Hempseed Peptides Exert Hypocholesterolemic Effects with a Statin-Like Mechanism

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ABSTRACT

This study had the objective of preparing a hempseed protein hydrolysate and investigating its hypocholesterolemic properties. The hydrolysate was prepared treating a total protein extract with pepsin. Nano HPLC-ESI-MS/MS analysis permitted identifying in total 90 peptides belonging to 33 proteins. In the range 0.1-1.0 mg/mL, it inhibited the catalytic activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) in a dose-dependent manner. HepG2 cells were treated with 0.25, 0.5, and 1.0 mg/mL of the hydrolysate. Immunoblotting detection showed increments in the protein levels of regulatory element binding proteins 2 (SREBP2), low-density lipoprotein receptor (LDLR), and HMGCoAR. However, the parallel activation of the phospho-5'-adenosine monophosphate-activated protein kinase (AMPK) pathway, produced an inactivation of HMGCoAR by phosphorylation. The functional ability of HepG2 cells to uptake extracellular LDL was raised by 50.5± 2.7%, 221.5 ± 1.6%, and 109 ± 3.5%, respectively, versus the control at 0.25, 0.5, and 1.0 mg/mL concentrations. Finally, also a raise of the protein level of proprotein convertase subtilisin/kexintype 9 was observed. All these data suggest that the mechanism of action has some similarity with that of statins.

INTRODUCTION

Hypercholesterolemia is one of the main risk factors for the development of cardiovascular disease (CVD). In the presence of a moderate deviation from normal values, diet changes may represent a first tool for cholesterol control in order to delay the use of statins [1-3]. A possible solution is the shift from a diet based on animal foods to a plant-based diet, in which protein-rich seeds are valuable sources of nutrients and bioactive phytochemicals. In this scenario, hempseed is certainly an underexploited non-legume seed that would deserve a greater attention.

34 The cultivation of industrial hemp is currently legalized worldwide, since most countries accept the
35 distinction between industrial hemp, i.e. the *Cannabis sativa* varieties that have a very low content of
36 Δ9-tetrahydrocannabinol (THC), and marijuana or hashish, i.e. the psychoactive varieties with THC
37 contents falling in the range between 1-20%. In spite of this fact, the research of the potential health
38 benefits provided by hempseed is still penalized by the negative reputation of marijuana, which daunts
39 interests and investments.
40 This is a pity considering that hempseed contains 35.5% oil, 24.8% protein, 27.6% carbohydrates,
41 27.6% total fiber (5.4% digestible and 22.2% non-digestible fiber), and 5.6% ash [4], and in addition
42 the content of major antinutritional factors, such as phytic acid, condensed tannins, and trypsin
43 inhibitors, is inferior than in other seeds [5]. Hempseed protein mainly consists of a storage protein,
44 edestin, which accounts for 60-80% of the total protein content, with albumin accounting for the rest
45 [6]. Interestingly, the protein digestibility-corrected amino acid scores (PDCAAS) of dehulled
46 hempseed protein is equal to 61%, i.e. superior than lentil (52%), whole wheat (40%), or almond
47 (23%), although inferior than soy protein (71%) [7].
48 Recently, we have conducted an improved proteomic characterization of this seed using advanced
49 analytical techniques (Aiello et al., 2016), as a first step of a research aimed to valorize the potential
50 health benefits provided by hempseed. In fact, available literature indicates that peptides, obtained
51 through hydrolysis of hempseed protein with different enzymes, may function as hypotensive agents
52 [8, 9] and antioxidants [8, 10, 11]. On the contrary, literature reports only a few evidences on the ability of
53 hempseed to modulate the lipid profile [12-14].
54 The inhibition of cholesterol biosynthesis is the most efficient way to reduce serum cholesterol levels.
55 Since intracellular cholesterol production is a multistep pathway in which 3-hydroxy-3-
56 methylglutaryl coenzyme A reductase (HMGCoAR) mediates the rate-limiting step, this enzyme is
57 an important drug target. In fact, statins are able to reduce the *de novo* cholesterol production by
58 inhibiting HMGCoAR in the liver and increasing the low-density lipoprotein receptor (LDLR) ability
59 to uptake extracellular low-density lipoprotein (LDL).
60 A transcription factor known as sterol-responsive element binding protein 2 (SREBP2) plays a crucial
61 role in HMGCoAR mRNA expression [15, 16]. Among SREBP2 gene targets, the LDLR is particularly
62 important. In fact, the majority of plasma cholesterol is transported by the LDL fraction and the
63 cellular uptake of LDL is mediated by the LDLR. The circulating level of LDL is determined in large
64 part by its uptake rate through the hepatic LDLR pathway [17, 18]. In general, the LDLR expression is
65 finely tuned by changes in intracellular cholesterol [19].
66 Stimulated by the increasing use of hempseed in human nutrition, the final goal of our research is
67 improving the knowledge of the health benefits potentially provided by this seed. Starting from the

68 hypothesis that the activity should depend on specific peptides encrypted in the protein sequences, as
69 a first approach, we decided to work on a protein hydrolysate obtained by treating with pepsin a total
70 protein extract from hempseed and to investigate their hypocholesterolaemic properties using human
71 hepatic HepG2 cells as model system. More specifically, the present work had three main goals: a)
72 the preparation of a peptic hydrolysate from hempseed protein; b) a detailed characterization of its
73 composition by nano HPLC-MS/MS; and c) the elucidation of the mechanism through which these
74 peptides mediate a cholesterol-lowering effect at HepG2 cells, by molecular and functional
75 investigations on the LDLR-SREBP2 pathway.
76
77 **MATERIALS AND METHODS**
78
79 **Chemicals.** Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS),
80 phosphate buffered saline (PBS), penicillin/streptomycin, chemiluminescent reagent, and 96-well
81 plates were purchased from Euroclone (Milan, Italy). Bovine serum albumin (BSA), RIPA buffer,
82 and the antibody against β-actin and pepsin from porcine gastric mucosa (P7012, lyophilized powder,
83 ≥ 2,500 units/mg protein) were bought from Sigma-Aldrich (St. Louis, MO, USA). The antibody
84 against HMGCoAR was bought from Abcam (Cambridge, UK). The antibody against phospho-
85 HMGCoAR (Ser872) was purchased from Bioss Antibodies (Woburn, MA, USA). The antibody
86 against proprotein convertase subtilisin/kexin type 9 (PCSK9) were bought from Cayman Chemical
87 (Ann Arbour, MI, USA). Phenylmethanesulfonyl fluoride (PMSF), Na-orthovanadate inhibitors, and
88 the antibodies against rabbit Ig-horseradish peroxidase (HRP), mouse Ig-HRP, and SREBP-2 were
89 purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibody against the
90 LDLR was bought from Pierce (Rockford, IL, USA). The antibody against phospho-5'-adenosine
91 monophosphate-activated protein kinase (AMPK) (Thr172) was bought from Assay Biotech
92 (Sunnyvale, CA, USA) and the inhibitor cocktail Complete Midi from Roche (Basel, Swiss). Mini
93 protean TGX pre-cast gel 7.5% and Mini nitrocellulose Transfer Packs were purchased from Bio-
94 Rad (Hercules, CA, USA).
95
96 **Preparation and analysis of the peptic peptides from hempseed protein.** Hempseeds (*C. sativa*
97 cultivar Futura) were provided by the Institute of Agricultural Biology and Biotechnology, CNR
98 (Milan, Italy). The isolation of hempseed protein was carried out applying a method previously
99 applied to other seeds with some modifications [20]. Briefly, 2 g of defatted hempseed flour were
100 homogenized with 15 mL of 100 mM Tris-HCl/0.5 M NaCl buffer, pH 8.0. The extraction was
101 performed in batch at 4 °C overnight. The solid residue was eliminated by centrifugation at 6800 *g*

102 for 30 min at 4 °C and the supernatant was dialysed against 100 mM Tris-HCl buffer, pH 8.0 for 36
103 h at 4 °C. The protein content was assessed according to the method of Bradford, using BSA as
104 standard. The hydrolysis was performed on the total protein extract, changing the pH from 8 to 2 by
105 adding 1 M HCl. The enzyme solution (4 mg/mL in NaCl 30 mM) was added in a ratio 1:50
106 enzyme/hempseed protein (w/w). The mixture was incubated for 16 h and then the enzyme inactivated
107 changing the pH to 7.8 by adding 1 M NaOH. Samples were purified by ultrafiltration, using
108 membranes with a 3-kDa molecular weight cut-off (MWCO) (Millipore, USA). Filtered peptide
109 mixtures were acidified with 0.1 % of formic acid, and then analyzed on a SL IT mass spectrometer
110 interfaced with a HPLC Chip Cube source (Agilent Technologies, Palo Alto, CA, USA). Separation
111 was carried out in gradient mode at a 300 nL/min flow. The LC solvent A was 95% water, 5% ACN,
112 0.1% formic acid; solvent B was 5% water, 95% ACN, 0.1% formic acid. The nano pump gradient
113 program was as follows: 5% solvent B (0 min), 80% solvent B (0–40 min), 95% solvent B (40–45
114 min), and back to 5% in 5 min. The drying gas temperature was 300 °C, flow rate 3 L/min (nitrogen).
115 Data acquisition occurred in positive ionization mode. Capillary voltage was −1950 V, with endplate
116 offset −500V. Full scan mass spectra were acquired in the mass range from m/z 300 to 2000 Da. LC-
117 MS/MS analysis was performed in data dependent acquisition AutoMS(n) mode. The MS/MS data
118 were analyzed by Spectrum Mill Proteomics Workbench (Rev B.04.00, Agilent Technologies, Palo
119 Alto, CA, USA) consulting NCBI_ *Cannabis sativa* (531 sequences) protein sequences database.
120 Two missed cleavages were allowed to pepsin; peptide mass tolerance was set to 1.2 Da and fragment
121 mass tolerance to 0.9 Da. Threshold used for peptide identification score ≥ 6; Scored Peak Intensity
122 SPI% ≥ 70%; Autovalidation strategy either in peptide mode and in protein polishing was performed
123 using FDR cut-off ≤ 1.2%.
124
125 **Cell culture conditions.** The HepG2 cell line was bought from ATCC (HB-8065, ATCC from LGC
126 Standards, Milan, Italy). The HepG2 cell line was cultured in DMEM high glucose with stable L-
127 glutamine supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (complete
128 growth medium) and incubated at 37 °C under 5% CO_2 atmosphere. HepG2 cells were used for no
129 more than 20 passages after thawing, because the increase of the number of passages may change the
130 cell characteristics and impair assay results.
131
132 **MTT assay.** A total of 3 x 10^4 HepG2 cells/well were seeded in 96-well plates and treated with 0.1,
133 0.25, 0.35, 0.5, 1.0, and 2.0 mg/mL of peptic peptides, respectively, or vehicle (H_2O) in complete
134 growth media for 48 h. Subsequently, the treatment solvent was aspirated and 100 μL/well of 3-(4,5-
135 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) filtered solution added. After 48 h of

136 incubation, 0.5 mg/mL solution was aspirated and 100 μL/well of MTT lysis buffer (8 mM HCl +
137 0.5% NP-40 in DMSO) added. After 5 min of slow shaking, the absorbance at 575 nm was read on
138 the Synergy H1 fluorescence plate reader (Biotek, Bad Friedrichshall, Germany).
139
140 **HMGCoAR activity assay.** The assay buffer, NADPH, substrate solution and HMGCoAR were
141 provided in the HMGCoAR Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). The experiments were
142 carried out following the manufacturer's instructions at 37 °C. In particular, each reaction (200 μL)
143 was prepared adding the reagents in the following order: 1 X assay buffer, 0.1, 0.25, 0.35, 0.5, and
144 1.0 mg/mL of peptic peptides or vehicle (C), NADPH (4 μL), substrate solution (12 μL) and finally
145 HMGCoAR (catalytic domain) (2 μL). Subsequently, the samples were mixed and the absorbance at
146 340 nm read by a microplate reader Synergy H1 fluorescence plate reader (Biotek) at time 0 and 10
147 min. The HMGCoA-dependent oxidation of NADPH and the inhibition properties of lupin peptides
148 were measured by the absorbance reduction, which is directly proportional to the enzyme activity.
149
150 **Western blot analysis.** A total of 1.5 x 10^5 HepG2 cells/well (24-well plate) were treated with 0.25,
151 0.5, and 1.0 mg/mL of peptic peptides for 24 h. After each treatment, cells were scraped in 40 μL ice-
152 cold lysis buffer [RIPA buffer + inhibitor cocktail + 1:100 PMSF + 1:100 Na-orthovanadate] and
153 transferred in an ice-cold microcentrifuge tube. After centrifugation at 16,060 *g* for 15 min at 4 °C,
154 the supernatant was recovered and transferred into a new ice-cold tube. Total proteins were quantified
155 by Bradford method and 50 μg of total proteins loaded on a pre-cast 7.5% Sodium Dodecyl Sulphate
156 - Polyacrylamide (SDS-PAGE) gel at 130 V for 45 min. Subsequently, the gel was pre-equilibrated
157 with 0.04% SDS in H_2O for 15 min at room temperature (RT) and transferred to a nitrocellulose
158 membrane (Mini nitrocellulose Transfer Packs) using a Trans-blot Turbo at 1.3 A, 25 V for 7 min.
159 Target proteins, on milk blocked membrane, were detected by primary antibodies as follows: rabbit
160 anti-SREBP2, rabbit anti-LDLR, anti-HMGCoAR, anti-phospho-AMPK (Thr172), anti-phospho-
161 HMGCoAR (Ser872), anti-PCSK9, and anti-β-actin. Secondary antibodies conjugated with HRP and
162 a chemiluminescent reagent were used to visualize target proteins and their signal was quantified
163 using the Image Lab Software (Bio-Rad). The internal control β-actin was used to normalize loading
164 variations.
165
166 **Fluorescent LDL uptake cell based assay.** A total of 3 x 10^4 HepG2 cells/well were seeded in 96-
167 well plates and kept in complete growth medium for 2 d before treatment. On the third day, cells were
168 treated with 0.25 mg/mL peptic peptides or vehicle (100 mM Tris) for 24 h. At the end of the treatment
169 periods, the culture medium was replaced with 50 μL/well LDL-DyLight™ 550 working solution.

170 The cells were additionally incubated for 2 h at 37 °C, then the culture medium was aspirated and
171 replaced with PBS 100 μL/well. The degree of LDL uptake was measured using the Synergy H1
172 fluorescent plate reader from Biotek (excitation and emission wavelengths 540 and 570 nm,
173 respectively).
174
175 **Statistically Analysis.** Statistical analyses were carried out by One-way ANOVA (Graphpad Prism
176 6) followed by Dunnett's test. Values were expressed as means ± sem; P-values < 0.05 were
177 considered to be significant.
178

179 # RESULTS

180
181 **Preparation and analysis of peptic peptides from hempseed protein.** Hempseeds were extracted
182 to produce a total protein extract, which was hydrolyzed with pepsin. The complex peptide mixture
183 obtained was analyzed by nano LC-MS/MS. **Figure 1** shows the total ion current (TIC) of MS and
184 MSn, respectively, of these peptic peptides. The identification was carried out through MS/MS ion
185 search using the SpectrumMill search engine. Despite the number of acquired spectra was very high,
186 the use of a non-exhaustive database permitted the identification of 90 peptides belonging in total to
187 33 *C. sativa* proteins as reported in **Table 1**. The highest number of detected peptides belongs to the
188 main storage proteins in hempseed: 6 peptides were shared by all isoforms of Edestin 2 (ede2A; 2B;
189 2C), 6 peptides were shared by the isoforms of Edestin 1 (ede1A,B; 1B) and only 1 by the isoforms
190 of Edestin 1 (ede1A,B; 1D). Numerous peptides belonged to the most heterogeneous protein family
191 identified here, i.e. the acyl-activating enzyme superfamily: 5 unique peptides belonged to DNA-
192 directed RNA polymerase subunit beta, Photosystem I P700 chlorophyll, apoprotein A2, and Protein
193 Ycf2, whereas 4 peptides to (+)-alpha-pinene synthase. This protein is involved in the terpene
194 metabolism and naringenin-chalcone synthase and is better known as flavanone synthase. In total 3
195 peptides belonged to Putative LysM domain containing receptor kinase and THCA synthase, whereas
196 2 unique peptides belonged to ATP synthase (alpha and beta subunit), cannabidiolic acid synthase-
197 like 1, Delta 12 desaturase, Hypothetical chloroplast RF1, (-)-limonene synthase, MatR, NADH-
198 plastoquinone oxidoreductase (4 and 5 subunit), 4-coumarate:CoA ligase, and Polyketide synthase
199 family, respectively.
200
201 **HepG2 cell viability.** MTT experiments were performed in order to exclude the treatment doses with
202 potential toxic effects on the HepG2 cell line. No significant cell mortality was observed in the peptide
203 concentrations ranging from 0.1 to 1.0 mg/mL after a 48 h treatment *versus* vehicle (C, H_2O),

204 suggesting that hempseed peptides do not induce cell mortality in this dose range (**Figure 2**). For this
205 reason, all the following experiments, aimed at investigating the molecular and functional effects of
206 these peptides, were carried out using this range of doses.
207
208 **Hempseed peptides inhibit the catalytic activity of HMGCoAR.** In order to evaluate the ability of
209 these peptides to inhibit the catalytic activity of HMGCoAR, an *in vitro* assay was performed using
210 the purified catalytic domain of this enzyme. Peptide concentrations ranging from 0.1 to 1.0 mg/mL
211 were tested. The catalytic activity of HMGCoAR was inhibited in a dose-dependent manner (**Figure**
212 **3**). In particular, after incubation with 0.1, 0.25, 0.35, 0.5, and 1.0 mg/mL the activity of HMGCoAR
213 activity was inhibited by 13.1 ± 5.1% ($p<0.05$), 24.5 ± 1.7% ($p<0.001$), 45.5 ± 1.7% ($p<0.001$), 61.1
214 ± 0.7% ($p<0.001$), and 80.0 ± 4.0% ($p<0.001$), respectively, *versus* the control.
215
216 **Effects of hempseed peptides on the LDLR pathway modulation.** HepG2 cells were treated with
217 0.25, 0.5, and 1.0 mg/mL of hempseed peptides and each sample was investigated with
218 immunoblotting experiments. The treatment with hemp peptides induced an up-regulation of the
219 protein level of the N-terminal fragment of SREBP2 (mature form with a molecular weight of 68
220 kDa) by 34.8 ± 19% ($p < 0.001$), 29.1 ± 7.7% ($p < 0.001$), and 33.5 ± 6% ($p < 0.001$) at 0.25, 0.5,
221 and 1 mg/mL, respectively, *versus* the control (**Figure 4A-C**). As a consequence, up-regulations of
222 the LDLR and HMGCoAR protein levels were also observed. In particular, as shown in **Figure 4A-**
223 **C**, after the treatment with peptic hemp peptides at 0.25, 0.5, and 1.0 mg/mL, the LDLR protein level
224 was increased by 35.6 ± 10.4% ($p <0.01$), 54.7 ± 41.4% ($p< 0.0001$), and 63.0 ± 41.3% ($p< 0.0001$),
225 respectively, *versus* the control, whereas the HMGCoAR protein level was augmented by 32.2 ±
226 15.6% ($p <0.05$), 54.1 ± 10.4% ($p <0.01$), and 67.7 ± 38.9% ($p< 0.0001$), respectively, *versus* the
227 control (**Figure 4B-C**).
228
229 **Effects of hempseed peptides on AMPK pathway activation.** Suitable immunoblotting
230 experiments were performed in order to evaluate the effect of the treatment with hempseed peptides
231 on AMPK activation and HMGCoAR inactivation (AMPK substrate). The lysates from treated and
232 untreated HepG2 cells were therefore analyzed using specific antibodies for AMPK phosphorylated
233 at threonine 172 (**Figure 4D**) and for HMGCoAR phosphorylated at serine 872 (AMPK
234 phosphorylation site) (**Figure 4E**). **Figure 4F** shows that treatment with hempseed peptides
235 significantly increased AMPK phosphorylation by 95.2 ± 38.7% (0.50 mg/mL, $p <0.0001$) and by
236 120.3 ± 18.4% (1 mg/mL, $p <0.0001$) *versus* the control. As a consequence of the AMPK activation,

the phosphorylation levels of HMGCoAR were also increased by 67.0 ± 15.8% at 0.5 mg/mL ($p < 0.0001$) and 56.0 ± 37.3% at 1 mg/mL ($p<0.0001$), *versus* the control (**Figure 4 F**).

Modulation of the LDL-uptake in HepG2 cells. The functional ability of HepG2 cells to uptake extracellular LDL after the treatments with the same peptides was investigated by performing fluorescent LDL uptake experiments. As shown in **Figure 5**, hempseed peptides increased the LDL-uptake in a statistically significant way *versus* the control. In fact, after treatments with 0.25, 0.5, and 1.0 mg/mL, the LDL-uptake was increased by 50.5± 2.7% ($p < 0.001$), 221.5 ± 1.6% ($p < 0.001$), and 109 ± 3.5% ($p < 0.001$), respectively (**Figure 5**).

Modulation of the PCSK9 protein level. Immunoblotting experiments were carried out in order to evaluate the effects of the treatments on the modulation of PCSK9 in HepG2 cells. **Figure 6 A-B** shows that the treatment with hempseed peptides induced a 56.0 ± 40.5% (p < 0.01) increase of PCSK9-M protein level at a 0.5 mg/mL concentration and a 201.2 ± 13.5% (p < 0.001) augmentation at 1.0 mg/mL *versus* the control (**Figure 6 B**).

DISCUSSIONS

Preparation and characterization of a peptic hydrolysate of hempseed protein. As indicated in the introduction, the first objectives of the work were the preparation and the characterization of the peptic hydrolysate from hempseed protein. In total, it was possible to identify 90 peptides belonging to various protein families of *C. sativa* (Table 1). All identified peptides were very heterogeneous, being constituted by 7 - 28 amino acid residues and falling within an 859-3210 Da mass range. The most abundant (41%) contained predominantly basic residues, whereas 32% were acidic and 27% were neutral, respectively, with a net charge of the total peptide mixture of 0.22. Moreover, among all identified peptides, 42 had a calculated pI < pH 7 and 48 had a calculated pI > 7 (**Table 1**). Based on the hydrophobicity of each residue, the hydrophobicity of the total peptic hempseed peptide mixture was 44%, suggesting that about one half of the peptides are hydrophilic and one half hydrophobic.

Molecular and cellular investigation of the hypocholesterolemic properties of the hempseed hydrolysate. Another objective was the characterization of the bioactivities of these peptides focusing the attention on cholesterol metabolism. For the first time, this paper provides evidence of the hypocholesterolaemic effects mediated by hemp peptides suggesting also a mechanistic explanation. HMGCoAR is the rate-controlling enzyme of cellular cholesterol biosynthesis pathway

271 and therefore it constitutes the target of numerous investigations aimed at lowering the rate of
272 cholesterol biosynthesis [21-23]. Initially, *in vitro* experiments, performed using the purified catalytic
273 domain of the enzyme, showed that peptic hempseed peptides were able to function as direct
274 inhibitors of the activity of HMGCoAR (**Figure 3**).
275 The LDLR expression and the receptor protein localization at cellular membranes are strictly
276 correlated to the intracellular cholesterol biosynthesis pathway. In facts, the transcription of the LDLR
277 and the genes required for cholesterol and fatty acid synthesis are controlled by membrane-bound
278 transcription factors called SREBPs [24], and intracellular cholesterol acts with a negative feedback
279 inhibition mechanism [25]. The SREBP2 isoform is responsible for the LDLR and HMGCoAR
280 transcription and the SREBP2 maturation is regulated by the intracellular cholesterol homeostasis.
281 Thus, the up-regulation of LDLR represents a useful strategy to control plasma LDL cholesterol
282 levels. Our findings demonstrate that hempseed peptides are able to up-regulate the LDLR protein
283 levels through an increase of SREBP2 protein.
284 In addition, a detailed investigation of the LDLR pathway revealed that these peptides increase the
285 HMGCoAR protein levels in a significant way *versus* the control (**Figure 4A-C)**. However, this does
286 not mean an increase of cholesterol synthesis, since they are also able to inactivate HMGCoAR,
287 increasing its phosphorylation mediated by the activation of the AMPK pathway (**Figure 4D-F**).
288 Finally, in agreement with immunoblotting results, the increase of LDLR protein levels leads to an
289 increase of LDL uptake (**Figure 5**). The induction of the LDL clearance is strictly correlated to an
290 increase of LDLR protein level.
291 The cholesterol-lowering effects of hempseed peptides in human hepatic HepG2 cells have some
292 similarities with the behavior of lupin peptides [26]. In facts, also the peptides obtained by the hydrolysis
293 of a total lupin protein extract with pepsin, are able to mediate hypocholesterolemic effects in the
294 same cells through the activation of the LDLR pathway. Other hypocholesterolemic peptides with a
295 statin-like mechanism may be found in soy [27].
296 Another innovative result of this investigation is the demonstration that hempseed peptides modulate
297 the PCSK9 levels, increasing them (Figure 6). In light of this observation, these findings clearly
298 support the hypothesis that the cholesterol-lowering effect of hempseed peptides occurs through a
299 mechanism of action similar to that of statins. In fact, statins increase the transcription of both LDLR
300 and PCSK9 [28], since both gene expressions are co-regulated by SREBP-2 activation. Similarly,
301 hempseed peptides are able to increase the PCSK9 protein levels through an up-regulation of the
302 SREBP-2 pathway. According to our knowledge, this is the first demonstration that hempseed
303 peptides mediate a hypocholesterolemic effect with a statin-like mechanism.

304 Previous investigations have shown that hempseed protein and/or peptides bestow numerous useful
305 biological activities, such as ACE-inhibitory activity [9, 29], antioxidant activity [30], and
306 neurodegenerative activity [31] and cardiovascular disease modulation [32]. In this context, the present
307 work offers new insight on the bioactivity of these underexploited food ingredients.
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309
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319 AA. Manuscript writing: CL, GA & AA.
320
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324
325
326 **ABBREVIATIONS USED**

327 AMPK, phospho-5'-adenosine monophosphate-activated protein kinase; BSA, bovine serum
328 albumin; CVD, cardiovascular disease; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal
329 bovine serum; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HRP, Ig-
330 horseradish peroxidase; LDL, low density lipoprotein; LDLR, low-density lipoprotein receptor;
331 MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MWCO, molecular weight
332 cut-off; PBS, phosphate buffered saline; PCSK9, proprotein convertase subtilisin/kexintype 9;
333 PDCAAS, digestibility-corrected amino acid scores; PMSF, phenylmethanesulfonyl fluoride; SDS-
334 PAGE, sodium dodecyl sulphate – polyacrylamide; SREBP2, regulatory element binding proteins
335 2; THC, Δ9-tetrahydrocannabinol; TIC, total ion current.

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Captions of Figures

Figure 1. A) TIC of nano-LC MS; B) TIC of nano-LC MS/MS of hempseed protein hydrolysate.

Figure 2. HepG2 cell viability after hempseed protein hydrolysate **treatments**. Bar graphs indicate the results of MTT cell viability assay of HepG2 cells after hempseed peptide treatments for 24 h. The data points represent the averages ± SEM of three independent experiments in triplicate. C: control.

Figure 3. Inhibitory effect of hempseed peptides on HMGCoAR activity. The HMGCoAR, physiologically, catalyzes the four-electron reduction of HMGCoA to coenzyme A (CoA) and mevalonate (HMGCoA + 2NADPH + 2H+ > mevalonate + 2NADP+ + CoA-SH). In this assay, the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMGCoAR in the presence of the substrate HMGCoA, was measured spectrophotometrically for each dose tested. Each point represents the average ± SEM of three experiments in duplicate. (*) $p < 0.05$ and (***) $p < 0.0001$ *versus* control (C).

Figure 4. Effects of hempseed peptides on SREBP2-LDLR pathway. HepG2 cells (1.5×10^5) were treated with peptic hempseed peptides (0.25, 0.5, 1.0 mg/mL) for 24 h. SREBP2, LDLR, HMGCoAR, phospho-AMPK (Thr172), phospho-HMGCoAR (Ser872), and β-actin immunoblotting signals were detected using specific anti-SREBP2, anti-LDLR, anti-HMGCoAR, anti-phospho-AMPK (Thr172), anti-phospho-HMGCoAR (Ser872), and anti-β-actin primary antibodies, respectively (A-B-D-E). Each protein signal was quantified by ImageLab software (Bio-Rad) and normalized with β-actin signals (C-F). Bars represent averages ± SEM of six independent experiments (two duplicates per sample). (*) $p < 0.05$, (**) $p < 0.001$, and (***) $p < 0.0001$ *versus* control (C).

Figure 5. Fluorescent LDL-uptake assay after treatment of HepG2 with hempseed peptides. Cells (3×10^4) were treated with hempseed peptides (0.25, 0.5, 1.0 mg/mL) for 24 h. LDL-Dylight 550 (10 µg/mL) was incubated for an additional 2 h. Excess LDL-Dylight 550 was removed and cells were washed two times with PBS. Specific fluorescent LDL-uptake signal was analyzed by Synergy H1 (Biotek). Data points represent averages ± SEM of three independent experiments in triplicate. (***) $p < 0.0001$ *versus* control (C).

465 **Figure 6. Effects of hempseed peptides on PCSK9 protein levels.** HepG2 cells (1.5×10^5) were
466 treated with hempseed peptides (0.5, 1.0 mg/mL) for 24 h. PCSK9 and β-actin immunoblotting
467 signals were detected using specific anti-PCSK9 and anti-β-actin primary antibodies, respectively
468 (A). PCSK9-M represents the cleaved mature form of PCSK9 and its signals were quantified by
469 ImageLab software (Bio-Rad) and normalized with β-actin signals. Bars represent averages ± SEM
470 of six independent experiments (two duplicates per sample). *(**)* $p < 0.001$ and *(***)* $p < 0.0001$
471 *versus* control (C).
472

Table 1. LC-ESI-MS/MS based identification of peptic hydrolysate of hempseed proteins.

Accession n. [a]	Protein Name	Start-end	Sequence	pI[b]	Net Charge [b]	Hydrophobicity % [c]	m/z (Da) (charge)	$[M+H]^+$ (Da)
A0A090CXP8	Edestin 2	176-187	(D)WVYNNGDSPLVL(I)	0.7	-1	50	688.60 (2)	1376.69
		361-370	(V)LYKNGMMAPH(F)	9.7	1.1	50	581.74 (2)	1161.56
		380-403	(I)YVTRGSARLQVVDDNGRNVFDGEL(R)	4.3	-1	33.3	894.16 (3)	2680.34
		435-447	(N)DNAMRNPLAGKVS(A)	10.2	1	46.2	458.55 (3)	1372.70
		235-247	(R)RESGEQTPNGNIF(S)	4.2	-1	23.1	724.68 (2)	1448.67
A0A090CXP7	Edestin 1	450-460	(A)WVSPLAGRTSV(I)	10.7	1	54.6	586.80 (2)	1172.64
		178-187	(L)LDTSNVNNQL(D)	0.7	-1	30	559.84 (2)	1117.55
		279-288	(D)LVSPLRSSQE(H)	6.9	0	40	558.08 (2)	1115.61
		63-73	(L)IESWNPNHNQF(Q)	5.1	-0.9	36.4	693.73 (2)	1385.62
		461-469	(V)IRALPEAVL(A)	6.9	0	77.8	491.16 (2)	981.61
A0A090DLH8	Edestin 1	392-409	(M)YVLRGRARVQVVNHMGQKC(F)	11.2	4	36.8	738.37 (3)	2214.19
H9A1V3	Acyl-activating enzyme 1	625-639	(I)ERVCNEVDDRVFETT(A)	3.8	-3.1	26.7	623.95 (3)	1868.84
		170-190	(G)GYLNSAKNCLNVNSNKKLNDT(M)	9.6	1.9	23.8	789.33 (3)	2367.17
H9A1V4	Acyl-activating enzyme 2	280-298	(H)IFDRVIEELFILHGASIGF(W)	4.3	-1.9	57.9	725.95 (3)	2176.18
		28-44	(Y)RSMYAKDGFPPPIDGLD(C)	4.0	-1	47.1	627.21 (3)	1878.91
		442-453	(G)PPVPNVDVCLES(V)	0.7	-2.1	58.3	442.71 (3)	1325.64
H9A1V5	Acyl-activating enzyme 3	293-312	(L)ALSKNSMVKKFNLSSIKYIG(S)	10.8	4	40	743.33 (3)	2228.25
		360-382	(N)SGSAGMLASGVEAQIVSVDTLKP(L)	3.9	-1	47.8	739.89 (3)	2217.14
H9A1V7	Acyl-activating enzyme 5	253-266	(G)YTWGTAAVGATNVC(L)	3.1	-0.1	42.9	491.20 (3)	1470.67
		497-512	(F)VTLKKGAVRVTVTEKE(I)	10.4	2	37.5	586.93 (3)	1758.05
		54-62	(T)RCLRVASCI(E)	8.8	1.9	44.4	511.27 (2)	1020.55
H9A1W0	Acyl-activating enzyme 8	352-373	(D)QNGSAQLAGVSGEVCIRGPNVT(K)	6.1	-0.1	36.4	738.96 (3)	2214.09

		166-189	(D)VALFLHTSGTTSRPKGVPLTQLNL(A)	11.4	2.1	45.8	850.97 (3)	2550.44
H9A1W2	Acyl-activating enzyme 10	138-154	(Q)NIAAKTSAQFSLIPSVP(S)	9.7	1	58.8	582.30 (3)	1743.96
H9A1W3	Acyl-activating enzyme 11	260-268	(F)EMKKMVELI(E)	7.0	0	55.6	560.8 (2)	1120.61
		8-14	(F)IFRSKLP(D)	11.4	2	57.1	430.58 (2)	860.54
H9A8L2	Acyl-activating enzyme 13	160-180	(P)GAVLNIAECCLLPTSYPRKDD(D)	4.2	-1.1	42.9	760.06 (3)	2278.12
		289-309	(P)LYSRVVEAAPDRVIVLPATGS(N)	6.9	0	57.1	738.54 (3)	2213.23
		535-547	(Y)PDDQACTGEVGLI(P)	0.5	-3.1	38.5	459.05 (3)	1374.62
H9A8L3	Acyl-activating enzyme 14	374-392	(A)IPWTQLSPIRCAAESWAHM(D)	7.1	0	57.9	752.00 (3)	2254.09
		598-615	(I)KRTVGGYFIVQGRADDTM(N)	9.5	1	33.3	672.24 (3)	2014.02
		631-652	(V)CDRADESIVETAAVSVSPVDGG(P)	3.3	-4.1	40.9	744.98 (3)	2234.02
A7IZZ2	(+)-alpha-pinene synthase, chloroplastic	270-283	(I)RAEAKWFIEEYEKT(Q)	4.6	-1	35.7	600.89 (3)	1799.90
		592-606	(G)DGHASQDSHSRKRIS(D)	10.1	1.2	13.3	560.90 (3)	1680.82
		319-336	(H)SELGKNKMVYARDRLVEA(F)	9.4	1	38.9	693.56 (3)	2079.10
		185-201	(I)FNDFKDETGKFKASIKN(D)	9.5	1	29.4	663.93 (3)	1989.01
A0A0C5ARX6	ATP synthase subunit alpha	123-131	(I)STSESRLIE(S)	4.2	-1	22.2	511.23 (2)	1021.52
		134-154	(P)APGIISRRSVYEPLQTGLIAI(D)	9.9	1	52.4	751.82 (3)	2254.29
A0A0C5ARS5	ATP synthase subunit beta	382-405	(G)EEHYETAQRVKQTLQRYKELQDII(A)	5.5	-0.9	25	1007.12 (3)	3018.56
		144-158	(D)TKLSIFETGIKVVDL(L)	6.6	0	46.7	554.85 (3)	1662.97
E5DK51	ATP synthase subunit alpha	151-166	(E)TLYCVYVAIGQKRSTV(A)	9.4	1.9	37.5	620.24 (3)	1857.99
		287-309	(D)VSAYIPTNVISITDGQICLETEL(F)	0.6	-3.1	43.5	846.10 (3)	2536.29
A6P6W0	Cannabidiolic acid synthase-like 1	504-522	(A)RIWGEKYFGKNFNRLVKVK(T)	11.1	5	36.8	794.75 (3)	2382.36
		91-104	(V)SHIQGTILCSKKVG(L)	9.7	2	28.6	491.22 (3)	1470.81
A0A088MFF4	Delta 12 desaturase	179-196	(P)PGRVLSLFVTLTLGWPLY(L)	10.3	1	61.1	677.78 (3)	2032.16
		331-345	(Y)NAMEATKAVKPILGE(Y)	6.6	0	53.3	524.23 (3)	1571.85

A0A0C5ARQ8	RNA polymerase subunit beta	1047-1063	(L)RSLALELNHFLVSEKNF(Q)	7.5	0.1	47.1	672.75 (3)	2017.09
		549-568	(M)QRQAVPLSRSEKCIVGTGLE(S)	8.6	0.9	35	743.36 (3)	2228.18
		743-752	(L)TPQMAKESSY(A)	6.5	0	30	380.81 (3)	1141.52
		358-377	(T)STTLTTTFESFFGLHPLSQV(L)	5.1	-0.9	40	738.8 (3)	2213.11
		17-26	(N)QIQFEGFCRF(I)	6.1	-0.1	40	666.86 (2)	1331.62
A0A0C5AS14	Hypothetical chloroplast RF1	341-355	(Q)ENSKLEILNEKKGVN(Y)	7.1	0	26.7	572.56 (3)	1714.93
		259-279	(T)DVEIETTSETKGTKQEQGGST(E)	3.9	-3	9.5	742.45 (3)	2225.04
A7IZZ1	(-)-limonene synthase, chloroplastic	180-200	(L)RQYGFEVPQEIFNNFKNHKTG(E)	9.4	1.1	28.6	851.36 (3)	2553.26
		349-360	(G)VRFEPQFSYFRI(M)	9.8	1	50	794.79 (2)	1588.83
E5DKP2	MatR	382-400	(G)VQLAETLGTAGVRGPQVSV(L)	6.8	0	47.4	627.62 (3)	1882.04
		242-250	(R)KLAAPLKTH(Y)	10.7	2.1	55.6	489.57 (2)	978.61
A0A0C5AUJ6	NADH-plastoquinone oxidoreductase subunit 5	603-622	(M)DWNWYEFLTNATFSVSIASL(G)	0.6	-2	50	788.88 (3)	2364.12
		256-269	(E)GPTPISALIHAATM(V)	7.8	0.10	64.3	690.19 (2)	1379.74
A0A0C5APZ1	NAD(P)H-quinone oxidoreductase chain 4	234-257	(W)LPDTHGEAHYSTCMLLAGILLKMG(A)	6.1	-0.9	45.8	876.6 (3)	2628.30
		230-238	(P)LHTWLPDTH(G)	6.0	-0.8	44.4	373.88 (3)	1119.56
Q8RVK9	Naringenin-chalcone synthase	353-368	(K)CVEDGLNTTGEGLEWG(V)	0.5	-4.1	25	560.86 (3)	1679.72
		301-324	(W)IAHPGGPAILDQVESKLALKTEKL(R)	7.8	0.1	50	843.79 (3)	2528.45
		236-250	(P)IFELVSAAQTILPDS(D)	0.7	-2	60	535.27 (3)	1603.86
		183-201	(K)GARVLVVCSEITAVTFRGP(N)	8.9	0.9	52.6	678.37 (3)	2032.10
V5KXG5	4-coumarate:CoA ligase	262-281	(G)ATILIMPKFEIGSLLGLIER(Y)	7.1	0	60	738.78 (3)	2214.29
		17-23	(I)IFRSKLP(D)	11.4	2	57.1	430.58 (2)	860.54
F1LKH7	Polyketide synthase 2	371-385	(G)LTVERVVLRSVPINY(-)	9.8	1	53.3	586.93 (3)	1758.03

		303-310	(A)ILDKVEEK(L)	4.3	-1	37.5	487.31 (2)	973.56
F1LKH8	Polyketide synthase 4	2-16	(M)NHLRAEGPASVLAIG(T)	7.4	0.1	53.3	502.22 (3)	1504.82
		256-266	(A)GLIFDLHKDVP(M)	5.0	-0.9	54.6	627.28 (2)	1253.69
A0A0C5APZ4	Protein Ycf2	1630-1650	(P)FSLRLALSLSRGILVIGSIGT(G)	12.1	2	52.4	725.26 (3)	2173.31
		1902-1921	(Q)DHGILFYQIGRAVAQNVLLS(N)	7.8	0.1	50	738.97 (3)	2214.20
		1092-1102	(T)ISPIELQVSNI(F)	0.9	-1	54.6	404.94 (3)	1212.68
		536-547	(S)ENKEIVNIFKII(T)	7.0	0	50	487.41 (3)	1459.85
		143-152	(L)YLPKGKKISE(S)	10.1	2	30	581.39 (2)	1162.68
A0A0C5ARZ4	Photosystem I P700 chlorophyll a apoprotein A1	436-454	(I)SHLNWVCIFLGFHSFGLYI(H)	7.2	0.1	52.6	751.46 (3)	2253.13
		102-122	(W)LSDPTHIGPSAQVVWPIVGQE(I)	3.9	-1.9	52.4	744.39 (3)	2230.15
		561-572	(L)IPDKANLGFRFP(C)	10.1	1	58.3	458.58 (3)	1374.75
A0A0C5APY0	Photosystem I P700 chlorophyll a apoprotein A2	188-207	(S)LAWTGHLVHVAIPGSRGESV(R)	8.0	0.2	50	695.75 (3)	2086.12
		247-257	(T)SQGAGTSILTL(L)	3.4	0	36.4	524.2 (2)	1047.57
		241-258	(S)SHLFGTSQGAGTSILTLL(G)	7.5	0.1	38.9	601.63 (3)	1802.97
		352-369	(H)MYSLPAYAFIAQDFTTQA(A)	0.7	-1	55.6	680.29 (3)	2037.96
		695-708	(R)DKPVALSIVQARLV(G)	10.2	1	64.3	503.33 (3)	1508.92
U6EFF4	Putative LysM domain containing receptor kinase	398-417	(H)LRGSGRDPLTWSSRVQIALD(S)	10.5	1	40	743.19 (3)	2227.20
		125-144	(V)HRVNMFKPTRIPAGSPINVT(V)	12.1	3.1	50	745.35 (3)	2235.22
		115-142	(A)FANLTTEDWVHRVNMFKPTRIPAGSPIN(V)	9.9	1.1	50	1071.23 (3)	3211.65
A0A0E3TIL1	THCA synthase	87-102	(T)PSNNSHIQATILCSKK(V)	10.3	2	31.3	580.67 (3)	1740.91
		29-47	(A)NPRENFLKCFSKHIPNNVA(N)	9.6	2	42.1	743.33 (3)	2228.14
		504-522	(A)RIWGEKYFGKNFNRLVKVK(T)	11.1	5	36.8	794.75 (3)	2382.36

a) According to “UniProtKB” (http://www.uniprot.org/).

b) According to “Protein Peptide Calculator” (http://pepcalc.com/)
c) According to “Peptide2.0” (http://peptide2.com/)

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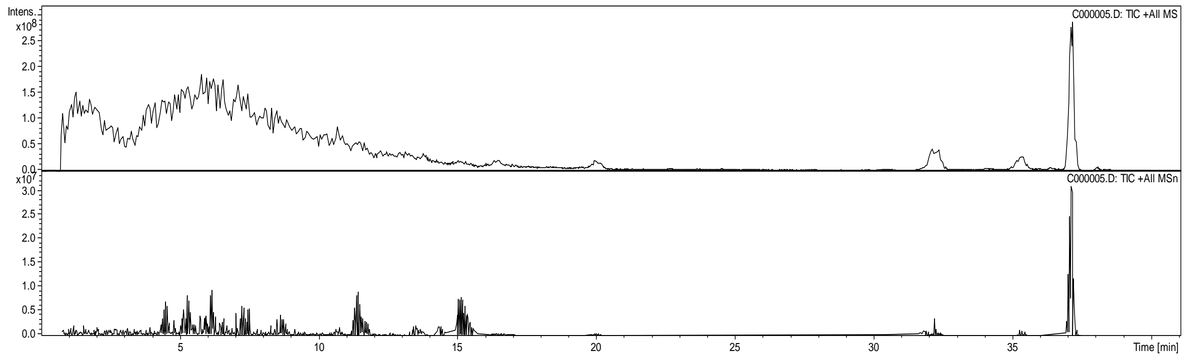

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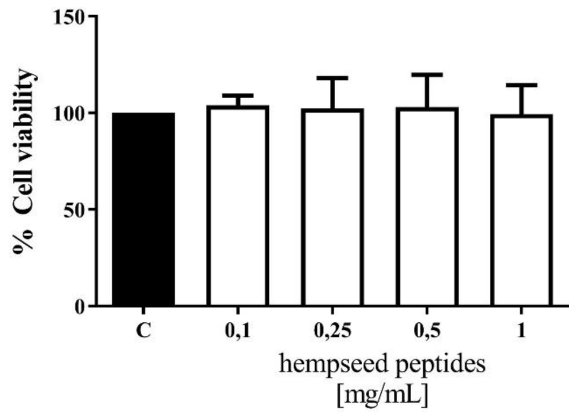

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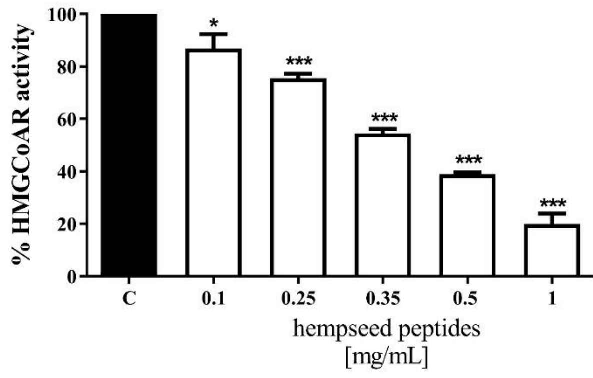

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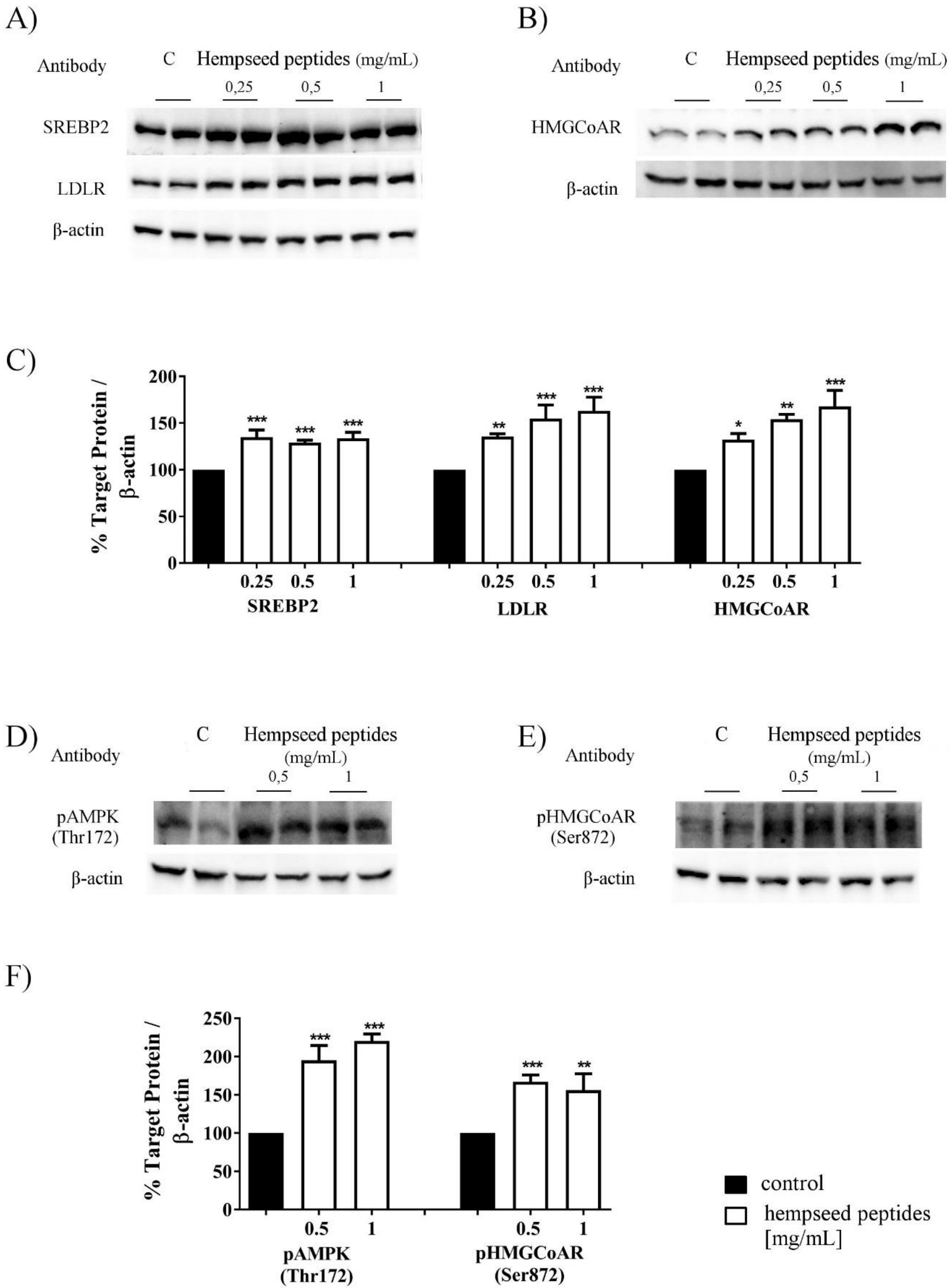

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38 Figure 4
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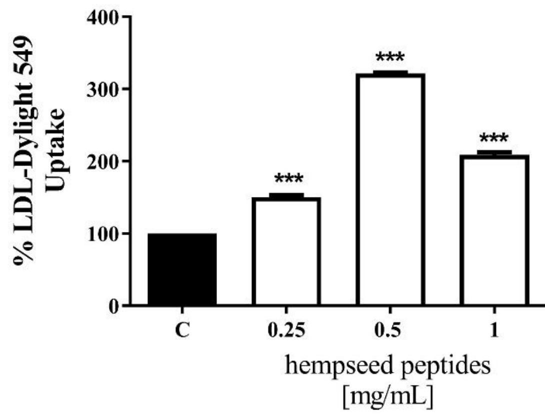

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42 Figure 5
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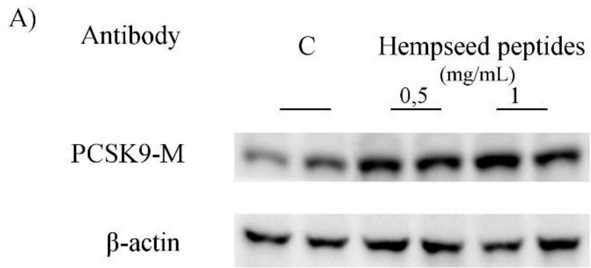

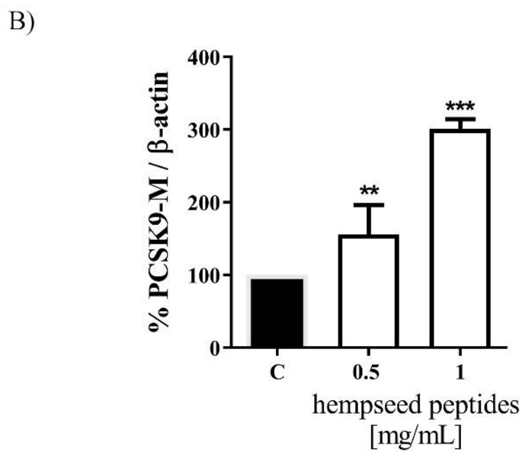

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52 Figure 6
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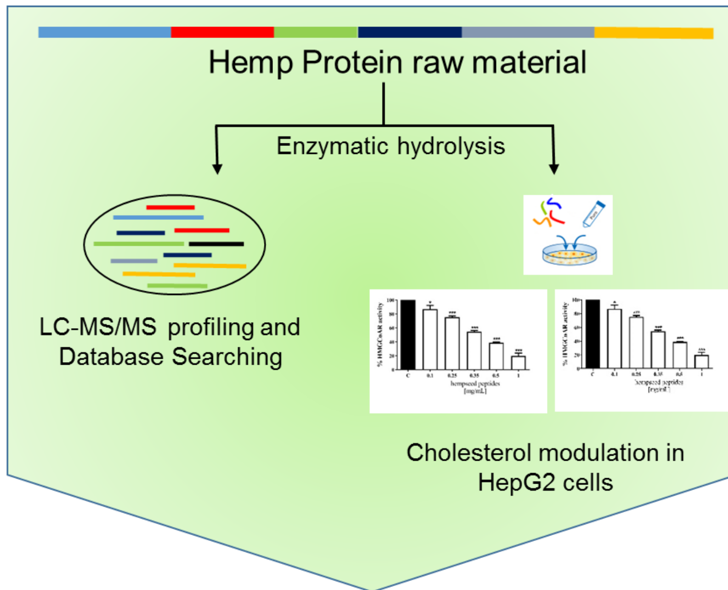

Hypocholesterolemic hempseed peptides: Statin-Like Mechanism of action

TOC