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Applied Biochemistry

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Industrial Biochemistry I

- Enzymes in cosmetic industry

อุตสาหกรรมเครื่องสำอาง มีการหาวิธีการสังเคราะห์แบบใหม่มาทดแทนวิธีทางเคมี เพราะว่า

- Environmental safety
- Sustainability ยั่งยืน
- สังคมสนใจผลิตภัณฑ์ที่เป็น natural และ chemical-free

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- Biocatalysis vs chemical route

การเร่งปฏิกิริยาทางชีวภาพ มีข้อดีกว่าวิธีเคมี เพราะ

- Process simplification กระบวนการง่ายขึ้น
- Quality of product
- ลดการเกิดของเสีย (waste)

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Lipases

- Lipases are the most commonly used enzymes in cosmetic industry.
- สามารถจดจำ (recognize) substrates ได้กว้างขวาง หลากหลาย
- เร่งปฏิกิริยาต่าง ๆ ได้มากมาย

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Beauty market

- แบ่งออกได้เป็น 5 ส่วน

1. Skincare
2. Hair care
3. Color (makeup)
4. Fragrances
5. Toiletries

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Esters

Esters เป็นสารอินทรีย์ที่ใช้มากที่สุดใน cosmetics เช่น

- Emollients ในครีม สร้างความชุ่มชื้นแก่ผิว
- Surfactants ในแชมพู (ลดแรงตึงผิว)
- Antioxidants ใน anti-aging creams
- Fragrances ในน้ำหอม (perfumes)
- Flavors ใน lip cosmetics

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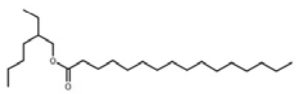
Emollient esters

เป็น ester ที่ใช้วิธี biocatalysis มากที่สุด

- การสังเคราะห์ 2-ethylhexyl palmitate



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Biocatalytic process

วิธีการทางเคมี ใช้

- อุณหภูมิสูง 150-240 °C
- ใช้ตัวเร่งที่เป็นกรดหรือเบส
- ทำให้เกิด poor quality products
- ไม่พึงประสงค์ในการใช้กับผิวหนัง
- สิ้นเปลืองค่าใช้จ่ายในการ treatment

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Biocatalytic process

วิธีการทางเอนไซม์ ใช้
- อุณหภูมิต่ำ 30-70 °C
- ความดันต่ำ
- ได้สารที่มีความบริสุทธิ์สูง (ultrapure)
- ไม่มีสี (colorless)
- ไม่มีกลิ่น (odorless)
- จัดเป็นผลิตภัณฑ์ 'green' ผู้บริโภคยอมรับ

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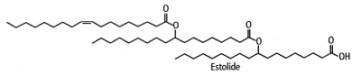
Isopropyl ricinoleate

วิธีการดั้งเดิม ใช้อุณหภูมิสูง
- ทำให้เกิด estolides เป็น by-product



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Isopropyl ricinoleate

การสังเคราะห์โดยใช้เอนไซม์
- ใช้ lipase จาก *Candida antarctica*
- ได้ yield มากกว่า 90% ที่อุณหภูมิ 40 °C
- ไม่เกิด estolide
- ใช้เป็นสารปรับสภาพผิว (skin conditioning agent) หรือ emollient

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Biosurfactant esters

ผลิตโดยใช้เอนไซม์หรือจุลินทรีย์
- เป็นสาร amphipathic
- ใช้เอนไซม์เร่งปฏิกิริยาแทนวิธีทางเคมี
- ใช้จุลินทรีย์โดยวิธีการหมัก (fermentation)
- วิธีการหมัก มักใช้กับวัสดุทางการเกษตร
- เป็นสาร glycolipid ประเภท carbohydrate fatty acid ester
- ใช้ใน อาหาร เครื่องดื่ม เครื่องสำอางและยา

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Biosurfactant esters

ในอุตสาหกรรมเครื่องสำอาง
- เป็น emulsifiers
- เป็นสาร foaming agents
- ที่ใช้มากคือ sorbitan esters และ sucrose esters
- Galactose oleate สังเคราะห์โดยใช้เอนไซม์ Lipozyme RM IM

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Industrial Biochemistry II

- Cutinases in industrial application
- cutinases เรียกอีกชื่อว่า cutin hydrolases
- รหัส EC 3.1.1.74
- พบครั้งแรกจากเชื้อราโรคพืช สามารถใช้ cutin เป็น sole C source
- cutin เป็น biopolymer เชิงซ้อน ประกอบด้วย hydroxy และ epoxy fatty acid
- cutin เป็นองค์ประกอบของ cuticle ในพืชชั้นสูง

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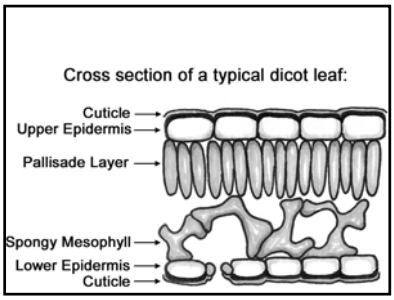
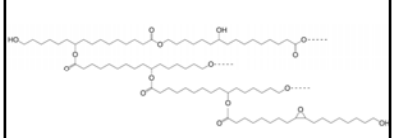


โครงสร้างทั่วไปของ cutin



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- cutinases มีสมบัติเป็นทั้ง lipases และ esterases
- active แม้จะมีลักษณะเป็น oil-water interface
- น่าสนใจที่จะนำมาประยุกต์ในอุตสาหกรรม
- เร่งปฏิกิริยาได้ทั้งแบบ hydrolysis, esterification และ trans-esterification

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- cutinases มีความเสถียรในตัวทำละลายอินทรีย์ และใน ionic liquid
- สามารถประยุกต์ในอุตสาหกรรมหลายชนิด เช่น

Food industry
Cosmetics
Fine chemicals
Pesticide and insecticide degradation
Treatment and laundry of fiber textiles
Polymer chemistry

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ตัวอย่างรายงานการประยุกต์ cutinase

TABLE 4.1 Reported applications of cutinase in biocatalysis

Publication Number	Publication Date	Author	Name	Reference
WO8809367	01-12-1988	Kolattukudy *et al.*	Methods and compositions regarding the use of cutinase in industrial cleaning processes	European Patent Office
JP3088897	15-04-1991	Enzaseido *et al.*	Method for the utilization of lipases, cutinases, and surfactants in cleaning processes	
NZ337239	28-09-2001	Reinhard and Henrik	Method for the enzymatic degradation of biodegradable polymers	
EP1694903	30-08-2006	Cavaco-Paulo *et al.*	Method for surface modification of polyacrylonitrile and polyamide fibers	
JP2005058228	10-03-2005	Yoshinobu *et al.*	Method for the production of esters in the absence of organic solvents	
CA 2480912	23-10-2003	Salmon *et al.*	Method intended to increase the tensile strength and abrasive ability of cotton fibers	Canadian Patents Database
CA 1262860	14-11-1989	Yuichi *et al.*	Method to enhance the effect of agricultural pesticides	
CA 2060510	10-01-1991	Ayrookaran *et al.*	Method to increase the permeability of fruits and vegetables surface	
CA 2465250	15-05-2003	Shi *et al.*	Method to remove the excess dye in industrial textile dyeing processes	

A. Oil and dairy products
- ปฏิกิริยา trans-esterification ปรับสภาพของไขมันหรือน้ำมันให้เหมาะสมกับอุตสาหกรรมต่างๆ
- ย่อยไขมันในนม ทำให้ได้กลิ่นรสที่หลากหลาย

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B. Flavor compounds

สารแต่งกลิ่นในอุตสาหกรรมอาหาร ยาและเครื่องสำอาง เป็นพวก terpenic esters ของ short chain fatty acids โดยทั่วไปได้จากการสังเคราะห์ทางเคมี การหมัก และการสกัดจากธรรมชาติ แต่มีต้นทุนสูง และได้ปริมาณน้อย

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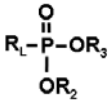
C. การย่อยยาฆ่าแมลงและยาปราบศัตรูพืช
- ย่อยสลายสารพวก organophosphate



Organophosphate
"insecticide"

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D. อุตสาหกรรมสิ่งทอและสารซักฟอก
- ปรับปรุงคุณสมบัติของใยสังเคราะห์ ให้มีความสบายในการสวมใส่มากขึ้น
- การใช้ด่าง NaOH และความร้อนสูง ส่งผลกระทบต่อสิ่งแวดล้อม ใช้เอนไซม์แทน
- ผสมในผงซักฟอก ช่วยกำจัดรอยเปื้อนไขมัน

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E. การย่อยสลาย biodegradable plastics

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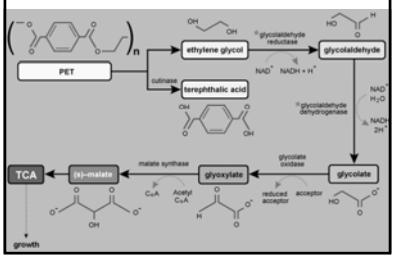

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Chestnut (*Castanea sativa* Mill.) industrial wastes as a valued bioresource for the production of active ingredients

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ABSTRACT

In the present study, by-products from chestnut peeling processing were used for the production of active ingredients. A blend of inner and outer chestnut shells (IOCS), and inner chestnut shells (ICS) were extracted through an eco-friendly method. IOCS extract contained the highest amount of phenolic molecules (205.99 ± 13.10 mg of Gallic Acid Equivalents/g of dry extract), and gallic acid was the most abundant compound among those identified by HPLC (63.51 ± 1.32 mg/g of dry extract). Condensed tannins represented the main phenolic fraction, accounting for 78.88% and 59.14% of the total phenolic compounds in IOCS and ICS extracts, respectively. Both extracts decreased the production of oxidized lipids in HaCaT keratinocytes after H_2O_2 exposure. They showed protecting activity against inflammation as well, because the production of NO and iNOS, selected as inflammatory markers, was attenuated. IOCS extract (0.002%) showed greater activity with a reduction of 58% of NO and 43% of iNOS levels. The extracts also exhibited hydration capacity and protection against collagen degradation in HaCaT keratinocytes. All the results suggest that chestnut shell extracts can be potential active ingredients for cosmetic formulations devoted to the skin protection.

1. Introduction

Plant Kingdom represents an inexhaustible source of natural bioactive compounds that can find application in several areas, such as food, pharmaceutical and cosmetic industries. Even though the initial research on active molecules was mostly directed towards isolated pure compounds, an alternative tendency focuses on mixtures of natural molecules. In fact, it has been ascertained that blends of natural compounds are more active than their isolated forms thanks to their synergic activity [1]. Moreover, the increasing demand for biologically active molecules, has encouraged the research of new sources of natural molecules [2]. In this perspective, the large amount of wastes generated by the anthropic activities represents an exploitable and valuable resource for the production of bioactive compounds [3].

European chestnut (*Castanea sativa* Mill.) belongs to the angiosperm family of *Fagaceae*. It is considered a significant tree in the agricultural and forestry economy, and chestnut seeds have represented one of the most important food resources of rural areas for many centuries. Nowadays, a large part of seeds is used either for fresh consumption and for preparation of products such as chestnut purée, marron-glacé, and chestnut flour which finds application in gluten-free diets.

In 2014, about 123,000 tonnes of chestnuts were produced and processed in South Europe, with Italy providing almost 43% of the whole European chestnut production (FAOSTAT, Food and Agriculture Organization of the United States: http://www.fao.org/faostat/en/#data). This activity generates large amounts of wastes (bur, shell, curing

Abbreviations: AA, ascorbic acid; AQP3, aquaporin-3; BHT, butylhydroxytoluene; C, catechin; CAE, caffeic acid equivalents; CE, catechin equivalents; DE, dry extract; DMEM, dulbecco's modified eagle's medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FBS, fetal bovine serum; GA, gallic acid; GAE, gallic acid equivalents; HEPES, *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); ICS, inner chestnut shells; IOCS, inner and outer chestnut shells; LPS, LipoPolySaccharide; MMP, metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide; iNOS, nitric oxide synthase; NO, nitric oxide; PCA, protocatechuic acid; RA, retinoic acid; ROS, reactive oxygen species; RSA, radical scavenging activity; TPCK, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone

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wastewater) which, nevertheless the problems of their disposal, are still a valuable source of bioactive molecules.

In Italy, the chestnut peeling processing produces about 5300 tonnes of shell/year that are usually burned to be used as fuel in the factories. More in detail, the peeling process known as "Brulage" produces two types of shell residues. In the first phase of the process, which consists in heating the chestnuts up to 900 °C for few seconds, a blend of inner and outer chestnut shells (IOCS) is produced. In the successive step, the remaining inner skin is removed from the kernel by a treatment with water steam. The residue represents the inner chestnut shells (ICS). An attractive solution for the exploitation of the chestnut shells is represented by their utilization for the production of biologically active compounds. In fact, the shells contain 36% of sugars that can be used for biofuel production [4], and phenolic molecules such as gallic and ellagic acids that are well-known for their antioxidant and anticancer properties [5].

Phenolic compounds contained in extracts from natural sources have been employed in formulas for skin care applications as well, and their protection activities on skin cells have been investigated. The activity of these substances has been studied by evaluating the reduction of the Reactive Oxygen Species (ROS), the skin damages associated to the heavy metals and UV light exposure, and the protection against inflammation [6–8].

According to this, the aim of the present work was the valorisation of the by-products from the "Brulage" peeling process, namely IOCS and ICS, through the investigation and the characterization of their extracts, as potential active ingredients in the skin care sector. In details, the authors describe a simple and eco-friendly extraction procedure of the bioactive compounds from IOCS and ICS. The resulting extracts were analysed in terms of phenolic families composition and biological activities toward ROS protection, inhibition of inflammatory mediators, as well as induction of genes responsible for hydration in skin cells. To the best of our knowledge, this is the first paper describing the potential application of the extracts from chestnut industrial wastes as active ingredients for skin care.

2. Materials and methods

2.1. Chemicals

Chemicals needed for phenolic families determination (Folin-Ciocalteu reagent and Na_2CO_3 for phenolic compounds; $NaNO_2$, $AlCl_3{\cdot}6H_2O$, and NaOH for flavonoids; HCl, and Na_2MoO_4 for *ortho*-diphenols; cinchonine hemisulfate and formaldehyde for tannins), 2,2-diphenyl-1-picrylhydrazyl (DPPH˙), ascorbic acid (AA), butylhydroxytoluene (BHT), catechin (C), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), LipoPolySaccharide (LPS), and HPLC standards (gallic acid, protocatechuic acid, epicatechin, ellagic acid, *p*-coumaric acid, and scopoletin) were purchased from Sigma-Aldrich Co. (Milano, Italy). All solvents for HPLC analyses were from Carlo Erba Reagents (Milano, Italy). 4,4-Difluoro-3a,4a-diaza-s-indacene (BODIPY), and the Griess reagent [N-(1-naphthyl)ethylenediamine and sulfanilic acid] were purchased from Invitrogen-Life Technologies (Carlsbad, California).

2.2. Extraction of phenolic compounds from chestnut shells

IOCS and ICS were kindly provided by Ingino s.r.l. food factory (Montoro Inferiore, Avellino, Italy). They were dried in oven at 55 °C until reaching constant weight and powdered using a food homogenizer (type 8557-54, Tefal, France). The bioactive molecules were extracted as follows: 5 g of chestnut shells (IOCS or ICS) were suspended in 100 mL of deionized water, and boiled for 1 h under continuous stirring. The mixture was cooled on ice and centrifuged at 3220 *g* for 1 h at 4 °C (Eppendorf 5810R). After the recovery of the supernatant, the solid residue was rinsed with the same volume of water lost during the boiling procedure. Then, the mixture was centrifuged as above, the supernatant was recovered and added to the previous one in order to restore the original volume. The obtained solution (extract) was lyophilized in an Edwards Modulyo freeze-dryer (Edwards, Cinisello Balsamo, Milano, Italy), and the powder (dry extract-DE) was stored at room temperature.

2.3. Characterization of the extracts

For IOCS and ICS extracts characterization, 2.6 and 3 mg of DE, respectively were dissolved in 1 mL of deionized water. All tests were conducted in triplicate and results were expressed as mean ± Standard Deviation (SD).

2.3.1. Total phenolic content

Total phenolic content was measured by the Folin-Ciocalteu method [9]. Aliquots of phenolic extract, diluted to 150 µL with deionized water, were mixed with 750 µL of Folin-Ciocalteu reagent (diluted tenfolds with deionized water) and 600 µL of 7.5% (w/v) Na_2CO_3. The reaction was developed at 25 °C for 2 h in the dark, and the absorbance was read at 765 nm against a blank prepared with 150 µL of deionized water (Varian spectrophotometer, model DMS-200, Varian Analytical Instruments, Leini, Torino, Italy). The total phenolic content was estimated by a calibration curve built with increasing quantities of a standard solution of gallic acid (range 1.5–10 µg). The results were expressed as mg of GAE (Gallic Acid Equivalents)/g DE.

2.3.2. Total flavonoid content

Total flavonoid content was determined following the method of Barreira et al. [10] with some modifications. Briefly, 250 µL of phenolic extract were mixed with 1.25 mL of deionized water and 75 µL of 5% (w/v) $NaNO_2$. After 5 min, 150 µL of 16% (w/v) $AlCl_3 \cdot 6H_2O$ were added. After 1 min, 500 µL of 1 M NaOH and 275 µL of deionized water were added, and the resulting solution was vigorously mixed. The absorbance was read at 510 nm versus a blank containing 250 µL of deionized water. The flavonoid amount was determined by a calibration curve obtained with increasing quantities of a standard solution of catechin (range 5–75 µg). The results were expressed as mg of CE (Catechin Equivalents)/g DE and subsequently converted, through a conversion factor, to mg of GAE in order to compare the amount of flavonoids with the total phenolic content. One mg of CE corresponded to 0.93 mg of GAE.

2.3.3. Total ortho-diphenolic content

Total *ortho*-diphenols were measured as described by Arnow [11]. Briefly, 100 µL of phenolic extract were diluted to 400 µL with deionized water. Then, 400 µL of 0.5 M HCl, 400 µL of 1.45 M $NaNO_2$ and 0.4 M Na_2MoO_4, and 400 µL of 1 M NaOH were added in sequence. The resulting mixture was immediately read at 500 nm using a blank made of 400 µL of deionized water. The quantification was carried out by a calibration curve obtained with increasing quantities of a standard solution of caffeic acid (range 5–50 µg). The results were expressed as mg of CAE (Caffeic Acid Equivalents)/g DE, and subsequently converted, through a conversion factor, to mg of GAE in order to compare the amount of *ortho*-diphenols with the total phenolic content. One mg of CAE corresponded to 0.86 mg of GAE.

2.3.4. Total tannin content

Total tannin content was estimated according to Peri and Pompei [12], with some modifications. Briefly, 0.8 mL of phenolic extract were added to 0.8 mL of 0.5% (w/v) cinchonine hemisulfate in a 2 mL Eppendorf tube. The solution was mixed and left overnight at 4 °C in order to achieve a better precipitation. After centrifugation at 16,100*g* for 5 min at 4 °C, the supernatant contained the non-tannin fraction, and the pellet represented the tannin fraction. The Folin-Ciocalteu assay was carried out on the supernatant in order to calculate the non-tannin

content. The total tannin fraction was determined by difference between the total phenolic content and the non-tannin content.

2.3.5. Hydrolysable and condensed tannin content

Hydrolysable and condensed tannin fractions were determined applying the procedure described by Peri and Pompei [12], and the formaldehyde precipitation method described by Scalbert et al. [13], with some modifications. The tannin residue, obtained after precipitation with the cinchonine hemisulfate, was dissolved in the original sample volume (0.8 mL) with ethanol/water (1:1 v/v). Then, 0.5 mL of this solution were mixed with 0.25 mL of 37% HCl/water (2:5 v/v) and 0.25 mL of 4.8% formaldehyde. The resulting mixture was mixed vigorously, incubated overnight at room temperature, and centrifuged at 16,100*g*, for 30 min at 4 °C. The supernatant (150 μL) was assayed by the Folin-Ciocalteu method, and the value obtained represented the hydrolysable tannin fraction. The condensed tannin content was determined by the difference between the total tannin content and the hydrolysable tannin content.

2.3.6. Reversed-phase HPLC analysis

High Performance Liquid Chromatography (HPLC) analysis was conducted with a 250 × 4.6 mm, 5.0 μm, reversed-phase (RP) Luna C18 (2) column (Phenomenex Inc. Castelmaggiore, Italy), equipped with a SecurityGuard™ pre-column containing a C18 cartridge. The HPLC system used was a Dionex UltiMate® 3000 with a quaternary pump and an UltiMate® diode array detector (Dionex, California, USA). The dry extracts were dissolved in water at concentration of 10 mg/mL; then, they were filtered through a Chromafil syringe filter, cut-off 0.45 μm (Macherey-Nagel GmbH & Co., Duren, Germany), and 50 μL were loaded onto the column. The elution was performed using 2% acetic acid (solvent A), 0.5% acetic acid in 50% acetonitrile (solvent B), and 100% acetonitrile (solvent C) as described in Table S1 (Supplementary data). The peak elution was monitored at 280 nm. Phenolic compounds were identified by comparing the retention times (RT) with those of pure commercial standards, and by co-injection with the corresponding standards. Quantification was obtained by calibration curves obtained injecting increasing quantities of the pure standards.

2.4. Antioxidant activity

The free radical scavenging activity of the phenolic extracts was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) assay according to Barreira et al. [10], with some modifications. A volume of extract containing 2 μg GAE of phenolic compounds was opportunely diluted to 150 μL with deionized water, and mixed with 1.35 mL of 60 μM DPPH· methanolic solution. The antioxidant activity, evidenced by the discoloration of the purple solution, was followed kinetically by reading the absorbance at 517 nm for 30 min versus a control containing 150 μL of deionized water. The Radical Scavenging Activity (RSA) was calculated according to the following formula:

$$\text{RSA}\ (\%) = \left(\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \right) \times 100$$

and compared with the following recognized antioxidant compounds assayed under the same conditions: AA, BHT, and C.

2.5. Cytotoxicity assay

HaCaT keratinocytes were plated into 96-well plates at a density of 1.3×10^3 cells/well, grown for 8 h at 37 °C, 5% CO_2, and treated for 48 h with different concentrations of the extracts (range 0.5%-0.0004%). After treatments, cells were washed with PBS and incubated with 100 μL per well of reaction buffer (10 mM HEPES, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM glucose and 0.5 mg/mL of the colorimetric substrate MTT in PBS buffer at pH 7.4). After 3 h, cells were solubilized by the addition of 100 μL of solubilisation solution (10% Triton X-100, 0.1 M HCl in isopropanol), and the plate was incubated for 4 h at room temperature. The number of healthy cells, proportional to the level of the formazan produced, was quantified at 595 nm by the multiwell plate reader Victor3 (Perkin Elmer, Waltham, MA, USA).

2.6. Reactive oxygen species determination

For the determination of lipid peroxides (membrane ROS), HaCaT keratinocytes were seeded in 96-well plates for 20 h. Then, the cells were incubated for 2 h with the extracts or with 100 μM α-Tocopherol, used as positive control. Successively, they were washed in PBS and incubated with the reagent BODIPY for 45 min at 37 °C. After an additional wash in PBS, the cells were treated for 1 h with 450 μM H_2O_2, and the fluorescence of the samples was measured at 535 nm (excitation 490 nm), using the instrument EnVision (Perkin Elmer).

2.7. Aquaporin and matrix metalloproteinases gene expression analysis

HaCaT cells were seeded in 6-well plates at a density of 1.5×10^4 per well and incubated for 6 h at 37 °C, 5% CO_2, with the extracts or with 1 μM retinoic acid (RA), used as positive control. The samples for analysis of the metalloproteinase (MMPs) expression were also treated for 3 h with 900 μM $NiSO_4$, as stressing agent. Total RNA was extracted with the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich Co., Milano, Italy), according to the manufacture's instructions and treated with DNAse I at 37 °C for 30 min to eliminate any contaminating genomic DNA. The first strand cDNA was synthesized from 1 μg of RNA using the RevertAid™ First Strand cDNA Synthesis (ThermoFisher Scientific). RT-PCR was performed using gene specific primers and the Quantum RNA™ 18S internal standard (Ambion™). The amplification reactions were performed with the following general scheme: 2 min at 94 °C followed by 35 cycles of 94 °C for 30s, annealing temperature (specific for each gene) for 30s, and 72 °C for 30–60s, with a 10 min final extension at 72 °C. The PCR products obtained were loaded on 1.5% agarose gel, and the amplification bands were quantified by the instrument Geliance 200 Imaging system (Perkin Elmer). The amplification band corresponding to the analysed gene was normalized to the amplification band corresponding to the 18S, and the measure obtained was converted into a percentage value by considering the untreated control as 100%. All the semi-quantitative RT-PCRs were repeated three times to ensure quality of reproduction. For the amplifications the following primers were used:

AQP3-FW: 5′gatcaagctgcccatcta 3′
AQP3-RV: 5′tgggccagcttcacattct 3′
MMP1-F: 5′ tttgggctgaaagtgactgg3′
MMP1-R: 5′gggataacctggatccatag3′
MMP3-F: 5′ccattggatggagctgcaag3′
MMP3-R: 5′aagtgggcatctccattaatcc3′
MMP9-F: 5′gtaccgctatggttacactcg3′
MMP9-R: 5′tactgcaccagggcaagccg3′

2.8. Anti-inflammatory activity assay

The RAW 264.7 murine macrophages were utilized to analyse the expression of the inducible Nitric Oxide Synthase (iNOS) gene, and to evaluate the Nitric Oxide (NO) concentration. For iNOS gene expression, the cells (1.5×10^5 cells/well) were grown at 37 °C in DMEM medium supplemented with 10% FBS for 24 h in 6-well plates. Cells were then incubated for 2 h with the extracts or with 10 μM TPCK, used as positive control, and treated with 1 μg/mL of the inflammatory agent LPS for 4 h. The RNA was extracted and processed as described in the paragraph 2.7. The oligonucleotides used in the PCR reactions were: iNOS-F (5′acaacatcctggaggaagtg3′) and iNOS-R (5′tccatgcagacaaccttgg3′).

To measure the Nitric Oxide (NO) concentration, the macrophages, seeded at a concentration of 1.5×10^5 cells/well in 96-well plates for 24 h, were pre-treated with the extracts or with 10 μM TPCK for 2 h, before the treatment with 1 μg/mL LPS for 18 h. The amount of NO, readily converted into nitrite, was measured by adding 75 μL per well of Griess reagent [solution of N-(1-naphthyl)ethylenediamine and sulfanilic acid, Invitrogen-Life Technologies], and measuring the absorbance at 540 nm by the multiwell-plate reader, Victor3 (Perkin Elmer), after 30 min incubation at room temperature.

2.9. Statistical analysis

Each value in the graphs was expressed as mean ± Standard Deviation (SD) of three independent experiments, conducted in triplicates. One-way ANOVA test was used for multiple comparisons using Microsoft Excel program. A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Chestnut shells extraction

In the present work, IOCS and ICS were extracted in boiling water for 1 h; 260 and 300 mg of dry extracts were obtained from 5 g of IOCS and ICS, respectively, with an extraction yield of 5.2 ± 0.1% and 6.0 ± 0.1% (Table 1). Under our experimental conditions, IOCS and ICS extracts contained 205.99 ± 13.10 and 43.69 ± 1.91 mg GAE/g DE, corresponding to 10.70 ± 1.29 and 2.62 ± 0.03 mg GAE/g dry matter, respectively.

Usually, the yield of aqueous extracts from chestnut shells is low, and our values were in agreement with those reported by other authors. Nazzaro et al. [14] reported a yield of 2.2% after 5 days of extraction at room temperature, while Barreira et al. [10] obtained a yield of 4.98% after boiling the shells in water for 30 min. Alternative solvents (methanol/water, ethanol/water) and conditions (temperature and solid/liquid ratio) were previously tested in our labs, and boiling water was the most effective extraction solvent [15]. A further advantage in choosing water is represented by its non-toxic character, its low environmental impact, and its safety in all the health care applications. Data concerning the amount of phenolic compounds extracted from chestnut shells vary greatly in the scientific literature. Extraction conditions may be different for temperature, time of contact, and solid/liquid ratio and moreover, the majority of papers reports the phenolic concentrations in the extract rather than in the original biomass; consequently, a comparison among results from different research groups is very difficult [14,5]. Even in the case of quite similar extraction conditions, the origin of the residue (fresh or from an industrial process) is a factor that may affect the extraction yield, making the comparison quite hard [10].

3.2. Characterization of IOCS and ICS extracts

The total phenolic content of the IOCS extract was almost 5-folds higher than that of the ICS extract (Table 1). The composition of the total phenolic compounds in both extracts was investigated taking into consideration different phenolic families, such as tannins, *ortho*-diphenols, and flavonoids. The condensed tannins were the main class of phenolic compounds present in the extracts, with162.49 ± 5.94 mg GAE/g DE for IOCS and 25.84 ± 1.19 mg GAE/g DE for ICS. The hydrolysable tannins were the less represented chemical class. *Ortho*-diphenols were the second class of phenolic molecules for their abundance in IOCS and ICS extracts with 98.06 ± 2.61 and 19.55 ± 1.01 mg CAE/g DE, respectively. To the best of our knowledge, the *ortho*-diphenolic content in chestnut shell extracts has never been indicated until now. We chose to estimate it because such type of molecules is provided with strong antioxidant power. In fact, the presence of the hydroxyl group in *ortho* position (catechol structure) increases the antioxidant activity of the compounds through the stabilization of the phenoxyl radical [16]. Similarly to the total phenolic content, the IOCS extract contained an amount of flavonoids 5-folds higher than that of the ICS extract.

The percentage of every phenolic family with respect to the total phenolic content was reported in Fig. S1 (Supplementary data). The findings indicated that the representativeness of the chemical families increased in the following order: hydrolysable tannins < flavonoids < *ortho*-diphenols < condensed tannins in both extracts; moreover, the water boiling procedure had the same extraction selectivity for both chestnut residues.

Despite the Folin–Ciocalteu is usually affected by some interferences due to the low specificity of the reagent toward the phenolic compounds, we chose this assay for the quantification of the phenols because it still represents the most common method used for the total phenols determination. In fact, it has been widely applied for the analysis of a large variety of natural extracts [17,18]. Unfortunately, the method did not provide qualitative indications on the phenolic molecules present in the extracts, and the molecular characterization of the compounds in the extracts was performed by RP-HPLC. The following molecules: gallic acid (GA), protocatechuic acid (PCA), catechin, epicatechin, ellagic acid, *p*-coumaric acid, and scopoletin were identified in both samples by comparison of their RT with those of the available standards. The findings indicated that there were no significant qualitative differences between the extracts. The concentration of all phenolic molecules identified under our experimental conditions was reported in Table 2. Among the detected compounds, GA was the most abundant with 63.51 ± 1.32 and 29.62 ± 0.71 mg/g DE in IOCS and ICS extracts, respectively. PCA was the second most represented compound in both extracts with 11.24 ± 0.30 and 3.43 ± 0.12 mg/g DE. The presence and the amount of these molecules varied according to the chestnut shell extracts described in the literature, depending on the extraction conditions selected. However, GA was almost always detected, and it was among the most abundant phenolic compounds identified in aqueous extracts [14,19].

Table 1
Extraction yield, total phenols and phenolic families in dry extracts from chestnut shells.[a]

	Extraction yield (%)	Total phenols	Flavonoids	*Ortho*-diphenols	Condensed tannins	Hydrolysable tannins
		(mg GAE/g DE)[b,c]	(mg CE/g DE)[d]	(mg CAE/g DE)[e]	(mg GAE/g DE) [b,c]	(mg GAE/g DE) [b,c]
IOCS	5.2 ± 0.1	205.99 ± 13.10	40.98 ± 1.22	98.06 ± 2.61	162.49 ± 5.94	12.94 ± 0.96
ICS	6.0 ± 0.1	43.69 ± 1.91	7.94 ± 0.15	19.55 ± 1.01	25.84 ± 1.19	2.02 ± 0.03

[a] All determinations were conducted in triplicate and results were expressed as mean ± SD values.
[b] GAE: Gallic acid equivalents.
[c] DE: Dry extract.
[d] CE: Catechin equivalents.
[e] CAE: Caffeic acid equivalents.

Table 2
Phenolic compounds determined by RP-HPLC in IOCS and ICS dry extracts.[a]

Compound	IOCS	ICS
	(mg/g DE)[b]	(mg/g DE)[b]
Gallic acid	63.51 ± 1.32	29.62 ± 0.71
Protocatechuic acid	11.24 ± 0.30	3.43 ± 0.12
Catechin	0.70 ± 0.06	0.30 ± 0.01
Epicatechin	0.71 ± 0.05	0.32 ± 0.01
Ellagic acid	0.81 ± 0.06	0.63 ± 0.02
p-Coumaric acid	0.22 ± 0.01	0.32 ± 0.01
Scopoletin	0.11 ± 0.01	0.41 ± 0.01

[a] All determinations were conducted in triplicate and results were expressed as mean ± SD values.
[b] DE: Dry extract.

3.3. Free radical scavenging activity

The antioxidant power of the chestnut extracts was investigated as a function of their radical scavenging activity (RSA) through the evaluation of the discoloration of DPPH˙, a free radical that accepts an electron or a hydrogen radical to become a stable molecule. This method has been the choice of many scientists to evaluate the RSA of natural compounds, because of its simplicity and reproducibility [20,21].

The kinetic profile of RSA was identical in both extracts. The scavenging activity rapidly increased during the first minutes of assay, slowing down as the reaction time increases (Fig. 1). The addition of 2 μg GAE of IOCS and ICS extracts to DPPH˙ caused a 35% reduction of the free radical after 5 min.

The RSA of the extracts was compared with those of other antioxidants, selected as reference compounds for the following reasons: C is a natural antioxidant detected in the chestnut shell extracts, while BHT and AA were chosen because they had been already used as antioxidant ingredients in cosmetic formulations [22]. After 5 min of assay the radical scavenging effect of the extracts was higher in comparison to 2 μg GAE of the reference compounds (AA 29%, C 23.5%, BHT 1.2%). At the plateau, the RSA of IOCS and ICS extracts was 44%, whereas the reference compounds were less powerful (AA 29%, C 37.2%, BHT 7.2%). Although the same quantity of samples was tested, the stronger radical scavenging effect showed by IOCS and ICS extracts could be explained by the presence of different phytochemicals that acted in a synergic manner [5]. This finding confirms the advantage of using natural mixtures of phytochemicals in comparison to pure antioxidant compounds. Moreover, it explains the current tendency of consumers to prefer natural preparations instead of single antioxidants to achieve health benefits [23].

3.4. MTT assay

Before performing the cellular assays, we determined the proper concentrations of the extracts to be used in the tests by analysing the cell cytotoxicity. Different concentrations of IOCS and ICS extracts (ranging from 0.5% to 0.0004%) were tested on cultured keratinocytes (HaCaT), and the vitality rate was determined. The results indicated that all the doses below 0.01% did not have any significant toxic effect on the exponentially growing cells (data not shown). The IC_{50} calculated for IOCS and ICS extracts were 0.14 and 0.35%, respectively. On the basis of these measures, we chose the concentrations of 0.002% and 0.0004% for both extracts as doses to use in the cell bioassays.

3.5. Protecting activity from oxidative stress

To test the ability of the extracts to scavenge the Reactive Oxygen Species (ROS) produced in the skin cells after oxidative stress, we measured the lipid peroxide formation in the membranes of HaCaT keratinocytes after treatment with 450 μM H_2O_2 by using a specific fluorescent dye. Lipid peroxides are unstable compounds formed at the level of the cell membranes in response to a strong oxidative stress. They give rise to soluble oxidant species that rapidly diffuse throughout the cytoplasm [24]. The results indicated that the increase of oxidized lipids in the cells stressed by H_2O_2 was attenuated by both extracts (Fig. 2). Treatment of keratinocytes with 0.002% IOCS extract reduced the ROS production at a level similar to the non-stressed cells. More in detail, the IOCS extract determined a dose dependent ROS reduction of 20% and 27% at the concentrations of 0.0004% and 0.002% respectively. No dose-dependent activity was observed with the ICS extract that caused about 8–17% of inhibition. Our hypothesis to explain the result obtained with the ICS extract is that some unidentified substances may act as pro-oxidants instead of antioxidants. In fact, although phenolic molecules are commonly considered to protect from oxidative damages, in some cases they can react with other components, such as metal ions, and produce cytotoxic metabolites that have pro-oxidant capacity [25,26].

3.6. Hydration capacity and collagen protection

To investigate the potential effect of the extracts on hydration and

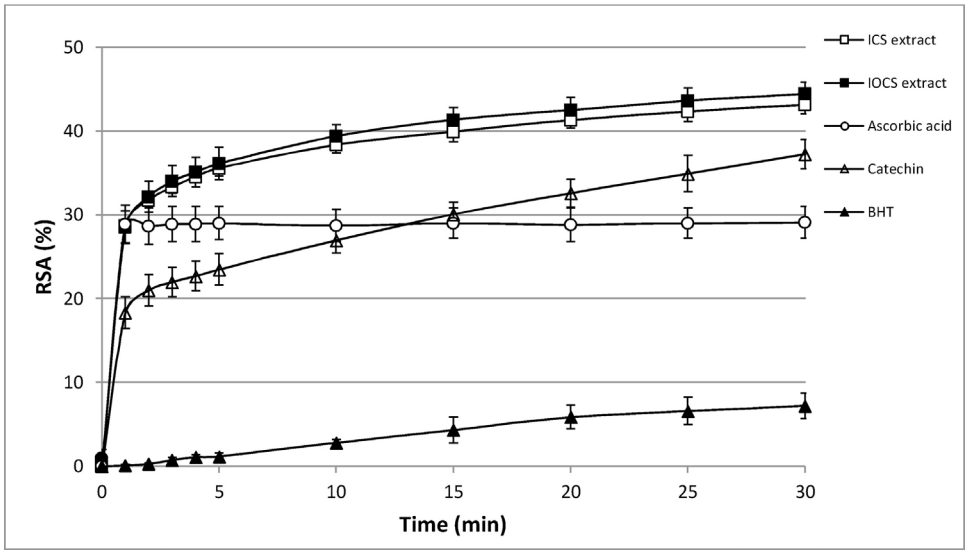

Fig. 1. Free radical scavenging activity of ICS and IOCS extracts, and antioxidant reference compounds. DPPH assay was performed with 2 μg GAE of phenolic extracts or pure compounds as described in the paragraph 2.4. All determinations were conducted in triplicate and results were expressed as mean ± SD.

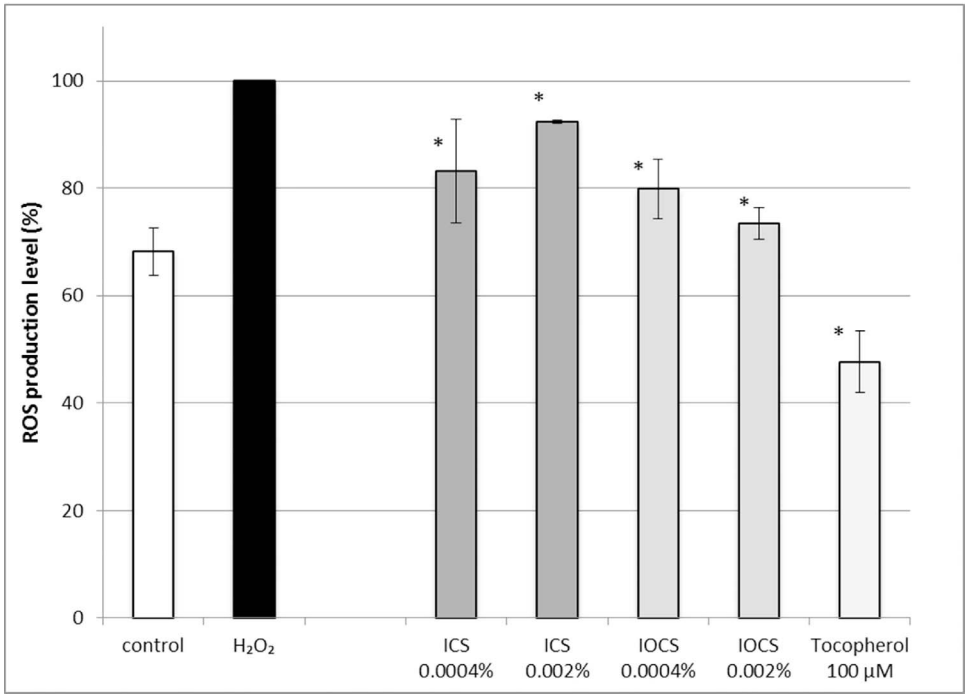

Fig. 2. Protection against oxidative stress. The amount of ROS production was measured in HaCaT keratinocytes treated with ICS and IOCS extracts before oxidative stress induced by 450 µM H_2O_2 as described in the paragraph 2.6. α-Tocopherol was used as positive control. All determinations were conducted in triplicate and results were expressed as mean ± SD. * $p < 0.05$.

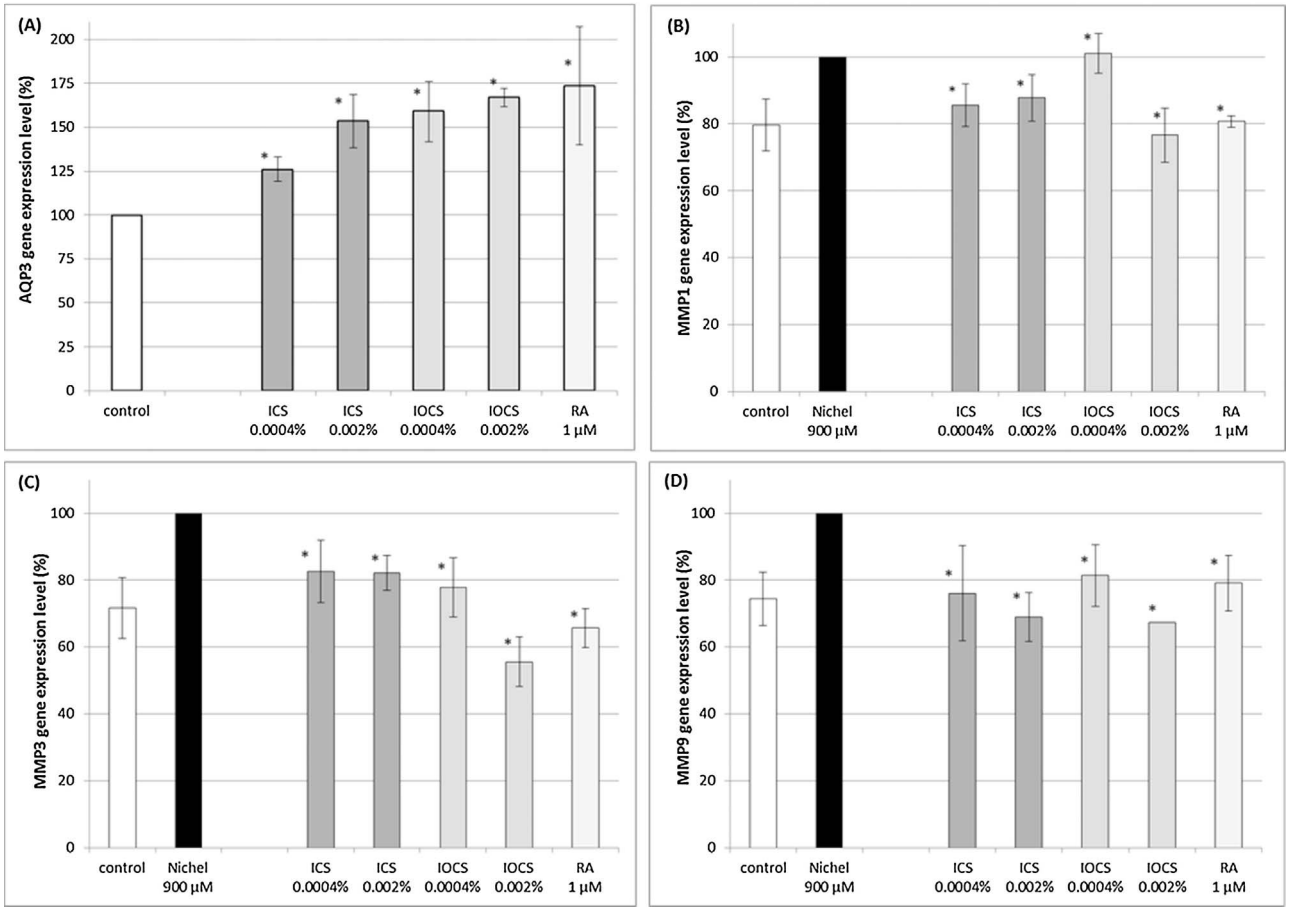

Fig. 3. Measurement of hydration capacity and collagen degradation protection. The gene expression level of AQP3 (A) and metalloproteinases MMP1 (B), MMP3 (C), and MMP9 (D) was measured in HaCaT keratinocytes incubated with ICS and IOCS extracts as described in the paragraph 2.7. RA was used as positive control. All determinations were conducted in triplicate and results were expressed as mean ± SD. * $p < 0.05$.

collagen protection, we evaluated the expression of proteins specifically involved in the maintenance of water balance and collagen stability in human keratinocytes. Aquaporin-3 (AQP3) is a channel protein involved in the glycerol/water transport through membranes, and in the maintenance of the water-proof capacity of the epidermis [27]. The matrix metalloproteinases (MMP1, 3 and 9) are produced by keratinocytes in response to stress, and they are responsible for the degradation of the skin collagen [28]. The results of our trials showed that ICS and IOCS extracts increased the expression of AQP3 gene in comparison to the control (taken as 100%) (Fig. 3A). More in detail, the gene expression level of AQP3 after treatment with 0.002% IOCS extract reached 167%, similarly to RA, used as positive control and known for its capacity to induce AQP3 gene expression [29]. Studies on phenolic compounds already demonstrated their capacity in modulating the synthesis of AQP3. Chrysin, a flavonoid occurring in honey, was able to revert the down-regulation of AQP3 gene in HaCaT keratinocytes after UVB exposure, and Fiorentini et al. reviewed the role of polyphenols in modulating the production and the activity of some AQPs [30,31]. Our results proved the capacity of the phenolic compounds contained in the chestnut shell extracts to act on AQP3 synthesis and to be part of the skin hydration modulating agents.

IOCS and ICS extracts were also able to inhibit the production of MMPs caused by heavy metal stress. Considering as 100% the MMP expression levels measured after exposure of HaCaT cells to Ni^{2+} ions, the reduction of the gene expression levels ranged from 12% to 45% (Fig. 3B, C and D). Also in this case, RA was used as positive control, since it is effective in the inhibition of MMPs gene expression [32].

MMP3 and MMP9 gene levels were reduced by 45% and 33%, respectively after treatment with 0.002% IOCS extract, while they were decreased by 34% and 21%, respectively after exposure to 1 μM RA (Fig. 3C and D).

Several studies have described the role of the phenolic compounds in the expression of MMPs. Hwang et al. [33] showed that the levels of MMP1 were decreased, compared to the untreated cells, after treatment of UVB irradiated NHDF cells with 10 μM GA.

In general, polyphenols have already been considered for the capacity to protect skin cells from the effects of dehydration and photoaging caused by external agents, such as UV radiations. Heinrich and co-workers [34] carried out a study on female volunteers whose skin was irradiated by a solar light simulator. They demonstrated that the consumption of a beverage containing green tea polyphenols had a positive effect on the skin structure by improving elasticity and hydration.

Two of the most important claims associated with cosmetic active ingredients are the hydrating activity and the anti-ageing potential that can be quantified as capacity to protect collagen, the most important component of the extracellular matrix in the dermis, from degradation. However, no investigations about the effect of phenolic extracts from chestnut shells on hydration capacity and protection against collagen degradation have been reported to date. Our findings highlight the potentiality of the extracts to improve skin homeostasis and integrity by keeping the water balance on the one hand, and preserving extracellular matrix from aberrant degradation on the other.

3.7. Protecting activity against inflammation

The presence of flavonoids and tannins in the extracts and the ability to reduce ROS formation in keratinocytes suggested a potential role of the extracts in the inhibition of the inflammatory response [35]. Moreover, the effect of phenolic compounds in the repression of inflammation-related genes has been reported in several types of cells [36]. As in the skin the macrophages are the main cells responsible for the initiation of the inflammatory cascade, we treated RAW 264.7 murine macrophages with different concentrations of the extracts; then, we measured the amount of the main pro-inflammatory second messenger, Nitric Oxide (NO) [37], and analysed the expression of the iNOS gene involved in the synthesis of NO under LPS-induced stress. As expected, the NO synthesis was strongly inhibited by the TPCK (10 μM), an anti-inflammatory drug used as positive control (Fig. 4A). However, the production of NO caused by the bacterial inflammatory agent LPS was attenuated by the presence of the ICS and IOCS extracts. The strongest effect was obtained by the IOCS extract at 0.002% concentration with 58% of NO reduction. Joo et al. [38] measured in RAW 264.7 macrophages a comparable level of inhibition (60%) with an ethyl acetate fraction obtained from a hot water extract of *Ulmus pumila* L stem bark. However, this fraction contained a quantity of phenols 35 times higher than those present in the IOCS extract, proving that the bioactive molecules in chestnut shell extracts were highly active. Similarly to what observed in the protection from oxidative stress in keratinocytes, no dose-dependent activity was observed with the ICS extract. A reduction of the NO levels of 40% and 32% was obtained at concentrations of 0.0004% and 0.002%, respectively.

To confirm that the NO decrease was due to the inhibition of the enzyme responsible for its synthesis, namely the inducible Nitric Oxide Synthase (iNOS), we verified the expression of the iNOS gene after treatment of the cells with the extracts, and quantified the specific mRNA produced in the cells. Inhibitory activity was observed for the iNOS expression as well, and the IOCS extract showed a dose-dependent effect (Fig. 4B). GA is recognized as a molecule with multiple and remarkable therapeutic properties, included antioxidant and anti-inflammatory activities [39]. Mard et al. [40] observed that the pretreatment of a gastric mucosal lesion with GA reduced the inflamed zone, and they demonstrated that this effect was due to a decreased production of iNOS. PCA is another phenolic compound able to suppress inflammatory effects. Min and co-workers [41] studied the anti-inflammatory response of the cyanidin-3-*O*-beta-D-glycoside and its metabolites (PCA and cyanidin) against inflammation induced by LPS in RAW 264.7 cells. The production of NO and the expression level of the iNOS gene were reduced by all compounds, and PCA was the most effective. As GA and PCA were the major phenolic components identified in our chestnut shell extracts, it was likely that the protection against inflammation was mainly due to the action of these substances.

4. Conclusion

Valorisation of chestnut shells, which are presently an undervalued industrial by-product produced in large amount, was achieved through the production of active molecules. IOCS and ICS residues were used to produce phenolic extracts with the purpose to test their suitability as active ingredients for cosmetic formulations. A very simple, low cost, and eco-friendly extraction method was accomplished, and the extracts obtained were rich in gallic acid and tannins. IOCS and ICS extracts were endowed with remarkable radical scavenging activity, and they were able to scavenge ROS generated in skin cells after oxidative stress. The extracts showed anti-inflammatory activity, capacity of skin hydration and ability to protect the collagen from degradation as well. All the observed results suggested an application of the chestnut shell extracts as potential active ingredients for the development of cosmetic preparations to use in the skin care industry.

Conflicts of interest

None.

Aknowledgement

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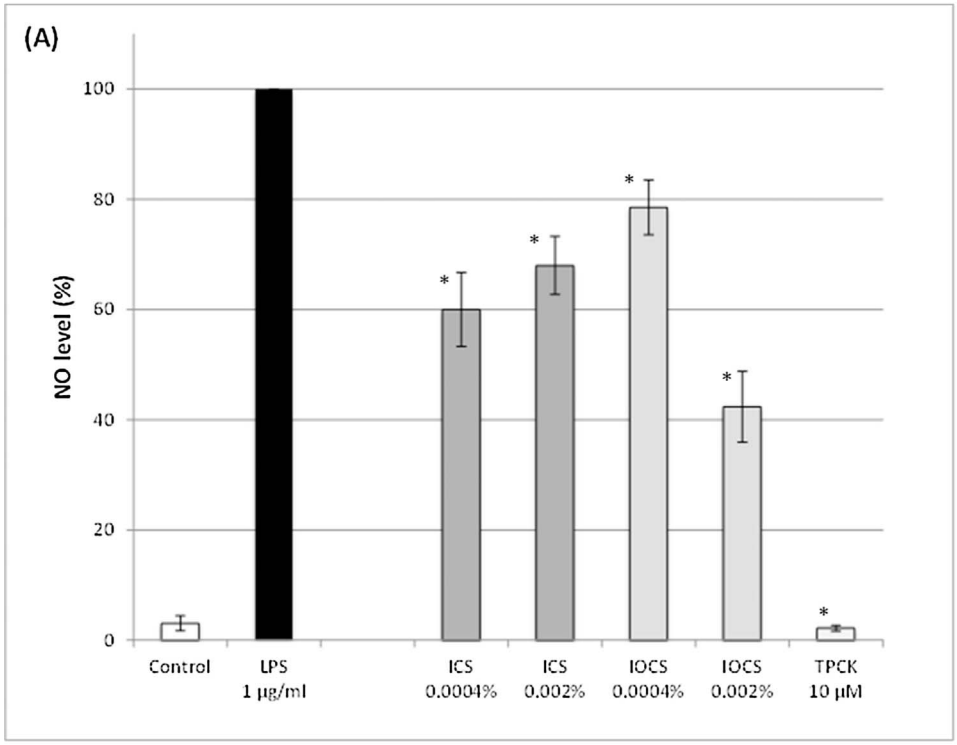
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the



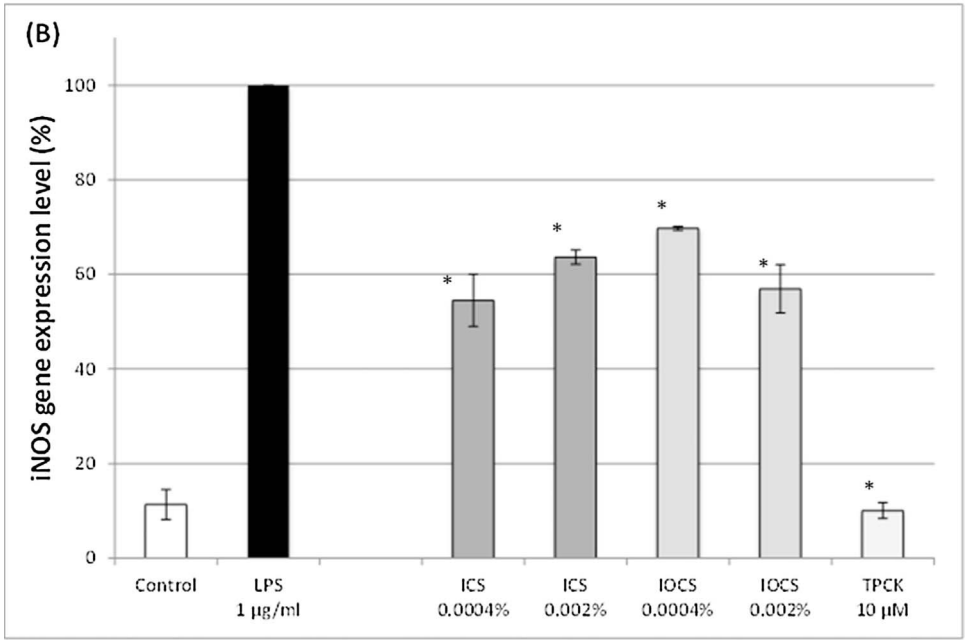


Fig. 4. Protection against inflammation. The level of NO production (A) and iNOS gene expression (B) was measured in RAW 264.7 macrophages treated with ICS and IOCS extracts before addition of bacterial LPS as described in the paragraph 2.8. TPCK was used as positive control. All determinations were conducted in triplicate and results were expressed as mean ± SD. * $p < 0.05$.

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High-fold improvement of assorted post-consumer poly(ethylene terephthalate) (PET) packages hydrolysis using *Humicola insolens* cutinase as a single biocatalyst

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Humicola insolens

ABSTRACT

The dissemination of technologies for poly(ethylene terephthalate) (PET) recycling is of paramount importance in the context of the plastics circular economy. One of the most promising alternatives is to use enzymes as catalysts for PET depolymerization to its monomers, but this route still needs improvement, especially regarding titer and productivity. In the present work, a sequential approach comprised of fractional factorial and central composite rotatable designs, the path of steepest ascent and one-way evaluation of variable effect, was performed to address these limitations, during assorted post-consumer PET (PC-PET) hydrolysis catalyzed by *Humicola insolens* cutinase. The highest terephthalic acid concentration and productivity during PC-PET hydrolysis were 100.9 mM (16.8 g/L) and 14.4 mM/day, corresponding to overall improvements of 10-fold and 20-fold, respectively. These data are among the best results described so far for enzyme-catalyzed hydrolysis of used PET packages. Also, the use of a single enzyme system, instead of multiple biocatalysts to achieve final conversion of PET to its monomers, lowers the process complexity and costs.

1. Introduction

The need for a circular plastics economy has been addressed with increasing importance by many countries since its global concept was launched in 2010 [1,2]. The packaging value chain, specifically, drags more attention, since it is currently a linear unsustainable economy, which utilizes 95% of finite feedstocks with a recycling rate of only 5% [3]. Poly(ethylene terephthalate) (PET) is one of the main synthetic plastics, with annual production of circa 50 million tons [4]. Among the technologies available or under development for its recycling (which includes mechanical and chemical, via different routes), the use of enzymes is an interesting option, for their renewability, non toxicity, high specificity and action under mild temperature and pressure conditions, comprising then a benign reaction media [5].

Although its environmental advantage for PET depolymerization, the enzyme-catalyzed reaction still does not compare favorably with chemical catalysts in terms of reaction rate and product concentration [6], therefore being characteristics to be improved in the enzymatic route in order to reach industrial feasibility. Further optimization of the biodepolymerization process is then needed for its competitiveness.

Hydrolysis is the main enzyme-catalyzed route for PET depolymerization, yielding mostly the final monomers, terephthalic acid (TPA) and ethylene glycol (EG). It is usually evaluated using thermophilic microorganism, such as *Thermobifida cellulosilytica* [7], *T. alba* [8] and *T. fusca* [9] strains, to allow reaction conditions as closer as possible to the glass transition temperature (Tg) of the polymer (circa 75–80 °C) [10]. Even though, hydrolysis of post-consumer samples takes days to weeks to reach TPA concentrations not higher than 15 mM [7].

The Humicola *insolens* cutinase (HiC) is among the most efficient biocatalysts described for PET depolymerization so far. It was successfully used for the hydrolysis of low-crystallinity (7%) PET into free-carboxyl group products [11], as well as for a fast hydrolysis of residual PET fiber oligomers released in a previous chemically-catalyzed reaction [12]. Still, the concentration and the productivity of TPA release should be further increased for future large scale applications of this enzyme.

Therefore, in the present work, a sequential approach for the

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