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In vitro approach for evaluation of carob by-products as source bioactive ingredients with potential to attenuate metabolic syndrome (MetS)

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Abstract

The potential bioactivities for alleviating Metabolic Syndrome associated risk factors were evaluated in carob (*Ceratonia siliqua* L.) fruit by-products, i.e. seed peel, germ and pod. Carob germ and seed peel showed higher phenolic content than pod (99.72, 80.24 and 47.06 μmol GAE g^{-1}, respectively). Pod mostly contained gallic acid and gallotannins; seed peel and germ's showed as most abundant polyphenols quercetin and apigenin derivatives. Carob pod and seed peel revealed stronger antioxidant capacities compared to germ. The strongest antihypertensive activity was found in seed peel, followed by pod and germ. Anti-inflammatory activity showed inhibition of NO production in LPS-induced macrophages, although only pod was able of reducing pro-inflammatory

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mediators (TNF-α andPGD_2). Finally, fat accumulation on mature adipocytes was reduced by carob seed peel and pod extracts. This work shows the potential use of pod carob by-products as food ingredients with special relevance of carob pod for attenuating metabolic syndrome.

Keywords: Food science, Food analysis

1. Introduction

Metabolic syndrome (MetS) is a complex multifactorial disorder associated to abdominal obesity, hyperglycemia, increased blood pressure, as well as atherogenic dyslipidemia, which are considered increase the risk of cardiovascular disease and type 2 diabetes. There are other non-metabolic components of MetS such as abnormalities in fibrinolysis and coagulation, chronic inflammation and endothelial dysfunction (Martínez-Larraz et al., 2016).

MetS prevalence is rising partly attributed to unhealthy dietary patterns, including energy dense food consumption and frequent snacking (Martínez-Larraz et al., 2016). Appropriate dietary habits are recommended by health organisations as one of the primary interventions for the prevention and treatment of the MetS. Healthy snacking patterns could improve quality diet by providing appropriate nutrients, satiety and appetite control, as well as regulating postprandial glycemic response (Leidy et al., 2015).

Carob fruit is used in food industry to produce carob and locust bean gum, which is added as flavouring, stabiliser and thickener in food (Dakia et al., 2007). Seeds are considered the most valuable part of the fruits, carob's pulp (pod) and seed peel could be considered as by-products susceptible of valorisation. Carob fruit is mainly composed of sugars, including sucrose, glucose and fructose (48%−56%), fibres such as cellulose, hemicellulose and lignin (30−40% in carob's pulp), gum (85% of carob seed is composed of galactomannan), amino acids and minerals (mainly potassium and calcium) (Albanell et al., 1996).

The concentration of phenolic compounds in carob fruit ranges from 45−5,376 mg gallic acid equivalents (GAE) 100 g^{-1} depending on genetic and environmental factors (Goulas et al., 2016); the main groups are phenolic acids, hydrolysable and condensed tannins (Stavrou et al., 2018). Papagiannopoulos et al. (2004).

Recent studies have revealed several physiological effects of carob fruit and its by-products due to the presence of bioactive compounds that may be relevant to the promotion of human health and the prevention of chronic diseases (Goulas et al., 2016; Benković et al., 2017). Nevertheless, at present, there is no study focused on the evaluation of the antioxidant, angiotensin I converting enzyme-inhibitory, anti-inflammatory and fat-lowering potential of carob by-products extracts and the

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relationship of bioactivity with phenolic composition. The objective of this work was to determine the above mentioned bioactivities of different carob by-products, in order to identify potential health benefits from their consumption.

2. Materials and methods

2.1. Material

Carob seed peel, germ and pod were gently provided in flour form by Adín Alimentaria (Paterna, Valencia, Spain). Seed peel was obtained during grinding of carob seeds and germ from the decortications and calibrated grinding of carob seeds. All flours were sieved to ensure particle size was under 300 μm.

2.2. Nutritional parameters

Protein, fat, moisture, ash, total dietary fibre and carbohydrate by difference content were determined as detailed in Martín-Diana et al. (2017). Data were expressed as % in dry weight basis (d.w.b.).

2.3. Phenolic compounds

Total Phenols (TP) was measured using the Folin-Ciocalteu method as described by Martín-Diana et al. (2017). Results were expressed as μmol gallic acid equivalents (GAE) g^{-1} of sample using a calibration curve with gallic acid as standard (9.8−70 mM).

Phenolic compound profile was determined using HPLC-DAD-ESI/MS chromatography, as described in Dueñas et al. (2015). The standards, gallic acid, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside, myricetin 3-*O*-glucoside, apigenin 6-*C*-glucoside, were obtained from Extra synthese (Genay, France). Galloyl glucose derivatives were quantified using gallic acid standard curve; derivatives of apigenin, quercetin, kaempferol, isorhamnetin and myricetin were quantified using apigenin 6-*C*-glucoside, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside and myricetin 3-*O*-glucoside standard curves, respectively. Results were expressed as μg g^{-1} sample (d.w.b.).

2.4. Antioxidant

Antioxidant activity was measured in methanolic extracts using different assays, namely Oxygen Radical Absorbance Capacity (ORAC), FRAP (Ferric Reducing Antioxidant Power), DPPH radical scavenging activity (DPPH) and Trolox Equivalent Antioxidant Capacity (TEAC). These last two methods were also applied on solid samples without previous extraction (DPPH- and TEAC-QUENCHER).

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Extract Preparation. Sample extracts were prepared as follows. One gram of sample was homogenised in 10 mL of methanolic solution (1:1, methanol:water). The solvent mixture chosen has previously shown high extraction yields of bioactive compounds in pulses (Rostagno et al., 2003; Hashim et al., 2016). The extraction was accelerated using a ceramic homogeniser's on the test tubes and stirring for 30 s. Sample was centrifuged at 1,635 x *g* for 10 min at 4 °C and the supernatant collected, filtered and stored at -80 °C until further analysis.

ORAC. The procedure was carried out as described by Martín-Diana et al. (2017), with a microplate reader (Fluostar Omega, BMG, Ortenberg, Germany), and results were expressed as μmol Trolox Equivalent (TE) g^{-1} sample (d.w.b.).

FRAP was determined according to the procedure described by Pereira et al. (2008). Results were expressed as μmol Fe equivalent g^{-1} sample (d.w.b.).

DPPH radical scavenging activity was estimated following the procedure described by Martín-Diana et al. (2017). Results were expressed as inhibition percentage (%).

TEAC was determined as described previously (Martín-Diana et al., 2017). Results were expressed as μmol Trolox Equivalent (TE) g^{-1} sample (d.w.b.).

DPPH-QUENCHER and TEAC-QUENCHER activities were estimated according to the procedure described by Serpen et al. (2007). DPPH was expressed as percentage of inhibition of the DPPH radical and TEAC results were expressed as mmol TE/g sample (d.w.b.).

2.5. ACE-inhibitory activity

Angiotensin converting enzyme (ACE) inhibitory activity was determined as described in Khiari et al. (2014). The ACE activity was expressed as IC_{50} (mg ml^{-1}).

2.6. Anti-inflammatory capacity

The effect of carob by-product's extracts on inflammatory markers was evaluated on macrophage cell line 264.7, as explained in Rico et al. (2018). Cytotoxicity, nitrite accumulation, and indicator of NO synthesis, PGD_2 and TNF-α levels were determined.

2.7. Triacylglyceride content

Measurement of triacylglycerol content in 3T3-L1 pre-adipocytes (American Type Culture Collection, Manassas, VA, USA) was carried out as described in Eseberri et al. (2015).

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2.8. Statistics

Data represent the mean and standard deviation of three independent replicates analysed at least twice (n = 6). Significance was tested by one-way ANOVA (Statgraphics v16.1.17, Statistical Graphics Corp., Rockville, USA). Differences between samples by Duncan's multiple-range test ($P < 0.05$). In the case of triacylglyceride-lowering activity, comparisons between each treatment with the control sample were analysed by Student's *t*-test.

3. Results and discussion

3.1. Proximal analysis

The content of humidity, ash, nitrogen, protein, carbohydrates, fat and fibre are given in Table 1. Germ showed higher content in ash, protein and fat than seed peel and pod, meanwhile seed peel showed higher content in fibre and pod higher content in carbohydrates. The results were consistent with results reported by Durazzo et al. (2014), in regard to higher protein, fat and ash content of germ, when compared to seed peel.

3.2. Phenolic compounds

Total phenolic compounds were evaluated in seed peel, pod and germ. Seed peel and pod had significantly higher TP values than germ (Table 2). These values were similar to those reported in carob pod by Huma et al. (2017) (4.01−61.38 mg GAE g^{-1}), but lower than Kumazawa et al. (2002) (192mg GAE g^{-1}). The variability found between our results and previous studies for total phenolics content of carob by-products might respond to differences in the origin of the carob source,

Table 1. Nutritional content of the different by-products of carob (*Ceratonia siliqua* L.).

	Carob (*Ceratonia siliqua* L.)		
	Pod	**Germ**	**Seed peel**
Humidity (%)	6.48 ± 0.11 a	9.94 ± 0.45 b	12.22 ± 4.35 b
Ash (%, d.w.b.)	3.68 ± 0.01 a	5.14 ± 0.18 c	3.73 ± 0.04 b
Nitrogen (%, d.w.b.)	0.74 ± 0.01 a	5.24 ± 0.16 c	0.92 ± 0.22 b
Protein (%, d.w.b.)	4.65 ± 0.01 a	32.74 ± 0.97 c	5.81 ± 1.34 b
Carbohydrates (%, d.w.b.)	84.99 ± 0.10 c	49.26 ± 1.10 a	77.88 ± 2.91 b
Fibre (% d.w.b.)	52.35 ± 0.35 a	57.7 ± 0.71 b	60.26 ± 0.39 c
Fat (%, d.w.b.)	0.18 ± 0.01 a	2.92 ± 0.14 c	0.34 ± 0.05 b

Data are the mean ± standard deviation of three replicates analyzed in duplicate. Different lowercase letters indicate statistical differences among treatments ($P < 0.05$. Duncan's test).

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Table 2. Total phenolic content, ACE inhibitory and antioxidant activities of the different carob by-products (*Ceratonia siliqua* L.).

	Carob (*Ceratonia siliqua* L.)		
	Pod	**Germ**	**Seed peel**
Total Phenolics (μmol GAE g^{-1} d.w.b.)	80.24 ± 2.53 b	47.06 ± 0.68 a	99.72 ± 1.01 c
ACE IA: IC_{50} (mg ml^{-1})	0.423	0.672	0.113
DPPH (% reduction)	93.46 ± 0.38 b	92.59 ± 0.47 a	93.55 ± 0.19 b
TEAC (μmol TE g^{-1}d.w.b.)	257.85 ± 2.63 b	142.0 ± 4.0 a	234.25 ± 8.74 b
FRAP (μmol Red. Fe g^{-1}d.w.b.)	6.10 ± 0.13 b	4.46 ± 0.07 a	5.91 ± 0.09 b
ORAC (μmol TE g^{-1}d.w.b.)	192.63 ± 2.25 a	284.94 ± 1.94 b	310.86 ± 3.21 c
DPPH-QUENCHER (% reduction)	55.32 ± 4.13 c	22.13 ± 4.62 b	14.99 ± 1.25 a
TEAC-QUENCHER (μmol TE g^{-1}d.w.b.)	61.64 ± 5.08 a	94.81 ± 11.46 c	68.41 ± 0.42 b

Data are the mean ± standard deviation of three replicates analyzed in duplicate. Different lowercase letters within a row indicate statistical differences ($P < 0.05$. Duncan's test).

growing conditions, or also solvent and temperature used during the extraction procedure. Extraction of phenolics using solid-liquid extraction methods requires transport of the solvent into the matrix, and the phenolics released to the solvent phase is modified by different aspects. Temperature increases the speed of the solutes in the extract, hence producing greater separation energy of the solutes from the solid matrices (Rodriguez-Rojo et al. (2012).

Twenty-five phenolic compounds were identified (HPLC-DAD-ESI/MS) belonging to hydroxybenzoic, gallotannins, flavones and flavonols phenolic classes (Table 3).

Hydroxybenzoic acids: *p*-hydroxybenzoic acid was the only hydroxybenzoic acid identified, which was confirmed by comparison of their UV-vis spectra and retention time with its commercial standard.

Gallotanins: Peaks 2, 4, 8 and 14 presented a fragmentation pattern characteristic of dimer, trigalloyl and tetragalloyl glucose, according to Sanz et al. (2010). These compounds presented a precursor ion $[M-H]^-$ at *m/z* 483, 635 and 787 and different fragments corresponding to the loss of one or more galloyl groups (152 mu) and/or gallic acid (170 mu). Similar compounds were previously reported in carob pods and derived products (Papagiannopoulos et al., 2004; Rached et al., 2016; Rakib et al., 2010). Compound 3 presented pseudo molecular $[M-H]^-$ at *m/z* 443 and a fragmentation pattern similar to those gallotannins. This compound was tentatively assigned as galloyl glucose derivative. This compound was also identified in carob pulp by Rached et al. (2016).

Flavones: Peaks 5−7 and 9−13 presented all similar UV-spectra (λ_{max} at 324−338 nm) and a fragmentation pattern characteristic of *C*-hexosyl-flavones, and similar to those observed by Picariello et al., 2017. Peaks 5, 6 and 7 showed a UV-vis spectra

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Table 3. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification of individual phenolic compounds (μg/g d.w.b) in *Ceratonia siliqua* L.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Pod	Germ	Seed peel
1	4.27	272	169	-	Gallic acid	1518.38 ± 230.03 c	213.78 ± 19.46 b	137.90 ± 4.86 a
2	4.73	280	483	331, 313, 271, 211, 169	Digalloyl-glucose	nd	179.63 ± 38.57 c	85.18 ± 3.65 b
3	5.74	282	443	331, 313, 271, 211, 169	Galloyl-glucose derivative	nd	583.24 ± 95.22 c	106.09 ± 6.41 b
4	5.99	278	483	331,313,271,169	Digalloyl-glucose	1218.50 ± 68.60 c	404.14 ± 36.39 b	162.21 ± 13.57 a
5	6.39	324	725	635,605,545,383,353	*O*-hexosyl-6-*C*-hexosyl-8-C-pentoside apigenin	nd	259.30 ± 11.65	nd
6	7.41	324	725	635,605,545,383,353	*O*-hexosyl-6-*C*-hexosyl-8-*C*-pentoside apigenin	nd	464.13 ± 50.30	nd
7	8.11	324	725	635,605,545,383,353	*O*-hexosyl-6-*C*-hexosyl-8-*C*-pentoside apigenin	nd	23.22 ± 5.89	nd
8	10.29	278	635	483,465,313,297,169	Trigalloyl-glucose	nd	224.98 ± 20.34 a	449.41 ± 19.10 b
9	10.75	338	593	503,473,383, 353	6-*C*-hexosyl-8-*C*-hexoside apigenin	nd	38.46 ± 8.90 c	21.18 ± 0.51[b]
10	14.64	334	563	473, 443, 383,353	6-*C*-hexosyl-8-*C*-pentoside apigenin	nd	478.09 ± 54.87 b	t
11	14.92	332	563	473, 443, 383,353	6-*C*-hexosyl-8-*C*-pentoside apigenin	nd	1119.68 ± 106.99 b	7.32 ± 1.44 a
12	16.25	336	563	473, 443, 383,353	6-*C*-hexosyl-8-*C*-pentoside apigenin	nd	129.02 ± 21.22 b	nd
13	16.44	334	563	473, 443, 383,353	6-*C*-hexosyl-8-*C*-pentoside apigenin	51.36 ± 3.37 b	76.77 ± 8.08 c	nd
14	16.68	286	787	635, 617,465,313,169	Tetragalloyl-glucose	124.51 ± 24.44b	221.33 ± 33.05 c	t
15	19.94	356	463	317	Myricetin *O*-hexoside	44.18 ± 3.40 b	t	t

(*continued on next page*)

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Table 3. (*Continued*)

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Pod	Germ	Seed peel
16	20.32	354	463	301	Quercetin 3-*O*-glucoside	54.10 ± 1.17 a	382.78 ± 35.71 c	293.89 ± 18.16 b
17	22.02	356	433	301	Quercetin *O*-pentoside	10.82 + 0.96 a	146.29 ± 33.49 b	119.94 ± 11.55 b
18	22.91	354	433	301	Quercetin *O*-pentoside	5.83 + 0.29 a	129.36 ± 36.28 b	158.05 ± 7.24 b
19	23.17	336	739	431, 341, 311	*C*-hexoside-*O*-rhamnosyl-hexoside apigenin	nd	258.76 ± 17.87 c	189.43 ± 22.54 b
20	23.90	348	447	301	Quercetin *O*-rhamnoside	63.19 ± 3.06 a	2167.31 ± 146.62 b	2669.06 ± 136.10 c
21	24.87	354	477	315	Isorhamnetin 3-*O*-hexoside	nd	88.30 ± 7.90 b	107.79 ± 22.19 b
22	26.67	348	417	285	Kaempferol *O*-pentoside	nd	3.21 ± 0.45 b	t
23	28.39	356	447	315	Isorhamnetin *O*-pentoside	nd	102.21 ± 9.90 c	40.01 ± 3.85 b
24	28.64	344	431	285	Kaempferol *O*-rhamnoside	nd	78.90 ± 8.60 b	115.02 ± 7.54 c
25	29.25	350	461	315	Isorhamentin *O*-rhamnoside	nd	136.35 ± 7.71 c	86.45 ± 2.87 b
					Total hydroxybenzoic acids	1518.38 ± 230.03 b	213.78 ± 19.46 a	137.90 ± 4.86 a
					Total gallotannins	1270.48 ± 4.91 b	1616.08 ± 217.92 c	844.74 ± 12.00 a
					Total flavones	51.36 ± 3.37 a	2768.26 ± 203.60 b	209.46 ± 32.35 a
					Total flavonols	140.28 ± 33.13 a	2461.81 ± 162.64 b	3563.26 ± 204.54 b
					Total phenolic compounds	3065.71 ± 210.49 a	7292.95 ± 700.63 c	4593.12 ± 357.84 b

Data are the mean ± standard deviation of three replicates. Different lowercase letters indicate statistical differences between values within a row ($P < 0.05$). nd: non detected; t: trace amount.

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with a maximum wavelength of absorption at 324 nm, a precursor ion $[M-H]^-$ at *m/z* 725 and different fragments at *m/z* 635 and 605, that corresponded to the loss of 90 and 120 mu, which are characteristics of *C*-hexosyl flavones (Ferreres et al., 2003). The fragment ion at *m/z* 545 ($[M-H-180]^-$) was associated to an *O*-glycosylation on the phenolic hydroxyl of sugar moiety from *C*-glycosylation. In addition, it released two fragments at m/z 383 and 353, that corresponded to the apigenin aglycone bearing some sugar residues [apigenin +113 mu] and [apigenin +83] that remained attached to it (Ferreres et al., 2003). These compounds were tentatively identified as *O*-hexosyl-6-*C*-hexosyl-8-*C*-pentoside apigenin, which were only found in carob germ.

Peak 9 presented a pseudo molecular ion $[M-H]^-$ at 593, releasing four MS^2 fragment ions at *m/z* 503 and 473, corresponding to the loss of 90 and 120 mu, characteristic of *C*-hexosyl flavones, and at *m/z* 383 ([apigenin + 113 mu] and 353 [apigenin + 83 mu] that might correspond to the apigenin aglycone plus the linked sugar residues (Ferreres et al., 2003).

This compound was tentatively identified as 6-*C*-hexosyl-8-*C*-hexoside apigenin, which was found in carob germ and seed peel.

Peaks 10, 11, 12 and 13 were tentatively identified was 6-*C*-hexosyl-8-*C*-pentoside apigenin. These peaks presented a pseudo molecular ion $[M-H]^-$ at 563, and the same fragmentation pattern that peak 9.

Peak 19 identified as *C*-hexoside-*O*-rhamnosyl-hexosideapigenin, possessed a molecular ion $[M-H]^-$ at *m/z* 739 that released three MS^2 fragments ions, one fragment at *m/z* 431 ($[M-308]^-$ (loss of a rhamnosyl-hexoside moiety), and two at *m/z* 341 [(M-308-90]$^-$ and 311 $[M-308-120]^-$, characteristics of flavone-*C*-glycosides. This compound was present in carob germ and seed peel.

Flavonols: quercetin derivatives (peaks 16, 17, 18 and 20) were present in all samples, which presented similar UV spectra (λ_{max} at 348−356 nm) to quercetin glycosides. Peak 16 (quercetin 3-*O*-glucoside) was identified according to their retention time, mass and UV-vis spectra by comparison with commercial standard. Quercetin *O*-pentoside (peaks 17, 18) and quercetin *O*-rhamnoside (peak 20) were identified according to $[M-H]^-$ at *m/z* 433, 447, respectively. Fragment ion at *m/z* 301 corresponded to quercetin.

Isorhamnetin derivatives (peaks 21, 23, 25) were also observed according to their UV-vis and mass spectra with fragment ions at *m/z* 315. Peak 21 was identified as isorhamnetin 3-*O*-glucoside by comparison with commercial standard.

Compounds 15, 22, 24 showed a pseudo molecular ion [M-H]- at *m/z* 463, 417 and 431, respectively, and similar MS^2 fragmentation patterns releasing one fragment 317 (myricetin) and 285 (kaempferol) from the loss of hexoside ($[M-162]^-$),

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pentoside ([M-132]$^-$) and rhamnoside ([M-146]$^-$). Different flavonol glycosides have been previously reported in carob samples (Rached et al., 2016; Rakib et al., 2010).

Results showed important differences in phenolic composition of carob germ, seed peel and pod (Table 3). Flavones were the most abundant class in carob germ, accounting for 38% of the total phenolic compounds. However, flavonols (77%) and hydroxybenzoic acid (50%) were the major phenolic groups detected in seed peel and pod, respectively. The sum of individual phenolic concentrations was significantly different ($P < 0.05$) between samples, showing seed peel the highest total phenolic content. The compound with the highest concentration in germ and seed peel was quercetin *O*-rhamnoside, presenting significant differences. The sample with the lowest concentration of total phenolic compounds was carob pod (3065.71 μg g^{-1}), where gallic acid and digalloyl glucose were the main compounds identified. Rakib et al. (2010) studied different carob pods from six Moroccan regions, and found gallic acid as the principal compound. Rached et al. (2016) observed gallotannins as the most abundant phenolics in carob pulp.

3.3. Antioxidant

Antioxidant capacity of carob extracts is mainly related to phenolic content. The results showed (Table 2) seed peel and pod extracts holding higher antioxidant capacity than germ, with the exception of the ORAC results, where germ showed higher antioxidant activity than pod, but lower than seed peel ($P < 0.05$). To the antioxidant activity of germ might also contribute the presence of antioxidant lipids (Henry et al., 2002). It was observed an effect due to the extraction procedure, and the total antioxidant capacity of the samples showed different trends in the methods carried out without extraction (QUENCHER). Germ fraction (solid, without extraction) showed highest and intermediate activity, as compared to pod and seed peel, in TEAC- and DPPH-QUENCHER assays, respectively. The higher antioxidant capacity of the germ in the direct methods (QUENCHER) might be explained by the differences in the amount of fibre of the fractions, which was higher in the case of germ (Table 1), fibre that has previously shown antioxidant activity (Owen et al., 2003).

3.4. ACE-inhibitory activity

Anti-hypertensive activity (Table 2) was measured through the inhibition of ACE (IC_{50}). It was observed that seed peel extract had a IC_{50} four times lower than pod and six times lower than germ; these results are in line with the data observed for antioxidant activity. Flavonols, especially quercetin and kaempferol rhamnosides, present in higher concentration in the seed peel fraction (Table 3), have shown a synergistic effect in their bioactivities (Hansen et al., 1996), which could explain in part the higher anti-hypertensive activity of the seed peel fraction.

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3.5. Anti-inflammatory capacity

The viability of RAW 264.7 cells incubated for 24 h with different concentrations of carob extracts (0.5, 0.1, and 0.05 mg mL^{-1}), is shown in Fig. 1 B. The highest concentration tested (0.5 mg mL^{-1}) did not provoke changes in cell viability (≥90% viability) in the case of germ extract; however, this concentration significantly ($P < 0.05$) reduced cell viability in the case of seed peel and pod extracts (29.2 and 48.3% surviving cells, respectively). The intermediate concentration tested (0.1 mg mL^{-1}) was significantly cytotoxic only for the seed peel extract, resulting in 76.6% of remaining living cells. On the other hand, the lowest concentration tested (0.05 mg mL^{-1}) for all carob extracts showed no significant changes, indicating no cell integrity damage. Due to the toxicity of the intermediate

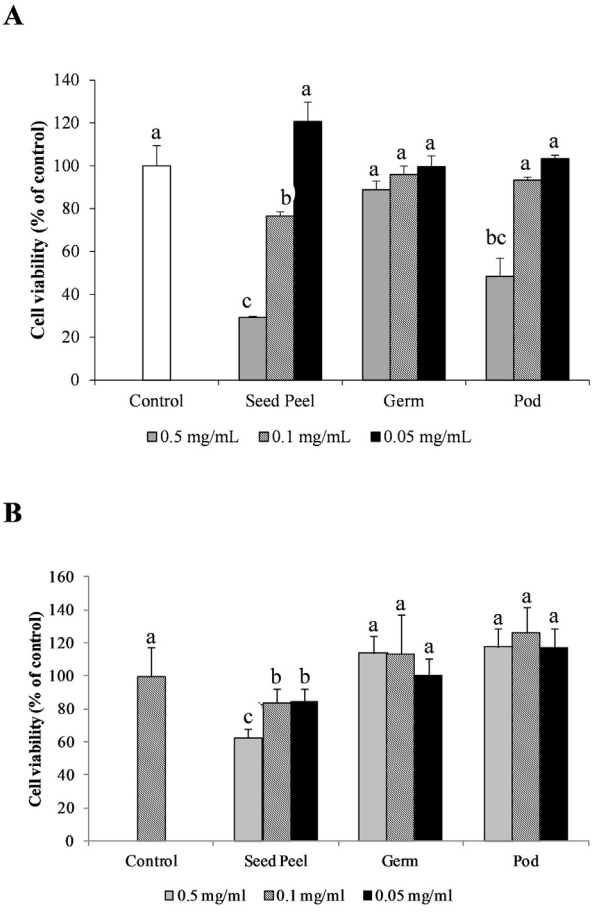

Fig. 1. Cell viability of RAW 264.7 cells (A) and 3T3-L1 mature adipocytes (B) cultured in the presence of carob extracts at three different concentrations (0-control, 0.5, 0.1, and 0.05 mg/mL) for 24 h. Values are expressed as a percent relative to the control condition by mean ± SD (n = 3 for RAW 264.7 cells and n = 6 for 3T3-L1 mature adipocytes). Asterisk indicate statistically significant differences ($P < 0.05$) compared with control condition.

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concentration for seed peel, and in order to compare all extracts in similarity of conditions, 0.05 mg mL^{-1} extract dose was selected to examine the potential anti-inflammatory effects.

To investigate whether carob extracts have anti-inflammatory properties, RAW 264.7 cells were pre-treated with 0.05 mg $mL^{-1}$of carob seed peel, germ or pod extracts for 24 h, and subsequently stimulated with 10 μg mL^{-1} lipolysaccharide (LPS) for 24h. Production of nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and prostaglandin D_2 (PGD_2) were measured in the culture medium. When LPS was administered to macrophages the NO, TNF-α, and PGD_2 production increased dramatically after 24h incubation (Fig. 2), indicating the successful activation of cells by LPS treatment.

In RAW 264.7 cells, LPS stimulation has been demonstrated to induce nitric oxide synthase (NOS) transcription, with a corresponding increase in NO production, considered a marker of active pro-inflammatory responses (Yang et al., 2009). Therefore, this cell system is an excellent model for evaluation of anti-inflammatory activity of samples. Our results (Fig. 2) showed that carob extracts from all by-products at 0.05 mg mL^{-1} significantly ($P < 0.05$) reduced NO production in LPS-activated macrophages. These results suggest seed peel, germ and pod carob extracts exert anti-inflammatory activities, which could be attributed to the presence of phenolic compounds. As with our results, enriched phenolic extracts have been previously shown to inhibit NO production (Benayad et al., 2014), through down regulation of inducible NOS expression in LPS-activated macrophages. This effect was most notorious in the presence of pod extract (51% of inhibition) than seed peel and germ (about 31% and 47% of inhibition, respectively). The higher concentration of gallic acid and gallotannins in carob pod could be related to stronger inhibition of NO production. Previous studies support this idea, showing isolated gallotannins and gallic acid from plant extracts able to reduce NO production in LPS-induced 264.7 macrophages through inactivation of nuclear factor-κB (Lin et al., 2017).

Other of the recognised anti-inflammatory activities is the suppression of pro-inflammatory cytokines, such as TNF-α and PGD_2, a potent inflammatory mediator. Macrophages are known to generate TNF-α and PGD_2 in response to various stimuli (Reis et al., 2011). Thus, elevation of TNF-α and PGD_2 levels after LPS stimulation reflect a pro-inflammatory process. In our *in vitro* experiment, carob pod only, and pod and germ extracts at 0.05 mg mL^{-1} produced a weak but significant reduction of TNF-α and PGD_2, respectively (Fig. 2). Similar to other bioactivities, the seed peel was not effective in the inhibition of TNF-α and PGD_2 production in LPS activated macrophages. These results could be explained by differences in the phenolic profile among carob by-products. Carob pod was mainly constituted by gallic acid, which

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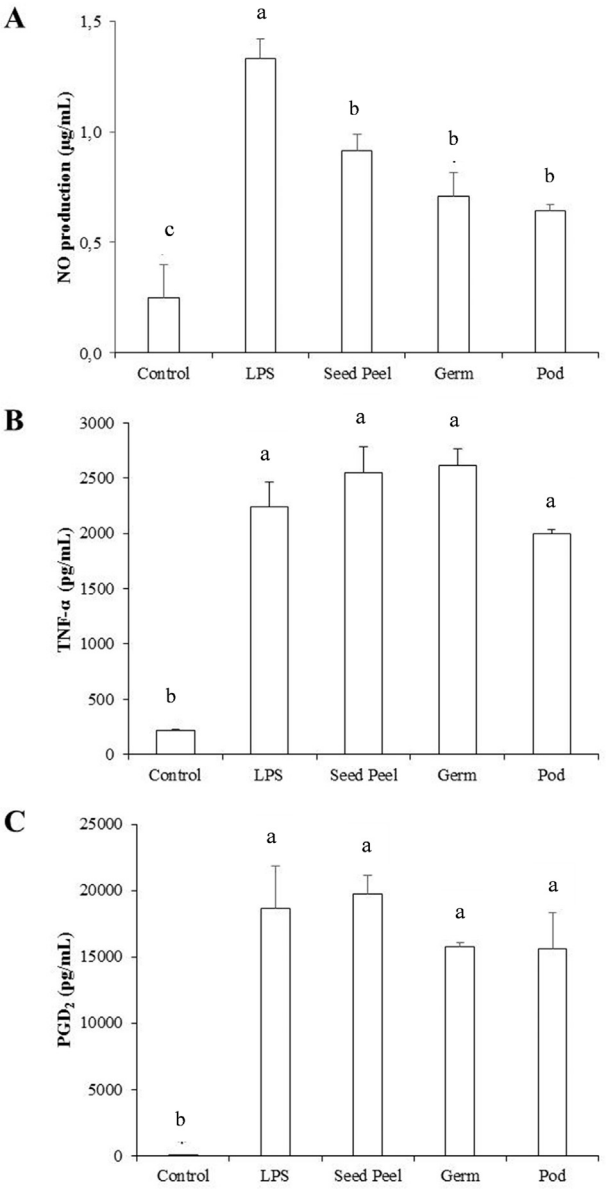

Fig. 2. NO (A), TNF-α (B) and PGD2 (C) production of RAW 264.7 cells stimulated by LPS after treatment with carob extracts (0.05 mg/mL) for 24h. Data represent the mean ± SD (n = 3). Asterisk indicate statistically significant differences ($P < 0.05$) compared with LPS-stimulated group.

has shown to exert a moderate inhibition of TNF-α production in LPS/IFN-γ-activated macrophages (Lin et al., 2017). Quercetin glucosides were dominant phenolic compounds of germ and seed peel. Earlier studies have shown that glycosylation of quercetin significantly decrease the inhibitory effects of cytokine production in LPS-induced macrophages. Moreover, kaempferol glucoside, a minor compound of carob seed peel and germ, has shown to increase the levels of TNFα in LPS-induced RAW 264.7 (Wang and Mazza, 2002).

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3.6. Triacylglyceride-lowering

Seed peel presented a toxic effect in 3T3-L1 mature adipocytes, whereas cell viability reduction was not observed for germ and pod extracts at doses of 0.5 mg mL^{-1} (Fig. 1B). Hyperplasia, hypertrophy and adipocyte apoptosis are physiological events at the cellular level closely linked to the increase in adipose tissue size and, consequently, to overweight and obesity. As far as the adulthood of species is concerned, adipogenesis has low impact, while the control of cell size in mature adipocytes is more related to the increase in fat accumulation. Numerous studies have shown that resveratrol, quercetin, genistein, epigallocatechin or curcumin, among other polyphenols, reduce lipid accumulation in mature adipocytes (Wang et al., 2014). The effective dose of isolated polyphenols is commonly set at 1−100 μM range (Eseberri et al., 2015; Lasa et al., 2012).

In this context, the present study demonstrates the reduction in fat accumulation, or triacylglyceride (TG) production, induced by seed peel and pod extract obtained from carob on mature adipocytes, at a dose of 0.1 mg mL^{-1} (Fig. 3), which might be related to an *in vivo* anti-obesity effect. By contrast, the lower dose of these extracts, as well as the set of phenolic compounds included in the carob-germ extract, has no potential anti-obesity activity, in terms of fat accumulation. These results are in line with those published with water-soluble fraction of the ethanol extract of *Smilax china* L. leaf (Kang et al., 2015). In this study, although a dose of 0.05 mg mL^{-1} was insufficient to produce any effect on fat accumulation in mature adipocytes, at a dose of 0.1 mg mL^{-1} the extract rich in polyphenols and flavonoids promoted an increase in the release of glycerol by the adipocytes, which suggests that the reduction in triacylglycerol was due, at least in part, to increased lipolysis.

Other studies carried out with plant extracts, such as *Kaempferia parviflora* or *Hibiscus sabdariffa*, have been effective at lower doses than those of *C. siliqua* L. (0.01

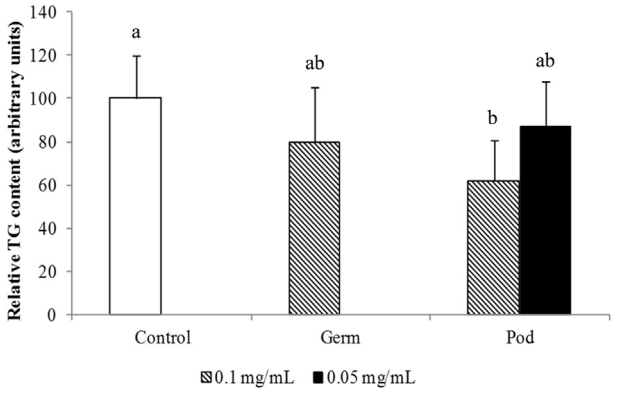

Fig. 3. Triacylglycerol (TG) amounts in 3T3-L1 mature adipocytes treated with 0.1 or 0.05 mg/mL germ or pod for 24 h. Data represent the mean ± SD (n = 6). Asterisk indicate statistically significant differences ($P < 0.05$) compared with control condition.

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and 0.04 mg mL^{-1}, respectively) (Herranz-López et al., 2012; Okabe et al., 2014). However, the difference in treatment length must be emphasised. In the present experiment, the treatment with the extract rich in polyphenols was 24 h, while for those with *K. parviflora*, *H. sabdariffa* and *L. citriodora*, the treatment took at least 48 h. This fact is even more important, given the low availability of most polyphenols, so that it is highly unfeasible for a phenolic compound to be in contact with adipocytes for more than 24 hours (D'Archivio et al., 2010).

4. Conclusions

The present results provide strong evidence that carob extracts could be useful for the attenuation of processes that are related to various chronic diseases, such as type 2 diabetes, obesity, and the metabolic syndrome. Thus, carob by-products demonstrated inhibitory activities against NO production and ACE. Moreover, they showed lowering triglycerides capacity and high antioxidant activity, which make these by-products promising candidates with therapeutic potential for prevention and/or treatment of metabolic syndrome. Pod showed a better balance between all the activities tested, which may be related to higher gallic acid content.

Declarations

Author contribution statement

Daniel Rico, Cristina Martinez-Vilalluenga: Conceived and designed the experiments; Performed the experiments.

Ana B. Martin-Diana: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Leixuri Aguirre, Daniel A. de Luis, Lasa Arrate: Analyzed and interpreted the data.

Jose Manuel Silvan, Montserrat Dueñas: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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