nowadays, *C. sativa* shells are applied as fuel (15). However, this by-product is a lignocellulosic material containing cellulose, hemicelluloses and lignin as major constituents (25) which could be used as a promising substrate to obtain non digestible oligosaccharides already reported as potent prebiotics (26). In addition, the antioxidant activity and richness of *C. sativa* shells in polyphenols support their promising value as antioxidant (26). Therefore, chestnuts are an added value resource in producing countries with an increased economic value due to their nutritional composition and health-promoting properties.

The antioxidant activities of chestnut fruit and its by-products, particularly flowers, leaves and shells, were studied by Barreira *et al.* (16, 27) and Vázquez *et al.* (28). According to Barreira *et al.*, the highest extraction yield was obtained for inner shell (21.6 ± 2.38%), while the lowest result was found for outer shell (4.98 ± 0.19%) (16). The highest antioxidant efficiency was determined for inner shells (DPPH: EC_{50} = 32.7 ± 0.38 µg/mL; FRAP: EC_{50} = 68.7 ± 0.01 µg/mL; bleaching inhibition: EC_{50} = 164 ± 13.8 µg/mL; hemolysis inhibition: EC_{50} = 47.5 ± 0.43 µg/mL; lipoperoxidation (LPO) inhibition: EC_{50} = 11.5 ± 4.57 µg/mL) and outer shells (DPPH: EC_{50} = 39.7 ± 1.11 µg/mL; FRAP: EC_{50} = 55.1 ± 0.05 µg/mL; bleaching inhibition: EC_{50} = 133 ± 11.0 µg/mL; hemolysis inhibition: EC_{50} = 91.4 ± 1.52 µg/mL; LPO

inhibition: EC_{50} = 7.87 ± 0.15 µg/mL), as well as the highest total polyphenols contents (TPC) (inner shells: 475 ± 27.04 mg GAE/g dw; outer shells: 510 ± 8.70 mg GAE/g dw) and total flavonoids contents (TFC) (inner shells: 330 ± 6.13 mg of catechin equivalents (CE)/g dw; outer shells: 503 ± 11.21 mg CE/g dw). The polyphenols and flavonoids contents were found in the following order: outer shells > inner shells > flowers > leaves > fruits. This work suggested that shells are the richest *C. sativa* by-product in bioactive compounds and the most efficient antioxidant, considering this disposable residue as a promising raw material for industrial purposes (16). In another study, Barreira *et al.* compared the antioxidant capacity of chestnut (leaves and shells) and almond (husk) by-products from different cultivars (27). Although shells had the lowest extraction yield (6.43 – 12.59%), they also showed the highest TPC (533.81 – 805.74 mg GAE/g dw) and considerable TFC (49.92 – 146.08 mg CE/g dw). The best antioxidant properties were displayed for shells with the lowest EC_{50} values for LPO inhibition (27.29 – 49.07 µg/mL), DPPH (82.41 – 159.99 µg/mL) and FRAP (79.25 – 117.58 µg/mL) assays. The TPC for shells were in the same range to the ones obtained in the previous study cited (16), whereas TFC was significantly lower (27). Likewise, Vázquez *et al.* compared the antioxidant potential of chestnut shells and eucalyptus bark, reporting higher extraction yield, TPC and antioxidant activity in *C. sativa* shells (28). According to the authors, shells presented 33.82% of sugars including cellulose (19.20%), lignins (acid-insoluble lignin: 29.15%; acid-soluble lignin: 2.83%) and five monosaccharides (32.70%), namely glucose (19.23%), galactose (2.98%), xylose (6.45%), arabinose (2.52%) and mannose (1.52%). The best results were obtained for the extracts prepared with 2.5% Na_2SO_3 aqueous solution (extraction yield: 25.62%; TPC: 13.4 g GAE/100 g dw; FRAP: 80.7 mmol ascorbic acid equivalents (AAE)/100 g dw). Moreover, the high TPC was also confirmed by the prevalence of high molecular weight compounds through Fourier-transform infrared (FTIR) spectroscopy (28). Overall, *C. sativa* shells might be used as a new source of natural antioxidants and nutraceuticals, providing a novel strategy to stimulate the application of this crop waste as new supplier of useful bioactive compounds.

A study performed by de Vasconcelos *et al.* evaluated the potential of chestnut inner and outer shells from different cultivars as sources of tocopherols, pigments and polyphenols (15). The inner shell displayed higher TPC (76.04 – 126.75 mg GAE/g of fresh weight (fw)) and gallic acid contents (0.22 – 0.35 mg/g fw) in the extracts prepared with 70% aqueous methanol, while the pericarp showed higher ellagic acid levels (0.14 – 0.19 mg/g fw). Considerable amounts of low molecular weight phenolics (gallic and ellagic acid), condensed tannins and ellagitannins (namely castalagin, vescalagin, acutissimin A and acutissimin B) were also identified in 70% methanol extracts. The 70% acetone extract

obtained from *C. sativa* inner shell presented the highest TPC levels (91.02 – 136.35 mg GAE/g fw), total procyanidins (64.14 – 110.35 mg procyanidin polymer equivalents/g fw) and condensed tannins. On the other hand, the outer shell exhibited the highest contents of low molecular weight phenolics (gallic acid: 1.07 ± 0.20 mg/g of fw; ellagic acid: 0.30 ± 0.01 mg/g fw). In addition, neither tocopherols, nor carotenoids and chlorophylls were detected (15).

Another study analysed the influence of solvent (water, Na_2SO_3 and NaOH at different concentrations, namely 1, 2, 4 and 8%) and extraction time (30, 60, 120, 240, 480 and 960 min) at fixed temperature (85 °C) on the extraction properties and phytochemicals profile of *C. sativa* shell extracts through a factorial design (29). These solvents are usually applied at industrial scale for the extraction of tannins from agro-residues. The solvent 1% NaOH was more effective to extract hydrolysable tannins, while the condensed tannins were efficiently extracted with 1% Na_2SO_3. An efficient, simple and low cost procedure using moderate concentrations of alkali solvents for long periods was determined as the best one for tannins extraction from *C. sativa* shells. Gallic acid (7.9 – 584.9 µg/g dw), vescalagin (67.5 – 109.4 µg/g dw), castalagin (49.6 – 100.4 µg/g dw), ellagic acid (47.6 – 3542.6 µg/g dw), epigallocatechin (13.6 – 213.4 µg/g dw), catechin (151.1 – 259.9 µg/g dw) and epicatechin (9.0 – 66.8 µg/g dw) were the main polyphenols. Organic acidic compounds contribute to the astringency and capacity of these extracts to be mixed with other substances in tanning industry. This study emphasized the high potentiality of chestnut shells to produce pomaces with high tannins levels and their capability to be reused in high-value applications such as phenols substitutes in the formulation of wood adhesives, pigments in tanning industry, and natural antioxidants or supplements in food, pharmaceutical and cosmetic industries (29).

Furthermore, Comandini *et al.* reported approximately 60% of active tanning substances in chestnut shells, including castalagin, castalin, vescalagin and vescalin that are glucosidic tannins easily hydrolysable (30). The main polyphenols present in shells were castalagin, vescalagin and 1-*O*-galloyl castalagin. Gallic acid, ellagic acid and 1-*O*-galloyl castalagin were also identified (30).

Gullón *et al.* used a hydrothermal technology to solubilize hemicellulosic oligosaccharides and antioxidant compounds from *C. sativa* shells (26). The extracts presented carbohydrates in oligomeric and monomeric forms, sugar degradation products and polyphenols. The extracts obtained at 180 °C had the highest amount of oligosaccharides (18.3 g/L), TPC (4.7 g GAE/L) and TFC (4.8 g of rutin equivalents/L), as well as low monosaccharides (2.4 g/L) and degradation products levels (0.5 g/L of acetic

acid). Lignin was the major component (44.59%) of shells, followed by hemicelluloses (21.4%). Glucan, xylan and arabinan were the main sugars. A considerable amount of uronic acids (5.99%) was also determined. Moreover, these extracts exhibited a remarkable antioxidant activity probably due to the phenolic profile, particularly lignin. The main sugar was 2-furoic acid (7.12%), whereas pyrogallol was the most abundant polyphenol (13.20%) followed by 3,4-dihydroxybenzaldehyde (5.04%). Pyrogallol probably resulted from the decomposition of gallic acid at temperatures between 105 °C and 150 °C. Otherwise, 3,4-dihydroxybenzaldehyde could be originated from the degradation of protocatechuic acid. This study also evaluated the potentiality of water-soluble compounds as nutraceuticals based on the structural characterization of oligosaccharides, since their molecular weight influences biological properties. However, a broad molecular weight distribution of oligosaccharides was observed, emphasizing their heterogeneous molecular nature. The thermal stability of oligosaccharides strongly supports their compatibility with industrial processes. Additionally, the solid fraction obtained by autohydrolysis was rich in cellulose and lignin that might be used for biorefinery. Thus, Gullón *et al.* highlighted the potentiality of shells to be reused to recover oligosaccharides and antioxidants that could be applied as functional ingredients (26).

Chestnut shells from different producing regions of Portugal (Minho, Trás-os-Montes and Beira-Alta) were analysed in a study conducted by Rodrigues *et al.* (24). According to the authors, shells were characterized by high carbohydrate (56.51 – 74.06 g/100 g dw) and moisture contents (21.29 – 38.61 g/100 g dw) and low fat levels (0.15 – 0.52 g/100 g dw). Low protein (2.77 – 3.13 g/100 g dw), ash (1.08 – 1.60 g/100 g dw) and fat levels were expectable taking into consideration the higher amounts of other components, namely dietary fibers mainly composed by cellulose, hemicellulose and lignin. Concerning the amino acid profile, arginine (355 – 721 mg/100 g dw) and leucine (159 – 208 mg/100 g dw) were the main essential amino acids. Among the non-essential amino acids, glutamic and aspartic acids were the predominant. Noteworthy, the nutraceutical industry is constantly looking for novel supplements rich in amino acids due to their ability to promote protein synthesis and improve immunity and cerebral functions (31). Thus, *C. sativa* shells might be a promising natural and economical source of amino acids with promising applications in nutraceutical field. The richness of shells in vitamin E (481.5 – 962.8 mg/100 g) was also described, being γ-tocopherol the predominant vitamer accounting for more than half of the total content. Therefore, shells also revealed to be an excellent source of vitamin E, the major lipid-soluble antioxidant in cell antioxidant defense system only obtained from diet (32). This vitamin is involved in the promotion of cardiovascular health, improvement of immune system functions, skin repairing and protection of cell membranes from

radicals-mediated damage (32). Moreover, the TPC ranged between 143.0 and 796.8 mg GAE/g dw, while TFC varied from 31.4 to 43.3 mg CE/g dw (24). The highest antioxidant activity was also reported for Trás-os-Montes shells (DPPH: EC_{50} = 31.8 ± 1.1 µg/mL; FRAP: 8083.5 ± 164.8 µmol ferrous sulphate equivalents (FSE)/g dw). The strong antioxidant ability of this by-product is probably related to its high polyphenols and vitamin E levels, reinforcing the importance of these compounds for the antioxidant capacity. No antimicrobial activity was reported. Overall, the use of shells as source of functional ingredients and their potential application in different industries, particularly pharmaceutical, food and cosmetic, comprises a promising field of research (24).

Several authors have already reported some innovative applications of chestnut shells as heavy metal adsorbent, phenol substitutes in adhesive formulations, chrome substitutes in leather tanning and as source of antioxidant compounds (22, 33, 34). For example, Vázquez *et al.* investigated the potential of these shells as an adsorbent for the removal of cadmium (Cd^{2+}) ions from aqueous solutions (33). Generally, the wastes produced by industries containing heavy metals are extremely toxic and highly contribute to water pollution. Therefore, it is important to remove the metals from wastewaters before their release for the environment, fulfilling the regulation applied to residues discharge. Additionally, adsorption process has been outstanding in this field due to its high efficiency, easy handling, cost effectiveness and availability of adsorbents (33). In another study, Vázquez *et al.* performed an alkali pre-treatment of shells to improve its capacity for metal cation removal, such as cadmium, copper, lead and zinc (34). The authors obtained the highest adsorption capacity and percentage of Cd^{2+} removal for the shells pretreated with 4% NaOH aqueous solution at 25 °C for 4h. These results were in line with the ones obtained for Cu^{2+}, Pb^{2+} and Zn^{2+} adsorption. Furthermore, the affinity proceeded in the following order: $Cd^{2+} > Cu^{2+} \approx Zn^{2+} > Pb^{2+}$, since the maximum uptake ability was reported for Cd^{2+} (9.9 mg/g) (34).

The use of chestnut shells could be of high economic interest due to their richness in bioactive compounds with notable biological properties (e.g. antioxidant activity). Based on the bioactivity, this crop waste might be used as a natural source of bioactive compounds for industrial purposes, improving the triad of economic, environmental and social sustainability, lowering the pollution and generating more profits. Taking into account the sustainability concerns, the reuse of underexploited chestnut wastes, particularly shells, might comprise a novel challenge for several industries, particularly pharmaceutical, food and cosmetic, in order to design new added value products and to develop promising applications, simultaneously increasing the economic resources.

1.3 Bioactive compounds from *C. sativa* shells as potential ingredients for cosmetics

As previous detailed, during the chestnut production a huge amount of by-products are generated probably leading to a major environmental issue due to disposal problems. The richness in bioactive compounds responsible for several biological activities, mainly antioxidant activity, as well as the interesting profile of vitamin E and amino acids, support the interest in the valorization of *C. sativa* shells for cosmetic purposes (15, 24, 35). In this sense, recent studies have focused on alternatives for the valorization of this agro-residue, particularly on a sustainable recovery of valuable natural compounds, being cosmetic application a promising way to recycle this by-product (35, 36).

On the other hand, skin care companies exerted huge environmental impacts due to the use of unsustainable sourcing of raw materials and pollution in the manufacturing phase and disposal of packaging and products. According to Cosmetics Europe – The Personal Care Association, 450 million of Europeans daily use a large variety of cosmetic products (e.g. soap, shampoo, hair conditioner, toothpaste, deodorant, shaving cream, skin care, perfume and make-up) (37). For these reasons, cosmetic companies have attempted to create greener formulations using sustainable raw materials.

The human skin is responsible for many functions such as regulation of body heat and water loss, and prevention of the entry of toxic substances and microorganisms. Since a long time ago, natural compounds, phytonutrients, microbial metabolites, minerals and animal protein components have been considered beneficial for a healthy skin aging. In cosmetics, the efficacy of active compounds depends on their diffusion capacity through the skin and their concentration in the formulations. Indeed, small molecules with lipophilic and hydrophilic properties showed a high ability to pass through the *stratum corneum* (38).

Nowadays, the main concerns of consumers are their health and appearance. Therefore, in the cosmetic field, the demand for anti-aging products has increased in the last decades. Aging is a natural, complex and multifactorial process that can be classified as intrinsic and extrinsic (39, 40). The intrinsic aging, also known as biological aging, could be affected by a wide range of internal factors, such as genetic predisposition, hormonal disorders and vitamin deficiencies (39, 40). Otherwise, extrinsic aging depends on external factors such as ultraviolet (UV) radiation, environmental pollution and improper skin care (39, 40). In fact, photoaging provides the loss of structural integrity and physiological function of the skin. The clinical signs of skin aging are thinning skin with exaggerated expression lines, wrinkles, age spots and actinic keratosis (39, 40). Among all factors, the

major cause of skin aging is the chronic exposure to UV rays that can originate several harmful effects on skin, such as wrinkling, dryness, pigment abnormalities and skin cancer (39-41). The UV rays include short wavelengths UVC (200 – 280 nm), medium wavelengths UVB (280 – 320 nm) and long wavelengths UVA (320 – 400 nm). Moreover, UVB rays are the most harmful UV radiation since it can penetrate deeply into the epidermis and dermis (41). The skin exposure to UV radiation initiates an imbalance between the generation of pro-oxidant reactive species (reactive oxygen species (ROS) and reactive nitrogen species (RNS)) and the antioxidant defense capacity of cells (including enzymes, namely catalase and superoxide dismutase, and nonenzymatic antioxidants, such as ascorbic acid, glutathione, and α-tocopherol), resulting in oxidative *stress* (38). Table 1 refers the different antioxidant defense systems used by human body against pro-oxidant reactive species.

Table 1. Examples of different antioxidant defense systems, including natural (enzymatic and nonenzymatic) and synthetic antioxidants.

Natural antioxidants		**Synthetic antioxidants**
Enzymatic	***Nonenzymatic***	
Catalase	Vitamins (Vitamin C and E)	Buthylated hydroxyanisole (BHA)
Superoxide dismutase	Glutathione	Butylated hydroxytoluene (BHT)
Glutathione peroxidase	Polyphenols	Ethylenediamine tetraacetic acid (EDTA)
	Carotenoids	
	Minerals (zinc and selenium)	

The UVB and UVA rays might interact with endogenous chromophores and photosensitizers, providing the generation of ROS and RNS. The pro-oxidant reactive species have deleterious effects, such as lipid oxidation, protein oxidation, deoxyribonucleic acid (DNA) damage and modulation of gene expression (41). Figure 7 represents the skin damage process induced by UV-mediated oxidative *stress*.

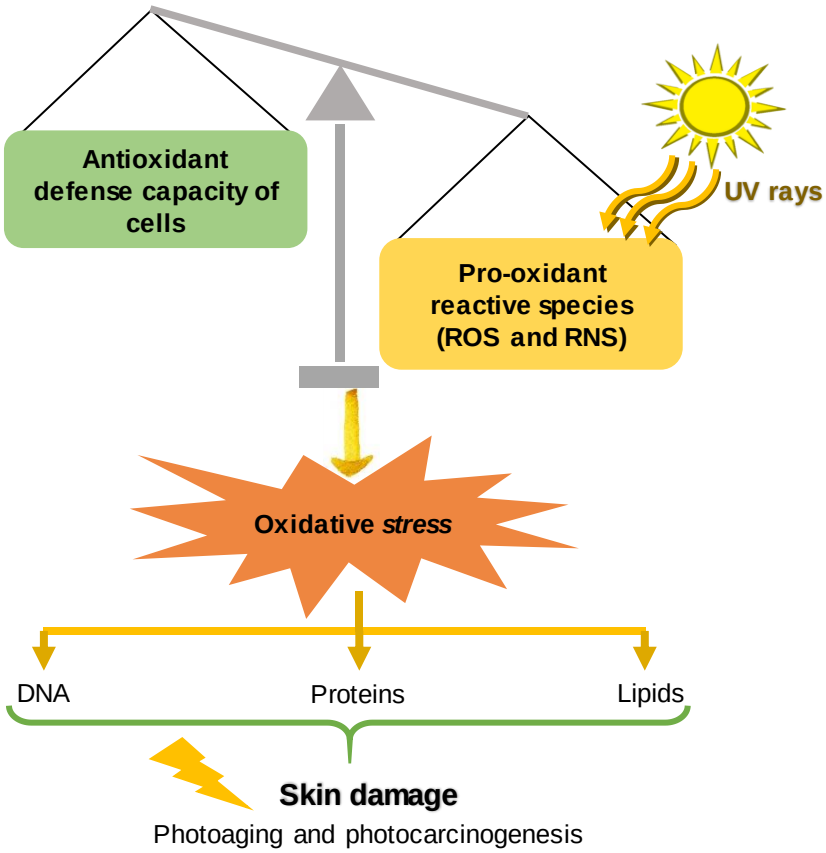

Figure 7. Schematic representation of the skin damage and its consequences induced by UV-mediated oxidative *stress*.

The overproduction of ROS induces the activation of several signal transduction pathways related to growth, differentiation, senescence and connective tissue degradation. The three major skin aging cascades activated by UVB rays are: (1) matrix metalloproteinase (MMP)-1 mediated aging; (2) mitogen-activated protein kinases (MAPKs) signaling and the transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) – AP-1/NF-κB – tumor necrosis factor (TNF-α)/Interleukin (IL)-6, iNOS, and cyclooxygenase (COX)-2-mediated inflammation-induced aging; and (3) p53–Bax–cleaved caspase-3–cytochrome C-mediated apoptosis-induced aging (40, 42, 43). Nevertheless, an upregulation transcription of inflammatory mediators, namely NO, iNOS, COX-2 and pro-inflammatory cytokines (TNF-α and IL-6) was also detected (40, 41). These inflammatory mediators lead to collagen degradation through promoting apoptosis in dermal fibroblasts, increasing the expression of matrix metalloproteinases (MMP-1, MMP-3 and MMP-9) and preventing the expression of procollagen (40, 41). In aging, the dermis suffers modifications due to the elastosis, with deposition of abnormal elastic fibers, collagen degradation and dilated microvasculature (39). Overall, the damages on DNA molecules and other lipid components can be caused by UVA rays, while UVB radiation can induce

oxidative *stress* and, consequently, pro-mutagenic DNA injuries and mutations in epidermal cells (41).

Antioxidants are innovative ingredients for skin care formulations that may attenuate the effects of skin aging through the mitigation of the biochemical consequences of oxidation. Therefore, plant secondary metabolites can act as UV blockers since some compounds, such as phenolic acids and flavonoids, are able to prevent the penetration of UV rays into the skin (44, 45). The interest in these compounds could be explained by their bioactivity and skin health effects, such as antioxidant and antimicrobial activities, protective effect from UV mediated-damage and inhibition of dermal proteinases (44, 45). Furthermore, a cosmetic active ingredient should regulate the skin protection against mechanical and chemical injuries and restore cell renewal in aged epidermis (40). Several plant-derived extracts have been employed in skin care formulations already marketed on a large scale. Table 2 presents different commercial cosmetic products with active ingredients prepared from polyphenols-enriched extracts.

Table 2. Some commercial skin care products rich in polyphenols obtained from natural extracts available on the market.

Commercial skin care products	Natural extracts/Active ingredients extracted from natural sources	Website
Beauty by earth	*Aloe vera* leaf juice, *Vaccinium angustifolium* (blueberry) extract, *Punica granatum* (pomegranate) extract, *Calendula officinalis* (calendula) flower extract, *Camellia oleifera* (japanese green tea) leaf extract, *Mentha piperita* (peppermint) essential oil, *Vanilla tahitensis* (vanilla) fruit extract, *Olea europaea* (olive) leaf extract, *Argania spinosa* kernel (argan) oil, *Laminaria digitata* (sea kelp) extract, *Theobroma cacao* (cocoa) seed butter, *Coffea arabica* (coffee) seed extract	https://www.beautybyearth.com/
Caudalie	*Vitis vinifera* (grape) fruit extract, juice and seed oil, *Vitis vinifera* grapevine shoot extract, *Coriandrum sativum* (coriander) seed oil, *Simmondsia chinensis* (jojoba) seed oil, *Opuntia ficus-indica* (prickly pear) seed oil, *Rosmarinus officinalis* (rosemary) leaf oil, *Melissa officinalis* (balm mint) leaf oil, *Rosa damascena* (Damask rose) flower oil	https://pt.caudalie.com/

Clarins	*Dipsacus sylvestris* flower extract, *Coffea robusta* (coffee) seed extract, *Avena sativa* (oat) kernel extract, *Olea europaea* (olive) fruit extract, *Ginkgo biloba* leaf extract, *Silybum marianum* (milk thistle) seed oil, *Actinidia chinensis* (kiwi) fruit extract, *Lycium barbarum* (goji berry) fruit extract	https://www.clarins.pt/
Estée Lauder	*Punica granatum* (pomegranate) extract, *Cucumis sativus* (cucumber) fruit extract, *Panax ginseng* (ginseng) root extract, *Cola Acuminata* (kola) seed extract, *Anthemis nobilis* (chamomile), *Adansonia digitata* seed extract, *Avena sativa* (oat) kernel extract, *Citrullus vulgaris* (watermelon) fruit extract , *Laminaria digitata* extract, *Lens esculenta* (lentil) fruit extract, *Pyrus malus* (apple) fruit extract, *Lavandula angustifolia* (lavender) oil	https://www.esteelauder.com/
Galénic	Dandelion extract (*Taraxacum officinale*), seaweeds, argan oil, castor oil, *Uncaria tomentosa* plant extract, yellow melilot plant extract (*Melilotus officinalis*), Brazilian peppertree extract (*Schinus terebinthifolius*)	https://www.galenic.pt/
Skinceuticals	Phytic Acid (from leguminous and seeds), phloretin (from apple tree bark), resveratrol (from *Fallopia japonica* plant), baicalin (from *Scutellaria baicalensis* roots), *Artemia salina* (plankton extract), *Vaccinium angustifolium* (blueberry) extract, silymarin (from *Silybum marianum*), allantoin (from a plant extract), *Aloe Vera* extract, chamomile extract (*Matricaria chamomilla*), Liquorice root extract (*Glycyrrhiza glabra*), thyme extract (*Thymus vulgaris*), cucumber extract (*Cucumis sativus*)	https://www.skinceuticals.pt/
Sukin	*Rosa canina* (Rose Hip) fruit oil, *Aloe vera* leaf juice, *Theobroma cacao* (cocoa) seed butter, *Persea gratissima* (avocado) oil, *Spirulina platensis* extract, *Chlorella vulgaris* extract, *Petroselinum crispum* (parsley) extract, *Brassica Oleracea* (kale) leaf extract, *Camellia sinensis* leaf extract, *Eucalyptus citriodora* oil, *Punica granatum* (pomegranate) fruit extract, *Adansonia digitata* (baobab) fruit extract, *Hibiscus sabdariffa* flower extract, *Cucumis sativus* (cucumber) fruit extract	https://sukinnaturals.com/

Chestnut shells are rich in polyphenols, vitamin E and amino acids which support its potential as an excellent source of novel active ingredients for cosmetic purposes due to their antioxidant and chemoprotective abilities as well as low toxicity and few side effects (24, 29, 35). Polyphenol-enriched extracts could be effective in cosmetic formulations for the prevention or treatment of premature skin aging induced by oxidative *stress*, playing an important role in skin photoprotection and aging and inflammation signals (44). Polyphenols have sunscreen effects as UV blockers, anti-inflammatory activity through the reduction of erythema and edema, antioxidant effect, ROS and RNS scavenging ability, and protective effect against UV-induced DNA damage (44, 45). Therefore, these compounds could be promising chemopreventive agents for skin disorders. Natural polyphenols were proposed as active components in sunscreen formulations to complement UV filter due to their similar structures with chemical UV filters and, consequently, similar reaction mechanisms (40). Gallic and ellagic acids are natural antioxidants with anti-inflammatory, antimicrobial and anti-cancer properties (44, 46). Gallic acid and its derivatives were tested as adjuvants in therapeutic formulations, such as a substitute of hydrocortisone in formulations for atopic dermatitis and other skin conditions, namely hyperpigmentation and wound healing, and as cosmetic active ingredient due to their skin anti-aging benefits (46). Otherwise, ellagic acid is a well-popular cosmetic ingredient applied in skin care products due to its skin-whitening effect, antioxidant activity, inhibitory effect of tyrosinase activity and blocking effect of melanin formation (47). Among flavonoids, catechin and its derivatives have been described as anti-oxidant, anti-inflammatory and anti-aging agent, also providing high UV-protection (45). In addition, tannic acids protect the skin from inflammatory responses induced by external irritation, while condensed tannins showed antioxidant, anti-cancer and anti-tyrosinase activities (35, 44). According to Aires *et al.*, chestnut shell extract could be a promising cosmetic ingredient due to its high polyphenols, tannins and ellagitannins contents, supporting its use in cosmetic field (29).

Furthermore, vitamins are employed in oral hygienic products and cosmetics for skin and hair treatment (e.g. creams, lotions, hair care formulations) (48). Vitamin E is present in the *stratum corneum* at low levels, being released by sebum (32). This vitamin is one of the most frequently used antioxidants in anti-aging products. α-tocopherol and α-tocopheryl acetate are the most used vitamin E-derivatives in cosmetics (49). The topical treatment with vitamin E might enhance the hydration of the *stratum corneum*, provide protection against UV-induced skin injuries, decrease the wrinkle amplitude and skin roughness, reduce erythema, edema, and UV-induced skin cancers (50). Indeed, vitamin E can be applied in sunscreen lotions as UV-filter due to its photoprotective effect against UV-

mediated skin damage (51). Moreover, vitamin E could be incorporated in dermatological formulations used in the treatment of skin inflammatory disorders (52). Previous studies reported that oxidative *stress* also plays an important role in the delay of diabetic wound healing and suggested that the topical application of vitamin E significantly improves the wound healing (53). The oral supplementation of vitamin E was described as a promising therapeutic adjunct for atopic dermatitis, while the combined application of vitamins C and E seems to ameliorate chloasma and pigmented contact dermatitis (50). Noteworthy, vitamin E application prior to UV exposure considerably attenuates acute skin responses, namely erythema and edema, sunburn cell formation, lipid peroxidation, DNA-adduct formation, immunosuppression and UVA-induced binding of photosensitizers (48, 50). Additionally, chronic skin reactions due to prolonged UVB/UVA-exposure, such as skin wrinkling and skin tumor incidence, were also reduced by topical vitamin E formulations (50).

Amino acids could be useful ingredients for the treatment of chronic diabetic foot ulcers and traumatic wounds or burns (54). Besides the antimicrobial activity, arginine also improves the collagen synthesis and the blood flow through NO synthesis (55). Glutamine is a good energy source and protects tissues from inflammatory damages (56), whereas hydroxy methylbutyrate (leucine metabolite) stimulates the protein synthesis and reduces the apoptosis (57). Furthermore, amino acids are hair and skin-conditioning agents and might help to improve the osmotic balance and the hydration of skin due to their ability to bind water with the horny layer of skin and its annexes (58). The *stratum corneum* is mainly composed of water, sebum, and a group of substances called Natural Moisturizing Factor (NMF). NMF is a moisture-retaining substance naturally derived from the epidermis that contains amino acids as main components (59). Non-canonical amino acids are not only applied in protein/enzyme engineering, but also in the development of novel biomaterials, drugs and cosmetics, showing a promising potential to be employed as skin moisturizers due to their great water retention even higher than hyaluronic acid, glycerin and urea (60).

Lignin could be used in formulations as dispersant, adhesive and surfactant and can also be transformed in polyphenols through synthesis (61). These phenol lignin-derivatives might be incorporated in sunscreens, hair coloring and skin lightening preparations (61). Based on the chemical structure, lignin also have antioxidant and UV-protection properties. Recently, Bajwa *et al.* reported that lignin-nanoparticles used in sunscreens have a similar absorbance region to UVB and UVC rays (61). In addition, lignin has a brown colour that provides a pleasant tone to the skin (62). Recently, Qian *et al.* introduced lignin in a commercial pure cream and a sunscreen lotion as a natural sun blocker and observed that lignin can boost significantly the sunscreen performance (63, 64). Moreover, lignin can be

used in organic hydrogels, providing a good gel strength and mechanical properties. The bio-based renewable hydrogels have been broadly used in food, cosmetics, pharmaceuticals, and biomedical implants (65, 66).

In a study performed by Li *et al.*, cellulose was also used to develop a bio-hybrid film which consisted of renewable materials that can potentially be exploited in sustainable applications such as biocomposites and packaging materials (67).

Only two studies focused on the valorization of chestnut shells for cosmetic purposes (35, 36). Squillaci *et al.* investigated the bioactivity of inner chestnut shells (ICS) and a blend of inner and outer chestnut shells (IOCS) through an eco-friendly extraction method in boiling water and tested their suitability as active ingredients for cosmetics (35). The IOCS extract showed the highest TPC (205.99 ± 13.10 mg GAE/g dw), TFC (40.98 ± 1.22 mg CE/g dw), ortho-diphenols (98.06 ± 2.61 mg of caffeic acid equivalents/g dw), condensed tannins (162.49 ± 5.94 mg GAE/g dw) and hydrolysable tannins contents (12.94 ± 0.96 mg GAE/g dw). The main class of polyphenols in both extracts was condensed tannins, representing 78.88% (IOCS) and 59.14% (ICS) of TPC. The polyphenols identified were gallic acid, protocatechuic acid, catechin, epicatechin, ellagic acid, *ρ*-coumaric acid and scopoletin. Gallic acid was the main phenolic compound in IOCS (63.51 ± 1.32 mg/g dw) and ICS extracts (29.62 ± 0.71 mg/g dw), followed by protocatechuic acid (IOCS: 11.24 ± 0.30 mg/g dw; ICS: 3.43 ± 0.12 mg/g dw). These extracts were endowed with a remarkable radical scavenging capacity (44% of inhibition), being even higher than the reference compounds (ascorbic acid: 29%; catechin: 37.2%; butylated hydroxytoluene (BHT): 7.2%). Indeed, BHT and ascorbic acid were selected as reference compounds since they are usually applied as antioxidants in cosmetic formulations. Therefore, the presence of several phytochemicals in *C. sativa* shells with synergic actions could explain their strong radical scavenging power. Through cell viability studies, the extracts at 0.01% were considered as safe for keratinocytes (HaCaT). In addition, these extracts reduced the generation of oxidized lipids in keratinocytes after H_2O_2 exposure, meaning that they are able to scavenge ROS produced in skin cells after oxidative *stress*. A protective effect against inflammatory processes was observed through the attenuation of nitric oxide (NO) production and reduction of inducible nitric oxide synthase (iNOS) expression levels. A higher anti-inflammatory activity was observed for IOCS extract that promoted a decrease of 58% on NO production and 43% on iNOS levels. Furthermore, both extracts presented a notable skin hydration capacity and ability to protect keratinocytes from collagen degradation. In conclusion, these authors suggested that *C. sativa* shell extracts could be interesting active ingredients for the development of skin care formulations (35). The antioxidant and deodorizing activities of polyphenols extracted from chestnut inner shells were evaluated

by Ham *et al.* (36). In this sense, different extractions were performed using aqueous alcohols and alkaline solutions (50% ethanol, 50% methanol, 1% NaOH and 2% NaOH) at different temperatures (25 – 90 °C). The TPC and antioxidant activity increased with the temperature increase. Therefore, aqueous ethanol (50%, w/v) was selected as the best solvent since it was the most effective in the recovery of polyphenols (294.02 – 558.12 mg GAE/g dw) and showed the highest DPPH radical scavenging activity (170.20 – 174.61 mM TE/g dw). On the other hand, alkaline solutions exhibited the highest condensed tannins (228.13 – 244.63 mg CE/g extract) and flavonoids contents (139.85 – 166.28 mg CE/g extract). It is noteworthy that the condensed tannins content was considerably higher than TFC for all the extracts. The deodorizing activity was assessed using two odor compounds as comparison, *trans*-2-nonenal and methyl mercaptan, which have unpleasant greasy odors. The volatile compounds of these odor components were reduced in the presence of ICS extracts. The deodorizing activity was also proportional to the percentage of extract used. The alkaline extracts revealed higher deodorizing activities than alcoholic ones. The 1% NaOH extract prepared at 90 °C was tested in a concentration range of 1 – 100 mg/mL and showed a deodorizing activity of 27.84 – 63.67% against *trans*-2-nonenal and 33.98 – 66.50% against methyl mercaptan. Furthermore, the deodorizing potential of ICS extract obtained with 1% NaOH at 90 °C was similar to epicatechin gallate isolated from green tea. A positive correlation was observed between deodorizing activity and tannins and flavonoids contents, meaning that these classes of compounds were the major contributors to deodorizing potential. Regarding methyl mercaptan, TFC displayed a higher correlation coefficient than condensed tannins content (R^2 = 0.93 vs. 0.81), indicating that flavonoids were more active deodorizing agents than *trans*-2-nonenal. Otherwise, condensed tannins seems to be more active in deodorizing against *trans*-2-nonenal due to the highest correlation coefficient (R^2 = 0.94). Thus, the authors suggested that this by-product might be used as an effective source of natural antioxidants and deodorant compounds based on its remarkable TPC, particularly tannins and flavonoids (36).

Overall, the current trend in cosmetic field focus on the development of novel skin care products using natural extracts as active ingredients. These extracts are rich in natural antioxidants that may replace the synthetic ones classified as toxic and with potential carcinogenic effects. The possibility to valorize this by-product through extraction of valuable compounds and their incorporation in cosmetics, enhancing the profitability of agro-wastes for industries. Thus, the application of *C. sativa* shells as novel active ingredients for skin care formulations comprises a huge challenge for cosmetic industry, representing a solution for this discarded by-product.

1.4 Green extraction techniques for recovery of bioactive compounds

Nowadays, the current society has demonstrated a high awareness and responsibility concerning to environmental issues. In addition, the interest for bioactive compounds extracted from natural sources has increased due to their potential application as active ingredients in several industries, particularly cosmetic, food and pharmaceutical. The solid samples are rarely analysed by direct methods with an appropriate level of sensitivity and accuracy (68). For this, the bioactive compounds need to be recovered through an extraction process that is composed by several unit operations. Noteworthy, the sample treatment is one of the most challenging steps and could include digestion, mineralization, extraction, filtration and distillation (68).

In 1998, Paul Anastas defined the concept of green chemistry and its 12 principles to make a greener chemical, process or product. The list of principles comprises: 1) prevention; 2) atom economy; 3) less hazardous chemical synthesis; 4) designing safer chemicals; 5) safer solvents; 6) design for energy efficiency; 7) use of renewable feedstocks; 8) reduce derivatives; 9) catalysis; 10) design for degradation; 11) real-time analysis for prevention of pollution; and 12) safer chemistry for accident prevention (69). Later, the scientific community formulated six principles of green extraction that are cited in Figure 8.

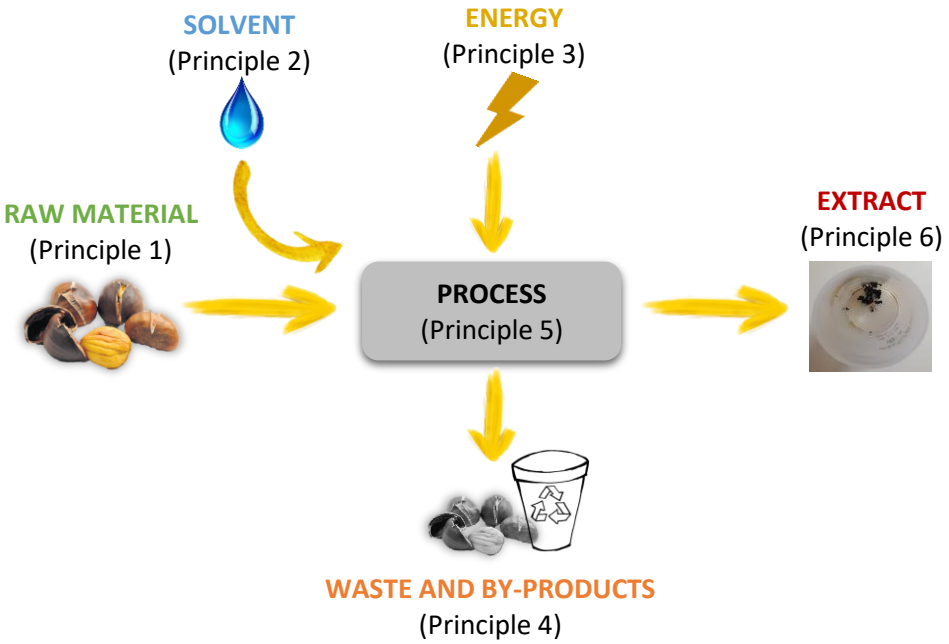

Figure 8. Schematic representation about the six principles of green extraction.

The first principle for green extraction focuses on raw materials, prioritizing the use of renewable plant resources (70). The selection of solvent is implied in the second principle (70). Besides the great extraction efficiency reported for organic solvents, they are hazardous solvents with several toxic effects on human health and environment, being easily released to the atmosphere and acting as air pollutants promoting the global warming (70). For this reason, these solvents should be avoided and replaced for greener alternatives such as water. Particularly, should be preferred the use of renewable solvents with high rate of recyclability or biodegradability (to limit the environmental impacts), low emissions of volatile organic compounds, suitable for industrial purposes, and that ensure the maximal solvent recovery (70). The principle 3 establishes a reduction of energy consumption since an energy-consuming process leads to environmental issues, increase of the production costs and loss of profitability (70). On the other hand, the principle 4 focuses on wastes and by-products, encouraging their reuse and valorization for the production of co-products towards bio-refinery concepts and stimulating the exploitation of by-products and the reduction of effluents and wastes in terms of quantity and harmfulness (70). Bio-refinery is a concept that settles in economic, environmental and social sustainability aspects and consists in the transformation of biomass into constituents and, consequently, in their use to produce biofuels, chemicals, energy or materials with high added-value and promising industrial applications (71). The main subject of principle 5 is the process, considering the reduction of the number of unit operations and the development of eco-friendly, efficient and safe extraction methodologies as priorities (70). This approach is included in the process intensification. A complete extraction process involves usually four main stages: 1) pre-treatment of plant material; 2) solid-liquid extraction with suitable solvent; 3) solid-liquid separation; and 4) solvent removal and recycling (68). Figure 9 represents the different stages for extract preparation.

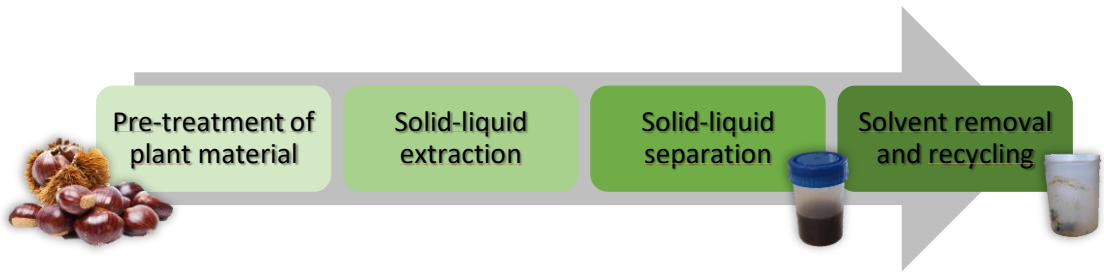

Figure 9. The four steps of a complete extraction process.

The first step is the pre-treatment of plant material that might include drying and grinding (68). Afterwards, the extraction technology should be selected based on the type of plant material and final application (68). The solid-liquid extraction is the most crucial unit

operation in the recovery of bioactive compounds. The main key factors of an extraction process are temperature, pressure, solvent and extraction time (69). In the past, conventional extraction techniques were widely used. Some of the most used conventional extraction methods consisted of maceration, heat reflux and Soxhlet extraction, and required a large amount of solvents and long extraction times (72). However, these extraction techniques present considerable disadvantages, namely time and energy consuming as well as the use of large amounts of solvents, frequent use of harmful solvents for human health and environment (e.g. organic solvents), inefficient recovery of extracts, and production of high amounts of wastes (70). Furthermore, the extract obtained might not be safe since it could contain residual solvent, contaminants from raw materials, or denatured compounds from the drastic extraction conditions applied (70).

Regarding the extraction of polyphenols, most of the studies reported the use of water and alcohols (mainly ethanol and methanol) as solvents (24, 73, 74). Taking into consideration the environmental, economic and safety concerns, alternative greener and cleaner extraction methodologies are emerging with the commitment of performing a more selective extraction. In a green point of view, many extraction techniques have been employed as potential candidates to replace conventional methods, such as ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pulse electric field extraction, and enzyme assisted extraction (68). These are some promising methodologies already reported in the extraction of polyphenols from different plant parts (e.g. roots, leaves, fruits) (70). The implementation of these innovative technologies will provide higher extraction efficiency and higher quality extracts than conventional methods, as well as reducing extraction time, energy consumption, amount of solvents, number of unit operations, amounts of wastes generated, environmental impacts and economical costs (70). Lastly, a post-treatment of liquid extract should be performed, including the solid-liquid separation (through filtration or centrifugation) and the removal of solvent (by freeze-drying, concentration, evaporation under vacuum and purification) (68). Since these procedures are frequently time and energy-consuming, intensification is applied to overcome these problems, providing faster and more effective use of energy, improving mass and heat transfer, reducing the number of processing steps and the size of the equipment, and eliminating the use of organic solvents (70). The development of novel and sustainable extraction technologies will contribute to a remarkable growth of circular economy. The principle 6 refers to the final extract that should be natural, green, biodegradable and without contaminants, in other words its molecular structure must remain the same independently of the process employed (70). The essential properties of an “eco-extract” are: natural, high

quality with active compounds, high functionality, low environmental footprint and being in accordance with legislation of the sector of industrial application (70).

Besides the principles defined for green chemistry and the huge number of proposed theories, there is no consensus in the scientific community regarding how to evaluate the green character of analytical methodologies (70). The use of eco-friendly and green extraction techniques of natural products using reusable and GRAS (Generally Recognized as Safe) solvents is a novel concept that focuses on the protection of human health and environment from deleterious side effects and enhances the competition between industries, encouraging the ongoing research to create more ecologic, economic and innovative techniques (70). In the future, it is also imperative to design carefully scale-up procedures as the key step for industrial applications (75).

1.4.1 Subcritical water extraction

Subcritical Water Extraction (SWE), also known as pressurized hot water extraction, is an environmentally friendly extraction technology that has gained more attention due to its unique properties and broad range of applications. In this methodology, water is employed as solvent at high temperature (100 – 374 °C) and pressure (10 – 60 bar) enough to maintain the water in the liquid state (76, 77). At a temperature above 374 °C and pressure above 220 bar, water is in supercritical state (70). Water is called subcritical, near-critical or pressurized hot water at a temperature range between 100 °C and the critical temperature (70). At this range of temperature and high pressure, a considerable drop on the water dielectric constant with the increasing of temperature leads to a decrease on the polarity of water, being suitable for the extraction of polar and non-polar compounds (70, 77). The dielectric constant of water lowers due to the weakening of hydrogen bonds. Thus, at high temperatures, liquid water is a solvent of low polarity (77, 78).

A significant change in the physicochemical properties of water might be observed at different extraction temperatures and pressures (79). The extraction efficiency could be influenced by mass transfer, matrix effect and solubility (77). In SWE, these interrelated factors are mainly limited by temperature, pressure, extraction time, flow rate and particle size (79). Temperature is the key parameter that most affects the extraction efficiency and selectivity (77). Under subcritical water conditions, as the temperature rises, there is a decrease in water dielectric constant, permittivity, viscosity and surface tension, an increase in the diffusion rate and, consequently, changes in water polarity. However, it is noteworthy that an increase of extraction temperature above a certain value leads to the degradation of compounds of interest. Likewise, an increase in flow rate results in an increase of

superficial velocity and quick mass transfer (70, 77). According to Khajenoori *et al.*, a particle size of 0.5 mm affords a much higher extraction yield than 1 mm and no differences were found compared to a particle size of 0.25 mm (80). Therefore, a low particle size of at least 0.5 mm is enough to provide a great extraction efficiency (80). Moreover, type of analysis, extraction vessel characteristics and use of modifiers or additives are also important parameters that could affect SWE (79).

At room conditions, water is considered a polar solvent and it is appropriate for the extraction of polar compounds, being its dielectric constant approximately 80. The polar compounds have high solubility in water, being most efficiently extracted at low temperatures (70). In the subcritical state, an extraction with water is similar to some organic solvents (e.g. methanol), at ambient conditions, used to extract compounds of moderate polarity since the water dielectric constant decreases (70). Under subcritical conditions, the water dielectric constant decrease from 80 (room temperature) to about 30 (250 °C) (76). Therefore, moderate polar and non-polar compounds are easily recovered in a less polar medium induced at high temperatures (70). Furthermore, the low polarity at high temperatures in SWE contributes to achieve high extraction yields and short extraction times for hydrophobic compounds (70, 77). The surface tension, polarity, and water viscosity and density considerably decreases in subcritical state at high temperatures, whereas the dissociation constant (K_w) of water increases. A reduction of surface tension of water promotes the wetting of plant material and the dissolution of bioactive compounds, while the decrease of water viscosity enhances its penetration in plant material, improves the diffusion rate and mass-transfer kinetics and, consequently, accelerates the extraction. In addition, the pH changes from about 7.0 to 5.5 (70, 77).

Besides the positive aspects of SWE, an increase of temperature could also provide some disadvantages such as: i) the extraction might become less selective due to the increase of solubility of desired analytes and other matrix components; ii) degradation of thermolabile compounds; and iii) other chemical reactions in the sample matrix (e.g. Maillard reactions) (77). The huge influence of temperature in SWE efficiency demands the determination of the optimal extraction conditions in order to improve the solubility and mass transfer and minimize the undesirable disadvantages (77). Table 3 summarizes the main advantages and disadvantages of SWE.

Table 3. Advantages and disadvantages of subcritical water extraction (SWE) (77).

Advantages	Disadvantages
• SWE is characterized by a higher diffusion rate into the plant materials and mass-transfer compared to other extraction methodologies; • SWE is employed in the extraction of low-polar and non-polar compounds; • SWE uses a low cost and easily available solvent and short extraction times to minimize the cost of extraction; • As a solvent, water is safe, non-toxic, non-flammable and environmentally friendly; • Extracts obtained are also safe; • Uncomplicated equipment.	• Loss of selectivity at high temperatures due to an increase of solubility of other matrix compounds; • High risk of hydrolysis reactions and degradation of thermolabile compounds at high temperatures; • Undesirable chemical reactions (e.g. caramelization, Maillard reactions) and formation of toxic compounds at high temperatures; • SWE equipment need to be made of expensive high quality materials to increase corrosion resistance and strength at high temperatures and pressures; • High investments costs.

The SWE equipment is composed by a solvent container, a high-pressure pump to provide the water input into the system, a valve that keep the pressure within the system, and a cell or column within an oven where the extraction occurs (Figure 10) (70).

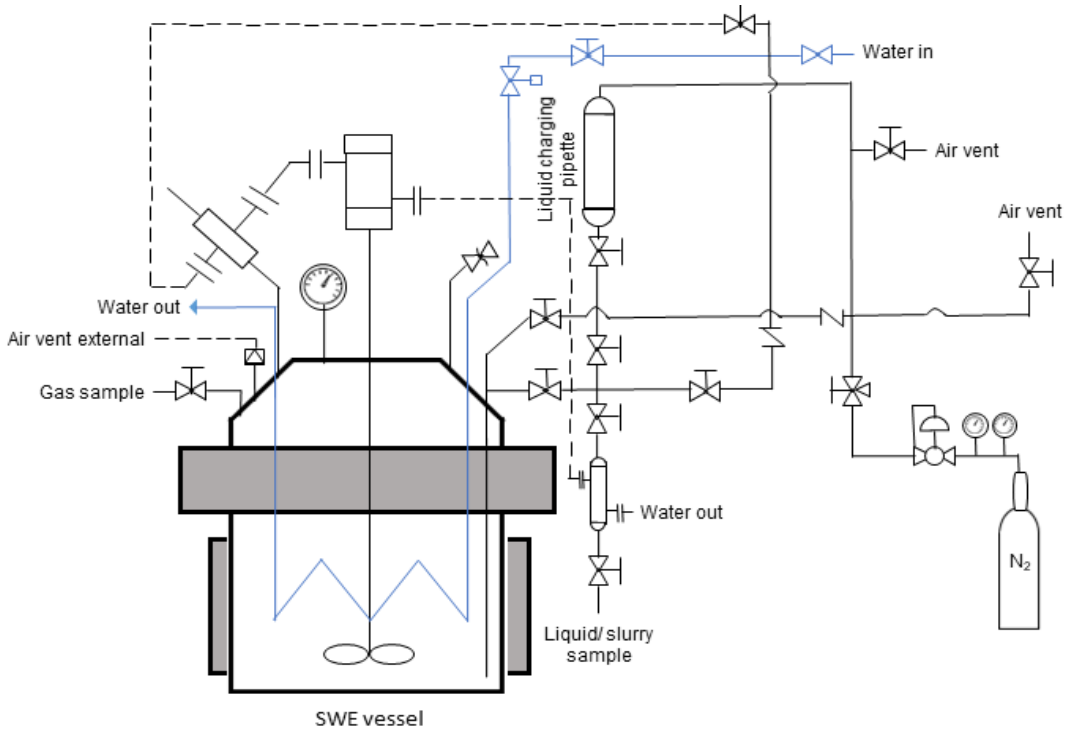

Figure 10. Schematic representation of an SWE equipment.

The SWE seems to have six sequential steps: 1) rapid fluid entry; 2) desorption of solutes from matrix active sites; 3) diffusion of solutes through organic components; 4) diffusion of solutes through static fluid in porous materials; 5) diffusion of solutes through layer of stagnant fluid outside particles; and 6) elution of solutes by the flowing mass of fluid (79). The diffusion of the dissolved solute within the solid is usually the limiting step of the extraction rate for most botanicals. Otherwise, partitioning of solute between the solid matrix and solvent has also been described as the rate-limiting mechanism for SWE (79).

Besides these unique properties of subcritical water, this solvent is considered a potentially green solvent since it is safe, non-toxic to human health and environment, non-flammable, naturally abundant, easily available and low cost. In addition, water has insignificant environmental impact and low disposal cost, being a good option to replace the hazardous organic solvents (77). Thus, water is an interesting solvent to be applied due to its physicochemical changes from ambient to near-critical conditions. The application of SWE on the recovery of bioactive compounds from plant sources and its derivatives has been extensively studied (81-91). This modern extraction technology has been applied for the extraction of antioxidants, carotenoids, essential oils, fatty acids, mannitol, oils, pectin, polysaccharides, sugars, among others (78). Muñoz-Almagro *et al.* extracted pectins from cacao (*Theobroma cacao* L.) pod husk using SWE (81), while Valizadeh *et al.* recovered curcumin from turmeric (*Curcuma longa* L.) (88). Indeed, polysaccharides were recovered from *Sagittaria sagittifolia* L. plant (90) and mushroom (*Lentinus edodes*) (91). Several authors suggested the extraction of polyphenols from natural sources such as 'Hayward' (*Actinidia deliciosa*) kiwifruit pomace (82), onion skin (84), lotus (*Nelumbo nucífera*) seed epicarp (87), and blackberry (*Morus nigra* L.) fruits (89). Also, flavanones, particularly hesperidin and narirutin, were obtained through SWE from orange peels (83). Moreover, anthocyanins were extracted from goji berry (*Lycium ruthenicum* Murr.) (86), while Gabaston *et al.* recovered stilbenes from grapevine canes, woods and roots (85).

In the last few years, a notable increase in the number of publications using water as a green extraction solvent and the use of SWE as a green extraction method has grown exponentially (78). The optimal SWE conditions for polyphenols greatly vary depending on the group of phenolic compound to be extracted (78). According to Plaza and Turner, polyphenols have usually been extracted through SWE up to temperatures of 150 °C and for an extraction time until 60 minutes, revealing a higher efficiency on the recovery of polyphenols compared to conventional extraction methodologies, as well as a higher heterogeneity and quality of the individual polyphenols extracted (92). However, most of the studies applied extraction temperatures of 80 – 150 °C and times of 1 – 60 min (70). Overall, SWE is a promising eco-friendly extraction method that allows a faster, cleaner and less

expensive extraction of bioactive compounds when compared to conventional techniques, also reducing the solvent employed and increasing the extraction rate and yield (76). This methodology comprises a step forward in the search for novel alternatives to the extraction using organic solvents.

2 Objectives

The present work focuses on the recovery of antioxidants and phenolic compounds from *C. sativa* shells through SWE using a mathematical tool (Response Surface Methodology - RSM) in order to study and characterize a new potential cosmetic ingredient.

The main objectives undertaken on the present dissertation were:

- To optimize the SWE of shells in order to obtain the highest antioxidant activity and phenolic content through Response Surface Methodology (RSM). The independent variables were time and temperature and the dependent variables were ABTS, DPPH, FRAP and TPC;
- To characterize the optimal extract regarding scavenging capacity against ROS, phenolic profile and cell viability in keratinocytes and fibroblasts;
- To develop and characterize a hydrogel with the optimal extract regarding organoleptic properties, colour, pH, texture and rheological characteristics;
- To perform the hydrogel stability study at different times (1, 7 and 30 days) and temperatures (25 and 40 °C).

3 Materials and Methods

3.1 Chemicals

All chemicals and solvents used were of analytical grade, used as received or dried by standard procedures and obtained from commercial sources. 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were obtained from Aldrich Chemical Co. (Steinheim, Germany). Folin–Ciocalteu's reagent, gallic acid, catechin and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, USA). Dihydrorhodamine 123 (DHR), sodium hypochlorite solution with 4% available chlorine, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), nitroblue tetrazolium chloride (NBT), α,α'-Azodiisobutyramidine dihydrochloride (AAPH), Trolox and fluorescein sodium salt were obtained from Sigma Aldrich (Steinheim, Germany). Dimethylsulfoxide (DMSO) was purchased from AppliChem (Darmstadt, Germany). Human foreskin fibroblasts (HFF-1) were acquired from ATCC (ATCC Number: SCRC-1041; ATCC, Manassas, VA, USA). Human immortalized non-tumorigenic keratinocytes cell line HaCaT (ethnicity, Caucasian; age, 62 years; gender, male and tissue, skin) was obtained from CLS Cell Lines Service, Germany. Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), GlutaMAX^{TM}, Hank's Balanced Salt Solution (HBSS), non-essential amino acids, penicillin, streptomycin and trypsin–EDTA were obtained from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). Trypan blue dye was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA USA). Tissue culture flasks were acquired from Orange Scientific (Belgium).

3.2 Sample

Castanea sativa shells were kindly supplied by Sortegel, located in Sortes, Bragança, Portugal (latitude 41°42'18.6"N and longitude 6°48'36.6"W), in October 2018. Shells were dehydrated (Excalibur Food Dehydrator, USA) at 41 °C during 24 hours and grounded in a miller (Ultra Centrifugal Mill ZM 200, Retsch, Germany) to a particles size of 1 mm. Afterwards, samples were thoroughly mixed and stored under light-free conditions until

extraction. Figure 11 presents the dried shells and the final powder obtained after milling the shells.

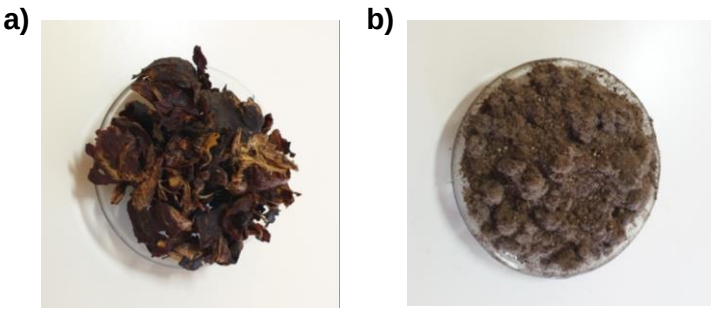

Figure 11. *C. sativa* shells: a) dried chestnut shells; b) powdered dried chestnut shells.

3.3 Experimental design and optimization of extraction conditions

The RSM mathematic design was applied using a central composite design (CCD) in order to optimize the SWE through the determination of the best extraction conditions that achieve the highest antioxidant activity. This procedure aims the modulation of the optimal conditions for an extraction based on a response of interest that is influenced by different variables. This experimental design analysed two independent variables, time (X1) and temperature (X2). A total of 13 experiments were performed using a range of five periods of time (6, 10, 20, 30 and 34 min) and five temperatures (51, 80, 150, 220 and 249 ºC) (Table 5, section 4.1). Following a randomized order, five centre points were selected and the extraction conducted. CCD comprises a complete 22-factorial design as cubic points, with four axial points at a distance of α = 1.414 from the design centre and five centre points. The responses studied were TPC (Y4) and the antioxidant activity analysed by ABTS (Y1), FRAP (Y2) and DPPH (Y3) assays. Through the determination of regression equations and the analysis of the response surface and contour plots using the predictive equations of RSM, it is possible to obtain the optimal values of responses Y. Afterwards, using the critical values optimized, a set of experiments was carried out to evaluate the accuracy of the models. Then, a *t*-test was done in order to compare the responses obtained under the optimized conditions with those predicted by the model. Statistical analysis was accomplished using the software Design Expert trial version 7 (Stat-Ease Inc., Minneapolis, MN, USA) (93).

3.4 Subcritical water extraction of bioactive compounds from *C. sativa* shells

SWE was carried out using a 400 mL Parr Reactor (Series 4560 high-pressure mini-reactors, Parr Instrument Company, Moline, Illinois, USA) equipped with a Parr Reactor Controller (Series 4848, Parr Instrument Company, Moline, Illinois, USA) to recover the bioactive compounds from *C. sativa* shells (Figure 12).

Figure 12. Equipment used for SWE.

This equipment have a temperature and pressure controller. For each extraction, 5 g of powdered dried shells were mixed with 100 mL of water (1:20, w/v) in the extraction vessel. After SWE ended up, a mixture of liquid extract and solid fraction was obtained. This mixture was filtered under vacuum through a Whatman no. 1 filter paper and centrifuged at 8000 rpm during 5 min (Thermo Scientific, model Heraeus Megafuge 16 R, Germany). The extracts were then lyophilized (Telstar, model Cryodos -80, Spain) in order to perform TPC, FRAP, DPPH and ABTS assays. The freeze-dried extracts were stored in plastic tubes under light-free conditions until further experiments. Figure 13 shows the liquid extracts obtained by SWE and after filtration and the freeze-dried extracts.

a) **b)**

Figure 13. *C. sativa* shell extract: a) liquid extract obtained by SWE after filtration and centrifugation; b) freeze-dried extract.

The extraction yield corresponding to the recovery of polyphenols was also determined for each extract. Through RSM, the extract selected as the best one, based on the mathematical model, was newly prepared. Thereafter, an aliquot of this extract was lyophilized in order to perform TPC, FRAP, DPPH and ABTS assays, ROS and RNS scavenging assays, cell viability studies and LC-DAD/ESI-MS analysis for characterization of the phenolic profile. The other portion of the liquid extract was kept at -80 °C until the hydrogel preparation.

3.5 Quantification of bioactive compounds

3.5.1 Determination of Total Polyphenols Content

The TPC was spectrophotometrically determined according to the *Folin-Ciocalteu* procedure with minor modifications (94). The methodology principle is based on a complex redox reaction between polyphenols and *Folin-Ciocalteu*'s reagent (containing phosphomolybdic and phosphotungstic acids). This assay was straightly made in a 96 wells microplate. All extracts (1 mg) were previously diluted in 1 mL of distilled water. Briefly, 30 µL of chestnut shell extracts (1 mg/mL) were mixed with 150 µL of *Folin-Ciocalteu*'s reagent (1:10, v/v) and 120 µL of 7.5% (w/v) Na_2CO_3 solution. The microplate was incubated at 45 °C for 15 min in a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) and after left to stand in the dark for 30 min, at room temperature. Then, the absorbance was measured at 765 nm using the microplate reader described above. A calibration curve prepared with gallic acid diluted with distilled water was used as standard (linearity range = 5 – 100 µg/mL, R^2 = 0.999). The TPC of the extracts was expressed as mg of GAE per gram of plant material on dw (mg GAE/g dw).

3.6 *In vitro* antioxidant activity

In vitro antioxidant activity of *C. sativa* shell extracts were evaluated by DPPH radical scavenging activity, FRAP and ABTS free radical scavenging assays as detailed in the following points.

3.6.1 DPPH free radical scavenging assay

The DPPH assay was used to evaluate the free radical scavenging activity of *C. sativa* shell extracts. DPPH is a stable free radical with a maximum absorption between 515 and 517 nm. This procedure was directly performed in a 96 wells microplate based on the

reduction of DPPH$^{\bullet}$ to a hydrazine compound which reacts with hydrogen donors, such as an antioxidant (74). The extracts (1 mg) were previously diluted in 1 mL of distilled water. The reaction mixture, in each well, consisted of 30 µL of extracts (1 mg/mL) and 270 µL of ethanol solution containing DPPH radicals in a final concentration of $6x10^{-5}$ M. This methodology was directly carried out in a 96 wells microplate. Afterwards, the microplate was left to stand in the dark and at room temperature, during 40 min. The absorbance was measured at 517 nm, in the microplate reader described above, to determine the DPPH$^{\bullet}$ reduction in which a low absorbance value corresponds to a high free radical scavenging activity. Trolox was used as reference to plot a standard curve (linearity range: 5 – 75 µg/mL, R^2 = 0.988). The antioxidant ability based on the DPPH free radical scavenging capacity of extracts was expressed as mg of Trolox equivalents (TE) per gram of plant material on dw (mg TE/g dw).

3.6.2 Ferric reducing antioxidant power (FRAP) assay

The FRAP of the extracts was determined according to the method described by Benzie *et al.*, with minor modifications, through the measuring of the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ) in the presence of antioxidants (95). This procedure requires a single electron reaction between $Fe(TPTZ)_2$ (III) and an electron donor ArOH (chemical structure presents in phenolic compounds). Briefly, 1 mg of extract was diluted in 1 mL of distilled water. In a 96 wells microplate, an aliquot (35 µL) of each extract (1 mg/mL) was added to 265 µL of FRAP reagent (composed by 10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3.6H_2O$ solution). After the incubation of the microplate at 37 °C for 30 min, the increasing of absorbance was measured at 595 nm using the microplate reader described above. A standard curve was prepared with ferrous sulphate ($FeSO_4 \cdot 7H_2O$) as reference (linearity range: 25 – 500 µM, R^2 = 0.998) and the results were expressed as µmol of FSE per gram of plant material on dw (µmol FSE/g dw).

3.6.3 ABTS free radical scavenging assay

The ABTS radical scavenging activity of extracts was evaluated according to the methodology described by Re *et al.*, with minor modifications, based on the reduction of ABTS radical by antioxidants (96). An aliquot (1 mg) of extracts were previously diluted in 1 mL of ultrapure water. Firstly, 7 mM ABTS and 2.45 mM potassium persulfate stock solutions were prepared. The working solution of ABTS$^{\bullet+}$ chromophore was then prepared by adding equal volumes of the two stock solutions and allowing it to react at room

temperature, in the dark for 16h, to obtain a dark green solution. Prior to use, the $ABTS^{\bullet+}$ solution was filtered using a nylon filter from 0.22 µm and diluted in ultrapure water to obtain an absorbance of 0.700 ± 0.02 at 734 nm. Afterwards, 20 µL of each extract was mixed with 180 µL of $ABTS^{\bullet+}$ diluted solution and the reaction mixture was kept in the dark for 6 min. Then, the ABTS radical cation discoloration was determined by reading the absorbance at 734 nm in the microplate reader described above. A calibration curve was obtained using known concentrations of ascorbic acid as standard (linearity range: 5 – 100 µg/mL, R^2 = 0.993). The results were expressed as mg of AAE per gram of plant material on dw (mg AAE/g dw).

3.7 Reactive oxygen species scavenging capacity assays

Regarding ROS assays, *C. sativa* shell extract and positive controls (catechin, gallic acid and Trolox) were previously dissolved in the phosphate buffer used for each assay and tested at different concentrations. These procedures were done in a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) equipped with a thermostat and used to chemiluminescence, fluorescence and UV/Vis measurements. For each assay, four independent experiments using six concentrations, in duplicate, were performed. The results were expressed as IC_{50} value (defined as the concentration required to obtain an inhibition capacity of 50%) and determined from the curves of inhibition percentage *versus* antioxidant concentration using the GraphPad Prism 7 software. Prior to the assays, the absorption of the extract was studied for the appropriated wavelengths for each procedure.

3.7.1 Superoxide radical scavenging assay

Following the method already described by Gomes *et al.*, the scavenging activities of *C. sativa* shell extract and positive controls against superoxide ($O_2^{\bullet-}$) were evaluated through a colorimetric methodology based on the reduction of NBT into a purple coloured diformazan as a result of the reaction with $O_2^{\bullet-}$ (97). This ROS was produced by the non-enzymatic NADH/PMS/O_2 system. The reaction mixture, in each well, consisted of 50 µL of extracts or positive controls, 50 µL of NADH 166 µM, 150 µL of NBT 43 µM and 50 µL of PMS 2.7 µM, in a final volume of 300 µL. Catechin and gallic acid were used as positive controls. The extracts and catechin were tested in a range of concentrations between 7.81 and 250 µg/mL, while the concentrations analysed for gallic acid were from 0.98 to 31.25 µg/mL. The assay was performed at room temperature. Then, the absorbance was measured at 560 nm for 6 min and the results were expressed as the inhibition, in IC_{50}, of the NBT reduction to diformazan.

3.7.2 Hypochlorous acid scavenging assay

Hypochlorous acid (HOCl) scavenging activity was measured using a fluorescent methodology previously described by Gomes *et al.*, based on the HOCl-induced oxidation of DHR to rhodamine (97). The positive controls used were gallic acid and catechin. A 1% (w/v) NaOCl solution was used to prepare the HOCl solution by adjusting the pH to 6.2 with addition of H_2SO_4 by dropwise. Through a spectrophotometric analysis at 235 nm and using the molar absorption coefficient of 100 $M^{-1}cm^{-1}$, the concentration of HOCl in solution was determined. The working solution of HOCl was prepared by proper dilution in 100 mM phosphate buffer at pH 7.4. After purging with nitrogen, a 2.89 mM stock solution of DHR in dimethylformamide was stored at -20 °C. The working solution of DHR was prepared by dilution in phosphate buffer and kept in the ice and under light-free conditions until perform the determinations. The positive controls used were catechin and gallic acid. The concentrations of catechin and extracts analysed ranged from 0.12 to 3.91 µg/mL, whereas a range of concentrations between 0.48 and 15.63 µg/mL were tested for gallic acid. In a 96-wells plate, each well contains the following reaction mixture in a final volume of 300 µL: 150 µL of phosphate buffer solution 100 mM solution at pH 7.4, 50 µL of extracts or positive controls, 50 µL of DHR 5 µM, and 50 µL of HOCl 5 µM. The fluorescence was immediately read at 37 °C, at the emission wavelength at 528 ± 20 nm and excitation wavelength at 485 ± 20 nm. Results were expressed as the inhibition, in IC_{50}, of HOCl-induced oxidation of DHR.

3.7.3 Peroxyl radical scavenging assay

The peroxyl ($ROO^{\bullet}$) scavenging ability was determined by monitoring the effect of *C. sativa* shell extract and positive controls on fluorescence decay as result of the $ROO^{\bullet}$-induced oxidation of fluorescein according to Ou *et al.* (98). $ROO^{\bullet}$ was generated by thermal decomposition of AAPH at 37 °C. Each well consisted of a reaction mixture containing 150 µL of fluorescein 61.2 nM, 25 µL of extracts or positive controls and 25 µL of AAPH 19.1 mM, in a final volume of 200 µL. Then, the microplate was incubated in the microplate reader at 37 °C during 2h. The fluorescence signal was monitored at each minute at the emission wavelength at 528 ± 20 nm and excitation wavelength at 485 ± 20 nm until the total decay of fluorescence. Catechin (0.006 – 0.2 µg/mL) and gallic acid (0.047 – 1.5 µg/mL) were used as positive controls, while Trolox (0.047 – 1.5 µg/mL) was employed as standard. The extracts were tested in a range of concentrations from 0.488 to 15.625 µg/mL. Using GraphPad Prism 7 software, the difference between the area under the fluorescence decay curve (AUC) in the presence of the sample ($AUC_{antioxidant}$) and in its

absence (AUC_{blank}) was determined. The regression equations between AUC and the concentration of the sample were calculated for extract, catechin, gallic acid and Trolox. The oxygen radical absorbance capacity (ORAC) values were calculated using the standard curve of each assay. Results were expressed as the ratio between the slope obtained for extract or positive controls and the slope of Trolox. In addition, the $ROO^{\bullet}$ scavenging capacity was also expressed as mg of TE per gram of plant material on dw (mg TE/g dw).

3.8 Phytochemical profile of *C. sativa* shell extract

The phytochemical characterization of the extract was carried out by LC-DAD/ESI-MS analysis according to Fernandes *et al.* (99). For the sample preparation, a solution of the freeze-dried shell extract with a concentration of 8 mg/mL was prepared using distilled water as solvent and filtered by a nylon filter of 0.45 µm. A Finnigan Surveyor series liquid chromatograph, equipped with a Thermo Finnigan (Hypersil Gold) reversed-phase column (150 mm x 4.6 mm, 5 µm, C18) thermostated at 25°C, was employed for this analysis. A photodiode spectrophotometer and mass spectrometry were used for the double-online detection. The mass detector was a Finnigan LCQ DECA XP MAX (Finnigan Corp., San Jose, CA) quadrupole ion trap equipped with an atmospheric pressure ionization (API) source, using electrospray ionization (ESI) interface. The vaporizer and the capillary voltages were 5 kV and 4V, respectively, and the capillary temperature was set at 325 °C. Nitrogen was employed as both sheath and auxiliary gas at flow rates of 80 and 30, respectively (in arbitrary units). The mass spectrometer was programmed to perform a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS-MS of the most intense ion using relative collision energies of 30 and 60. Spectra were obtained in the negative ion mode between *m/z* 120 and 2000. The samples were directly injected into the MS spectrometer with a pump at a flow rate of 3 µL/min. The capillary temperature and voltage used were 275 °C and 15 V, respectively, and spectra were obtained in the positive ion mode between *m/z* 120 and 2000. Results were expressed as mg per g of plant material on dw (mg/g dw).

3.9 Cell viability assays

3.9.1 Cell lines, primary cell isolation and culture conditions

HaCaT and HFF-1 cells were grown separately in tissue culture flasks in a complete medium composed by Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™ and supplemented with 10% (v/v) inactivated FBS, 1% (v/v) L-glutamine, 1% (v/v) non-essential

amino acids, 1% (v/v) antibiotic–antimitotic mixture (final concentration of 100 U/mL penicillin and 100 µg/mL streptomycin) and 0.25 µg/mL amphotericin B, and maintained in a 5% CO_2 environment at 37 °C under a water saturated atmosphere in an incubator (CellCulture® CO_2 Incubator, ESCO GB Ltd, UK). Passage 21–23 of HFF-1 cells and passage 83-85 of HaCaT cells were employed for MTT assay. Both cell lines were harvested at 90–95% of confluence using trypsin. In addition, the trypan blue dye exclusion assay was used to assess the viability and cell lines were re-plated in T125 flasks.

3.9.2 MTT assay

The effect of *C. sativa* shell extract on cell viability was evaluated by MTT assay through monitoring the uptake and metabolism of the vital mitochondrial dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by cell mitochondria (74). According to Rodrigues *et al.*, cells were incubated in the absence or presence of extracts dissolved in cell culture medium containing 0.1, 1, 10, 100 and 1000 µg/mL of extracts (74). Each concentration was tested in triplicate in three independent experiments. The extracts dissolved in culture medium were previously filtered using 0.45 µm Millex GV filters (Millipore, Nepean, ON, Canada) and exposed to HaCaT and HFF-1 cells. Briefly, in 96-well microplates, cells were seeded at a density of 25 x 10^3 cells per mL of culture medium for 24 h. Afterwards, the medium was changed and the cells were incubated with different concentrations of *C. sativa* shell extract for 24 h at 37 °C in a 5% CO_2 environment. DMEM was used as positive control, while the negative control was 1% (w/v) Triton X-100. Following the removal of extracts, the cells were washed with phosphate buffered saline solution (PBS pH 7.4). The number of viable cells was determined by adding the MTT reagent dissolved in medium to each well and incubating at 37 °C during 3 h to allow the formation of formazan crystals. Then, the medium was removed and 150 µL of DMSO was added to solubilize the blue formazan crystals. The absorbance was read at 490 nm with background subtraction at 630 nm.

3.10 Preparation of *C. sativa* shell hydrogel

Firstly, glycerin and methylparaben were dissolved in water, at room temperature. Glycerin affords emollient and moisturizing properties to the gel (100), while methylparaben protects the gel against the bacteria, fungi and yeast growth (101). *C. sativa* shell extract was dissolved in the previous mixture. Then, Carbopol® 940, a polyvinyl carboxy polymer acting as viscosity enhancer and gelling agent, was slowly dispersed under magnetic stirring (750 rpm) at 40 °C for 1 h. Carbopol® 940 is a non-irritant, non-toxic, and hydrophilic

cross-linked polyacrylic acid polymer. This polymer has been employed in drug delivery systems based on its high affinity for water, improved absorption capacity, reduced toxicity, fast swelling, and high biocompatibility (102). After cooling down, the formulation was homogenized and the triethanolamine added dropwise to complete the gelation process. The triethanolamine was used as base to adjust the acid/base balance and added in enough quantity to neutralize the carboxylic groups of the carbomer and allow the cross link of the carbopol and the formation of the gel (103). The composition of the hydrogel is described in Table 4.

Table 4. Composition of the hydrogel.

Compounds	*Quantity (%)*
Glycerin	10
Carbopol® 940	0.5
Methylparaben	0.2
C. sativa shell extract	50
Triethanolamine	0.15
Purified water	q.s.100

q.s. – *quantum satis*

A slow stirring of the mixture avoids air incorporation and provides a homogeneous appearance. Afterwards, the formulation was packed in glass jars and rested 24 h until analysis. Two sets of formulations were prepared to perform a stability study. The first set (3 glass jars) was kept at 25 °C and the second (3 glass jars) at 40 °C. Figure 14 represents the preparation of the hydrogel and *C. sativa* shell gels already prepared.

a) **b)**

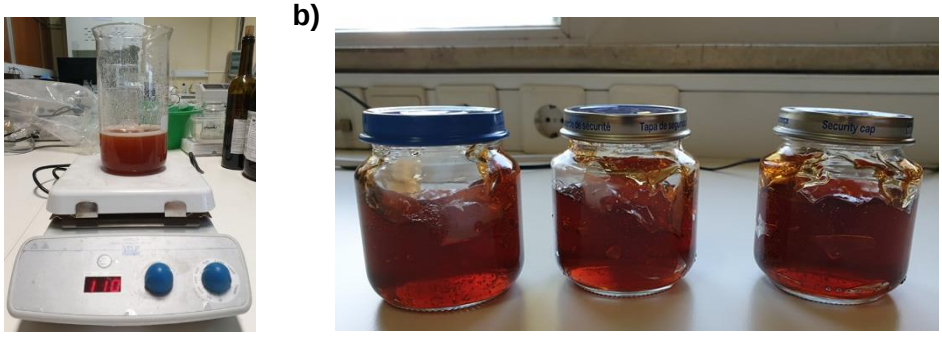

Figure 14. Preparation of hydrogels containing *C. sativa* shell extract: a) heating and dispersion of Carbopol® in the aqueous mixture containing the extract; b) gels prepared and packed in glass jars.

3.11 Characterization of *C. sativa* shell hydrogel

3.11.1 Colour evaluation

Colour was analysed using a colorimeter (Chroma Meter CR-410, Konica Minolta, Tokyo, Japan) (Figure 15). The equipment was previously calibrated with a white reference background. The determinations were performed using a light source D_{65} and an observation angle of 2°. An aliquot of gel was placed on a Petri plate with 5.5 cm diameter and a sample thickness of 5 mm. The results were determined based on the colour space CIE 1976 *L* a* b**, defined by the *Comission Internationale de l'Éclairage* (CIE). The three coordinates represent the lightness of the colour (*L**), its position between red and green (*a**) and its position between yellow and blue (*b**). Data was collected using software Spectra MagicTM NC software®. Results were expressed as the average of three measurements for all parameters.

Figure 15. Colorimeter used to analyse the colour of the hydrogels.

3.11.2 Determination of pH

A pHmeter (Basic 20+, Crison, Barcelona, Spain) with a glass electrode previously calibrated was used to determine the pH (Figure 16). The measurements were performed in triplicate.

Figure 16. pHmeter used to analyse the pH of the hydrogels.

3.11.3 Texture analysis

The hydrogel consistency was evaluated in a texture analyser (Stable Micro Systems TA-XT2i, Godalming, UK) under compression at 20 ºC using a cylinder penetration probe of 0.5 mm of diameter (P/0.5) that provides a contact area of 126.68 mm^2 (Figure 17). The trigger value force was 0.005 N and the probe penetration depth was 10 mm at a test speed of 1 mm/sec. The pre-test and post-test speed was 1 and 3 mm/sec, respectively. The equipment was previously calibrated for height and force. The units defined for force, distance and time were Newton (N), millimeter (mm) and seconds (sec), respectively. The consistency of the gel is evaluated in this analysis. Firmness (peak positive force, N) and adhesiveness (negative area, N/mm) were the parameters analysed. Results were collected using the Exponent Stable Micro Systems software® (version 6,1, 12,0).

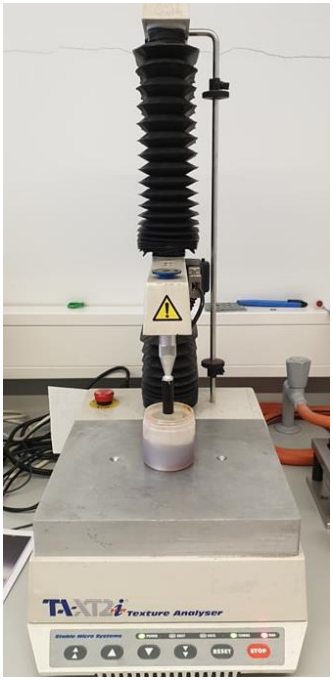

Figure 17. Texture analyser used to characterize the hydrogels.

3.11.4 Rheological behaviour analysis

The rheological properties were assessed on a rotational rheometer (Kinexus lab+, Malvern, Worcestershire, United Kingdom) equipped with a geometry of parallel plates, one of which rotates (20 mm, 60 mm with a gap of 1 mm) based on a rotational test (Figure 18). This equipment allows the study of the flow and deformation of matter, measuring the viscosity and elasticity of non-Newtonian fluids under a wide range of conditions and establishing an interrelation between deformation, force and time. The analysis was performed at 25 °C in a shear rate range from 0.1 to 100 s^{-1}, with 10 samples per decade. Data were collected using the rSpace software® (Kinexus 1,75: PSS0211-17). The final results were represented in a graph that relates the apparent viscosity, expressed as Pa.s, and the shear rate, expressed as s^{-1}. The yield *stress* and thixotropy of the hydrogel were also analysed.

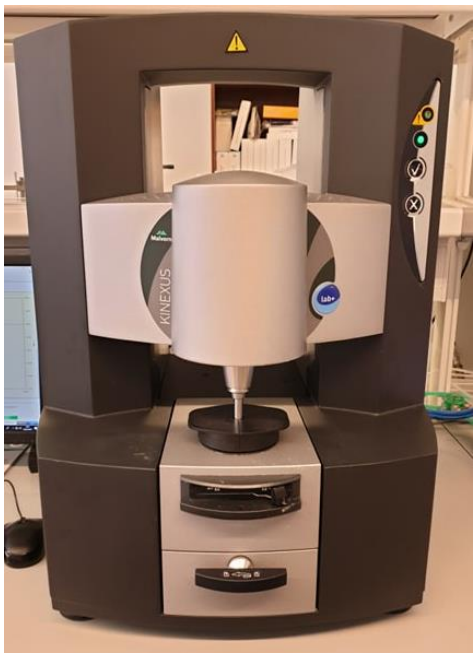

Figure 18. Rheometer used to analyse the rheological behaviour of the hydrogels.

3.12 Stability Study

The formulation was evaluated at different storage times, namely after 1, 7 and 30 days (T_1, T_7 and T_{30}, respectively), and different temperatures (25 °C and 40 °C). The parameters analysed were organoleptic properties, colour, pH, texture and viscosity.

3.13 Statistical analysis

All experimental analyses were carried out at least in triplicate. Results were reported as mean ± standard deviation of at least triplicate experiments. Design Expert trial version 7 (Stat-Ease Inc., Minneapolis, MN, USA) was used to the determination of the regression equations, analysis of the response surface and contour plots, and statistical analysis of the RSM. IBM SPSS Statistics 24.0 software (SPSS Inc., Chicago, IL, USA) was employed to perform the statistical analysis of the data. One way ANOVA was applied to investigate the differences between samples for all assays and *post hoc* comparisons of the means were performed with Tukey's HSD test. A denoting significance was accepted for $p<0.05$ in all cases. A correlation study was also performed, being selected the Pearson's regression coefficient 'R' with *p*-value. The IC_{50} values of ROS scavenging activity were determined from the curves of percentage of inhibition *versus* antioxidant concentration, using the GraphPad Prism 7 software (GraphPad, La Jolla, CA, USA).

4 Results and Discussion

4.1 Validation of the experimental design

Table 5 presents the SWE experimental conditions as well as the predicted and experimental values of ABTS, FRAP, DPPH and TPC assays and the extraction yield for recovery of bioactive compounds from *C. sativa* shell extracts. The bioactive compounds recovery yields ranged from 6.70% (run 5, 220 °C, 10 min) to 9.19% (run 1, 80 °C, 10 min). These results are in line with the ones reported by Barreira *et al.* for chestnut shell aqueous extracts prepared by conventional extraction (6.43 – 12.59%) (27). Otherwise, in another study performed by Barreira *et al.*, a lower extraction yield (4.98%) was obtained for chestnut outer shell extract (16). Squillaci *et al.* also prepared aqueous extracts from *C. sativa* shells and reported extraction yields slightly lower (5.2 – 6.0%) than the ones obtained in this study (35). Through SWE, Lachos-Perez *et al.* recovered flavanones from defatted orange peels and achieved similar extraction yields (4.44 – 10.63%) (83).

According to the results, TPC ranged between 315.21 mg GAE/g dw (run 13, 150 ºC, 20 min) and 496.80 mg GAE/g dw (run 6, 249 ºC, 20 min); ABTS ranged from 631.16 mg AAE/g dw (run 12, 150 ºC, 20 min) to 965.45 mg AAE/g dw (run 4, 220 ºC, 30 min); DPPH ranged between 549.23 mg TE/g dw (run 11, 150 ºC, 34 min) and 1125.68 mg TE/g dw (run 6, 249 ºC, 20 min); and FRAP ranged from 2793.95 mg FSE/g dw (run 12, 150 ºC, 20 min) to 11393.97 mg FSE/g dw (run 6, 249 ºC, 20 min). As shown in Table 6, the analysis of the variance (ANOVA) allows the evaluation of adequacy and significance of the models through means of Fisher's *F*-test.

Table 5. Experimental and predicted values of ABTS (mg AAE/g dw), FRAP (mg FSE/g dw), DPPH (mg TE/g dw) and TPC (mg GAE/g dw) of *C. sativa* shell extracts obtained by CCD.

	Independent variables		**Dependent variables**								
Point[a]	*SWE conditions*		*Y1, ABTS (mg AAE/g dw)*		*Y2, FRAP (mg FSE/g dw)*		*Y3, DPPH (mg TE/g dw)*		*Y4, TPC (mg GAE/g dw)*		**Extraction yield (%)**[a]
run	X1 (t, min)	X2 (T, ºC)	Exp[a]	Pred[b]	Exp[a]	Pred[b]	Exp[a]	Pred[b]	Exp[a]	Pred[b]	
1	10	80	813.48	839.00	7015.33	6978.80	915.19	935.26	383.79	372.85	9.19 ± 0.35
2	30	80	864.58	841.97	6421.25	5563.75	780.64	690.54	410.80	402.66	7.24 ± 0.75
3	20	51	893.51	893.44	7611.66	8173.54	904.95	958.22	429.25	448.67	7.42 ± 0.60
4	30	220	965.45	949.53	8769.93	8467.19	899.91	897.91	366.30	405.87	7.87 ± 0.37
5	10	220	738.30	770.50	7796.43	8314.66	755.65	863.82	333.00	369.77	6.70 ± 0.32
6	20	249	930.58	921.06	11393.97	11171.33	1125.68	1054.34	496.80	448.75	7.58 ± 0.73
7	20	150	675.12	697.23	3257.39	3196.10	844.15	933.73	333.72	344.95	7.04 ± 0.16
8	20	150	750.74	697.23	3149.49	3196.10	969.84	933.73	359.10	344.95	7.11 ± 0.38
9	20	150	739.66	697.23	3033.65	3196.10	961.95	933.73	359.96	344.95	9.01 ± 0.22
10	6	150	766.93	727.66	5809.73	5396.42	854.06	766.14	316.78	304.17	8.14 ± 0.54
11	34	150	825.57	855.05	3753.15	4512.65	549.23	618.70	366.91	350.31	8.14 ± 0.43
12	20	150	631.16	697.23	2793.95	3196.10	946.06	933.73	356.19	344.95	8.47 ± 0.57
13	20	150	689.27	697.23	3752.91	3196.10	946.27	933.73	315.21	344.95	7.66 ± 0.83

[a] Experiments were performed in a random order and are the average of triplicate determinations from different experiments.
[b] Based on CCD evaluation.

Table 6. Model summary and analysis of variance (ANOVA) of ABTS (mg AAE/G dw), FRAP (mg FSE/g dw), DPPH (mg TE/g dw) and TPC (mg GAE/g dw) of *C. sativa* shell extracts.

source	Sum of squares				Mean squares				F value				*P*-value			
	Y1	Y2	Y3	Y4	Y1	Y2	Y3	Y4	Y1	Y2	Y3	Y4	Y1	Y2	Y3	Y4
Model	109941	85034820	171704	22585	21988	17006964	34341	4517	10.59	43.87	4.70	4.06	0.0037*	< 0.0001*	0.0335*	0.0475*
X1. min	16395	789014	21960	2150	16395	789014	21960	2150	7.90	2.04	3.01	1.93	0.0261**	0.1967	0.1266	0.2071
X2. ºC	763	8986314	9240	0.01	763	8986314	9240	0.01	0.37	23.18	1.26	0.00	0.5636	0.0019*	0.2978	0.9979
X1.X2	7749	614326	19434	10	7749	614326	19434	9.89	3.73	1.58	2.66	0.01	0.0947	0.2485	0.1469	0.9275
$X1^2$	15567	5433183	102313	551	15567	5433183	102313	551	7.50	14.01	14.00	0.50	0.0290**	0.0072*	0.0072*	0.5042
$X2^2$	76805	73035399	9167	18746	76805	73035399	9167	18746	36.99	188.38	1.25	16.85	0.0005*	< 0.0001*	0.2996	0.0045*
Residual	14535	2713896	51145	7787	2076	387699	7306	1112								
Lack of fit	49545	2209825	40710	6225	1652	736608	13570	2075	0.69	5.85	5.20	5.31	0.6042	0.0605	0.0725	0.0702
Pure error	9580	504071	10435	1562	2395	126018	2609	3901								
Total	124476	87748716	222848	30372												

R^2 *pred* (Y1) - 0.8832	R^2 *adjust* (Y1) - 0.7998	*Ratio* - 8.15
R^2 *pred* (Y2) - 0.9691	R^2 *adjust* (Y2) - 0.9470	*Ratio* - 18.8
R^2 *pred* (Y3) - 0.7705	R^2 *adjust* (Y3) - 0.6065	*Ratio* - 7.50
R^2 *pred* (Y4) - 0.7436	R^2 *adustj* (Y4) - 0.5605	*Ratio* - 6.38

Y1, ABTS (mg AAE/g dw); Y2, FRAP (mg FSE/g dw); Y3, DPPH (mg TE/g dw) and Y4, TPC (mg GAE/g dw).
* significance at $p<0.01$; ** significance at $p<0.05$.

The independent variable X1 (time, min) showed a significant effect (p<0.05) on ABTS response, whereas the independent variable X2 (temperature, °C) had a significant effect (p<0.01) on FRAP response. The quadratic term for X1 exhibited a significant effect on the three antioxidant activity responses (Y1, Y2 and Y3), while the quadratic term for X2 showed a significant effect on Y1, Y2 and Y4 responses (ABTS, FRAP and TPC, respectively). The R^2 is useful for checking the model fitness, being in this study classified as good (R^2 adjust was relatively close to 1). The values obtained were, respectively, 88.32%, 96.91%, 77.05% and 74.36% for the variation of X1 and X2 on the responses of ABTS, FRAP, DPPH and TPC (Table 6). Considering all response surface models, the "lack of fit" was not significant (p>0.05) and the *Ratio* was higher than 4 (as desirable), indicating an appropriate signal-to-noise ratio. This confirms the adequacy of the model terms to represent the experimental data and to predict the four analysed parameters. The following equations (1) to (4) show, respectively, the dependence of ABTS (Y1), FRAP (Y2), DPPH (Y3) and TPC (Y4) on time (X1) and temperature (X2). The parameters of the equation were determined through multiple regression analysis of the experimental results.

$$Eq.1: \mathrm{ABTS} = 1448.16 - 24.09xX1 - 7.55xX2 + 0.06x\,X1xX2 + 0.48x\,X1^2 + 0.02X2^2$$

$$Eq.2: \mathrm{FRAP} = 21692.11 - 474.41xX1 - 194.29xX2 + 0.56x\,X1xX2 + 8.97x\,X1^2 + 0.66X2^2$$

$$Eq.3: \mathrm{DPPH} = 939.04 + 29.04xX1 - 3.73xX2 + 0.10x\,X1xX2 - 1.23x\,X1^2 + 0.01X2^2$$

$$Eq.4: \mathrm{TPC} = 520.71 + 4.93xX1 - 3.22xX2 + 0.01x\,X1xX2 - 0.09x\,X1^2 + 0.01X2^2$$

4.2 Analysis of response surface

The RSM model was used to generate 3D contour plots in order to represent graphically the relationship between independent (time and temperature) and dependent variables (ABTS, FRAP, DPPH e TPC). As shown in Figure 19 A, B and D, the temperature and the extraction time highly contribute to the ABTS, FRAP and TPC responses, respectively.

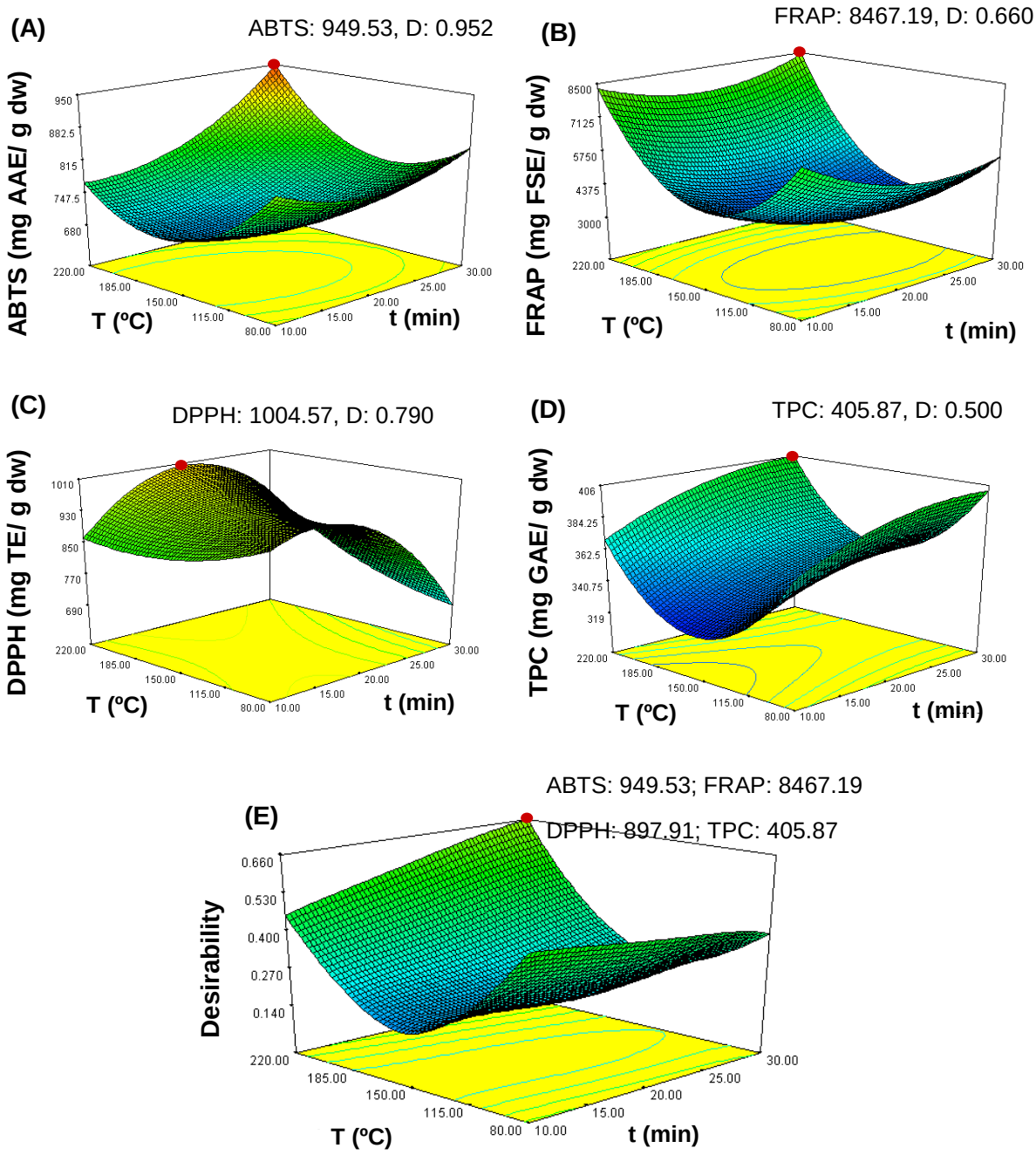

Figure 19. Response surface 3D plots for the interaction effects of independent variables of time (min) and temperature (°C) on dependent variables of ABTS (A), FRAP (B), DPPH (C) and TPC (D) and on the desirability index for combined responses (E) of *C. sativa* shell extract obtained through SWE. In the response surfaces were represented the optimal points.

According to Figure 19, the response profile of ABTS, FRAP and TPC were similar, while DPPH showed a distinctive response. At 80 °C/10 min, the responses in ABTS, FRAP and TPC assays were substantial since at these conditions some bioactive compounds with antioxidant activity were already extracted. However, the increase of temperature and

extraction time led to a decrease of the responses until around 150 °C. At 150 °C, the lowest responses were detected probably due to the degradation of some bioactive compounds with antioxidant activity. For temperatures higher than 150 °C, the responses increased significantly until achieving the optimal conditions at 220 °C/30 min. The extraction time also had a considerable influence on the responses observed in these assays since an increase of extraction time contributed to an increase of the antioxidant activity and TPC. Relatively to Figure 19 A, B and D, the optimal conditions for these assays corresponded to the maximum values of 949.53 mg AAE/g dw for ABTS, 8467.19 mg FSE/g dw for FRAP, and 405.87 mg GAE/g dw for TPC. Noteworthy, the results obtained for the extracts prepared at 220 °C/30 min and 80 °C/10 min were similar in all these assays.

Interestingly, the DPPH response was entirely different from the others. The temperature seems to have no effect on the antioxidant activity. On the other hand, the antioxidant activity increased with the extraction time, suggesting that the extraction time have a major influence on DPPH response. The highest DPPH response (1004.57 mg TE/g dw) was achieved at 220 °C/20 min.

According to the desirability index (Figure 19 E), the optimal extraction conditions to maximize antioxidant activity and TPC were 30 minutes at 220 °C (R^2 = 0.6600). The desirable results for these optimal extraction conditions were 949.53 mg AAE/g dw for ABTS, 8467.19 mg FSE/g dw for FRAP, 897.91 mg TE/g dw for DPPH, and 405.87 mg GAE/g dw for TPC. Nevertheless, a decrease on the responses was observed again at 150 °C, followed by a steady increase of responses with the increase of temperature. The experimental value for the four responses was similar (p<0.05) to the predicted value of the model, demonstrating once again the effectiveness of RSM for the optimization of the extraction of antioxidant compounds and polyphenols from chestnut shell extracted through SWE (Table 7).

Table 7. Antioxidant activity evaluated by ABTS, FRAP and DPPH assays and TPC of the optimized extract of *C. sativa* shells (220 °C/30 min).

	ABTS (mg AAE/g dw)	**FRAP (mg FSE/g dw)**	**DPPH (mg TE/g dw)**	**TPC (mg GAE/g dw)**
Experimental value[a]	901.16 ± 99.74	7994.26 ± 580.17	815.66 ± 30.57	417.30 ± 5.74
Predicted value	949.53	8467.19	897.92	405.87
p[b]	0.83	0.15	0.07	0.06

[a] results are expressed as mean ± standard deviation (n=3).

[b] indicate significant differences (p<0.05).

As shown in Table 5, the TPC ranged from 315.21 (run 13, 150 °C, 20 min) to 496.80 mg GAE/g dw (run 6, 249 °C, 20 min). These results were in line with the ones reported by Rodrigues *et al.* (24), Vázquez *et al.* (28) and Ham *et al.* (36) for chestnut shell extracts prepared by conventional extraction methodologies. However, lower TPC was obtained for shells by Squillaci *et al.* (43.69 mg GAE/g dw and 205.99 mg GAE/g dw for inner and a blend of inner and outer shells, respectively) (35) and Vázquez *et al.* (5.02 – 18.64 g GAE/100 g extract for different extracts prepared with alkaline and organic solvents) (22). Moreover, a study performed by Conde *et al.* obtained a similar TPC for chestnut burs (0.43 g GAE/g dw) (104), while lower results were reported by Pinto *et al.* (56.20 – 92.24 mg GAE/g dw for chestnut burs from different producing regions of Portugal) (105) and Vázquez *et al.* (11.54 – 35.98 g GAE/g dw for extracts prepared with alkaline solutions and organic solvents) (23). Regarding the ABTS assay, the results ranged between 631.16 (run 12, 150 °C, 20 min) and 965.45 mg AAE/g dw (run 4, 220 °C, 30 min). No studies about ABTS assay in chestnut shell extracts were published. Therefore, it was not possible to compare the results obtained in this assay. The antioxidant activity evaluated by FRAP assay reached from 2793.95 (run 12, 150 °C, 20 min) to 11393.97 mg FSE/g dw (run 6, 249 °C, 20 min). These results were substantially higher than the ones reported by Pinto *et al.* (835.3 – 4510.4 µmol FSE/g dw) (105) and Rodrigues *et al.* (6008.70 – 8083.50 µmol FSE/g dw) (24) for chestnut burs and shells, respectively. In DPPH assay, the extracts achieved between 549.23 (run 11, 150 °C, 34 min) and 1125.68 mg TE/g dw (run 6, 249 °C, 20 min). These results are in accordance with the ones reported by Vázquez *et al.* for different extracts of chestnut shells (0.92 – 3.24 mmol TE/g dw) (23). However, Ham *et al.* reported considerably lower values for shells (159.79 – 174.61 mM TE/g dw) (36). Noteworthy, there are just few studies about the antioxidant activity of these shells that express the results in the same unit than the present work. To the best of our knowledge, none of these studies used SWE to recover phenolic compounds from *C. sativa* shells.

One explanation for the low antioxidant activity and TPC observed at 150 °C might be the degradation of tannins and the formation of reaction products with significant antioxidant activity, such as pyrogallol and protocatechuic acid generated from gallic acid and catechin, respectively, at high temperatures. The extracts prepared at 80 °C/10 min and 220 °C/30 min had similar results for ABTS, DPPH, FRAP and TPC. In this way, the bioactive compounds of *C. sativa* shell extracts prepared at 80 °C/10 min and 220 °C/30 min were identified by LC-DAD/ESI-MS in order to better understand the differences in phenolic composition of these two extracts.

4.3 Phytochemical profile of *C. sativa* shell extracts

The phenolic compounds identified in the chestnut shell extracts prepared by SWE at the two best extraction conditions of desirability is detailed in Table 8. In Figure 20 is represented the LC-DAD/ESI-MS chromatogram of *C. sativa* shell extracts obtained at 280 nm.

Table 8. Identification and quantification of the phenolic compounds present in *C. sativa* shell extracts prepared at 80 °C/10 min and 220 °C/30 min by LC-DAD/ESI-MS analysis.

Compound	RT (min)	$[M-H]^-$	mg/g dw
Sample 80ºC/10 min			
Gallocatechin dimer 1	5.2	609	0.14
DiHHDP-Glucose isomer 1	5.2	784	0.51
Gallic acid	6.1	169	1.36
Gallocatechin dimer 2	6.3	609	0.23
Prodelphinidin dimer 1	6.4	593	0.12
DiHHDP-Glucose isomer 2	6.5	784	0.65
HHDP-NHTP-Glucose isomer 1 (vescalagin/castalagin)	7.3	933	0.70
Prodelphinidin dimer 2	7.5	593	0.04
DiHHDP-Glucose Isomero 2	7.7	784	0.65
Gallocatechin	8.2	305	0.87
HHDP-NHTP-Glucose isomer 1 (vescalagin/castalagin)	8.3	933	0.92
Galloyl-Hidroxychebuloyl-HHDP-Glucose	8.3	969	0.90
Prodelphinidin dimer 3	8.6	593	0.18
Gallocatechin dimer 3	8.7	609	0.04
Prodelphinidin dimer 4	9.4	593	0.05
Chebuloyl DiHHDP-Glucose isomer 1	9.6	951	1.22
Digallic procyanidin dimer	10.0	881	0.13
Gallocatechin dimer 4	10.1	609	0.04
Chebuloyl DiHHDP-Glucose isomer 1	10.2	951	0.93
DiHHDP-Glucose isomer 2	10.5	784	0.58
Procyanidin dimer 1	10.8	577	0.14
Digallate Procyanidin dimer	11.6	881	0.04
Digalloyl-HHDP-Glucose isomer 1	11.9	785	0.47
Procyanidin dimer 2	12.4	577	0.03

Galloyl-DiHHDP-Glucose isomer 1	12.6	934	0.90
Procyanidin trimer	13.0	865	0.06
Catechin	13.6	289	0.56
Galloyl-DiHHDP-Glucose isomer 1	13.6	934	1.31
Digalloyl-HHDP-Glucose isomer 1	14.9	785	0.51
Epicatechin	16.6	289	0.05
Procyanidin dimer 3	18.6	577	0.02
Quercetin-3-glucoside	22.5	463	0.06
Ellagic acid	22.8	301	1.19
Quercetin-3-glucoside	26.6	477	0.03
Methyl-Galloyl-DiHHDP-Glucose isomer 1	28.8	949	1.47
Isorhamnetin-3-O-glucoside	30.2	491	0.02
Myricetin 3-glucoside	31.7	493	0.05
Procyanidin dimer 4	33.4	577	0.02
Sample 220ºC/30 min			
Gallic acid	6.0	169	0.54
Pyrogallol	7.8	126	2.39
Protocatechuic acid	8.3	153	2.83
Unknown	10.0	167	0.40
Unknown	10.1	179	0.47
Unknown	13.2	197	0.52
Methyl gallate	14.1	183	0.77
Unknown	15.8	193	0.51
Unknown	19.5	275	0.75
Ellagic acid	23.1	301	2.86
Unknown	26.8	275	0.47

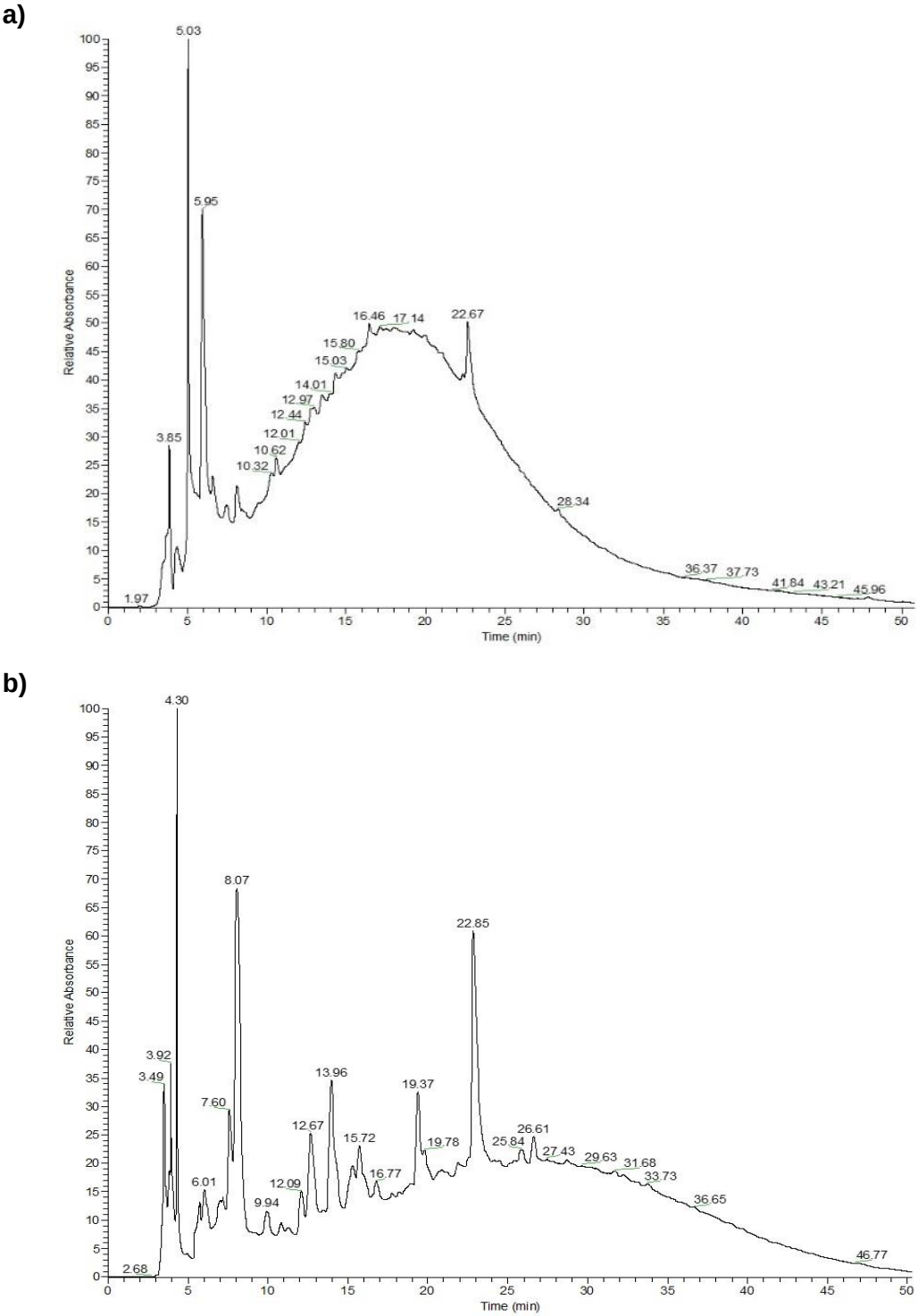

Figure 20. LC-DAD/ESI-MS chromatogram of *C. sativa* shell extracts obtained at 280 nm: a) extract prepared by SWE at 80 ºC/10 min; b) extract prepared by SWE at 220 ºC/30 min.

Although the RSM selected the extract prepared at 220 °C/30 min as the optimal, the phenolic profile of the extract prepared at 80 °C/10 min was also analysed based on the similarity of ABTS, DPPH, FRAP and TPC results for both extracts as well as the desirability index (R^2 = 0.5035). Likewise, the interest in a better understanding of the different phenolic

compounds present in these two extracts encouraged this analysis. A temperature of 80 °C in a SWE means that the water is not in the subcritical state, being extracted mainly polar compounds. Oppositely, water at 220 °C is in the subcritical state and might recover polar and nonpolar compounds. As shown in Figure 20 and Table 8, the extract prepared at 80 ºC/10 min exhibited a higher variety of polyphenols than the extract prepared at 220 ºC/30 min. The major class of polyphenols present in the extract obtained at 80 ºC/10 min was ellagitannins, a group of tannins derived from ellagic acid. Tannins are defined as "*naturally occurring water soluble polyphenolic compounds having a molecular weight between 500 and 3000, capable of precipitating alkaloids as well as gelatin and other proteins from aqueous solutions*" (106). After cellulose, hemicelluloses and lignins, tannins represent the most abundant compounds recovered from biomass (107). In addition, tannins are the second broadest source of polyphenols, after lignins (108). Concerning to the structural characterization, two main classes of tannins have been described: condensed and hydrolysable tannins. Condensed tannins are flavan-3-ol moieties extremely reactive and have the ability to self-polymerize, being classified as flavanols. The condensed tannins exclusively derived from (epi)catechins are defined as procyanidins, while tannins composed by (epi)fisetinidol, (epi)robinetinidol and (epi)gallocatechin units are classified as profisetinidin, prorobinetidin and prodelphindin, respectively. Moreover, procyanidins are the most abundant in plants. On the other hand, hydrolysable tannins are heteropolymers, including polyphenolic acids and derivatives, esterified to a polyol, such as β-D-glucose. Gallic and ellagic acids are the main phenolic acids found in the structure of hydrolysable tannins. Gallic acid moieties can generate other derivatives, namely hexahydroxydiphenic acid (HHDP) units (via oxidative coupling of at least two molecules). Otherwise, HHDP units might also lactonize to ellagic acid moieties through hydrolysis. Hydrolysable tannins have the ability to be cleaved into their basic units by hydrolysis, using weak acids or bases and generating gallic and ellagic acids. Therefore, hydrolysable tannins are classified into two major subclasses: gallotannins and ellagitannins. Gallotannins are composed by galloyl or digalloyl components attached to a polyol, whereas *e*llagitannins present at least one HHDP unit linked to a polyol. Two other types of tannins were already reported, namely complex tannins and phlorotannins. Complex tannins are formed by monomeric units of hydrolysable and condensed tannins, such as accutisim A (106).

According to Table 8, chestnut shell extract prepared at 80 ºC/10 min is particularly rich in tannins, whereas the extract obtained at 220 ºC/30 min is poor in these compounds. The absence of tannins in the extract prepared at 220 ºC/30 min is probably due to the degradation of these compounds at a temperature around 150 ºC (109). In SWE, the

temperature should be optimized taking into consideration the final application intended for the extract. Regarding tannins, high extraction temperatures could contribute to decrease its content (109). Mothé and de Freitas analysed the tannins profile of cashew gum and found three distinctive thermal regions: i) the first one ranged from 30.9 to 180 ºC; ii) the second one detected between 200 and 210 °C; and iii) the third one found from 380 to 600 °C that is the temperature range where the decomposition of compounds is complete (110). This study suggested that the complete degradation occurs around 240 °C (110). In a study performed by Grigsby and Gaugler, a thermogravimetric analysis was carried out in order to assess the relative thermal stability of condensed tannins and the thermal degradation behaviour of *Radiata pine* bark extracts (111). According to the results, the extracts with higher amounts of carbohydrates showed lower temperatures for degradation (approximately 150 °C) compared to the extracts without carbohydrate that had higher onset temperatures (111). Therefore, the purity and phytochemical profile of the extract rely on its final application. Thus, it is noteworthy that these parameters should be taken into consideration for the selection of the extraction temperature (111).

As shown in Table 8, gallic and ellagic acids were the only two polyphenols common to both extracts. Ellagic acid was present in higher amounts in the extract prepared at 220 °C/30 min (2.86 mg/g dw), while higher levels of gallic acid were detected in the extract prepared at 80 °C/10 min (1.36 mg/g dw). Only five phenolic compounds were accurately identified in the extract prepared at 220 °C/30 min. The major phenolic compounds in this extract were ellagic acid, pyrogallol and protocatechuic acid. The high amount of ellagic acid is possibly related to the thermal degradation of ellagitannins. Besides ellagic and gallic acids, the extract prepared at 220 °C/30 min also exhibited considerable levels of pyrogallol (2.39 mg/g dw) and protocatechuic acid (2.83 mg/g dw). Indeed, at high temperatures, gallic acid can generate pyrogallol via decarboxylation, being this compound a primary degradation product. On the other hand, the significant amounts of protocatechuic acid is probably due to the thermal decomposition of catechin, since protocatechuic acid is a catechin metabolite and heating might lead to the degradation of catechin into 3,4-dihydroxybenzaldehyde and protocatechuic acid (112).

In what concerns to the extract prepared at 80 °C/10 min, the different classes of polyphenols are present in the following order: tannins > phenolic acids > flavonoids > anthocyanins. A total of 37 phenolic compounds were identified in this extract. Tannins are the most abundant class of polyphenols, representing 12.49 mg/g dw from a total of 17.19 mg of phenolic compounds/g dw. Among the flavonoids, two subclasses were identified: flavanols (1.92 mg/g dw) and flavonols (0.16 mg/g dw). The main flavonoid identified in this

extract was catechin (0.56 mg/g dw), whereas the other flavonoids were present in low amounts. Moreover, this extract contained 0.82 mg/g dw of anthocyanins.

The chestnut shell extract prepared at 80 °C/10 min showed a similar phenolic profile to the ones reported by different authors, being rich in phenolic compounds and presenting a notable antioxidant activity (22, 28, 35). Tannins were indicated as the major class of polyphenols in these shells, particularly hydrolysable tannins that include ellagitannins and gallotannins (22, 28, 30, 35). Nevertheless, high amounts of ellagitannins, which form HHDP acid, were reported (22, 28, 30). According to Figure 20 and Table 8, these results are in line with the ones reported by Comandini *et al.*, revealing the presence of gallic acid, ellagic acid, castalin, castalagin, 1-*O*-galloyl castalagin, vescalin and vescalagin (30). In addition, commercial chestnut shell samples marketed for nutraceutical industry have a similar phenolic composition (30).

Based on the phenolic profile of the two extracts and the application intended in this study, the extract prepared at 220 °C/30 min was selected for the further experiments and to the incorporation on a cosmetic formulation due to its richness in polyphenols already described as effective cosmetic ingredients.

4.4 Reactive oxygen species scavenging capacity assays

The human organisms have several physiological mechanisms that play a key role in the production and depletion of ROS and RNS, such as cell signaling and homeostasis. The intervention of reactive species on living tissues highly contributes to the growing interest in the analysis of the scavenging ability of extracts against ROS and RNS. The production of reactive species occurs in cascade. The ROS primary source is $O_2^{\bullet -}$, being responsible for the production of other pro-oxidants such as H_2O_2. Indeed, H_2O_2 is one of the less reactive species. However, more powerful and toxic species are produced from ROS, including $HO^{\bullet}$ and HOCl (113). Table 9 summarizes the *in vitro* scavenging capacity of freeze-dried *C. sativa* shell extract prepared at 220 °C/30 min and positive controls against ROS.

Table 9. Superoxide anion radical ($O_2^{\bullet-}$), hypochlorous acid (HOCl) and peroxyl radical ($ROO^{\bullet}$) scavenging capacities of freeze-dried *C. sativa* shell extract. Values are expressed as mean ± standard deviation (*n* = 4).

	Reactive oxygen species			
	IC_{50} (µg/ml) *n* = 4		S_{sample}/S_{Trolox} [*a]	Trolox equivalents (µmol TE/mg dw)
	$O_2^{-\bullet}$	HOCl	$ROO^{\bullet}$	
C. sativa shell extract	12.92 ± 0.34	0.79 ± 0.06	0.084 ± 0.001	0.32 ± 0.01
Positive controls				
Catechin	48.99 ± 0.74	0.18 ± 0.01	7.592 ± 0.074	46.37 ± 0.20
Gallic acid	5.18 ± 0.19	1.25 ± 0.05	1.119 ± 0.005	5.94 ± 0.22

IC_{50} = *In vitro* concentration required to decrease in 50% the reactivity of the studied reactive species in the tested media (mean ± standard error of the mean).

[a] Results for $ROO^{\bullet}$ scavenging activity are expressed as slop ratio between samples or positive controls and Trolox. S_{sample} = slope of extract curves; S_{Trolox} = slope of the trolox curve.

The analysis of the results was carried out taking into consideration that low IC_{50} values indicate a high antioxidant activity. Among ROS studied, the extract exhibited the highest scavenging efficiency against HOCl (IC_{50} = 0.79 µg/mL). However, the scavenging power for $O_2^{\bullet-}$ was also considerably high (IC_{50} = 12.92 µg/mL).

The $O_2^{\bullet-}$ radicals were generated from dissolved oxygen by PMS/NADH coupling. The scavenging capacities of $O_2^{\bullet-}$ by chestnut shell extract and positive controls were determined through the reduction of NBT. As shown in Table 9, gallic acid displayed the highest ability to quench $O_2^{\bullet-}$ with an IC_{50} value of 5.18 µg/mL. Moreover, *C. sativa* shell extract exhibited a higher quenching power than catechin (IC_{50} = 48.99 µg/mL), but lower than gallic acid (IC_{50} = 5.18 µg/mL). According to Almeida *et al.* (114) and Diaz Reinoso *et al.* (115), *C. sativa* leaves extracts had a similar $O_2^{\bullet-}$ scavenging ability (IC_{50} values of 13.6 µg/mL and 15.1 µg/mL, respectively) to the shell extract analysed in this study. However, *C. sativa* shell extract showed a higher $O_2^{\bullet-}$ scavenging power than a coffee-like beverage prepared from *Quercus cerris* kernels (IC_{50} = 17.24 µg/mL) as reported by Pinto *et al.* (116).

Regarding the hypochlorous acid assay, catechin was the best scavenger (IC_{50} = 0.18 µg/mL), whereas gallic acid also showed a good ability to scavenge this radical (IC_{50} = 1.25 µg/mL). In contrast to superoxide assay, *C. sativa* shell extract had lower scavenging power of HOCl than catechin. However, the extract was a better quencher for this reactive species

than gallic acid. Moreover, the chestnut shell extract showed a significantly higher quenching capacity for HOCl than *C. sativa* leaves extract (IC_{50} = 63.8 µg/mL) and *Q. cerris* kernels beverage extract (IC_{50} = 9.25 µg/mL) (115, 116).

The ORAC assay was used to study the scavenging ability of chestnut shell extract against $ROO^{\bullet}$ based on the free radical damage to a fluorescent probe. The inhibition of free radical injuries as a result from the presence of antioxidants allows the preservation of the fluorescent signal. The results were expressed as a ratio between the slope of the AUC curve of extract or positive controls (S_{sample}) and the slope of the AUC curve of Trolox (S_{Trolox}) used as standard (S_{sample}/S_{Trolox}). Additionally, the results were also expressed as µmol of TE per mg of extract on dw (µmol TE/mg dw). The extract achieved a low quenching capacity against $ROO^{\bullet}$ with S_{sample}/S_{Trolox} lower than 1 (S_{sample}/S_{Trolox} = 0.084). Although this extract presented a low ability to quench $ROO^{\bullet}$, other studies reported no scavenging activity of the tested by-products for this radical (73, 113, 116). Notably, catechin exhibited the highest scavenging activity for $ROO^{\bullet}$ (S_{sample}/S_{Trolox} = 7.592), followed by gallic acid (S_{sample}/S_{Trolox} = 1.119). Concerning the results expressed as TE, this extract had lower quenching ability for $ROO^{\bullet}$ (0.32 µmol TE/mg dw) than *C. sativa* leaves as reported by Almeida *et al.* (1.24 µmol TE/mg dw) and Diaz Reinoso *et al.* (1.09 µmol TE/mg dw) (114, 115).

The chestnut shell extract prepared by SWE at 220 °C/30 min exhibited promising results on the scavenging ability against all tested ROS probably due to the phenolic profile, particularly the presence of ellagic acid, gallic acid, pyrogallol and protocatechuic acid, whose scavenging power has been described by several authors (117-120). Therefore, the chemical composition of *C. sativa* shells is possibly the first responsible for the obtained results (Figure 20; Table 8). Noteworthy, the presence of different compounds in this by-product might contribute to an increase on the scavenging power observed.

4.5 Effects of *C. sativa* shell extract towards fibroblasts and keratinocytes

Considering the final application intended, it is extremely important to evaluate the effects of the extract on skin cell lines. Two skin cell lines, keratinocytes (HaCaT) and fibroblasts (HFF-1), have been selected since they are the most representative skin cells. The results of cell viability evaluated by MTT assay are shown in Table 10.

Table 10. Effects of *C. sativa* shell extract prepared at 220 °C/30 min by SWE on the cell viability of fibroblasts and keratinocytes at a range of concentrations between 0.1 and 1000 µg/mL (mean ± standard deviation, *n* = 6).

Concentration (µg/mL)	Fibroblasts	Keratinocytes
Control	100.00 ± 18.00	101.04 ± 3.43
0.1	106.72 ± 24.46	82.70 ± 15.23
1.0	102.95 ± 1.92	77.52 ± 11.35
10.0	105.16 ± 20.93	77.92 ± 9.77
100.0	100.45 ± 7.24	73.17 ± 15.28
1000.0	99.66 ± 19.83	57.32 ± 11.92*

* indicate significant differences ($p<0.05$).

As shown in Table 10, it is noteworthy to conclude that *C. sativa* shell extract did not decrease the fibroblasts viability after exposure to all tested concentrations (0.1 – 1000 µg/mL), displaying a viability higher than 90% at the highest concentration tested (1000 µg/mL). Indeed, no significant differences ($p>0.05$) between the different concentrations tested were observed. On the other hand, the HaCaT viability significantly decreased ($p<0.05$) after exposure to the highest concentration of extract. Taking into account the results obtained for both cell lines, an optimal range of non-cytotoxic concentrations up to 1000 µg/mL and 100 µg/mL was selected for HFF-1 and HaCaT cells, respectively. These results are in accordance with Squillaci *et al.* that selected the optimal range of concentrations for HaCaT cells (below than 0.01%) after exposure to *C. sativa* shell aqueous extract (35). Pinto *et al.* also reported similar results on HaCaT cells for *C. sativa* bur hydro-alcoholic extract, selecting a range of concentrations from 0.1 to 100 µg/mL as safe for this cell line (105). However, at 1000 µg/mL the extract led to a decrease for 57.07% on cell viability (105). As shown in Table 10, the extract at 1000 µg/mL prepared in the present study also conducted to a cell viability of 57.32% in keratinocytes (105). In addition, *C. sativa* leaves hydro-alcoholic extract was more toxic for HaCaT cells than the extract prepared in this work, since the cell viability for a concentration of 100 µg/mL was lower than 50% (121).

4.6 Characterization of *C. sativa* shell hydrogel

4.6.1 Analysis of organoleptic properties

The organoleptic properties of the hydrogel containing *C. sativa* shell extract prepared by SWE at 220 °C/30 min were evaluated after 1, 7 and 30 days of the preparation. Table 11 summarizes the organoleptic features of the hydrogel at different times and temperatures.

Table 11. Evaluation of organoleptic properties of the hydrogel containing *C. sativa* shell extract as active ingredient after 1, 7 and 30 days of preparation.

Organoleptic properties	**Hydrogel containing *C. sativa* shell extract**				
	1 day (T1)	**7 days/25 °C (T7/25 °C)**	**7 days/40 °C (T7/40 °C)**	**30 days/25 °C (T30/25 °C)**	**30 days/40 °C (T30/40 °C)**
Photographic record					
Colour	Brown	Brown	Brown	Brown	Dark brown
Odour	Fruity	Fruity	Fruity	Fruity	Odorless
Appearance	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Heterogeneous (with lumps)

T1, after 1 day of preparation. T7/25 °C, after 7 days of preparation at 25 °C. T7/40 °C, after 7 days of preparation at 40 °C. T30/25 °C, after 30 days of preparation at 25 °C. T30/40 °C, after 30 days of preparation at 40 °C.

The hydrogel presented a typical brown colour, fruity odour and homogeneous appearance. The formulation showed similar organoleptic properties after 1 and 7 days of storage at both temperatures as well as after 30 days of storage at 25 °C. These results suggest the stability of the hydrogel at these storage conditions. Although the hydrogel showed similar organoleptic properties at T30/25 °C, it was observed distinctive organoleptic features in colour, odour and appearance after 30 days of storage at 40 °C (T30/40 °C), exhibiting a darker brown colour, heterogeneous appearance with apparent lumps, and odourless. The presence of lumps and heterogeneous appearance are probably due to the loss of water from the gel structure at 40 °C. Shawesh *et al.* also described a darker colour in Carbopol® hydrogels stored at 45 °C (122). Taking into consideration that the consumers prefer cosmetics with warm colours and mild odours (123, 124), the hydrogel containing *C. sativa* shell extract showed favorable organoleptic properties which might contribute to a good consumer acceptance. In addition, according to the results shown in Table 10, the storage of the formulation at 25 °C seems to be the best option to preserve the organoleptic properties. However, it will be important to evaluate the hydrogel stability for a longer storage time, for example 3 or 6 months.

4.6.2 Colour evaluation

Colour evaluation is a complementary technique employed to analyse the consumer acceptance of a cosmetic product. This technological parameter was evaluated at different storage times and temperatures. Table 12 summarizes the results of the colour (for each coordinate of colour system CIE L*a*b*) of the hydrogel containing *C. sativa* shell extract.

Table 12. Colour variation (L*, a* and b*) of the hydrogel containing *C. sativa* shell extract evaluated at different times and temperatures (mean ± standard deviation, *n* = 3).

Colour	**Hydrogel containing *C. sativa* shell extract**				
	1 day (T1)	**7 days/25 °C (T7/25 °C)**	**7 days/40 °C (T7/40 °C)**	**30 days/25 °C (T30/25 °C)**	**30 days/40 °C (T30/40 °C)**
L* (Lightness)	28.18 ± 1.20	28.41 ± 0.63	27.71 ± 0.86	29.31 ± 0.84	20.84 ± 1.47*
a* (Redness)	17.63 ± 0.69	17.18 ± 1.23	17.42 ± 0.79	18.30 ± 0.99	12.21 ± 0.33*
b* (Yellowness)	20.63 ± 1.32	19.97 ± 1.07	19.63 ± 1.22	22.30 ± 2.11	7.01 ± 0.91*

Concerning to L* parameter, the hydrogel showed low lightness at T1 (28.18). Relatively to a*, the formulation exhibited positive values (17.63) associated to redness. Likewise, the hydrogel also showed positive results for b* associated to the prevalence of

yellowness (20.63). No significant changes (p>0.05) were observed for the three coordinates of colour (L*, a* and b*) at T1, T7 for both temperatures, and T30/25 °C, suggesting the formulation colour stability at these storage conditions. However, significant changes (p<0.05) were observed at T30/40 °C in all colour coordinates, indicating a major influence of the temperature on colour. The hydrogels at T30/40 °C had a lower lightness, supporting the darker brown colour identified in the analysis of organoleptic properties.

Pinto *et al.* prepared hydrogels containing different percentages of *C. sativa* bur hydro-alcoholic extract, including one formulation with 50% of extract that corresponds to the same percentage incorporated in the hydrogel prepared in this work (100). The hydrogels with 50% of chestnut bur extract showed lower lightness (10.31) as well as lower prevalence of redness (0.43) and yellowness (3.03) compared to the hydrogel developed in this study. In Pinto *et al.* study, it was also prepared and characterized a base formulation (Carbopol® hydrogel without extract) with the same composition than the one prepared in this study. The base formulation showed similar results for L* and b*, but negative values for a* associated to the prevalence of greenness (100). Another study about a hydrogel containing *C. sativa* leaf extract as active ingredient showed considerably higher lightness (around 65), similar results for b* (around 20), and negative results for a* (around -4) that indicate the prevalence of greenness (125).

4.6.3 Determination of pH

The pH of the hydrogel containing *C. sativa* shell extract was analysed at different times and temperatures. The results are summarized in Table 13.

Table 13. pH of the hydrogel containing *C. sativa* shell extract evaluated at different times and temperatures (mean ± standard deviation, n = 3).

	Hydrogel containing *C. sativa* shell extract				
	1 day (T1)	**7 days/25 °C (T7/25 °C)**	**7 days/40 °C (T7/40 °C)**	**30 days/25 °C (T30/25 °C)**	**30 days/40 °C (T30/40 °C)**
pH	5.49 ± 0.05	5.54 ± 0.04	5.57 ± 0.07	5.57 ± 0.08	5.53 ± 0.06

The skin pH ranged between 4.2 and 5.6. The pH of the hydrogel containing chestnut shell extract was 5.49, being compatible with the skin pH. No significant differences (p>0.05) were observed on pH at different times and temperatures. Therefore, in what concerns to pH, the stability of the hydrogel was ensured and the formulation might be considered as suitable for cutaneous application. The pH of *C. sativa* shell extract was slightly lower and more acidic (4.78). According to Pinto *et al.*, the hydrogel containing 50% of *C. sativa* bur

extract showed a similar pH (5.67) to the formulation developed in this study, being also suitable for skin application (100). A study performed by Almeida *et al.* presented considerably lower pH values (4.73 – 4.76) for a hydrogel containing chestnut leaf extract, but this formulation was also compatible with skin pH (125).

4.6.4 Texture analysis

A suitable texture of the skin care formulation highly contributes to improve the consumer acceptance. The texture analysis allowed the evaluation of the firmness and adhesiveness of the hydrogel containing *C. sativa* shell extract. Table 14 summarizes the results of firmness and adhesiveness at different times and temperatures.

Table 14. Firmness and adhesiveness of the hydrogel containing *C. sativa* shell extract evaluated at different times and temperatures (mean ± standard deviation, *n* = 3).

Texture	**Hydrogel containing *C. sativa* shell extract**				
	1 day (T1)	**7 days/25 °C (T7/25 °C)**	**7 days/40 °C (T7/40 °C)**	**30 days/25 °C (T30/25 °C)**	**30 days/40 °C (T30/40 °C)**
Firmness (N)	0.088 ± 0.010	0.088 ± 0.003	0.098 ± 0.005	0.088 ± 0.004	0.209 ± 0.017*
Adhesiveness (N/mm)	-0.550 ± 0.062	-0.551 ± 0.028	-0.574 ± 0.023	-0.542 ± 0.011	-0.778 ± 0.113*

The firmness value was 0.088 N and the adhesiveness was -0.550 N/mm. No significant differences (p>0.05) were observed at T1, T7 for both temperatures, and T30/25 °C. On the other hand, the hydrogel at T30/40 °C showed significant differences (p<0.05) in these parameters compared to the hydrogel analysed at other times and temperatures. As shown in Table 12, a considerable increase of firmness (0.209 N) and adhesiveness (-0.778 N/mm) were observed in the hydrogel at T30/40 °C. Pinto *et al.* reported higher firmness (0.554 N) and adhesiveness (-1.480 N/mm) for the base formulation (100). These authors also obtained higher firmness (0.367 N) and adhesiveness (-0.932 N/mm) for the hydrogel containing 50% of *C. sativa* bur extract (100). Likewise, Almeida *et al.* described a significantly higher firmness (4.78 – 5.26 N) for a hydrogel containing *C. sativa* leaf extract (125). The graphics of force *versus* distance and force *versus* time are presented in the Annexes 1 and 2, respectively.

4.6.5 Rheological behaviour analysis

4.6.5.1 Viscosity

Viscosity is an important parameter to evaluate the stability of formulations intended to skin application (126, 127). This parameter can be described as the resistance that a product offers to deformation or flow and varies with the shear *stress*. The physicochemical properties and temperature might affect the viscosity of a formulation (104, 112). Based on the rheological behaviour, there are two types of systems: Newtonians and non-Newtonians. A constant viscosity independent of the shear rate characterizes a formulation with Newtonian behaviour, while non-Newtonians formulations are characterized by a variation of viscosity with shear rate, being known as apparent viscosity (113, 114). The non-Newtonian formulations can be classified as pseudoplastic (or shear thinning) and dilatant (or shear thickening). In the pseudoplastic formulations, the apparent viscosity decreases with the increase of shear rate, while the dilatant formulations show an increase of apparent viscosity with the increase of shear rate. Figure 21 represents the relationship between shear viscosity (Pa.s) and shear rate (s^{-1}) of the hydrogel containing *C. sativa* shell extract at different times and temperatures.

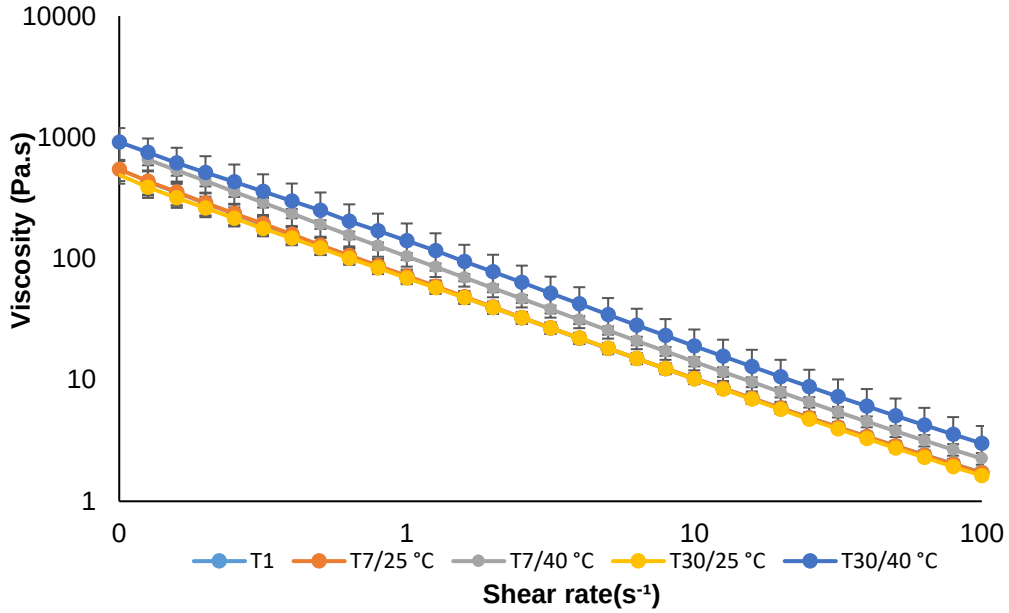

Figure 21. Graphic that represents the variation of shear viscosity (Pa.s) of the hydrogel containing *C. sativa* shell extract with the shear rate, at different times and temperatures (mean ± standard deviation, *n* =3).

As shown in Figure 21, the viscosity decreased with the increase of shear rate. Therefore, the hydrogel have a non-Newtonian behaviour, particularly pseudoplastic. The skin care formulations with pseudoplastic behaviour favor the skin application, since the

force needed to promote the spreading of the formulation is relatively low and decreases when it is spread into the skin. Nevertheless, this rheological behaviour is similar to gels developed with acrylic polymers (128). Almeida *et al.* and Pinto *et al.* described a similar rheological behaviour in hydrogels containing *C. sativa* leaf and bur extracts, respectively (100, 125). In this study, the formulation showed this rheological profile for all temperatures and times analysed. In addition, the hydrogel at T30/40 °C showed the highest viscosity, closely followed by the formulation analysed at T7/40 °C. A similar viscosity was observed at T1, T7/25 °C and T30/25 °C. Temperature seems to influence the viscosity of the hydrogel since at high temperatures (40 °C) the formulation was more viscous. Otherwise, time did not lead to significant changes in viscosity. Figure 22 represents graphically the variation of viscosity (Pa.s) of the hydrogel containing *C. sativa* shell extract, at different times and temperatures, at shear rates of 1, 10 and 100 s^{-1}.

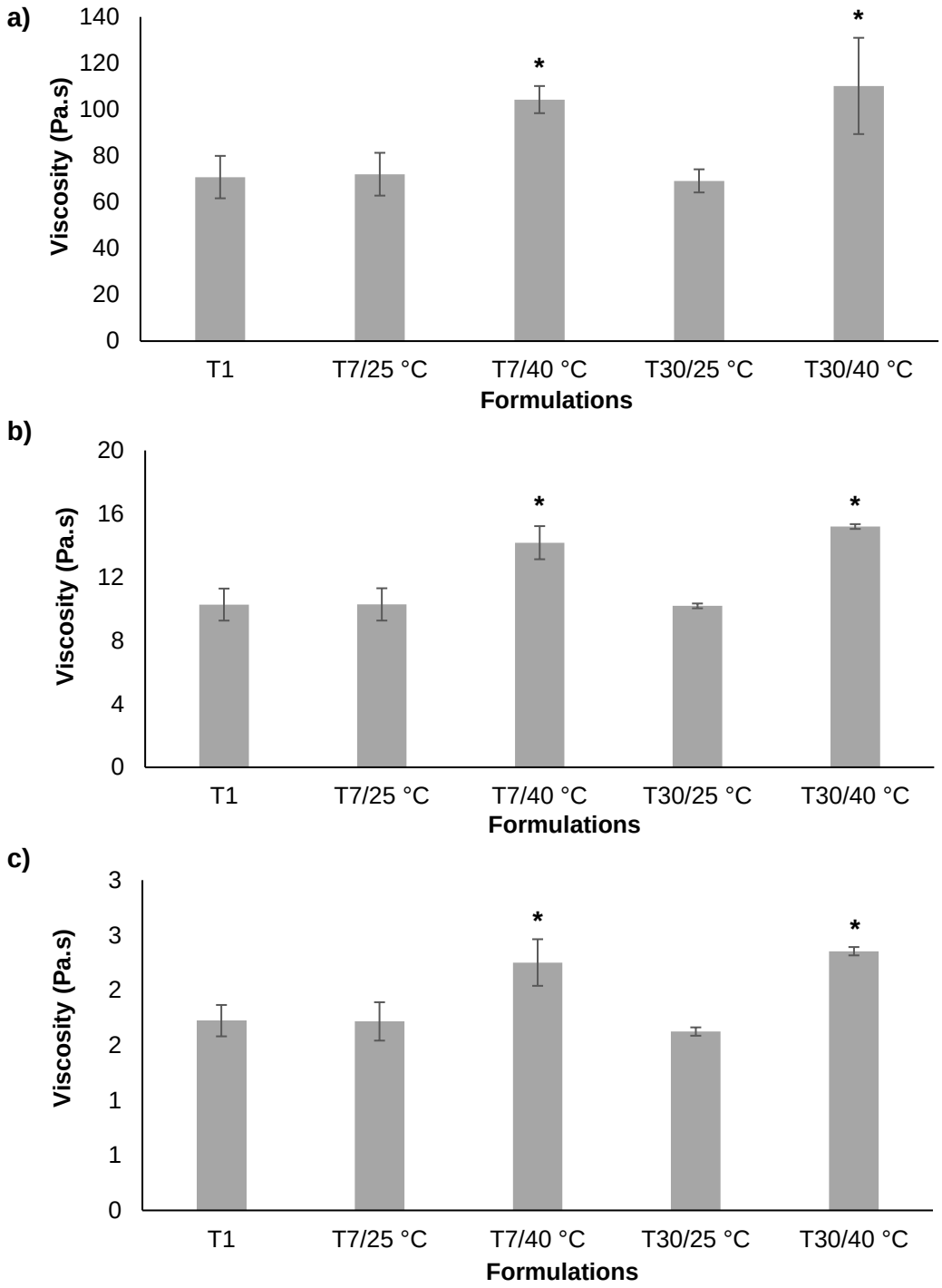

Figure 22. Graphics that represent the variation of viscosity (Pa.s) of the hydrogel containing *C. sativa* shell extract, at different times and temperatures: a) at shear rate of 1 s^{-1}; b) at shear rate of 10 s^{-1}; c) at shear rate of 100 s^{-1} (mean ± standard deviation, n = 3). * indicate significant differences (p<0.05).

According to Figure 22, the viscosity of the hydrogel at T1, T7/25 °C and T30/25 °C was similar for the three shear rates analysed, suggesting that the formulation was stable regarding viscosity during storage time at 25 °C. However, the hydrogel at T7/40 °C and T30/40 °C had a significantly (p<0.05) higher viscosity for all shear rates. Therefore, the storage of the hydrogel at 40 °C increased the viscosity, which might be due to the loss of water at high temperatures.

4.6.5.2 Analysis of thixotropy

Thixotropy is characterized by a reversible structural transition induced by changes on apparent viscosity, being considered a shear thinning property and time-dependent phenomenon. The decrease of apparent viscosity (or the corresponding shear *stress*) over time describes a formulation with thixotropic characteristics. Thixotropy is characterized by two stages: fluidification (under high shear rates) and stiffening (in rest or at low shear rates). The microstructure of a gel with thixotropic behaviour responds to the flow, becoming more fluid after application of a force and break down by deformation. However, when the formulation returns to the rest, it recovers the initial viscosity. On the other hand, the anti-thixotropy is characterized by an increase of the apparent viscosity after the application of a force. Besides the non-Newtonian behaviour, most of skin care formulations also have thixotropic characteristics. Figure 23 represents the relationship between shear viscosity (Pa.s) and time (s) of hydrogel containing *C. sativa* shell extract deformation.

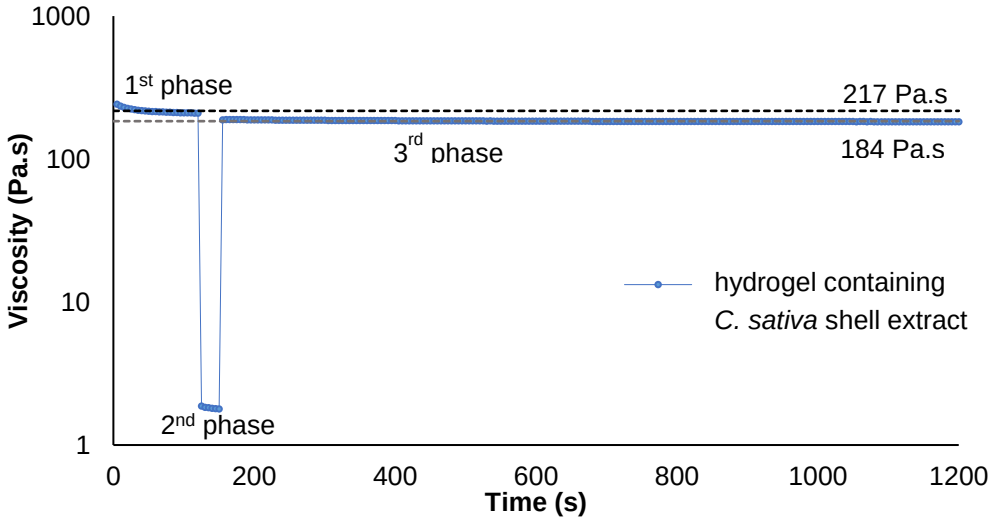

Figure 23. Graphic that represents the variation of the viscosity (Pa.s) with time of deformation (s) of the hydrogel containing *C. sativa* shell extract, at a shear rate from 0.1 to 100 and returning to 0.1 s^{-1}.

In Figure 23, three distinctive phases are observed. In the first phase, the hydrogel containing the *C. sativa* shell extract was slightly disturbed by the rotational movements of one of the two parallel plates, rotating at shear rate of 0.1 s^{-1}, since the viscosity maintained almost constant. Afterwards, in the second phase, the viscosity drastically decreased with the increase of the shear rate to 100 s^{-1}, becoming more fluid. After the shear rate decreased back to 0.1 s^{-1}, in the third phase, the formulation recovered 85% of its initial viscosity. Based on the Figure 23, it is possible to conclude that the hydrogel presented thixotropic behaviour. The skin care formulations with thixotropic behaviour allow an easier spreading of the product on skin and the recovery of the initial viscosity.

4.6.5.3 Yield *stress*

The yield *stress* is the minimal tension required to induce a plastic deformation without a significant increase in load. In other words, the yield *stress* of a formulation corresponds to the resistance that needs to be overcome in order to start the flow of the material. This concept is commonly related to the strength of the material inner structure. Table 14 presents the yield *stress* determined at T1.

Table 15. Yield *stress* of the hydrogel containing *C. sativa* shell extract determined at T1 (mean ± standard deviation, *n* = 3).

	Hydrogel containing *C. sativa* shell extract
	1 day (T1)
Yield *stress* (σ) (Pa)	12.43 ± 0.51

The yield *stress* of the hydrogel containing *C. sativa* shell extract was 12.43 Pa. This result was significantly lower than others reported for Carbopol® hydrogels (125, 129). A study performed by Shafiei *et al.* achieved a considerably higher yield *stress* (129). However, the Carbopol® concentration was four to six times higher than the one used in the hydrogel developed in this work. For a concentration of 2% of Carbopol®, the yield *stress* ranged from 61.7 to 273.1 Pa, while the hydrogels with 3% of Carbopol® had a yield *stress* between 146.9 and 348.6 Pa. This research suggested that Carbopol® concentration and pH might affect the yield *stress* of the hydrogel (129). Therefore, the lower yield *stress* obtained for the hydrogel developed in this study might be related to the lower Carbopol® concentration (0.5%) used. In another study, Almeida *et al.* also reported a higher yield *stress* (101.34 Pa at time 0) for a hydrogel containing *C. sativa* leaf extract prepared with 1% of Carbopol® (125).

5 Future perspectives

Besides the diversity of interesting bioactive compounds and therapeutic effects, the safety, stability, and quality of topical formulations incorporating *C. sativa* shell extracts need to be guaranteed. The main concern regarding the use of food by-products as active ingredients for different purposes is toxicity. Indeed, *in vitro* and *in vivo* assays should be carefully performed to evaluate the bioavailability and toxicity of food by-products. For instance, food by-products, particularly chestnut shells, are rich in antioxidants with promising applications in the cosmetic field such as antiaging ingredients. The European legislation applied to cosmetics (Regulation (EC) No. 1223/2009) is extremely restrictive and forbids the animal tests not only for cosmetic ingredients but also for final products. Likewise, new *in vitro* models are employed as alternatives to evaluate the safety of novel ingredients. The Scientific Committee on Consumer Safety (SCCS) describe the parameters that should be analysed in a new cosmetic ingredient: 1) acute toxicity; 2) corrosivity and irritation; 3) skin sensitization; 4) dermal/percutaneous absorption; 5) repeated dose toxicity; 6) reproductive toxicity; 7) mutagenicity/genotoxicity; 8) carcinogenicity; 9) toxicokinetics studies; 10) photo-induced toxicity; and 11) human data. For these reasons, the use of valuable natural compounds are substantially limited. Although cosmetic industry has demonstrated a huge interest for "green" formulations in the last decade, it is also important to clarify the efficacy of "green" formulations compared to others, for example in the skin photoprotection. This study encourages the opening of new fields of research for *C. sativa* shells, particularly in what concerns to cosmetic purposes since it guarantees the safety of this extract for skin cell lines. The reuse of *C. sativa* shell extracts as an active ingredient for skin care formulations comprises a great challenge for cosmetic industry. The increasing interest of consumers for natural products, the multiplicity of bioactive compounds isolated from shells, as well as the safety, interesting composition and remarkable biological activities are the main advantages for the valorization of this agro-residue as a cosmetic ingredient. On the other hand, some possible concerns must be clarified including the guarantee of a regular supply of this by-product to industries interested in its reuse as well as the selection of an eco-friendly extraction methodology, using non-toxic solvents. However, there are just few studies published about this crop waste and many doubts still need to be answered. *In vivo* studies, such as patch test and cell viability studies in skin 3D models must be performed in further researches to confirm the safety and efficacy of this extract as a cosmetic ingredient.

Phenolic compounds is one of the major class of antioxidants present in shells. However, the functionality and aesthetic properties need to be ensured after their incorporation in topical formulations and their applicability to humans has faced limited success. Polyphenols are very sensitive to environmental factors, such as light or oxygen,

being easily oxidizable, responsible for the progressive appearance of a brown colour and unwanted odours. Moreover, these bioactive compounds in cosmetic formulations might present a considerable loss of activity and low permeability. A limited water solubility in free form and low diffusion across barriers in glycosylated form are also some concerns related to polyphenols. Therefore, the *in vitro* release of polyphenols from formulation to the skin should be analysed. The low release of polyphenols might be solved by the use of novel technologies (e.g. encapsulation) that have been developed to overcome the sensitivity of natural compounds to environmental factors, as well to enhance the stability, increase the bioavailability and skin permeation, maintain the structural integrity, avoid incompatibilities, and provide the transport toward the physiological target. Another solution to overcome the solubility and bioavailability issues consists in the isolation of bioactive compounds using green extraction techniques employed to improve the extraction yields or to isolate specific compounds. In addition, the extraction methodology must be sustainable and easy to implement on an industrial scale in order to encourage industries to reuse this agro-residue. An industrial scale-up might be studied in order to investigate the viability of the process at large scale in economic terms. In addition, sensorial analysis of formulations containing chestnut shell extract should be carried out to evaluate the preferences of the market and consumers.

Overall, the scarcity of studies about cosmetics containing *C. sativa* shell extracts encourages further studies regarding their benefits for human skin. In the next years, to the best of our knowledge, formulations containing *C. sativa* shell extracts should be developed and proved their benefits in skin oxidative *stress*-related disorders based on the antioxidant activity already described for the extract.

6 Conclusion

The present work provided scientific evidences that support the valorization and reuse of *C. sativa* shell extract, obtained by an eco-friendly extraction technique – Subcritical Water Extraction - for cosmetic purposes. Chestnut is one of the most appreciated fruits in Europe, generating high amounts of by-products during processing, particularly shells Although *C. sativa* shell is an undervalued by-product, the challenge to discover promising applications for this agro-residue has gained more attention in the last few years due to its richness in bioactive compounds (e.g. polyphenols, vitamin E, amino acids). Since these bioactive compounds have a well-documented application in the cosmetic field, the interest on the valorization of this by-product for different industries, particularly cosmetic, has aroused, representing a solution with high economic value, particularly in Mediterranean countries that produce huge amounts of shells. Moreover, cosmetic industry is considered a field on the rise, constantly searching for novel active ingredients, mainly from natural sources, and encouraging the development of innovative formulations using agro-industrial wastes.

The optimal extraction conditions for *C. sativa* shells were determined by RSM as 220 °C/30 min based on the response surface profiles of ABTS, FRAP, DPPH and TPC. A considerable decrease on TPC and antioxidant activity was observed at 150 °C which could be related to the degradation of tannins and formation of other reaction products (e.g. pyrogallol and protocatechuic acid). The phenolic profile of the extracts prepared at 80 °C/10 min and 220 °C/30 min was analysed through LC-DAD/ESI-MS due to the similar results obtained in the previous assays. Only two phenolic compounds, gallic and ellagic acids, were common to both extracts. A higher variety of polyphenols and huge levels of tannins were detected in the extract prepared at 80 °C/10 min. The extract prepared at 220 °C/30 min was selected for further experiments based on their preferable phenolic profile, particularly ellagic acid, gallic acid, protocatechuic acid and pyrogallol, taking into consideration the final application intended. The highest scavenging efficiency was observed for HOCl, while the safety of this extract in HaCaT and HFF-1 cells was observed until a concentration of 100 and 1000 µg/mL, respectively. The chestnut shell extract was then incorporated in a hydrogel base. The pH of the hydrogel was compatible with the skin pH and the technological properties were suitable for skin application. The hydrogel was stable during 30 days at 25 °C.

Overall, this work supports the reuse of *C. sativa* shell extract as a useful active ingredient for skin care formulations due to its richness in bioactive compounds and pro-health properties, namely the prevention and treatment of skin oxidative *stress*-mediated disorders. The reuse of this by-product highly contributes to the profitability of agro-industries and, simultaneously, to the environmental and economic sustainability.

To the best of our knowledge, this is the first study that provides a comprehensive evaluation of the applicability of *C. sativa* shell extract in a cosmetic formulation using SWE as extraction technique.

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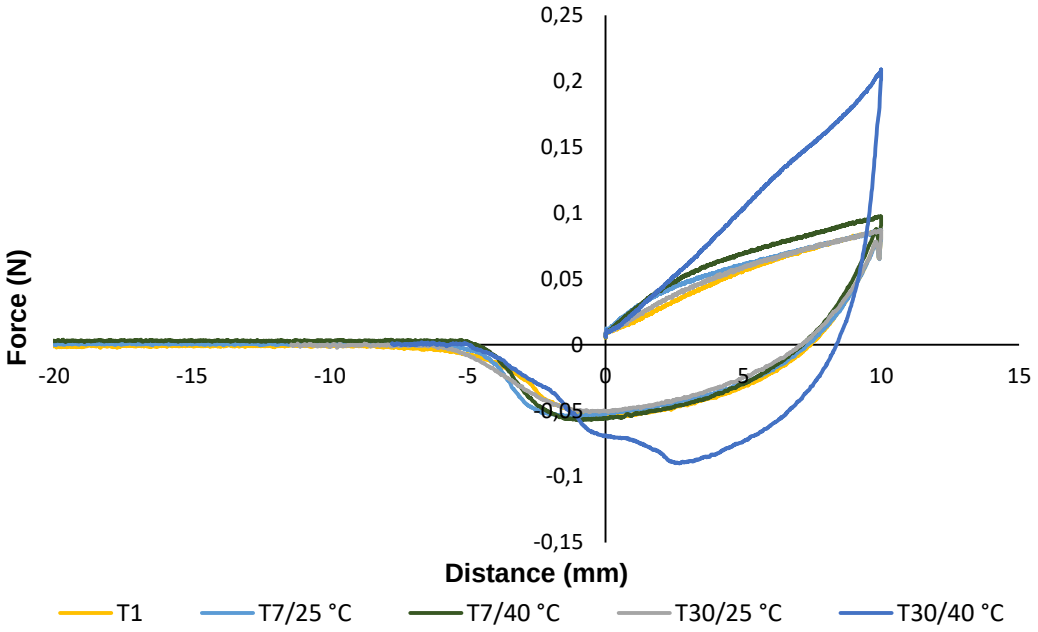
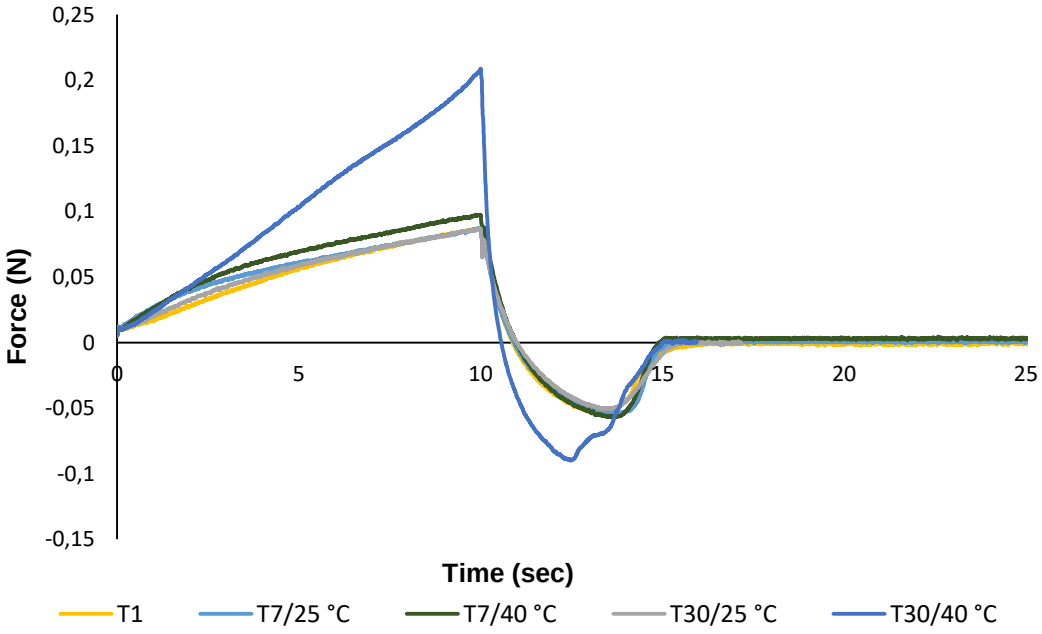
8 Annexes



Annex 1. Graphic force (N) *versus* distance (mm) plotted from the results obtained in the texture analysis.



Annex 2. Graphic force (N) *versus* time (sec) plotted from the results obtained in the texture analysis.

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