Isolation and functional characterization of hemp seed protein-derived short- and medium-chain peptide mixtures with multifunctional properties for metabolic syndrome prevention

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Abbreviations

ACE: angiotensin converting enzyme
ANOVA: analysis of variance
AP: apical
BCA: Bicinchoninic Acid Protein Assay
BL: basolateral

DPP-IV: dipeptidyl peptidase IV
DPPH: 2,2-Diphenyl-1-picrylhydrazyl
EFSA: European Food Safety Authority
FRAP: Ferric Reducing Antioxidant Power
GCB: graphitized carbon black
HMGCoAR: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
LDL: low-density lipoprotein
LDLR: low-density lipoprotein receptor pathway
M: medium-chain peptide mixture of the hemp seed hydrolysate
MetS: Metabolic Syndrome
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PCSK9: Proprotein convertase subtilisin/kexin type 9
PepT1: peptide transport 1
RYR: red yeast rice
S: short-chain peptide mixture of the hemp seed hydrolysate
SDC: sodium deoxycholate
SEC: size exclusion chromatography
T: total hemp seed hydrolysate
TFA: trifluoroacetic acid
UFM: ultrafiltration membrane
UHPLC-HRMS: ultra-high-performance liquid chromatography - high-resolution mass spectrometry

Abstract

This study aims to obtain a valuable mixture of short-chain peptides from hempseed as a new ingredient for developing nutraceutical and functional foods useful for preventing metabolic syndrome that represents the major cause of death globally.

A dedicated analytical platform based on a purification step by size exclusion chromatography or ultrafiltration membrane and high-resolution mass spectrometry was developed to isolate and

comprehensively characterize short-chain peptides leading to the identification of more than 500 short-chain peptides. Our results indicated that the short-chain peptide mixture was about three times more active than the medium-chain peptide mixture and total hydrolysate with respect to measured inhibition of the angiotensin-converting enzyme. The short-chain peptide mixture was also two times more active as a dipeptidyl peptidase IV, and two-fold more active on the cholesterol metabolism pathway through the modulation of low-density lipoprotein receptor.

Keywords: peptidomics, size-exclusion chromatography, LC-HRMS, ACE, DPP-IV, HMGCoAR

1. Introduction

Metabolic syndrome (MetS) comprises a cluster of metabolic abnormalities, including accumulation of lipids, insulin resistance, and chronic inflammation, leading to three main long-term medical conditions, i.e., type II diabetes, ischemic stroke, and cardiovascular diseases, which represent the first cause of death globally (Saklayen, 2018). The current management to address MetS-related abnormalities includes adopting a healthy lifestyle and following a long-term medication regimen, often involving administering several expensive drugs, which is challenging for patients (Lillich et al., 2021).

Nowadays, research and development in the nutraceutical field offer the opportunity to prepare patented proprietary combinations of chemically well-defined nutraceuticals to improve specific health issues as support to conventional drug treatments. Based on natural bioactive compounds, preventive and therapeutic methods are free from significant side effects and could represent a proper adjuvant treatment to reduce hospitalization and health costs and improve the quality of life of MetS patients (Santini & Novellino, 2017).

In this context, the administration of red yeast rice (RYR) extract, containing Monacolin K, represents an effective strategy for promoting vascular and metabolic health (Yuan et al., 2022). On the other side, the natural compound berberine can reduce cholesterolemia by increasing low-density lipoprotein (LDL) cholesterol receptors on the liver cell surface and inhibiting triglycerides biosynthesis by activating the adenosine monophosphate-activated proteokinase (Brusq et al., 2006). In addition, berberine modulates the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene expression by acting on the expression of its selective transcription factor HNF1-alpha, improving LDL receptor pathway (LDLR) stability (Dong et al., 2015). These considerations suggest that the combined administration of RYR and berberine may provide a broader range of health protection than the one afforded by prescribed statin therapy (McCarty et al., 2015). Despite the wide use of this product, due to the presence of monacolin K (MonK, the same compound sold with the commercial

name of lovastatin), RYR possesses specific adverse effects that are typical of statins, such as liver damage and muscle disorders (myopathy) (Mazzanti et al., 2017). Therefore, it is necessary to find new active ingredients to replace RYR in the formulation of dietary supplements to prevent hypercholesterolemia and cardiovascular diseases. Indeed, the European Food Safety Authority (EFSA) has just delivered a scientific opinion on the safety of monacolins, the active ingredients from RYR, and provided advice on the dietary intake of monacolins that does not give rise to concerns about harmful effects. Exposure to MonK does not lead to severe adverse effects on the musculoskeletal system, including rhabdomyolysis, and on the liver only at doses inferior to 3 mg/day. It is important to underline that in most commercial food supplements, the current MonK dosage is 10 mg/day, a level considered by the panel as a significant safety concern (EFSA Panel on Food Additives and Nutrient Sources added to Food 2018).

Considering all mentioned issues, discovering alternative nutraceutical products is a is a priority for the food and nutrition industry. Among the great diversity of functional phytochemicals, bioactive peptides have stood out as functional compounds due to their ability to reach the bloodstream and exert biological activity (Pérez-Gregorio et al., 2020). More recently, short-chain peptides (i.e., peptides with 2-4 amino acid long sequences) were becoming particularly interesting and showed advantages over longer peptide sequences; in particular, short-chain peptides have low cytotoxicity and the ability to maintain their biological properties unaltered upon absorption, as they are not subject to in-vivo transformation (Webb et al., 1992). In addition, multifunctional peptides represent a new concept in the field (Lammi, Aiello, et al., 2019). In particular, this definition indicates the peptides that have the capacity to impart more than one physiological outcome by affecting different targets. In the case of food hydrolysates, the multifunctionality could be due to: a) the presence of peptides that have different bioactivities even though they are monofunctional; b) the existence of truly multifunctional peptides; c) to synergistic or interaction effects between different peptides

Recently, it has been demonstrated that protein hydrolysates derived from the hydrolysys of Chlorella pyroneidosa and Spirulina (*Arthrospira platensis*) proteins with pepsin inbith both dipeptidyl

peptidase IV (DPP-IV) and angiotensin-converting enzyme (ACE), respectively (Aiello et al., 2019; Li et al., 2021).

In this work, an analytical platform based on high-throughput techniques was developed to purify and tentatively identify short peptide sequences produced from hemp seed proteins. Hemp seed samples were selected since they possess high nutritional content (about 30% proteins). Recent research based on *in-vitro* or *in-vivo* experiments has shown that hydrolysates obtained from hemp seed proteins possess several biological activities (Aguchem et al., 2022). More in detail, size exclusion chromatography (SEC) and membrane ultrafiltration (UFM) with a molecular cut-off < 1 kDa were tested to maximize the isolation yield of short-chain peptides, considering issues due to the wide range of polarity of these molecules and the complexity of the hempseed hydrolysates.

The composition of the purified extracts was investigated by a suspect screening untargeted metabolomics approach based on ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) and bioinformatics, devised explicitly for the profiling of short-chain peptides. The biological activity associated with the prepared hydrolysates was tested for the specific needs of MetS improvement. In particular, the anti-DPP-IV, anti- ACE, and anti-HMGCoAR, and antioxidant activities were investigated. Initially, in vitro studies were carried out to measure the inhibitory activity of the hydrolysates on relevant enzymes such as ACE, DPP-IV, and HMGCoAR. The cholesterol-lowering activity of hempseed peptides was characterized using HepG2 cells to assess their effects on the cholesterol metabolism pathway. Afterward, the human intestinal Caco-2 cells were employed to assess the effects of the hydrolysates on the DPP-IV activity modulation. Then, *ex vivo* experiments were performed using the human serum to evaluate the effect of hempseed short-chain peptide mixture on circulating DPP-IV. The bioactivity of food peptides depends not only on their biological effects but also on bioavailability at the intestinal level, reaching intact the organs where they can exert health-promoting activity (Lammi, Aiello, et al., 2019). In general, short-chain peptide mixture tend to be absorbed faster than medium- and/or long-chain ones by enterocytes (Lammi, Aiello, et al., 2019). Hence, differentiated Caco-2 cells were used

as a reliable model for assessing the ability of short-chain peptide mixture (S) to be transported at the intestinal level, addressing delicate issues related to their stability and bioavailability.

2. Materials and methods

2.1 Protein extraction and digestion

Hemp flour, obtained from ground seeds of the hemp variety Futura 75, was purchased in a local market. The protein content declared on the label was 40%.

Fifty mg of hemp flour was extracted with 2 mL of a buffer containing 50 mmol/L of Tris-HCl (pH 8.5) and 2% (w/v) of sodium deoxycholate (SDC). The sample was incubated on ice for 1 h with intermittent vortexing (1 min) every 15 minutes. After this step, samples were placed in an ultrasonic bath for 1 h; the entire cycle was repeated twice, and, finally, the insoluble material was removed by centrifugation at room temperature for 20 min at 20 000 × g. The extracted proteins were quantified by Bicinchoninic Acid Protein Assay (BCA) using bovine serum albumin as standard as previously described (Walker, 1996).

After quantification, hemp protein was hydrolysed by Alcalase®, as previously described (Montone et al., 2018). Briefly, a 15 mg protein aliquot was diluted with 50 mmol L^{-1} Tris-HCl (pH 8.8) to obtain a final urea concentration of 0.8 mol/L. Alcalase was added (1:10, enzyme: protein ratio), and samples were incubated at 60 °C for 4 h. Enzymatic hydrolysis was quenched by decreasing the pH to 2 with trifluoroacetic acid (TFA). The hydrolysed sample was centrifuged for 10 min at 20 000 x g at room temperature to remove SDC that precipitates in acid environment. The resulting peptide mixture was stored at −20 °C until analysis. More in details, following this procedure, three independent hydrolysates T were produced. The peptide concentration was determined by GoA assay, according to Lammi et al., (Lammi et al., 2016), based on chelating the peptide bonds by Cu (II) in alkaline media and controlling the change of absorbance at 330 nm. In brief, the reagent contained 0.6 M sodium citrate, 0.9 M sodium carbonate, and 0.07 M copper sulfate, 2.4 M NaOH at pH 10.6. A solution containing X µL peptide mixture, (500 − X) µL water, 500 µL 6% (w/w) NaOH in water,

and 50 µL active reagent was prepared. The optical density of the solution was measured at 330 nm. A sterile solution of peptone (10 mg/mL) in water was used as standard for the calibration curve.

2.2 Evaluation of the degree of hydrolysis (DH)

The DH was determined by the o-phthalaldehyde (OPA) assay, according to Nielsen et al., 2001 (https://doi.org/10.1111/j.1365-2621.2001.tb04614.x) with certain modifications. This assay is based on the formation of an adduct between the α-amino groups of peptides and the OPA reagent. 200 µL of OPA reagent were mixed with 26.6 µL of hydrolysates, the absorbance was assessed at 340 nm using the Synergy H1 fluorescent reader (Biotek, Bad Friedrichshall, Germany) after 1.5 min of incubation at 25 °C.

2.3 Peptide separation

The Alcalase hydrolysate was subjected to peptide separation using two different protocols, namely SEC and UFM systems.

2.3.1 Size exclusion chromatography

The total hydrolysate was subjected to peptide separation using a BIOBASIC SEC 120, 5 µm, 150 x 7.8 mm (Thermo, Waltham, Massachusetts, USA) connected to a Shimadzu Prominence LC-20A system, including a CBM-20A controller, two LC-20 AP preparative pumps and a DGU-20A3R online degasser. An SPD-20A UV with a preparative cell (0.5 mm) was used as a detector and was set at 214nm. An FRC-10A Shimadzu was employed as an auto collector. Data acquisition was performed by the LabSolution version 5.53 software (Shimadzu, Kyoto, Japan).

The sample was eluted in isocratic with a flow rate of 1 mL/min using ddH_2O/TFA (99.9/0.1, v/v). Two fractions were collected, as shown in Figure 1S. The fraction collected from 1 to 5 min contained medium-sized peptides, while the fraction collected from 6 to 10 min contained short peptides. The dry weight of the mixture was 14.7 mg (98% w/w).

205 ***2.3.2 Ultrafiltration***

206 The total hydrolysate was subjected to Ultrafiltration Discs, 1 kDa NMW (Ultracel®) regenerated
207 cellulose, 44.5 mm in diameter. The filter was initially pre-treated and rehydrated in H_2O for 1 h to
208 remove impurities from the manufacturing process or additives used for stabilization. After that, the
209 membranes were washed three times with Milli-Q water and stored before use. Freshly pre-treated
210 membranes were used in all experiments unless indicated otherwise.

211 Then, the filter was rinsed three times with 3 mL of MeOH, once with 1 mL of deionized H_2O/TFA
212 (99.9/01, v/v), and stored before use. Freshly pre-treated membranes were used in all experiments
213 unless indicated otherwise. Fifteen mg of the total hydrolysate was transferred to the membrane and
214 filtered by Millipore classic glass filter holder. The filtrate, containing short-chain peptide mixture,
215 was recovered and dried. The dry weight of the purified mixture was 6 mg (40% *w/w*).

216

217 **2.4 Analysis of medium-chain peptide mixture by nano-high performance liquid**
218 **chromatography-MS/MS**

219 Medium-chain peptide mixture obtained from SEC chromatography were analyzed by nano HPLC
220 on an Ultimate 3000 (Thermo Fisher Scientific, Bremen, Germany) coupled to an Orbitrap Elite mass
221 spectrometer (Thermo Fisher Scientific). According to the manufacturer's instructions, the mass
222 spectrometer was calibrated once a week using the Pierce LTQ Velos ESI Positive Ion Calibration
223 Solution (Thermo Fisher Scientific). Mass accuracy was <1.5 ppm without lock-mass. Medium-chain
224 peptide mixture was analyzed as previously described (Cerrato, Capriotti, et al., 2020), with some
225 modifications. Twenty µL were online preconcentrated on a µ-precolumn (Thermo Fisher Scientific,
226 300 µm i.d. × 5 mm Acclaim PepMap 100 C18, 5 µm particle size, 100 Å pore size) at 10 µL/min
227 flow rate of a premixed mobile phase H2O/ACN 98:2 (v/v) containing 0.1% (v/v) TFA. Then,
228 samples were separated on an EASY-Spray column (Thermo Fisher Scientific) 15 cm × 75 µm i.d.
229 PepMap C18, 3 µm particles, 100 Å pore size operated at 250 nL/min and 20 °C. A 55 min-long
230 gradient was employed with H_2O and ACN as mobile phases A and B, respectively, both with 0.1%

formic acid. The following linear gradient was used: 1% B for 5 min; 1–5% B in 2 min; 5–35% B in 38 min; 35–50% B in 5 min; 50–90% B in 5 min. Finally, the column was washed at 90% B for 10 min and then equilibrated at 1% B for 20 min.

Peptide spectra were acquired in the 380–1800 m/z range at 30,000 resolution (full width at half maximum, FWHM, at m/z 400) for the full scan. MS/MS spectra were acquired at 15,000 resolution (FWHM, at m/z 400) in top 10 data-dependent acquisition (DDA) mode with the rejection of singly charged ions and unassigned charge states. Precursors were fragmented by higher-energy collisional dissociation (HCD) with 35% normalized collision energy and 2 m/z isolation window (other fragmentation techniques, such as infrared multiphoton dissociation, could provide additional information due to the formation of more secondary fragments and reduced ion losses (Bianco et al., 2014). Dynamic exclusion was enabled with a repeat count of 1 and a repeat duration of 30 s with an exclusion duration of 20 s. For each sample, three technical replicates were performed. Raw data files were acquired by Xcalibur software (version 2.2, Thermo Fisher Scientific).

2.5 Analysis of short-chain peptide mixture by ultra-high performance liquid chromatography-MS/MS

Short-chain peptide mixture obtained from SEC and UFM were analyzed by LC-HRMS in a suspect screening fashion as previously described (Cerrato, Aita, et al., 2020) using a Vanquish binary pump coupled to a hybrid quadrupole-Orbitrap Q Exactive mass spectrometer (Thermo Fisher Scientific) through a heated electrospray source. Samples were separated by a Kinetex XB-C18 (100 × 2.1 mm, particle size 2.6 μm, Phenomenex, Torrance, CA, USA) operated at 40 °C. Spectra were acquired in the positive ion mode range m/z 150-750 with a resolution (full width at half maximum, FWHM, m/z 200) of 70,000. For each sample, two individual runs were acquired for natural and modified short peptides using two dedicated inclusion lists containing the exact m/z of the protonated ions of all unique short peptide masses. The inclusion lists were prepared using MatLab R2018, as previously described (Cerrato, Aita, et al., 2020). The acquisition of the higher collisional dissociation (HCD) MS/MS spectra was performed using the top 5 data-dependent acquisition (DDA) mode at 35%

normalized collision energy and 35,000 (FWHM, m/z 200) resolution. All samples were run in triplicate.

The identification of the short endogenous peptidome was achieved thanks to a dedicated data processing workflow implemented on Compound Discoverer 3.1 (Thermo Fisher Scientific) by our research group (Cerrato, Aita, et al., 2020). The strategy allowed to extract the m/z from the RAW data files, align the runs, and remove blank signals and masses not associated with at least one MS/MS spectrum. Moreover, the workflow allowed to filter out all masses not present in the mass list (the same employed for short peptide data acquisition). The identification of the short sequences was achieved after manual interpretation of the MS/MS spectra aided by the match of the in silico spectra generated by mMass (Strohalm et al., 2010).

2.6 Cell culture conditions

Human hepatic HepG2 cells were bought from ATCC (HB-8065, ATCC from LGC Standards, Milan, Italy) and intestinal Caco-2 cells were obtained from INSERM (Paris, France). Both cell lines were cultured in DMEM high glucose with stable L-glutamine, supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin (complete growth medium) with incubation at 37 °C under 5% CO2 atmosphere..

2.7MTT assay

A total of 3 x 10^4 HepG2 and Caco-2 cells/well were seeded in 96-well plates and treated with 0.1, 0.5, 1.0, and 2.0 mg/mL of S, M, and T peptide mixtures, or vehicle (H_2O) in complete growth media for 48 h at 37 °C under 5% CO_2 atmosphere. Subsequently, the treatment solvent was aspirated and 100 µL/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) filtered solution added. After 2 h of incubation at 37 °C under 5% CO_2 atmosphere, 0.5 mg/mL solution was aspirated and 100 µL/well of the lysis buffer (8 mM HCl + 0.5% NP-40 in DMSO) added. After 5 min of slow shaking, the absorbance at 575 nm was read on the Synergy H1 fluorescence plate reader (Biotek, Bad Friedrichshall, Germany).

2.8 HMGCoAR activity assay

283 The assay buffer, NADPH, substrate solution, and HMGCoAR were provided in the HMGCoAR
284 Assay Kit (Sigma). The experiments were carried out following the manufacturer's instructions at
285 37 °C. In particular, each reaction (200 μL) was prepared adding the reagents in the following order:
286 1 X assay buffer, S and M peptides mixtures (at final concentrations of 0.1, 0.12, 0.15, 0.175, 0.2, 0.3
287 mg/mL, and the total hydrolysate T (at final concentrations of 0.2, 0.25, 0.3, 0.4, 0.5 mg/mL) or
288 vehicle (C), the NADPH (4 μL), the substrate solution (12 μL), and finally the HMGCoAR (catalytic
289 domain) (2 μL). Subsequently, the samples were mixed and the absorbance at 340 nm read by a
290 microplate reader Synergy H1 from Biotek at time 0 and 10 min. The HMGCoAR-dependent
291 oxidation of NADPH and the inhibition properties of lupin peptides were measured by absorbance
292 reduction, which is directly proportional to enzyme activity.
293
294 **2.9 Western blot analysis**
295 A total of 1.5×10^5 HepG2 cells/well (24-well plate) were treated with S at a concentration of 0.5
296 mg/mL and with M and T at a concentration of 1 mg/mL, or with the reference compound MonK at
297 1 μM, or vehicle (H_2O), for 24 h. After each treatment, cells were scraped in 30 μL ice-cold lysis
298 buffer [RIPA buffer + inhibitor cocktail + 1:100 PMSF + 1:100 Na-orthovanadate] and transferred in
299 an ice-cold microcentrifuge tube. After centrifugation at 13300g for 15 min at 4 °C, the supernatant
300 was recovered and transferred into a new ice-cold tube. Total proteins were quantified by Bradford
301 method and 50 μg of total proteins loaded on a pre-cast 7.5% Sodium Dodecyl Sulphate -
302 Polyacrylamide (SDS-PAGE) gel at 130 V for 45 min. Subsequently, the gel was pre-equilibrated
303 with 0.04% SDS in H2O for 15 min at room temperature (RT) and transferred to a nitrocellulose
304 membrane (Mini nitrocellulose Transfer Packs,) using a trans-Blot Turbo at 1.3 A, 25 V for 7 min.
305 Target proteins, on milk or BSA blocked membrane, were detected by primary antibodies as follows:
306 anti-SREBP2, anti-LDLR, anti-HMGCoAR, anti-PCSK9, anti-HNF1-α and anti-β-actin. Secondary
307 antibodies conjugated with HRP and a chemiluminescent reagent were used to visualise target
308 proteins and their signal was quantified using the Image Lab Software (Biorad). The internal control

β-actin was used to normalize loading variations. The anti-PCSK9 and anti-HNF1-α primary antibodies were used after the complete removal of primary and secondary antibodies from membranes were the SREBP-2 and the HMGCoAR proteins, respectively, were previously detected, since the molecular wheigths of these proteins are similar.

2.10 Assay for evaluation of fluorescent LDL uptake by HepG2 cells

A total of 3×10^4 HepG2 cells/well were seeded in 96-well plates and kept in complete growth medium for 2 days before treatment. On the third day, cells were treated with S peptides mixture at a concentration of 0.5 mg/mL and with M and T peptides mixtures at a concentration of 1 mg/mL, or with the reference compound Monacolin K (MonK) at 1 μM or vehicle (H_2O) for 24 h. At the end of the treatment period, the culture medium was replaced with 50.0 μL/well LDL-DyLight™ 550 working solution. The cells were additionally incubated for 2 h at 37 °C and then the culture medium was aspirated and replaced with PBS (100 μL/well). The degree of LDL uptake was measured using the Synergy H1 fluorescent plate reader from Biotek (excitation and emission wavelengths 540 and 570 nm, respectively).

2.11 *In vitro* DPPIV activity assay

The *in vitro* experiments were carried out in duplicate in a half-volume 96-well solid plate (white). Each reaction (50 μL) was prepared adding the reagents in a microcentrifuge tube in the following order: 1 X assay buffer [20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, and 1 mM EDTA] (30 μL), S, M and T peptides mixtures at final concentrations of 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL or vehicle (10 μL) and finally the DPPIV enzyme (10 μL). Afterward, the samples were mixed and the 50 μL were transferred in each plate well. Each reaction was started by adding 50 μL of substrate solution (200 μM H-Gly-Pro-7-amido-4-methylcoumarin (AMC)) to each well and incubated at 37 °C for 30 min. Fluorescence signals were measured using the Synergy H1 fluorescent plate reader from Biotek (excitation and emission wavelengths 360 and 465 nm, respectively). The DPPIV enzyme and the substrate solution were provided by Cayman Chemicals (Michigan, USA).

2.12 Evaluation of the inhibitory effect of hempseeds peptides on cellular DPP-IV activity

335 A total of 5×10^4 Caco-2 cells/well were seeded in black 96-well plates with clear bottoms. On the
336 second day after seeding, spent media was discarded and the cells were treated with 1.0 and 2.0
337 mg/mL of S, M, and T peptides mixtures, or Sitagliptin (100 nM) as reference compound, or vehicle
338 (H_20) in growth medium for 3h at 37 ◦C. Subsequently treatments were removed and 40 µL of Gly-
339 Pro-AMC substrate were adde to each well at the concentration of 25 µM in PBS. Fluorescence signal
340 (excitation and emission wavelengths 350 and 450 nm, respectively) was recorded after 30 min using
341 a Synergy H1 microplate reader.
342
343 **2.13 *Ex vivo* DPP-IV Activity Assay**
344 Human serum sterile filtered commercially available from Sigma-Aldrich (Cat. N° H4522-100 mL)
345 were aliquoted 40 µL/well in black 96-well plates and 10 µL of S and M peptides mixtures (at final
346 concentrations of 1.0 and 2.0 mg/mL) and Sitagliptin (at a final concentration of 100 nM) were added
347 and the plate was incubated for 1h at 37 °C. Subsequently, 50 µL of Gly-Pro-AMC was added to each
348 well to achieve a final substrate concentration of 25.0 µM. Fluorescence signals (ex./em. 350/450
349 nm) were then recorded using a Synergy H1 microplate reader.
350
351 **2.14 *In vitro* measurement of the ACE inhibitory activity**
352 ACE inhibitory activity was tested by measuring with HPLC the formation of hippuric acid from
353 hippuryl-histidyl-leucine, used as a mimic substrate for ACE I. Test was performed in 100 mM Tris-
354 HCOOH, 300 mM NaCl pH 8.3 buffer, and using ACE from porcine kidney (Sigma-Aldrich, Milan,
355 Italy). S, M and T peptides mixtures were tested at 1.0 mg/mL and IC50 value is the concentration
356 needed to observe a 50% inhibition of ACE activity. All experimental details of samples preparation
357 and analyses conditions have been published elsewhere (Boschin et al., 2014a) (Boschin et al.,
358 2014b).
359
360 **2.15 Statistical analysis**

Three independent T hydrolysates from which three independent M and S fractions were produced. Each independent sample was analytical characterized, and the sequences of the identified peptides are the same among the three independent T, M, and S samples, respectively. For the biological characterization, a pool of the three independent hydrolysates was produced and tested performing all the biological experiments. Statistical analyses were carried out by One-way analysis of variance (ANOVA, Graphpad Prism 9) followed by Dunnett's test. Values were expressed as means ± s.d.; P-values <0.05 were considered to be significant.

3. Result and Discussion

3.1 Hempseed peptides production and peptidomics analysis

Hemp seeds contain 20–25% protein, rich in essential amino acids for maintaining good health (Farinon et al., 2020). Hemp seed proteins possess high bioavailability and digestibility, contain hypoallergenic peptides whose absorption is improved by the fiber complex, and have a subtle aromatic profile, a balanced food source for human nutrition (Nwachukwu & Aluko, 2021). The current trend is to relaunch this underexploited plant to obtain protein seeds with benefits for various diseases that have been medically proven (Hertzler et al., 2020). Recent investigations have demonstrated that peptides produced by enzymatic hydrolysis of hempseed proteins provide several biological activities, including antihypertensive activity (Malomo et al., 2015), anti-inflammatory activity (Cruz-Chamorro et al., 2022), hypocholesterolemic activity (Zanoni et al., 2017), antioxidant activity (Pontonio et al., 2020), inhibitory activity on HMGCoAR (Aiello et al., 2017), and anti-diabetic properties (Lammi, Bollati, et al., 2019). Most of these works focus on a limited number of medium-sized peptides, namely those with more than seven amino acids in their sequences.

In contrast, only one report has obtained an UFM extract in which the molecular weight distribution of hemp peptides estimated by MALDI-TOF/TOF mainly comprises 2–3 amino acid residues (Wei et al., 2021). It has been reported that short oligopeptides, especially dipeptides or tripeptides, are the best candidate since they can easily be absorbed, resisting to hydrolysis by gastrointestinal proteases and serum peptidases, and can be transported in their bioactive forms to target tissues (Wang et al., 2019). Short peptide sequences have been largely neglected to date, compared to medium- and long-chain peptides, which have long been investigated by borrowing the analytical technologies from bottom-up proteomics (Fricker, 2015). Short peptides show a more remarkable resemblance to polar metabolites than longer peptides for their low molecular weights and wide range of physicochemical properties (acid-base properties and polarity). An analytical platform was set up in our laboratory based on a purification step dedicated explicitly to the separation of short-chain peptide mixture, suspect screening data acquisition, and a customized data processing workflow on Compound

Discoverer software in a metabolomics-based fashion to deal with the untargeted identification of short peptide sequences (Cerrato, Aita, et al., 2020).

In our previous works, a solid phase purification based on graphitized carbon black (GCB) material was employed to enrich short peptides (Piovesana et al., 2019). For nutraceutical applications, which have the final purpose of introducing a potential new product with multifunctional activity on the market, the industrial scale-up of purification and separation processes is essential to treat a high amount of samples. Using material as GCB poses several limitations for routinary purification because the analytical process is laborious and time-consuming. For basic peptides, for instance, a backflushing elution is needed. Moreover, the GCB is produced at high temperatures, and some reproducibility problems could occur between two different preparation batches or manufacturers. For these reasons, the remarkable production volumes of peptide mixtures for nutraceutical purposes have generated a strong interest in alternative purification procedures, mainly due to their relevant impact on manufacturing costs. Because of its flexibility, preparative liquid chromatography is nowadays the method of choice in these applications, allowing one to choose case-by-case the experimental conditions that most suitably fit that particular purification problem (De Luca et al., 2021). In line with literature evidences (Malomo et al., 2015), the DH (%) of Alcalase derived total hydrolysate (T) was 27.8±2.2%, and the peptide concentration, which was determined by GoA assay (Lammi et al., 2016), is 308,03±10,5 µg/µL. Two analytical techniques were chosen and compared in this work: SEC and UFM. The use of SEC permitted obtaining two distinct fractions, as shown in Figure 1S, containing medium-chian (1-5 min) and short-chain peptides (5-10 min). In contrast, using UFM led to obtaining a single fraction consisting of only short-chain peptide mixture.

The obtained fractions were analysed differently; those containing short-chain peptide mixture was submitted to a suspect screening approach on UHPLC-HRMS instrumentation, while a typical proteomics experiment by nanoLC-HRMS was employed to analyse the fraction containing medium-chain peptide mixture.

420 The suspect screening approach was based on using inclusion lists in the mass spectrometric method
421 that bypasses the limitations of data-dependent acquisition mode when comprehensive lists of the
422 analytes are available (Cerrato, Aita, et al., 2020). Moreover, the data processing workflow allowed
423 extracting the m/z from the raw data files, aligning the features in the different samples, removing
424 compounds present in the black sample, predicting the molecular formulas from the accurate masses
425 and isotopic patterns, and associating the predicted formula to those of the short peptide sequences
426 listed in the short peptide databases that were also employed for data acquisition. After careful manual
427 validation of the putative peptides based on the peculiar short peptide fragmentation pathways, 559
428 short peptides and 557 peptides were tentatively isolated in hemp seed by SEC and UFM,
429 respectively. Table S1 reports, for short-chain peptides isolated by SEC and UFM, respectively,
430 detailed data on the tentatively identified sequences, including retention time, proposed formula,
431 experimental m/z, MS accuracy, and primary diagnostic product ions. Since Leu and Ile cannot be
432 distinguished by MS/MS (MS3 experiments are needed) (Cerrato, Aita, et al., 2020), the
433 nomenclature Xle was employed throughout the manuscript and Supplementary Materials for
434 indicating either Leu or Ile in a peptide sequence. Five hundred forty-eight peptides (98%) were
435 common in the two isolation procedures establishing no significant differences between the
436 techniques employed. Of the 568 annotated short peptides, 224 (39.3%) were dipeptides, 176 (31%)
437 were tripeptides, and 168 (29.7%) were tetrapeptides with a molecular weight in the range 174.1-
438 587.3 Da.
439 Due to its simplicity, UFM represents the most suitable technique for routinary analysis and industrial
440 scale-up. Still, at this stage, SEC had the advantage of purifying and recovering both short-chain and
441 medium-sized peptides for comparing the biological activity of the two distinct fractions and selecting
442 those more likely to be used to prepare nutraceuticals.
443 The identification of medium-chain peptides was achieved by using an established peptidomics
444 approach. Currently, techniques borrowed from shotgun proteomics are the most efficient methods
445 for identifying peptides in complex samples, such as food protein digests or native peptide extracts.

By this approach, 278 medium-chain peptides were identified; data are reported in Supplementary Material Table S2.

3.2 Multifunctional health-promoting effects of hemp seed peptides

3.2.1 Activity of hempseed peptides on cholesterol metabolism pathway

3.2.1.1. Hempseed peptides inhibit the *in vitro* HMGCoAR activity

In vitro experiments were carried out using the purified catalytic domain of the enzyme HMGCoAR, a known target of statins. The results suggested that the total hydrolyzate (T), the short- (S), and medium- (M) chain peptide mixtures can inhibit the enzyme activity in a dose-dependent manner with IC_{50} values equal to 0.38 ± 0.012, 0.18 ± 0.005, and 0.25 ± 0.009 mg/mL, respectively (Figure 1A-B). Improved inhibitory activity of S fraction appears evident since it is 2-fold more active than the total hydrolyzate. Comparison with the literature shows that the peptides obtained by hydrolyzing hempseed proteins with Alcalase are more active than those obtained by hydrolyzing these proteins with pepsin (IC_{50} 0.8 mg / mL) (Zanoni et al., 2017), trypsin (IC_{50} 0.65 mg/mL)(Aiello et al., 2017), whereas the peptides obtained using pancreatin are inactive as inhibitors of HMGCoAR (Aiello et al., 2017). The peptides obtained by co-digesting hemp seed proteins with pepsin, trypsin, and pancreatin can inhibit the HMGCoAR enzyme but not in a dose-dependent manner (Aiello et al., 2017).

3.2.1.2 Hempseed peptides modulate the cholesterol metabolism pathway

Based on the ability of S, M, and T peptides mixtures to inhibit the HMGCoAR enzyme, in-depth experiments (Figure 2S A-C) were performed to establish the mechanism of action through which they exert a cholesterol-lowering effect in human hepatic HepG2 cells.

Initially, MTT experiments were carried out to evaluate the effect of the S, M, and T peptides on hepatic cell viability. Results indicated that in the concentration range 0.1 - 2.0 mg/mL, any samples showed cell viability-reducing effects (Figure 1S A-C). Then, the HepG2 cells were treated with S peptides at the concentration of 0.5 mg/mL[1] and with M and T peptides at the concentration of 1

mg/mL. MonK (1 μM) was used as the reference compound, being responsible for the cholesterol-lowering activity in all nutraceuticals based on RYR extract. All tested peptides (S, M, and T) modulate cholesterol metabolism by activating the LDLR and confirming that S peptides are 2- fold more active than M and T (Figure 2). More in detail, S, M, and T induced an increase of mature SREBP-2 transcription factor (65 KDa isoform) of 34.7 ± 4.5%, 43.5 ± 17.2%, and 43.5 ± 16.5%, respectively (Figure 2A). The increase of this transcription factor determined an increase in LDL receptor levels by 55.2 ± 4.7%, 85.1 ± 21.4%, and 80.2 ± 20.3% for S, M, and T, respectively (Figure 2B). In agreement with these results, an increase in HMGCoAR protein levels of 30.2 ± 10.8%, 32.8 ± 10.6%, and 56.2 ± 7.2% was observed upon HepG2 treatments with S, M, and T peptides, respectively (Figure 2C). Results suggested that hemp seed peptides modulate cholesterol metabolism with a mechanism of action that is similar to MonK. Notably, MonK increases mature SREBP-2 protein levels by 41.3 ± 14.0%, LDLR by 48.0 ± 6.5% and HMGCoAR by 33.4 ± 21.5% (Figure 2 A-C).

From a functional point of view, S, M, and T increase the ability of HepG2 cells to absorb LDL from the extracellular environment with a final cholesterol-lowering effect. Also, in this case, S peptides are 2-folds more active than M and T (Figure 2D). In particular, the S increased the ability of cells to clear extracellular LDL by 183.8 ± 13.7% at 0.5 mg/mL, whereas both M and T up to 147.7 ± 1.9% and 211.4 ± 21.3% at 1.0 mg/mL, respectively (Figure 2D). In parallel, MonK increased the functional capacity of cells to uptake LDL by 126.9 ± 11.0 ± 12.6%, 39.3 ± 15.8%, and 42.7 ± 9.1%, respectively (Figure 2D). Indeed, Figure 2D clearly shows that the functional capacity of HepG2 cells to absorb extracellular LDL after treatment with M and T peptides and, in particular, S peptides, at half of the concentration of M and T, is precisely equal to the HepG2 cell capacity to absorb extracellular LDL after treatment with MonK at the concentration of 1 μM.

In addition, the PCSK9 protein levels decrease by 38.1 ± 12.6 %, 38.2 ± 15.8 %, and 42.6 ± 9.1%, after treatment with S, M and T peptides, respectively (Figure 2E). These reductions are due to the ability of these samples to decrease the levels of HNF-1alpha transcription factor by 24.1 ± 8.4%,

17.2 ± 17.7%, and 8.6 ± 10.9%, respectively (Figure 2F). In parallel, the experiments were carried out using MonK, confirming its ability to increase PCSK9 levels by 25.8 ± 2.4% and HNF-1alpha by 27.1% ± 5.7, with a negative effect on active LDLR levels localized on the surface of the hepatocytes. These results, therefore, highlight a new and different cholesterol-lowering mechanism from that exerted by MonK. The behavior of these peptides also differs significantly from what is known in the literature. The peptic hydrolyzate cannot modulate any effect on the PCSK9 pathway (Aiello et al., 2017).

3.2.2 Assessment of the DPP-IV inhbitiroy activity of hempseed peptides.

In vitro experiments were carried out using the recombinant form of the enzyme DPP-IV, a known target for treating type 2 diabetes. The results showed that the S, M, and T peptides inhibit the DPP-IV activity with IC_{50} values of 0.82 ± 0.12, 1.17 ± 0.09, and 1.36 ± 0.08 mg/mL, respectively (Figure 3A). These results suggest that the S peptides are about 2 times more active than the total hydrolyzate. Recent shreds of evidence indicated that tryptic and peptic hydrolysates (1.0 mg/mL) inhibit the enzyme activity by 17.5 and 32.0%, respectively (Lammi, Bollati, et al., 2019), clearly suggesting that the S, M, and T peptides are more active.

Based on these results, *in situ* experiments were carried out to evaluate the ability of hempseed peptides to inhibit the DPP-IV enzyme expressed on the membrane of human intestinal Caco-2 cells. Initially, MTT experiments were conducted to evaluate the potential cytotoxic effects of the samples on intestinal Caco-2 cells. The results showed no effects of reduction of cell viability were highlighted in the range of concentrations tested (Figure 2S (C-D-E)).

Then, Caco-2 cells were treated with S, M, and T peptides (1 and 2 mg / mL). The results showed that S, M, and T inhibit the DPP-IV enzyme expressed on the membranes of Caco-2 cells by 20.2 ± 6.3%, 15.4 ± 11.9%, and 19.7 ± 10.6%, respectively, at 1 mg / mL and by 47.6 ± 4.2%, 31.3 ± 6.3% and 35.4 ± 2.7% respectively. at 2 mg/mL (Figure 3B). In parallel, sitagliptin at 100 nM inhibits the enzyme by 46.8 ± 0.6% (Figure 3B).

523 Finally, the S and M peptides were tested *ex vivo* to evaluate their ability to inhibit the circulating
524 form of the DPP-IV enzyme present in human serum. Indeed, the results demonstrate that S and M
525 reduce the activity of the circulating form of DPP-IV by 47.1 ± 2.4% and 36.5 ± 4.7%, at 1 mg/mL,
526 respectively, and by 52.9 ± 1.6% and 38.1 ± 3.1%, at 2 mg / mL, respectively (Figure 3C).
527
528 **3.2.3 Anti-ACE and antioxidant activity of the hempseed peptide fractions**
529 *In vitro* experiments were carried out using the porcine kidney ACE enzyme, a known target for the
530 treatment of hypertension. The results showed that at the fixed concentration of 1 mg/mL, the S, M,
531 and T peptides inhibited the ACE enzyme by 57.5 ± 0.1%, 15.7 ± 0.3%, and 32.4 ± 0.5%, respectively,
532 towards control ($p < 0.0001$) (Figure 4). From the statistical analysis, it appears clear that the S
533 peptides are about three times more active than the M peptides ($p < 0.0001$) and that they clearly
534 represent the active component of the total hydrolyzate T ($p < 0.0001$) (Figure 4). Notably, since in
535 the T sample, M and S peptides are concomitantly available, the enzyme may recognize and bind
536 both short-chain and medium-chain peptides which can bind the ACE enzyme exerting the inhibitory
537 effect with an additive/synergistic behavior. These results suggested that the ACE inhibitory activities
538 exhibited by our samples are lower than hempseed peptide mixture obtained using Alcalase by Girgih
539 and co-workers (Girgih et al., 2014).
540 Two different assays were used to evaluate the antioxidant activity: the first was the 2,2-Diphenyl-1-
541 picrylhydrazyl (DPPH) assay, with which the ability of the samples to eliminate the DPPH radical
542 and, therefore, the scavenging ability of free radicals is assessed; the second assay was FRAP (Ferric
543 Reducing Antioxidant Power), which measures the ability of antioxidants to reduce the Fe^{3+} complex
544 to the reduced Fe^{2+} complex.
545 Figure 5 shows the *in vitro* effects of the direct antioxidant activity of S, M, and T peptides by DPPH
546 assay. The results clearly suggest that the samples express antioxidant power with a dose-response
547 trend (Figure 5A). In particular, at concentrations of 0.5, 1.0, 2.5 and 5.0 mg / ml, the S peptides have
548 scavenger activity equal to 10.9 ± 2.4%, 18.3 ± 2.9%, 26.8 ± 4.8% and 42.3 ± 4.6%; the M peptides

549 equal to 8.9 ± 0.5%, 12.7 ± 4.6%, 18.8 ± 2.6% and 25.8 ± 7.3%; while the T peptides have DPPH
550 radical removal activities equal to 27.3 ± 1.6%, 32.1 ± 1.2%, 39.3 ± 1.5% and 44.9 ± 0.9%,
551 respectively. Our results suggest that T peptides are more effective to scavenge the DPPH radicals
552 than S and M samples, respectively. This diffrence may be explained considering that in T sample
553 both short- and medium chains peptides are concomitantly present exerting the antioxidant activity
554 with a synergistic effects among peptides.
555 Figure 5B shows the *in vitro* effects of the FRAP assay's direct antioxidant activity of the S, M, and
556 T peptides. The results clearly indicate that the peptides express a reducing capacity in a dose-
557 response manner (Figure 5B). In particular, at concentrations of 0.1, 0.5, 1.0 and 2.5 mg / ml, S
558 peptides increase iron reduction by 528.9 ± 53.8%, 1936 ± 234.8%, 3284 ± 390.8%, 5238 ± 523.5%,
559 M peptides by 44.44 ± 9.6%, 197,2 ± 29.3%, 377.8 ± 81.8% and 838.9 ± 194.1%, finally the T
560 peptides increase the reduction of iron by 888.7 ± 62.7%, 1678 ± 133.8%, 2546 ± 181.0% and 4654
561 ± 405.5%, respectively. Our results suggest that T peptides exert a FRAP activity with a trend which
562 is equal to S clearly underlining that short-chain peptide mixture is the active components of the T
563 samples. This explaination is reforced by the fact that M peptide mixture is less active than M peptides
564 in FRAP activity.
565 Many physicochemical factors may influence the ability of peptides to exert antioxidant activity.
566 Although certain aspects of the structure-function relationship of antioxidant peptides are still poorly
567 understood, chain length, type, composition, sequence, and the location of specific amino acids in a
568 peptide chain have been suggested to be critical issues for exerting the antioxidant property (Gallego,
569 Mora, & Toldrá, 2018). In this context, short peptides may often be potent antioxidants. Literature
570 indicates that, besides containing hydrophobic amino acids, such as Leu or Val, in their N-terminal
571 regions, peptides containing nucleophilic sulfur-containing amino acid residues (Cys and Met),
572 aromatic amino acid residues (Phe, Trp, and Tyr) and/or the imidazole ring-containing His are
573 generally found to possess strong antioxidant properties (Santos-Sánchez et al., 2022).

574 Our results are in agreement with other studies demonstrating that hempseed peptides generated by
575 alcalase, alcalase + flavourzyme, and pepsin + pancreatin, exert radical scavenging activity and
576 increase the total antioxidant status (Santos-Sánchez et al., 2022). On the contrary, hempseed peptides
577 produced by neutrase, and proteases preparation [AFP 4000 (AFP), HT Proteolytic Concentrate (HT),
578 Protease G (Pro-G), actinidin, protamex, and zingibain] only show radical scavenging activity
579 (Santos-Sánchez et al., 2022).
580
581 **3.3 Assessment of the intestinal trans-epithelial transport of hempseed peptides**.
582 In the field of food bioactive peptides, one of the main limitations, which affects their fast exploitation
583 on the market as nutraceuticals and/or functional foods, is represented by their low metabolic stability
584 and bioavailability at the intestinal level (Amigo & Hernández-Ledesma, 2020). Short-chain peptide
585 are more easily transported to the bloodstream and the presence of bioactive peptides in the blood,
586 after ingestion of protein hydrolysates derived from both vegetal and animal food source, have been
587 detected (Ejima et al., 2018; Iwai et al., 2005; Segura-Campos et al., 2011). Indeed, intestinal cells
588 are the first physiological barrier encountered by food peptides and/or hydrolysates. In general, when
589 the protein hydrolysates and/or peptides come in contact with human enterocytes, two main
590 phenomena may occur: peptides are in part transported by the cells and in part metabolized by the
591 same cellular environment, even though the metabolic degradation of bioactive peptides does not
592 always mean that the mixtures lose the bioactivity (Bollati et al., 2022; Lammi et al., 2021). Hence,
593 the intestine is a very complex physiological environment that actively modulates the bioactivity of
594 protein hydrolysates and/or peptides through direct effects on their chemical compositions and
595 peptidomics profiles. Therefore, it is crucial to realize *in vitro* experiments for mimicking the
596 intestinal transport of food protein hydrolysates before performing expensive *in vivo* experiments to
597 confirm their health-promoting activity. Worldwide, differentiated human intestinal Caco-2 cells are
598 successfully employed as a reliable model for *in vitro* evaluation of food bioactive peptide propensity
599 to be transported by intestinal cells. Taking this into account, Caco-2 cells have been differentiated

and incubated on their apical (AP) side with S at the concentration of 1 mg/mL for 2 h. Then, AP and basolateral (BL) solutions were collected and analyzed by UHPLC-HRMS. Focusing on dipeptides, tripeptides, and tetrapeptides results clearly indicated that out of the 569 hempseed peptides which were identified in the starting S fraction of the hempseed hydrolysate, only 279 peptides had been identified in the BL solution, clearly indicating that only 49% of the short peptides aretransported intact by differentiated intestinal cells. In general, food-derived peptides may be transported across the intestinal brush-border membrane into the bloodstream *via* one or more of the following routes: (i) peptide transport 1 (PepT1)-mediated route, (ii) paracellular route via tight junctions (TJs), (iii) transcytosis route, and (iv) passive transcellular diffusion (Lammi et al., 2021).

Certainly, peptide size, charge, hydrophobicity, and degradation due to the action of peptidases are among the main factors influencing the absorption through one or more of these routes. In general, short peptides, such as dipeptides and tripeptides, are preferentially transported by PepT1, due to its high capacity, low affinity, and high expression in the intestinal epithelium (Lammi et al., 2020), whereas highly hydrophobic peptides are transported by simple passive transcellular diffusion or by transcytosis (Lammi et al., 2020).

Using the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/biopep-uwm/), most of the S peptides transported by intestinal cells display DPP-IV and ACE inhibitory properties as well as antioxidant activity.

Mature enterocytes develop microvilli that function as the gastrointestinal tract's primary surface of nutrient absorption. Their membrane is packed with enzymes that favor the breakdown of complex nutrients into simpler compounds that are more easily absorbed. The dynamic equilibrium between bioactive peptide degradation and transport is crucial from a physiological point of view. Possessing a wide range of membrane peptidases naturally expressed by the AP side of enterocytes, including DPP-IV and ACE (Lammi et al., 2021), a differentiated Caco-2 model is also a reliable tool for investigating the proteolytic activity of the brush border barrier. Under this hypothesis, our results indicate that out of the total 569 short peptides identified in the S fraction of total hydrolysate, 471

626 are present in the AP solution, suggesting that about 17.2% (15 dipeptides, 35 tripeptides, and 36
627 tetrapeptides) of peptides which were present in the original short peptide fraction are metabolized
628 by intestinal cells, or they are uptaken and intracellular used by Caco-2 cells during the 2 hr of
629 incubation with the cells. Interestingly, short peptides containing in their N-terminal methionine (M)
630 and tryptophan (W) residues are not able to be transported by intestinal cells; this particular aspect
631 may be explained considering that the brush border membranes express aminopeptidase N and
632 ectopeptidase and from its position which faces with intestinal lumen it can recognize peptides
633 containing in the N-terminal, M, and W metabolizing them (Röhnert et al., 2012).
634
635 **4. Conclusions**
636 Using a multidisciplinary approach, this bottom-up study clearly supports the pleiotropic activity
637 exerted by hempseed short-chain peptide mixture. Indeed, hempseed peptide mixture enriched with
638 short-chain ones is endowed with potential hypocholesterolemic, anti-diabetic, hypotensive, and
639 antioxidant activities, respectively. In this study, we have produced, analyzed, and detailed
640 characterized hempseed hydrolysate providing a comprehensive picture of its multifunctional power.
641 Of course, the bio-activity of short-chain peptide mixture reflects the composition of the fraction in
642 which being present hundreds of peptides some synergistic interaction may occur, clearly suggesting
643 that not only the size of the peptides contributes to the biological actvity but rather than the force of
644 the synergistic/additive interaction among the spicies within the fraction may play a key role in the
645 multifunctional effect. This preclinical assessment demonstrates that our hempseed peptide mixture,
646 which is easily scaling up at the industrial level, may be potentially exploited as new valuable
647 ingredients for the development of innovative functional foods and or dietary supplements useful for
648 the prevention of metabolic syndrome. Of course, to achieve this important goal, further experiments
649 have to be performed to confirm the multifunctional properties of these hempseed peptides using
650 animal models.
651

Fundings

This research was in part funded by Sapienza University of Rome – Progetto per Avvio alla Ricerca – Tipo 2 – "Development of innovative strategies for the untargeted identification of natural and modi-fied short-sized bioactive peptides", grant number AR22117A815792CD

Acknowledgements

The authors gratefully acknowledge Carlo Sirtori Foundation (Milan, Italy) for having provided part of the equipment used in this experimentation. In addition, The authors gratefully acknowledge the financial support from the China Scholarship Council for a fellowship to J.L.

Patent:

Italian Patent provisional number 102022000003347 " Metodo per la caratterizzazione di peptidi corti da canapa industriale" Inventors Anna Arnoldi, Chiara Cavaliere, Anna Laura Capriotti, Andrea Cerrato, Aldo Laganà, Carmen Lammi, Carmela Maria Montone, Susy Piovesana – property: Università degli Studi di Milano e Università di Roma La Sapienza.

CRediT authorship contribution statement

Andrea Cerrato: Investigation, Methodology, Data Curation. **Carmen Lammi**: Conceptualization, Supervision, Formal analysis, Writing - Original Draft, Writing - Review & Editing. **Anna Laura Capriotti**: Conceptualization, Supervision, Resources; Writing - Original Draft; Writing - Review & Editing. **Carlotta Bollati**: Investigation, Writing - Original Draft. **Chiara Cavaliere**: Writing - Review & Editing. **Carmela Maria Montone**: Investigation. **Martina Bartolomei**: Investigation. **Giovanna Boschin**: Investigation. **Jianqiang Li**: Investigation. **Susy Piovesana**: Visualization. **Anna Arnoldi**: Writing - Review & Editing. **Aldo Laganà**: Supervision; Project administration

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE CAPTIONS

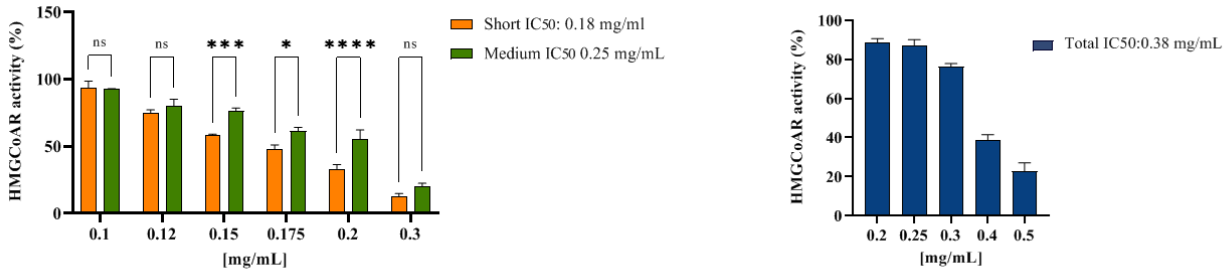
Figure 1. Effect of short-chain peptide mixture (S), medium-chain peptide mixture (M), and total hydrolysate on HMGCoAR activity. Bars represent the mean ±s.d. of three independent experiments performed in triplicate. *, $p<0.5$; ***, $p<0.001$; ****, $p<0.0001$; ns: not significant

Figure 2 A-C: Modulation of the cholesterol pathway by hempseed peptides. **D:** Effect of hempseed peptides on HepG2 ability to uptake LDL from the extracellular environment. **E,F:** Hempseed peptides modulate the PCSK9 pathway. S: short-chain peptide mixture, M: medium-chain peptide mixture; T: total hydrolysate; MonK: monacolin K. Bars represent the mean ±s.d. of three independent experiments performed in triplicate. *, $p<0.5$; **, $p<0.001$; ***, $p<0.001$;****, $p<0.0001$; ns: not significant.

Figure 3. Effect of hempseed peptides on DPP-IV activity *in vitro* (A), *in situ* (B), and *ex vivo* (C). S: short-chain peptide mixture, M: medium-chain peptide mixture, and T: total hydrolysate. Bars represent the mean ±s.d. of three independent experiments performed in triplicate. *, $p<0.5$; ***, $p<0.001$; ***, $p<0.001$;****, $p<0.0001$; ns: not significant

Figure 4. The ACE-inhibitory activity of short- (S) and medium- (M) -chain peptide mixtures and total hydrolysate (T) at 1mg/mL. Bars represent the mean ±s.d. of three independent experiments performed in triplicate. ****, $p<0.0001$.

Figure 5. Direct antioxidant activity of hempseed peptides measured by DPPH (A) and FRAP (B) assays, respectively. S: short-chain peptide mixture, M: medium-chain peptide mixture, and T: total hydrolysate. Bars represent the mean ±s.d. of three indipendent experiments performed in triplicate. *, $p<0.5$; ***, $p<0.001$; ***, $p<0.001$;****, $p<0.0001$; ns: not significant



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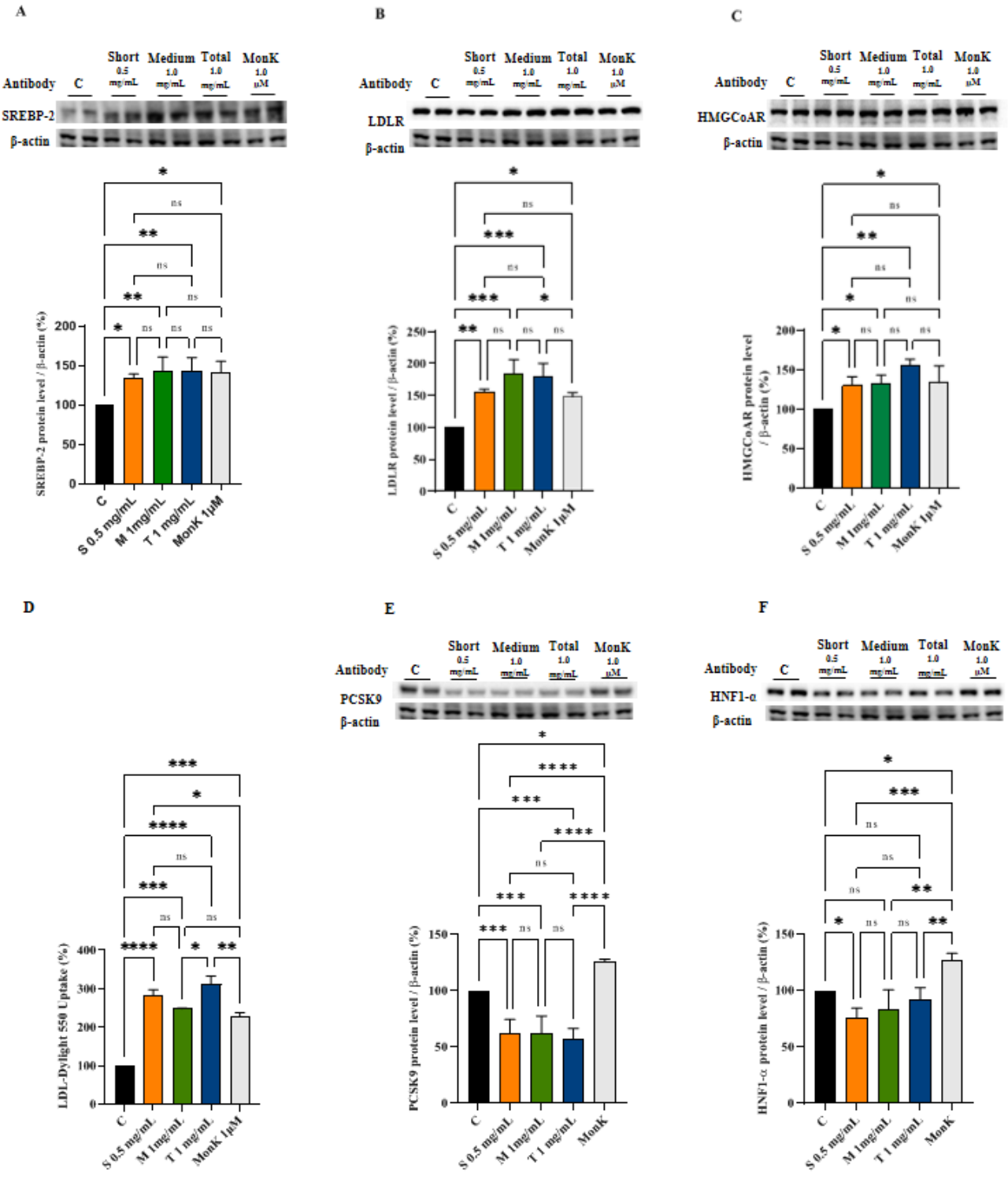

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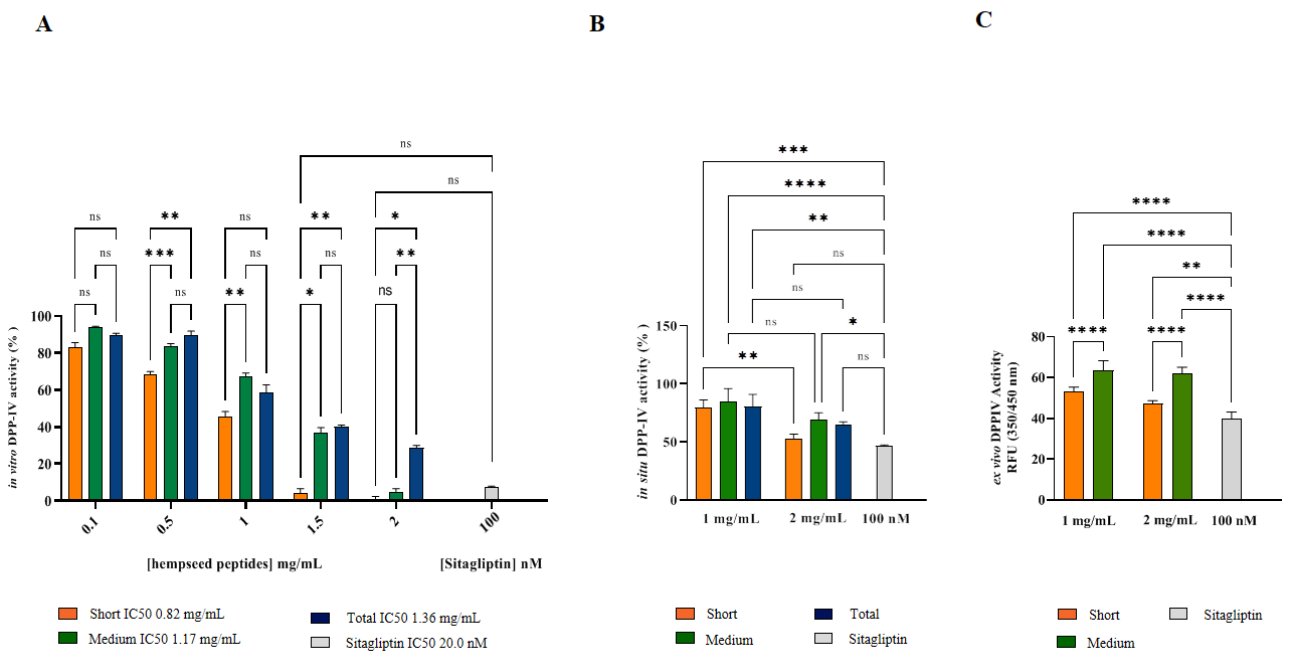

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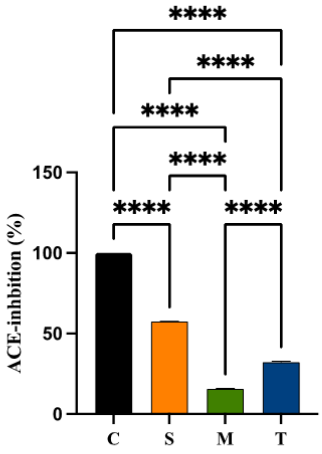

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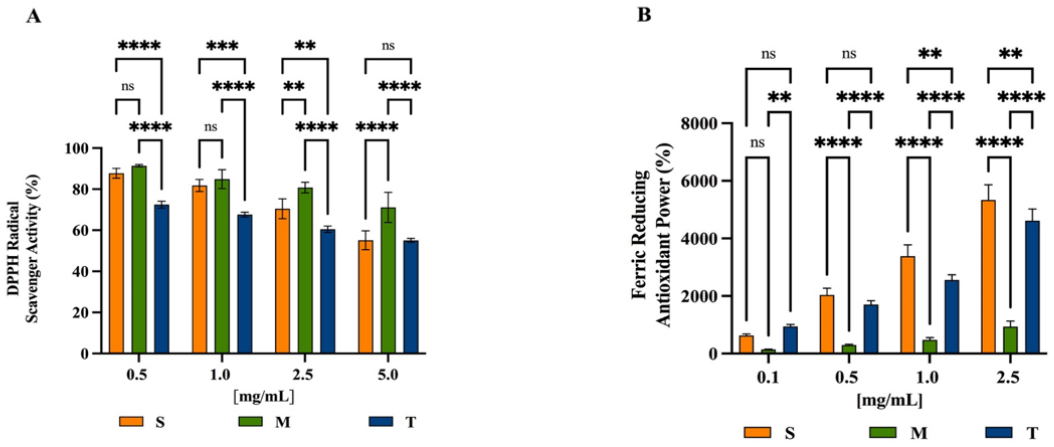

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880 Figure 5