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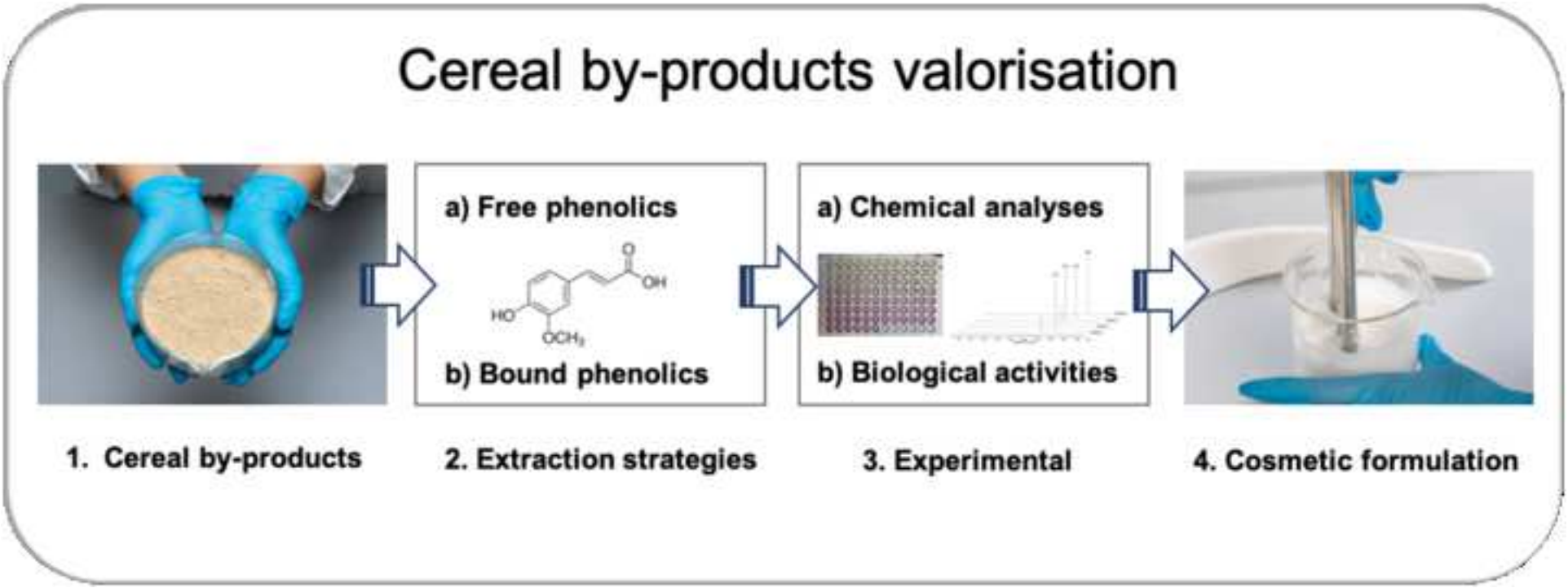
Highlights

1) Durum wheat and rice brans are sources of bound phenolic acids whose extraction can be improved by combining ultrasonication to alkaline hydrolysis;
2) Extracts obtained with ultrasound-assisted alkaline hydrolysis demonstrated higher antioxidant activity compared to the commonly used chemical hydrolysis, despite the reduction of extraction time;
3) Durum wheat extract obtained with ultrasound-assisted alkaline hydrolysis demonstrated to be a promising ingredient for cosmetic products.



Antioxidant and antimicrobial extracts obtained from agricultural by-products: strategies for a sustainable recovery and future perspectives.

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Abstract

Durum wheat and rice brans are by-products deriving from the milling industry and source of important phytochemicals, mostly phenolics in bound form. The objective of this work was to exploit the agricultural waste of two Italian cereal industries, in order to evaluate the most effective method for the extraction of phenolic acids with antioxidant and antimicrobial activity. Free phenolics were extracted with ultrasound-assisted extraction and bound phenolics with alkaline hydrolysis and ultrasound-assisted alkaline hydrolysis. Extracts were analyzed with HPTLC (high-performance thin-layer chromatography) and RPHPLC-DAD-ESIMS (reversed-phase high-performance liquid chromatography-diode array detector-electrospray ionization mass spectrometry); the content of *trans*-ferulic acid, *p*-coumaric acid and total phenolics were also quantified. The antioxidant activity was investigated using DPPH and ABTS radicals. The antimicrobial activity was evaluated against *Staphylococcus aureus* and *Staphylococcus epidermidis* strains. Results obtained using ultrasound-assisted alkaline hydrolysis showed the most interesting data, suggesting this method as the most effective to obtain active extracts from rice and durum wheat by-products. Durum wheat, extracted with this method, exhibited the highest content of *trans*-ferulic acid (406.14 ± 0.65 µg FA/mg extract), total phenolics (610.58 ± 57.60 mg GAE/g dried extract) and good antioxidant and antimicrobial activities and it has been selected to formulate oil-in-water cosmetic products.

Keywords

Cereals; by-products; bound phenolics; antioxidant; antimicrobial

Abbreviations[1]

[1] AH: Alkaline hydrolysis; FA: *trans*-ferulic acid: *p*-CA: *p*-coumaric acid; UAAH: Ultrasound-assisted alkaline hydrolysis; UAE: ultrasound-assisted extraction.

1. Introduction

Triticum durum (Desf.) and *Oryza sativa* L. are, after the primacy of maize, the two most produced crops worldwide and they contribute to more than a half of human dietary calories. Counting a production between 9 and 24 MMT in 2014, Europe contends the lead as the largest cereal waste producer together with USA and industrialized Asia (FAO, 2011); moreover, Italy is one of the major European cereal producers. Faced with the issue of food waste management, the exploitation of industrial wastes becomes a priority, as suggested by the European "Circular Economy Action Plan" (European Commission 2015). Cereal by-products, coming from the cereal supply chain, are residues from the milling industry and consist predominantly of brans and germs. The recovery of biomolecules from cereal wastes has become a hot topic in the last years but the extraction techniques proposed in literature are still unsustainable and require long working times. Liyana-Pathirana and Shahidi (2005) optimized the extraction of phenolics from wheat using response surface methodology, with the same e Zhu et al. (2011) used various ethanol concentration, but both techniques proposed are focused on the soluble phenolic fraction. nd phenolics from cereal by-products have been successfully extracted with acid and alkaline hydrolysis by Kim et al. (2006) and Zaupa et al. (2014) using strong hydrolysis conditions which can potentially be improved from the point of view of extractive timing and sustainability. Phenolics in cereal bran are, in fact, predominantly bound to cell wall arabinoxylans and *trans*-ferulic acid (FA) is the most abundant phenolic that characterizes cereal brans. Acosta-Estrada (2011) reported various bound phenolic percentages in wheat and rice brans which were calculated to be between 60 and 80% of the total phenolic content; the hydrolysis step is, thus, required to extract the majority of cereals bran phenols. Recent studies focused on the extraction of bound phenolic from plant matrices (Gonzales et al., 2014; Burlini et al., 2020) suggest the use of ultrasounds in association with alkaline hydrolysis in order to enhance bound phenolic extraction and shorten the extraction time. Ultrasonication causes the acoustic cavitation effect on the plant matrix and permits a greater penetration of the solvents into the sample with mechanical effects. The contribution of the sonication effect during hydrolysis conditions has been investigated in the present study in order to apply a more sustainable extraction method, from durum wheat and rice brans, compared to those commonly used. Phenolics from cereal brans exhibited strong antioxidant activity in various literature reports (Esposito et al., 2005; Abozed et al., 2014); moreover, a 0.5% of ferulic acid, in tion to 15% L-ascorbic acid and 1% alpha-tocopherol increased the stabilization of a UV protector topical preparation (Murray et al., 2008). The antimicrobial activity of various wheat brans has been demonstrated by Elhassan et al. (2017) towards gram-positive and gram-negative *bacteria*. In 2013, Borges et al. explained that ferulic acid antibacterial m anism of action is due to hydrophobicity, decrease of negative surface charge and occurrence of local rupture and pore formation which cause irreversible changes in the cell membranes. The antioxidant and antimicrobial activities of the extracts have been therefore evaluated to find possible applications of cereal by-products in the dermo-functional and cosmetic industries.

2. Materials and Methods

2.1. Plant material

Triticum durum (Desf.) bran was provided by Molino Grassi (Parma – Italy) and obtained after decortication of durum wheat caryopsis; *Oryza sativa* L. bran was provided by Grandi Riso S.p.a (Pontelangorino, Ferrara – Italy) and obtained after the rice husking phase. After being received, all plant materials were stocked at -20°C until used. Prior to any extraction, all plant materials were milled through a 2 mm sieving ring of a Variable Speed Rotor Mill (Fritsch, Germany).

2.2. Chemicals

All the solvents and reagents employed for analyses were chromatographic grade. *Trans*-ferulic acid standard, Trolox, DPPH (1,1-diphenyl-2-picrylhydrazil), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), methanol, ethyl acetate, ethanol, formic acid, acetic acid, toluene, natural products-polyethylene glycol reagents (NP/PEG), Folin & Ciocalteu's Reagent and gallic acid were purchased from Sigma-Aldrich Italy (Milano, Italy). Muller Hinton Broth (MHB) and Muller Hinton Agar (MHA) were obtained from Oxoid S.A. Madrid (Spain). Two clinical isolates of *Staphylococcus aureus* (Methicillin-Resistant Staphylococcus Aureus 185087 and Methicillin-Sensitive Staphylococcus Aureus 185960) and one clinical isolate of *Staphylococcus epidermidis* (185240) were obtained from the Hospital Universitario Reina Sofía (Córdoba, Spain). One reference strain of *Staphylococcus aureus* (ATCC 29213) Culti-Loops was obtained from (Oxoid S.A. Madrid, Spain). All antimicrobial agents were purchased from Oxoid (Oxoid, S.A. Madrid, Spain).

2.3. Ultrasound-assisted extraction (UAE)

In order to extract free phenolics from cereal by-products matrixes, 5 g of each bran were placed into a volumetric flask (100 mL), filled with a 65% ethanolic solution as extraction solvent and sonicated at room temperature. Ultrasound-assisted extractions were performed in an ultrasonic cleaning bath (Ultrasonik 104X, Ney Dental International, MEDWOW, Cyprus) under a working frequency of 48 kHz. Wheat bran extraction time was 25 minutes in according to Wang et al. (2008); rice bran extraction time was 45 minutes in according to Tabaraki et al. (2011). The obtained extracts have been filtered, the ethanol was removed with a rotary evaporator (100 rpm, 25°C) and finally extracts were lyophilized until use.

2.4. Bound phenolics extractions

Bound phenolic compounds were extracted as reported by Verma et al. (2009) from all UAE residues, which have previously had the free phenolics removed. Briefly, 2.5g of each matrix was hydrolyzed with 50 mL of 2M sodium hydroxide at room temperature for 1 h (alkaline hydrolysis, AH). After alkaline hydrolysis, the pH of the mixture was adjusted to 3 with 6N HCl. Bound phenolics were then extracted three times with ethyl acetate. The ethyl acetate extracts were evaporated to dryness under a stream of nitrogen at room temperature and the residue was dissolved again in ethyl acetate, centrifuged 10 minutes at 4000 rpm. All samples were evaporated to dryness under a stream of nitrogen at room temperature and finally weighed to calculate the extraction yields. Bound phenolics were also extracted coupling sonication to alkaline hydrolysis (ultrasound-assisted alkaline hydrolysis, UAAH) as described by Gonzales et al. (2014), thus following the same procedure described above for the alkaline conditions but for 30 min instead of 60. After the extraction, all samples were evaporated to dryness under a stream of nitrogen at room temperature and finally weighed to calculate the extraction yields.

2.5. High Performance Thin Layer Chromatography (HPTLC)

HPTLC silica gel 60 F_{254}-precoated high performance thin layer chromatographic plates (CAMAG, Muttenz, Swiss) were used for the analyses with the Linomat V automatic sampler (CAMAG). WinCATS Planar Chromatography Manager software (CAMAG) was used for the analyses. Twin Trough Chambers (20x10 cm) have been used and pre-saturated for 20 min with the eluent mixture.

8 µl of each free phenolics extracts (20 mg/mL in 60% ethanolic solution) were put on the chromatographic plate using the automatic sampler. The chromatographic separation of free phenolics have been performed following the guidelines

of Wagner and Bladt (2009) with some modifications. A two steps elution has been performed. First phase: ethyl acetate/ formic acid/acetic acid/water (100:11:11:20); second mobile phase: toluene/ethyl acetate/acetic acid (100:90:10). For the chromatographic separation of bound phenolic acids, the method described by Barberousse et al. (2008) was chosen with some modifications. 8 µl of each bound phenolics extracts (7 mg/mL in ethyl acetate) were put on the plate using the automatic sampler. The mobile phase was: chloroform:/ethyl acetate/methanol (7:2.5:0.5) + 1% acetic acid. Plates were captured at 254 nm and 366 nm before and after derivatization with NP/PEG in order to highlight the presence of phenolic compounds with TLC Visualizer (CAMAG).

2.6. (HP)TLC bioautographic assay for radical scavenging activity evaluation

HPTLC bioautographic assay was employed to determine antioxidant compounds of the extracts using DPPH radical and ABTS, following the method described by Rossi (Rossi et al., 2011). Each extract was applied to HPTLC plates as described for HPTLC analyses. After development, plates were sprayed with DPPH (i) and ABTS (ii).

(i) After development plates were sprayed with a methanolic solution of 2,2-diphenyl-1-picryl-hydrazyl radical (2 mg/mL) and photographed at visible light after 30 min;

(ii) After development, plates were sprayed with 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) solution obtained after the reaction of 2 mM ABTS and 70 mM of potassium persulfate ($K_2S_2O_8$) and immediately photographed at visible light.

2.7. RP-HPLC-DAD-MS Analyses

The analyses of phenolic acid extracts for the identification and the quantification of FA and *p*-CA acids were performed using a Waters modular HPLC system (MA, model 1525) coupled to a diode array detector (model 2998) and a FinniganMAT LCQ (TermoQuestCorp./FinniganMAT; San Jose, CA) mass spectrometer module linked to an injection valve with a 20µL sampler loop, following the method described by Robbins & Bean (2004). The separation of phenolic acids was achieved with a Luna C18 column (Phenomenex, 250 x 4.6 mm; particle size 5 µm); the mobile phase consisted of methanol (B) and 0.1% aqueous formic acid (A) as the binary solvent system. The solvent gradient in volumetric ratios was as follows: 5–30% B over 50 min, held at 30% B for an additional 15 min; at 65 min the gradient was increased to 100% B and held at 100% B for an additional 10 min to clean up the column. The column was thermostatically controlled at 30°C. The mass experiments were carried out on a FinniganMAT LCQ (TermoQuest Corp./FinniganMAT; San Jose, CA) mass spectrometer module, equipped with an ion trap mass analyzer and an ESI ion source electrospray, in negative ion mode. For ESI-MS and MS^2 experiments, the parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 20 psi, the capillary temperature was 300°C, the auxiliary gas (N_2) fow was 9 l/min, and the skimmer voltage was 40 V. The mass spectrometer was operated in the negative ion mode in the m/z range 100–1000. Injection volume was set to 20 µl. Dedicated JASCO software (ChromNAV ver 2.02.01) was used to calculate peak area by integration. Following chromatogram recording, sample peaks identification was carried out by comparison of UV spectra and retention time (RT) with those of the pure standard. Each tested extract was prepared in a methanolic solution (methanol/water, 80:20) at the concentration of 0.5 mg/mL. Four different concentration of *trans*-ferulic acid and *p*-coumaric standard were prepared in methanol (5 - 500 µg/mL) and each solution was injected in triplicate. The obtained calibration graphs allowed the determination of *trans*-ferulic and *p*-coumaric acids concentrations. Limit Of Detection (LOD) and Limit Of Quantitation (LOQ) were calculated following the approach based on the standard deviation of the response and the slope as presented in the "Note for guidance on validation of analytical procedures: text and methodology", European Medicine Agency ICH Topic Q2 (R1).

2.8. Total phenolic content

The Folin–Ciocalteu spectrophotometric assay was used to determine total phenolic content all extracts with a ThermoSpectronic Helios-γ spectrophotometer and performed according to previously described methods (Singleton et al., 1999; Tacchini et al., 2018). The mean of three readings was used and the total phenolic content was expressed as mg of gallic acid equivalents (GAE)/ g of dried extract. Bound phenolics percentage was calculated with the following formula: bound phenolics % = (TPC_{bound} x 100) / (TPC_{bound} + TPC_{free}).

2.9. DPPH radical scavenging activity

The method of Cheng et al. (2006) was followed to evaluate the radical scavenging activity of the extracts with DPPH (1,1-diphenyl-2-picrylhydrazyl) spectrophotometric assay. Extracts were tested at the following concentrations: UAE 48.88 - 3000 µg/mL; AH: 1.95 - 125 µg/mL; UAAH: 0.49 - 31.25 µg/mL. Eight different concentrations of Trolox (20 - 0.16 µg/mL) were prepared and used as positive control. After 40 min of incubation in the dark at room temperature, microplates were analyzed with a microplate reader (Biorad, 680 XL) and the absorbance was read at 515 nm in triplicate against a blank. The DPPH inhibition in percentage was determined by the following formula: IDPPH% = [1 – (A1/A2)] X 100; where A1 was the DPPH absorbance with the extracts and A2 without extracts. Antioxidant activity of the extract was expressed as IC_{50}, concentration providing 50% inhibition of the radical, and calculated as described by Nostro et al. (2016). All experiments were assessed in triplicate and values were reported as mean ± standard deviation.

2.10. ABTS radical scavenging activity

The ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), radical scavenging activity was performed using the method of Horswald and Andlauer (2011). The same concentrations prepared for the previously described DPPH assay were used for both extracts and Trolox. 7 mmol/L aqueous solution of ABTS (10 mL) and 51.4 $mmolL^{-1}$ aqueous solution of $K_2S_2O_4$ (0.5mL) were mixed to obtain a radical cation solution that has been adjusted spectrophotometrically to 0.7 ± 0.05 at 734 nm. After 6 min of incubation in the dark at room temperature, microplates were analyzed with a microplate reader (Biorad, 680 XL) and the absorbance was read at 734 nm in triplicate and against a blank. Antioxidant activity of the extract was expressed as IC_{50}, the concentration providing 50% radical inhibition, and calculated as described for DPPH. All experiments were assessed in triplicate and values were reported as mean ± standard deviation.

2.11. Antimicrobial susceptibility test

The antimicrobial susceptibility of the *bacteria* was determined on Mueller–Hinton agar (Oxoid S.A. Madrid, Spain) using the disk diffusion method. Six different antimicrobial agents, widely used in human clinical, were studied: ampicillin (10 µg/disk), penicillin (10 µg/disk), chloramphenicol (30 µg/disk), kanamycin (30 µg/disk), ciprofloxacin (5 µg/disk) and doxycycline (30 µg/disk). *Staphylococcus aureus* reference strain ATCC 25923 was used as a quality control. Each antimicrobial agent has been tested against all bacteria strains and incubated overnight at 37°C. The measurement and interpretation of growth inhibition diameters was performed following the CLSI guidelines for human antimicrobial susceptibility tests for human pathogens (CLSI, 2015).

2.12. Microdilution assay for MIC calculation

The antimicrobial activity test was performed on the most interesting extracts in terms of FA content because of its already well-known antimicrobial properties described in literature by Borges et al. (2013). Minimum Inhibitory Concentration

(MIC) of hydrolyzed extracts were determined using the microbroth dilution method. One reference strain (ATCC 29213), two clinical isolates strains of *Staphylococcus aureus* (Methicillin-Resistant *Staphylococcus aureus*, MRSA 185087 and Methicillin-Sensitive *Staphylococcus aureus* MSSA 185960) and one clinical isolate of *Staphylococcus epidermidis* (185240) were used for the antimicrobial activity assay of alkaline hydrolyzed extracts. The MICs were determined through microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2015). All strains have been sub-cultured overnight in Muller-Hinton Agar (MHA) before testing. An inoculum was prepared for each *bacterium* using a 0.85% saline solution and adjusting its OD_{595nm} to 0.08 ± 0.1 (1 x 10^8 CFU/mL). 100 µL of each extract was put in a sterile u-shaped 96-well PS-microplate (Greiner bio-one, USA) and twelve different concentrations (serial twofold dilutions) were tested (2048 – 1 µg/mL) and the same volume of *bacteria* has been put in the microplates; penicillin has been used as positive control and eight different concentrations of it (4 ± 0.06 µg/mL) were tested too. *Bacteria* suspensions without extracts and Muller-Hinton Broth (MHB) were used as controls. The microplates have been then incubated overnight at 37°C. MIC results were determined as the lowest concentrations of extracts at which no *bacteria* growth was detected. A re-count of *bacteria* concentration was performed after each test by diluting 10 µL of each bacteria suspension (negative control) in 10 mL of sterile water: 100 µL of this final suspension have been put on MHA plate at 37°C for 24 hours in order to count the CFU. All tests have been performed in triplicate and compared with controls.

2.13. Cosmetic formulation

Starting from the interesting antioxidant and antimicrobial activity results obtained, one extract has been selected to be inserted in a cosmetic formulation, in order to propose a practical application of cereal by-products in the cosmetic market. We decided to formulate the finished products using durum wheat bran UAAH extract in two concentrations: 0.3% (F1) and 0.5% (F2).

The phenolic extract was included in cosmetic formulations (oil-in-water emulsion) with the following INCI (International Nomenclature of Cosmetic Ingredients):

- *Aqua, Cocoglycerides, Coco-caprylate, Glycerine stearate citrate, Glyceryl stearate, Glycerin, Cetearyl alcohol, Benzyl alcohol, Ethylhexylglycerin, Durum wheat UAAH extract* (INCI*: Triticum turgidum durum seed extract*), *Xanthan gum.*

A common oil-in-water base protocol was used for all formulations. Phase 1 (hydrophilic) was prepared by weighting each raw material and heating the phase up to 70°C. Phase 2 (lipophilic) was prepared and heated up to 40°C and subsequently added to phase 1 by pouring the lipophilic phase into the hydrophilic one with the use of a turboemulsifier mixer (Silverson®). Finally, the emulsion obtained was divided into aliquots to add phase 3 (extract). The final pH was adjusted within the range 5- 5.5 to which all preservative systems work best and to make the preparations suitable for topical application. The stability of the cosmetic products was evaluated at both ambient temperature and at 40°C in oven for 1 month.

2.14. *Photochemiluminescence (PCL) method*

The PCL assay, based on the methodology of Popov and Lewin (1994), was used to measure the antioxidant activity of cosmetic products and of the selected extract in collaboration with Ambrosialab Srl (www.ambrosialab.it). The antioxidant activity was measured with a Photochem. apparatus (Analytik Jena,Leipzig, Germany) against superoxide anion radicals generated from luminol, a photosensitizer, when exposed to UV light (Double Bore. phosphorus lamp, output 351 nm, 3 mWatt/cm2), using ACL (Antioxidant Capacity of Liposoluble substance) kit designed to measure the

antioxidant activity lipophilic compounds (Benov and Lewin, 1994). In ACL studies, the kinetic light emission curve was monitored for 180 s and expressed as micromoles of Trolox per gram of dry matter. The areas under the curves were calculated using the PCL soft control and analysis software. As greater concentrations of Trolox working solutions were added to the assay medium, a marked reduction in the magnitude of the PCL signal and hence the area calculated from the integral was observed. This inhibition was used as a parameter for quantification and related to the decrease in the integral of PCL intensities caused by varying concentrations of Trolox. The observed inhibition of the signal was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve. The extract for ACL measurements was centrifuged (5 min at 16,000 g) prior to analysis. The antioxidant assay was carried out in triplicate for each sample, and 20 µL of the diluted extract (1:40, v/v) HPLC-grade methanol (ACL) was sufficient to correspond to the standard curve.

2.15. Statistical Analysis

All results were means of three independent experiments. The presented data show mean values ± SD. The results were evaluated for statistical significance using Student's t-test for two groups (GraphPad Software, San Diego, California, USA) considering a significant difference of $P < 0.05$ and univariate analysis of variance for more than 2 groups with Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA) and Tukey's post hoc test to investigate significant differences ($P < 0.05$) between the tested samples.

3. Results and Discussions

The yields obtained with the various extraction method applied are reported in Table 1: free phenolic extracts (UAE) gave the highest extraction yields (around 17-20%) due to the hydroalcoholic solvent used which enable the extraction of other molecules with a low extractive specificity. Bound phenolics (AH and UAAH) yields % are much lower but more selective for our phenolic target. In contrast to what was previously verified with maize germ (Burlini et al., 2020), no significant differences in % yields have been obtained coupling sonication to alkaline hydrolysis ($P > 0.05$).

Table 1 Phenolic extraction % yields. Different uppercase letters indicated significant differences in each group of data ($P < 0.05$) for durum wheat bran and rice bran (analysis of variance).

A slightly higher percentage of yield was found by extracting the rice (0.54%) than durum wheat bran (0.41-0.48%). Figure 1 shows the screening qualitative analyses performed with high-performance thin-layer chromatography (HPTLC): plates were captured at 254 nm (A) and 366 nm (B) after derivatization with NP/PEG solution to verify the presence of phenolic acids and of *trans*-ferulic acid (FA), in particular. UAE and hydrolyzed extracts have been eluted with two different eluent mixture and, for this reason, FA is reported in both HPTLC plates: HPTLC plate of UAE extracts, on the left, shows few phenolic spots mainly between R_f 0-0.3 but no spots were detected at FA R_f (0.78). The HPTLC plates on the right, are those related to hydrolyzed extracts which report a blue spot at R_f 0.55 corresponding to FA. This analysis was adopted as a rapid screening for the extracting efficacy of the techniques used, and from its results both type of hydrolysis allowed the extraction of FA.

Figure 1 (1.5 column fitting). HPTLC plates detected at 254 nm and 366 nm after derivatization with NP/PEG. Plates: (A) 254 nm; (B) 366 nm. Tracks: UAE-1 – durum wheat bran UAE; UAE-2 – rice bran UAE; 1- durum wheat bran AH; 2- rice bran AH; 3- durum wheat bran UAAH; 4- rice bran UAAH; FA- ferulic acid standard.

HPTLC bioautographic assays results are reported in Figure 2; this technique was performed to screen the antioxidant capacity of the extracts with DPPH (C) and ABTS (D). Extracts 1, 2, 3, 4 are characterized by a discoloration at R_f 0.55, corresponding to that of FA, suggesting that it could be the main molecule responsible for the radical scavenging activity of the extracts. The presence of CH=CH-COOH group in the hydroxycinnamic acids is, in fact, considered as the main responsible for the higher antioxidant activity compared to hydroxybenzoic acids characterized by the COOH (White & Xing, 1997). Moreover, the same extracts exhibited antiradical activity at R_f 0.1-0.3 for the presence of other phenolic compounds. UAE-1 and UAE-2 extracts shows some active molecules at R_f 0.9-0.95, particularly with ABTS.

Figure 2 (1.5 column fitting). HPTLC plates detected visible light after derivatization with DPPH (A, B) and ABTS (C, D). Tracks: UAE-1 – durum wheat bran UAE; UAE-2 – rice bran UAE; 1- wheat bran AH; 2- rice bran AH; 3- wheat bran UAAH; 4- rice bran UAAH.

The RP-HPLC-DAD analyses confirmed FA and revealed the presence of the *p*-coumaric acid (*p*-CA), previously not detected with HPTLC probably because of the dark blue color that characterizes its spot at 366 nm and for the R_f very similar to that of FA. The presence of the phenolic acids in the hydrolyzed extracts was performed thanks to the comparison of chromatograms obtained, UV spectra and retention times with those of the reference standards and literature (Kim et al., 2006; Tilay et al., 2008). Figure 3 reports RP-HPLC-DAD chromatograms of hydrolyzed extracts where it can be noticed a main peak characterizing all extracts at RT 37 min, corresponding to that of the FA and a second minor peak, corresponding to *p*-CA, at RT 31 min.

Figure 3 (single column fitting). RP-HPLC-DAD of hydrolyzed extracts. Peaks: FA- ferulic acid; p-CA: p-coumaric acid. Extracts: 1- durum wheat bran AH; 2- rice bran AH; 3- durum wheat bran UAAH; 4- rice bran UAAH.

Table 2. Phenolic acids identified with RP-HPLC-ESI-MS

Table 3. RP-HPLC-DAD FA and *p*-CA quantifications. Different uppercase letters indicated significant differences in each group of data ($P < 0.05$) for durum wheat bran and rice bran (Student's t-test).

FA and *p*-CA acids quantifications are reported in Table 3. Both matrices exhibited a higher concentration of FA when extracted with UAAH compared to the AH commonly used. The ultrasounds irradiation allowed the sodium hydroxide to better break the ester linkages of ferulic acid thanks to the several mechanisms involved in the sonication, described by Chemat et al. (2017) such as: fragmentation, erosion, sonocapillarity effect, sonoporation, local shear stress and destruction of plant structures. UAAH wheat bran extract exhibited a content of FA of 406.14 ± 0.65 µg FA/mg extract which is significantly higher than the quantity obtained with the commonly used alkaline treatment: 386.20 ± 10.44 µg FA/mg extract ($P < 0.05$). Similar results were obtained with rice bran that counted 387.36 ± 1.33 µg FA/mg extract with UAAH compared to 369.05 ± 4.91 µg FA/mg extract ($P < 0.05$). Durum wheat bran exhibited the highest difference among the two hydrolysis techniques and the highest *trans*-ferulic acid quantity. *p*-CA was found mainly present in

hydrolyzed rice bran extracts (82.90 ± 1.0 mg *p*-CA/g extract for AH; 92.20 ± 0.1 mg *p*-CA/g extract for UAAH) compared to wheat where the quantity found is much lower (1.50 ± 0.1 mg *p*-CA/g extract for AH; 1.10 ± 0.1 mg *p*-CA/g extract for UAAH).

Table 4. Total phenolic content (TPC) and bound phenolic (BP) percentages. Different uppercase letters indicated significant differences in each group of data ($P < 0.05$) for durum wheat bran and rice bran (analysis of variance).

The total amount of phenolics was quantified for UAE and hydrolyzed extracts and reported in Table 4. Both matrices showed a greater amount of bound phenols than the soluble ones. Free phenolics quantification showed that wheat and rice brans have a very similar free phenolics content (respectively of 80.90 ± 8.60 mg GAE/g dried extract and 79.02 ± 2.20 mg GAE/g dried extract). The quantification of total bound phenolics showed significant higher concentrations with the UAAH compared to the AH ($P< 0.05$): wheat bran hydrolyzed extract exhibited the highest difference in TPC between the two hydrolysis techniques and showed also the highest concentration. Wheat, in fact, showed a total of 437.58 ± 9.90 mg GAE/g of dried AH extract and 610.58 ± 57.60 mg GAE/g of dried UAAH extract. Bound phenolic percentage has been calculated for both wheat and rice matrices and also reported in Table 4: wheat brans exhibited a bound phenolics of 84.40% with AH and 88.3 % with UAAH; and rice bran exhibited percentages of bound phenolics of 86.51% with AH and 86.87% with UAAH. These numbers are in line with those of literature data that show a range of bound phenolics respectively of 62.12-83.18% and 62-88% (Adom & Liu, 2002; Acosta-Estrada, 2011).

Table 5 reports the IC_{50} results of the radical scavenging activity of the extracts that has been investigated both with DPPH and ABTS radicals. The tests have shown the same trend of activity with the highest IC_{50} obtained by UAE, particularly with wheat bran (1194.80 ± 44.93 µg/mL and 250.00 ± 18.79 µg/mL for DPPH and ABTS respectively). Results obtained with rice bran UAE gave a greater activity with both tests (275.10 ± 13.79 µg/mL and 63.45 ± 6.61µg/mL for DPPH and ABTS respectively) despite the content of calculated free phenolic molecules was equivalent. This result may be due to a lipophilic fraction contained in rice bran that, during the hydrolcoholic extraction process, may have contributed to the extract's activity. The lipophilic fraction of rice bran is, in fact, higher than that characterizing wheat bran (Jiang and Wang, 2005) and it is the food source with the highest phytosterol content, class of molecules that have demonstrated to be antioxidants (Lerma-Garcia, 2009; Wang et al., 2002). Regarding bound phenolic extracts, durum wheat bran exhibited the best results both with DPPH (IC_{50} of 3.61 ± 0.09 µg/mL) and ABTS (IC_{50} of 1.16 ± 0.1 µg/mL); in particular, DPPH activity of UAAH durum wheat extract is significantly higher than AH ($P< 0.05$). Moreover, the same result is comparable to that obtain with Trolox (DPPH: 3.43 ± 0.32 µg/mL) suggesting that the association of ultrasounds to alkaline hydrolysis can be a valid technique to effectively obtain antioxidant extracts from cereal wastes. Similar results have been obtained with rice bran extracts, although with slightly lower activity than durum wheat bran: bound phenolics obtained with AH and UAAH gave IC_{50} respectively of 55.00 ± 5.37 µg/mL and 38.01 ± 0.52 µg/mL with DPPH and IC_{50} respectively of 10.3 ± 0.96 µg/mL and 4.22 ± 0.09 µg/mL with ABTS.

Therefore, by-products from durum wheat and rice industry could be interesting sources of antioxidant compounds and FA has been demonstrated to be the main responsible for the activity of the hydrolyzed extracts; this can be inferred thanks to the results obtained through bioautographic and spectrophotometric assays and to the comparison with literature data (Anson et al., 2008; Zou, Laux and Yu, 2004).

Table 5. Radical scavenging activity with DPPH and ABTS. Different uppercase letters indicated significant differences in each group of data ($P < 0.05$) for durum wheat bran and rice bran (analysis of variance).

The antimicrobial susceptibility test was used to evaluate the susceptibility of the *bacteria* strains against six antimicrobial agents which are commonly used to treat human infections with *S. aureus* and *S. epidermidis*. Figure 4 shows *bacteria* growth diameters which has been used to decide whether the *bacterium* was susceptible (S), intermediate (I) or resistant (R) to each antimicrobial agent. In Table 6 results of the antimicrobial susceptibility tests are summarized for each antimicrobial agent: Ampicillin (AK30), Penicillin (P10), Chloramphenicol (C30), Kanamycin (K30), Ciprofloxacin (CIP 5), Doxycycline (DO30). *S. aureus* MRSA and *S. epidermidis* demonstrated to be resistant to two or more antimicrobial agents (CLSI, 2015).

Figure 4 (single column fitting). Bacteria growth diameter of each bacterium evaluate with disk diffusion method. (A): *S. aureus* ATCC; (B): *S. aureus* MRSA; (C): *S. aureus* MSSA; (D): *S. epidermidis*.

Table 6. Antimicrobial susceptibility test (R: resistant; S: sensitive; I: Intermediate)

The Minimum Inhibitory Concentration (MIC) results are reported in Table 7. The most interesting results were obtained against the *Staphylococcus aureus* MRSA strain with MICs of 16 µg/ml and 32 µg/ml for wheat and rice brans respectively. Regarding the *S. aureus* ATCC reference strain, UAAH extracts of wheat bran (128 µg/ml) and rice bran (128 µg/ml) showed higher activity than AH extracts (respectively 521 µg/ml and 256 µg/ml). The same results were obtained by comparing wheat bran UAAH extract on MSSA clinical isolated strain (128 µg/ml) and the AH one (256 µg/ml) even if a difference of one or two dilutions is not considered significant by the CLSI (CLSI, 2015). Extracts tested against *S. epidermidis* showed high MICs values (> 1024 µg/ml) suggesting that the clinical isolated *bacterium* was resistant to all treatments. Finally, according to CLSI guidelines (2015), all strains have been shown to be resistant to penicillin with MICs above 0.25 µg/ml, as previously verified by the antimicrobial susceptibility test (disk diffusion method). The antimicrobial activity test defined wheat and rice brans potentially good antimicrobial sources. They could be use as active ingredients in cosmetic or dermo-functional products with antimicrobial activity or as additives for microbiological stability and therefore as natural preservatives in finished preparations.

Table 7. Microdilution assay for MIC calculation

In order to propose a practical application of the most active extract, a cosmetic product has been formulated (O/W emulsions) and tested for its stability both at ambient temperature and at 40°C. The high quantity of FA, characterized by a deep yellow color, which was contained in durum wheat extract, caused a slight alteration of final preparation's color, in particular with 0.5% of extract concentration. However, a slight color variation did not affect the pleasantness of the finished product and it was not indication of a depletion of the product but enhance its naturalness. Its fragrance was characteristic and pleasant.

The cosmetic products showed excellent stability at room temperature while a slight alteration occurred at 40° C, with a slight surfacing of the oily phase, probably due to the reduced quantity of product taken for the investigation. Furthermore, in both stability tests, a slight modification in the pH has been noted (Table 7) which was in any case within the suggested limits for cosmetic formulations (pH: 3.5-6).

Since previous studies demonstrated that the type of cosmetic formulation, the pH and storage conditions significantly influence the antioxidant activity of a functional ingredient (such could be natural extracts), it is necessary to evaluate the antioxidant activity of a finished product in relation to that of the single active ingredient (Ziosi et al., 2010). Therefore, the antioxidant activity has been tested and compared with PCL method which can provide evaluation of both cosmetic formula and extract activities, consenting an effective comparison. The PCL results are reported in Table 8 and are expressed as μmoL equivalent of Trolox per gram of sample. The higher the value expressed in μmol TE/ gram the greater is the activity. The results verified durum wheat UAAH extract high activity (1728.20 ± 21.76 μmol Trolox/g), but its presence inside the final product performs in proportion to the concentration: having tested low concentrations of extract, the formulation activity exhibited average results. Even if such potencies are compatible with a potential efficacy on skin (Mota et al., 2014), it would be sufficient to increase the concentration of the extract by up to 2% to obtain a very active product.

Table 8. PCL and stability results of the cosmetic formulation

4. Conclusions

Cereal by-products are already well-known sources of interesting phytochemicals but, although their extraction has been the subject of numerous literature studies, precisely because of the great application potential of these matrices, the extraction techniques used up to now require long extraction times and methods that are still not sustainable. The overall results of the present study confirm the possibility to apply a more sustainable and effective method for the release of bound phenolic acids from cereal by-products. In particular, durum wheat and rice bran by-products are interesting sources of phenolic acids, mostly in bound form, whose extraction can be improved by combining ultrasonication to alkaline hydrolysis. Extracts obtained with this technique demonstrated higher content of FA and total phenolics compared to the commonly used chemical hydrolysis, despite the reduction of extraction time from 60 to 30 min. Moreover, the same extracts exhibited the highest antiradical and antimicrobial activities results. For this reason, durum wheat UAAH extract has been used for the formulation of an oil-in-water emulsion for cosmetic use. The extract and the formulations obtained have been tested for the antioxidant activity with PCL, obtaining interesting results given the application potential in the cosmetic field. Since FA is already widely used as an active ingredient and preservative in various topical cosmetic formulations, its extraction with this hybrid technique, makes it possible to boost its application in the health sector, such as in the cosmetic market, with a more sustainable, cheaper and therefore more advantageous scale-up for the industry.

5. Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure 1

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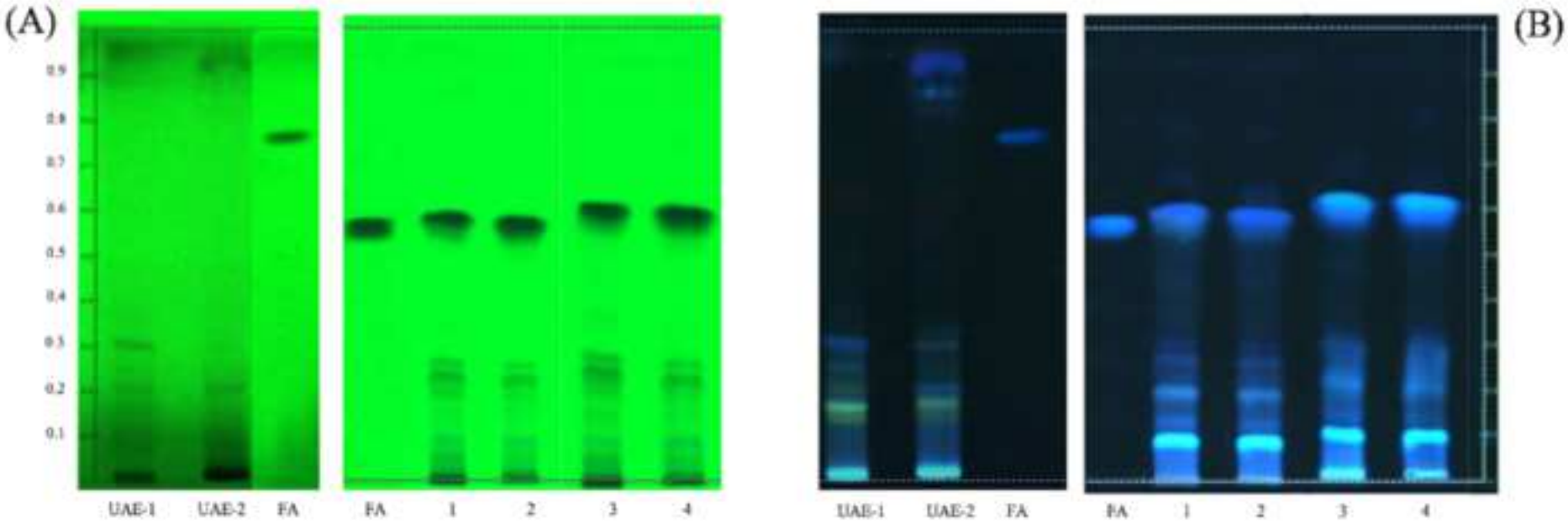

Figure 2

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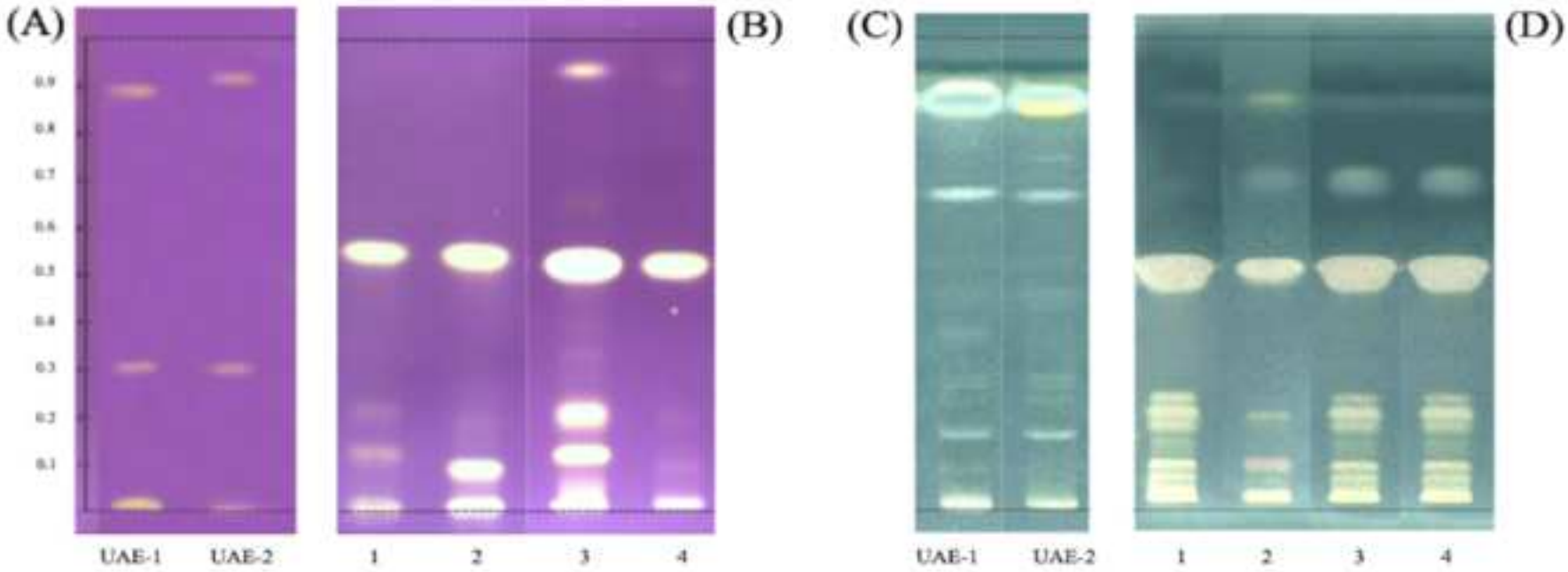


Figure 3

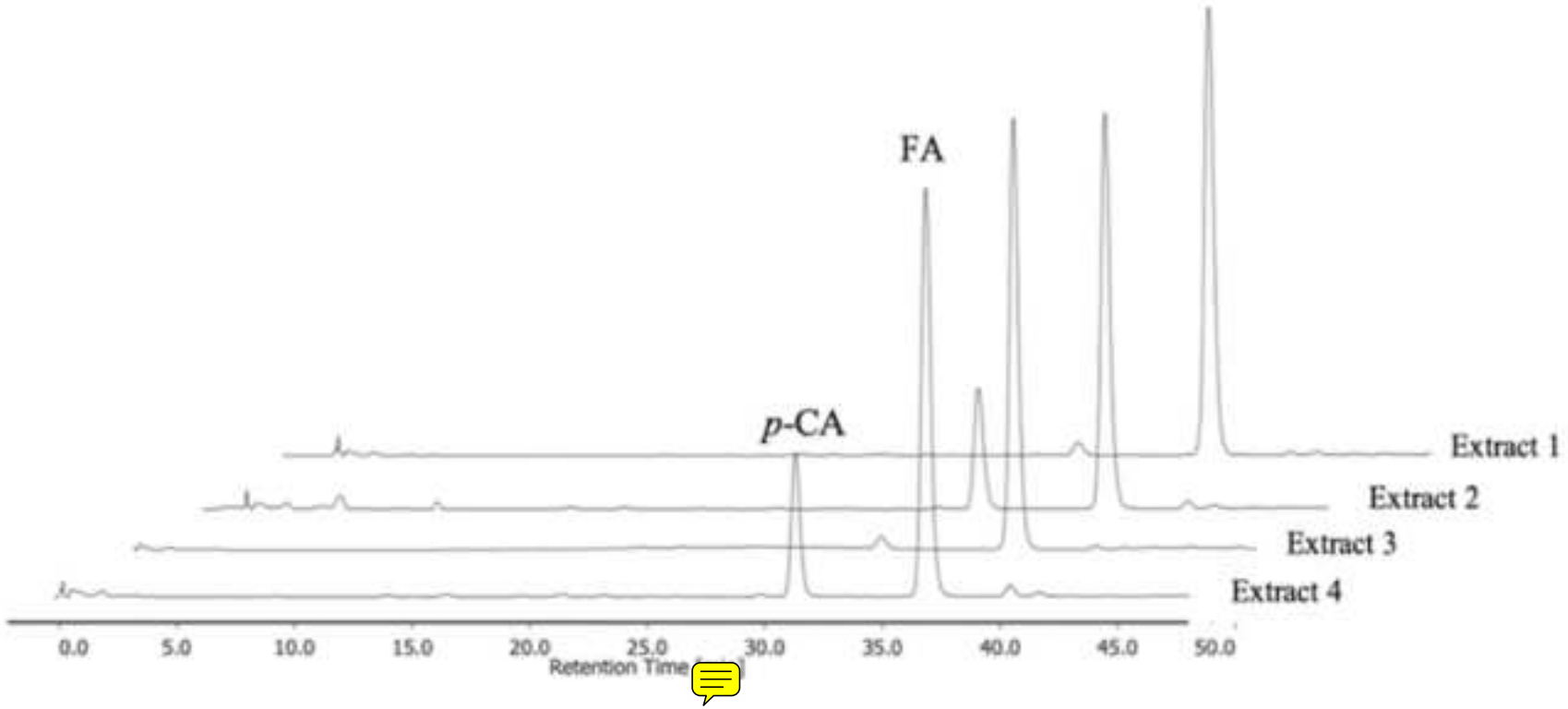
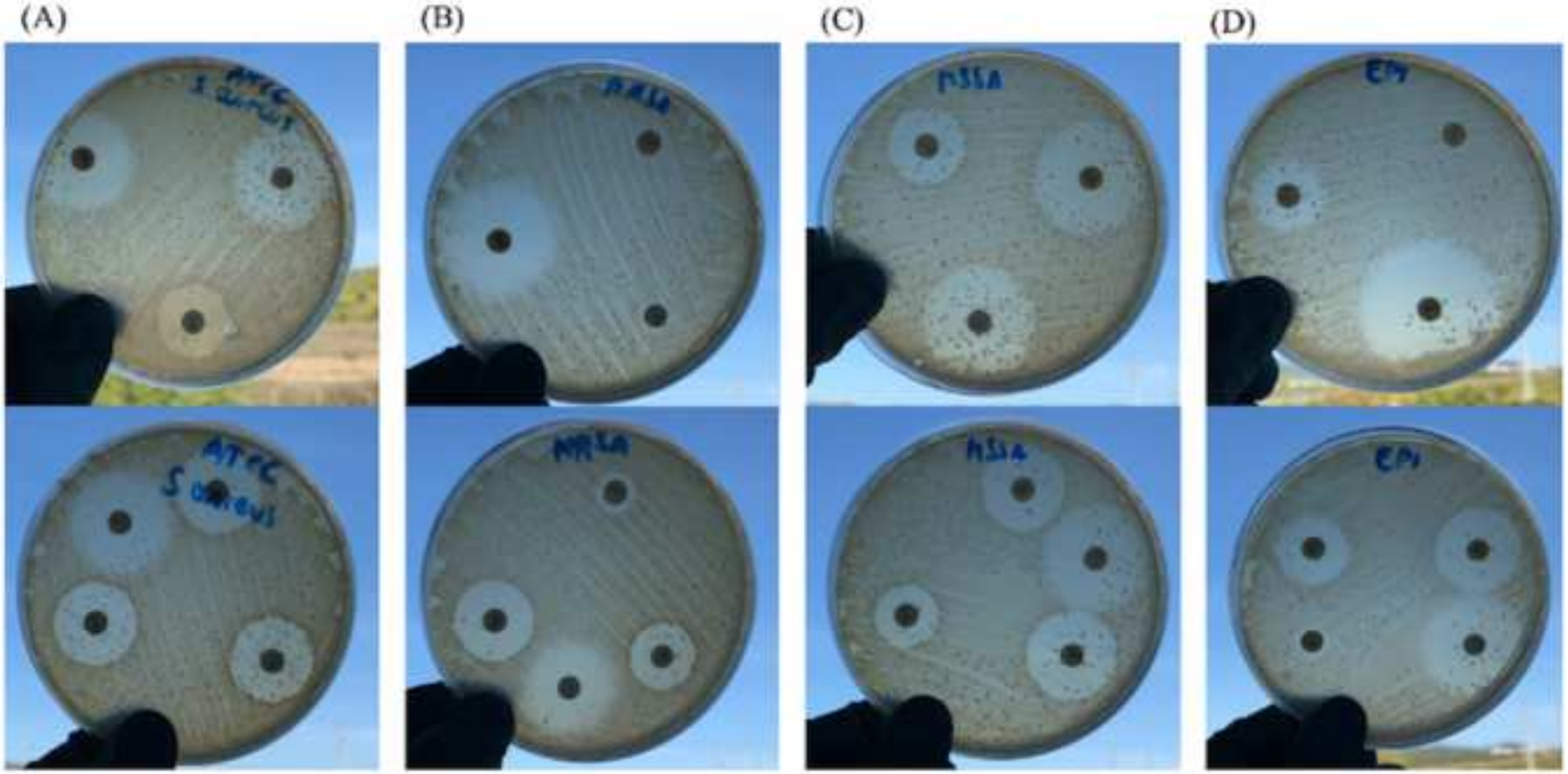


Figure 4



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