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DOI: 10.1039/D0FO00066C

ARTICLE

Characterization of bioactive compounds in defatted hempseed (*Cannabis sativa* L.) by UHPLC-HRMS/MS and anti-inflammatory activity in primary human monocytes

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Julio Rea Martinez [a,*], Sergio Montserrat-de la Paz [b], Rocío De la Puerta [a], M Dolores García-Giménez [a], M Ángeles Fernández-Arche

Hempseed (*Cannabis sativa* L.) has beneficial impact on human health mainly because of its wide variability of bioactive compounds. However, many of them are not fully characterized yet. In this work, hempseed was defatted and through a bio-guided studied, two fractions (F03 and F05) with the highest content of phenols, flavonoids and antioxidant capacity were selected. Fractions were chemically analyzed by UHPLC HRMS/MS. The anti-inflammatory capacities of these compounds were evaluated on human monocytes using flow cytometry, RT-qPCR and Elisa procedures. A high amount of phenolic compounds were identified, with the major compound being: *N-trans*-caffeoyltyramine (6.36 mg/g in F05 and 1.28 mg/g in F03). Both, F03 and F05 significantly reduced the inflammatory competence of LPS- treated human primary monocytes, decreasing TNF-α and IL-6 gene expression and secretion. These findings indicate that in the defatted fraction of the hempseed there are a wide number of compounds with beneficial potential to prevent and treat inflammatory disorders, as well as other processes caused by oxidative stress.

1. INTRODUCTION

Hemp, a multi-purpose crop is a good natural source of different group of components, corresponding to the specialized metabolism of the plants.[1] More than 545 cannabinoids and non-cannabinoids compounds has been isolated and identified in cannabis.[2] Cannabinoids are the most investigated hemp bioactive compounds due to the psychotropic effects and their wide pharmaceutical applications. Other groups of phytochemicals (terpenes, phenols, stilbenoids, lignans) have also shown pharmacological effects and there is increasing interest in them as health-promoting agents due to their possible future applications.[3] Hempseed typically contains over 30% oil and about 25% protein, with considerable amounts of dietary fiber, vitamins and minerals. Therefore, it has been used for thousands of years around the world as an important nutritional resource. Especially, it has a high concentration of polyunsaturated fatty acids (PUFAs) and a ratio of linoleic acid and α-linolenic acid of around (3:1) which is considered to be optimal for human health. [4] In the recent years, there has been an increase on the hemp cultivation in Europe, looking for increasing the production and benefits, updating the extraction process to obtain profitable bioactive components and nutraceutical products.[5] After the defatted process on seeds, several compounds with medium-high polarity such as phenolic acids, lignans or flavonoids, remain in the wastes and can be recovered using polar organic solvents.[6] Although the amount of bioactive compounds in hempseed is complex, are far to be completely characterized.

The aim of this work was to identify phytochemical compounds with bioactive potential from the defatted hempseeds. From two different extraction processes, six fractions were obtained. By a bio-guided study the two fractions of higher phenol and flavonoid contents and greater antioxidant capacity were selected for chemical characterization and assay of potential anti-inflammatory effects.

Fractions (F03 and F05) were analyzed using UHPLC-HRMS/MS. To evaluate the possible effects in inflammatory processes, both fractions were assessed (*in vitro*), in LPS-treated human primary monocytes.

2. MATERIALS AND METHODS

2.1 Chemical and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), $Na_2S_2O_8$, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2´2-Azino-bis(3-ethylbenzothiazoline-6 sulfonic acid) diammonium salt (ABTS), Folin-Ciocalteu´s phenol reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), LPS, gallic acid, salicylic acid,

[a] Department of Pharmacology, Faculty of Pharmacy, University of Seville, 41012 Seville, Spain
[b] Department of Medical Biochemistry, Molecular Biology and Immunology, School of Medicine, University of Seville, 41071 Seville, Spain

E-mail address: julreamar@alum.us.es; Telephone: +34665810329

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

caffeic acid, ferulic acid, vanillic acid, sinapic acid, *p*-coumaric, quercetin, naringenin, apigenin, catechin and other standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), vitexin, isovitexin, genistin, diosmetin from Extrasynthese (Genay-France), methanol, formic acid LC-MS grade from VWR chemicals (France), rutin standard (MERCK), RPMI 1640 medium from Biochrom AG (Berlin, Germany), TRIsure (Bioline).

2.2 Plant Material and preparation of extracts

The hempseed (Jarad–Seed; batch: 250816) was commercially acquired in the province of Seville, Spain.

2.2.1 Preparation of extracts and fractions

Hempseed (500 g) two portions, were crushed and defatted with n-hexane (two times, 750 mL x 24 h). After filtration and evaporation of n-hexane, defatted hempseed followed two different procedures. Extraction 1: Defatted hempseed was extracted with acetone (two times, 500 mL x 24 h) and ethanol 50% (two times, 500 mL x 24 h). Acetone and n-hexane extract were concentrated under vacuum until evaporation of the solvent and later, were extracted with a equal amount of ethanol 96% four times (**Fig. 1**), to obtain fractions denominated F01 (5.7 g), F02 (10.75 g) and F03 (16.1 g). Extraction 2: defatted hempseed was extracted with ethanol 75% (two times, 750 mL x 24 h), ethanol extract was concentrated under vacuum to reduce the solvent until 200 mL approximate volume, followed by a liquid–liquid extraction with ethyl acetate (200 mL x 4 times) and butanol (200 mL x 4 times) to obtain F04 (12.98 g), F05 (4.8 g), F06 (0.2 g) respectively. Samples were concentrated under vacuum, freeze-dried and stored in a dark glass bottles at 4ºC prior to analysis.

2.3 *In vitro* antioxidant activity

2.3.1 DPPH• Radical scavenging assay

Free radical scavenging assay was carried out based in the reduction capacity of DPPH with slight modifications adopted from the method described in literature.[7] Briefly, a solution of DPPH in absolute ethanol was prepared (0.022%) and kept into the dark at 4ºC. Stock solution of the fractions was prepared and a series of final concentrations was obtained by diluting with absolute ethanol (0.025, 0.05, 0.1, 0.2, 0.4 mg/mL). 100 µL each concentration were added to 96 well-plates, followed for 100 µL absolute ethanol and 50 µL DPPH solution. The mixture was shaken and incubated in the dark for 30 minutes. Then, the absorbance was measure at 515 nm in a microplate reader. The percentage of inhibition was calculated using the formula: Inhibition % $= [1 - (A - A')/A^{\circ})] * 100$; where A is the absorbance of sample, A´ is the absorbance of sample without DPPH solution and A° is the absorbance of DPPH solution. IC_{50} of each fraction was obtained through lineal interpolation regression analysis. Caffeic acid and Quercetin were used as antioxidant standards, results were expressed as µM Trolox equivalent (TE) per g of extract.

2.3.2 ABTS•+ Radical scavenging assay

Free radical $ABTS^{\bullet+}$ was generated following the method described in literature[8] with some modifications. ABTS was dissolved in deionized water to give a 7 mM solution. ABTS radical cation ($ABTS^{+}$) was produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate. The mixture was left to stand in the dark at room temperature (22 - 24°C) for 12-16 h before use. A concentrated $ABTS^{+}$ solution was diluted with ethanol, to a final absorbance of 0.70 ±0.03 at 734 nm at 30°C. A volume of 50 µL of each fraction at different concentrations (0.025, 0.05, 0.1, 0.2, 0.4 mg/mL) was added and mixed with 250 µL $ABTS^{\bullet+}$ solution in 96 well-microplates and incubated with continuous shake per 6 min in dark. The absorbance was measured at 740 nm, results were expressed as µM Trolox equivalent (TE) per g of extract.

2.4 Quantification of total phenols and flavonoids content

2.4.1 Total phenolic content

Total phenolic content was determined using Folin-Ciocalteu´s method[9], with slight changes to reduce the volume amount in reagents and samples.[10] Briefly, 50 µL of the fractions at different concentration was mixed with 430 µL of distilled water and 20 µL Folin-Ciocalteu´s reagent; it was shaken and left standing 5 minutes. Then, 50 µL of 7% Na_2CO_3 was added to the mixture and shaken again, the mixture was brought until 1mL adding 450 µL of distilled water and maintenance in a room temperature for 60 minutes. Finally, the absorbance was measured at 750nm. A standard curve was prepared using gallic acid at increasing concentrations (25 - 500 µg/mL). The results were expressed as mg gallic acid equivalent (GAE) per 100 mg of extract.

2.4.2 Total flavonoid content

Total flavonoids content in each fraction was assessed by following the detailed protocol.[11] In brief, 0.5 mL of sample was mixed with an equal volume of $AlCl_3$ (2%) solution, shaken and incubated for 15 min in a room temperature. The absorbance was read at 430 nm using a microplate reader, a quercetin stock solution was used as standard. The results were expressed as mg quercetin equivalents (QE) per 100 mg of extract.

2.5 Phytochemical characterization and quantification by UHPLC-HRMS/MS

Fractions (F03 and F05) were chromatographed using Ultra High Performance Liquid Chromatography (UHPLC, Dionex Ultimate 3000) Thermo Scientific® coupled with the Orbitrap Quadropole Q Exactive® hybrid mass spectrometer (Thermo Fisher Scientific). 5 mg of samples were diluted using 1mL methanol 50%, containing 0.1 % formic acid (v/v) and filtered by 0.2 µm. UHPLC conditions were as follow: Solvent A: water containing 0.1% formic acid (v/v); solvent B : methanol containing 0.1% formic acid (v/v). The gradient elution system was operated as follows: 0 - 5% B (0-1 minutes), 5 - 100% B (1-12 Minutes), 100 - 5% B (12.1-15 minutes). The separation was carried out on an ACQUITY UPLC BEH C18 Column (130Å, 1.7 µm, 2.1 mm X 100 mm) with injection volume 5µL, flow volume 0.5 mL/min, column temperature was 40°C, and total

run time 15 min. The HRMS/MS data were acquired in negative mode over a scan range 50-750 m/z. Full scan/All ion fragmentation mode / TIC (Total Ion current) chromatogram (scan time = 1micro scans and maximum inject time (IT) 500ms), with a resolution of 70000 were the equipment conditions. The HESI conditions were capillary temperature 320°C, spray voltage 3.0 kV, sheath gas (nitrogen) 60 Au and auxiliary gas 25 Au. The raw data was acquired and processed with Xcalibur 4.0. Software from Thermo Scientific, the peaks were identified by comparison of their retention times, exact mass, fragment ions (deviation not exceed 5ppm) of the corresponding standards and free database.

The quantification of compounds in the fractions was performed by the external standard method, using reference standard or the calibration curve of structurally related substance for the respective quantification, stock known solution of standard were injected (0.02 – 1.0 µg/mL, six points), compounds: salicylic acid (y = 185705x + 803652; R^2=0.999), gallic acid (y = 62284x - 515772; R^2=0.998), caffeic acid (y = 100380x + 341813; R^2=0.999), *p*-coumaric acid (y = 71295x - 121063; R^2=0.999), *N-trans*-caffeoyltyramine (y = 47200x + 406801; R^2=0.996), *N-trans*-coumaroyltyramine (y = 80369x - 119852; R^2=0.999), naringenin (y = 78254x + 227589; R^2=0.998), isovitexin (y = 50957x – 1E06; R^2=0.996), quinic acid (y = 56733x + 500860; R^2=0.997). The samples were analyzed by triplicate and results were expressed in mg compound/g extract, as mean ± SD.

2.6 Blood collection and isolation of human monocytes

To perform this study, we proceeded according to Good Clinical Practice Guidelines and in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Centrifugation over Ficoll-Histopaque gradient isolated Peripheral blood mononuclear cells (PBMCs) from buffy coat donated by the Regional Blood Transfusion Center (Seville, Spain. Agreement #33130099). Monocytes were isolated from PBMCs using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotec, Madrid, Spain) following manufacturer´s instructions. The purity for CD14 monocyte isolations was routinely > 95% by flow cytomety (FACScanto II flow cytometer and FACSDiva software, BD). Following isolation, monocytes were suspended in RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin and 10% heat-inactivated fetal bovine serum. For *in vitro* study, 5 x 10^5 of purified monocytes, were exposed to LPS (100 ng/mL) for one hour, prior to their treatments with F03 and F05 at 50 and 100 µg/mL for 24 hours.

2.7 Cell viability assay (MTT)

Monocytes were incubated with different concentrations (up to 200 µg/mL) of F03 and F05 in a 96- well plate (1x10^4 cells/well) during 24 h. Afterwards, the MTT solution (Sigma) was incubated in the well until a purple precipitate was visible. MTT-formazan crystals were solubilized with DMSO (Sigma) and then measured using a microplate reader at 570 nm corrected to 650 nm. Cell survival was expressed as the percentage of absorbance compared with that obtained in control, non-treated cells.

2.8 Immunostaining of human monocytes by FACS

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Monocyte membrane expression of CD16 (PE anti-human CD16, Miltenyi), CD14 (APC-Cy7 anti-human CD14, Miltenyi), and CCR2 (APC anti-human CCR2, Vitro) was assessed by flow cytometry. According to the manufacturer's instructions, cells were incubated with antibodies at room temperature and in the dark for 15 min; erythrocytes were removed with FACS lysing solution (BD). Mean fluorescence intensity (MFI) was measured by using a FACSCanto II flow cytometer (BD) and calibrated by using FACSDiva software (BD). MFI of 10^4 counted cells was assessed for each sample. Monocytes were gated as forward scatter^{high} (FSC^{high})-side scatter^{high} (SSC^{high})-cells. Expression levels are presented as MFI corrected for nonspecific binding of isotope control antibodies.

2.9 RNA isolation and RT-qPCR analysis

Total RNA was extracted by using Trisure Reagent (Bioline), as instructed by the manufacturer. A*260*/A280 ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Madrid, Spain) was used to determinate DNA quality. Momentarily, RNA (1µg) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain). An amount of 10 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT) as housekeeping genes **(supplementary Table 1).** All the amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (GAPDH and HPRT) gene content and expressed as percent was expressed as percentage of controls.

2.10 Cytokine quantification

Levels of TNF- α and IL-6 in culture supernatants were determined by enzyme-linked inmunosorbent assay (ELISA), following the manufacturer instructions (Diaclone, Besancon, France). Cytokine concentrations were expressed in pg per mL, as calculated from the calibration curves from serial dilution of human recombinant standards in each assay.

2.11 Statistical analysis.

All values are expressed as arithmetic means ± standard deviations (SD) or means ± standard error (SEM) depending of the obtained results. The data was evaluated with Graph Pad Prism Version 6.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), following Tukey multiple comparisons test as post hoc test. P values less than 0.05 were considered statistically significant.

3. RESULT AND DISCUSSION.

3.1 *In vitro* antioxidant activity

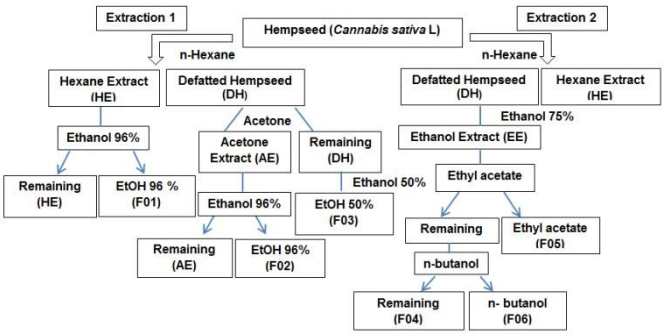

Fig. 1 Extraction procedures to obtain the different fractions from hempseed

In this study hempseeds have been extracted with solvents of different polarity: ethanol, acetone, ethyl acetate, n-butanol or mix of solvents and six fractions were obtained **(Fig. 1)**. These fractions were assayed for their scavenging capacity by DPPH and ABTS assays (Fig. 2). The results of the bio-guided study indicated a significant scavenging activity of DPPH at 400 µg/mL by F03 (78.08 ± 2.07 %), F05 (89.46 ± 1.33 %) and of ABTS by F03 (98.73 ± 0.43 %), F05 (99.64 ± 0.16 %), and F06 (99.19 ± 0.18 %). Other authors has reported low scavenging values for ABTS radicals (11.08 ± 2.08 µmol TE/g) in the methanol extracts from hempseed oil[12], likely because the phenols content in defatted seeds is higher than oil and more recently, it has been reported that the inhibitions ranges from 40 - 52 % at DPPH assays, with highest antioxidant capacity in sprouts than in seeds of hemp.[13]

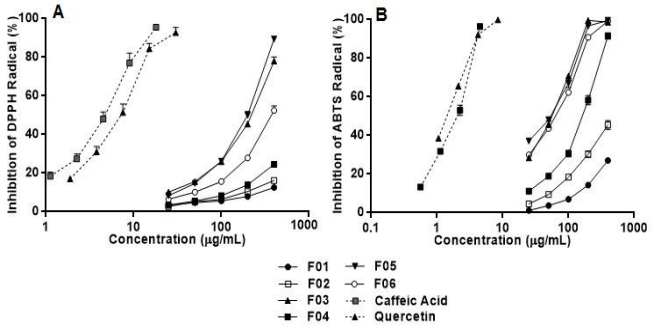

Fig. 2 DPPH and ABTS radical scavenging activity of Hempseed fractions (25 – 400 µg/mL): (**A**) inhibition of DPPH radical and (**B**) inhibition of ABTS radical. Caffeic acid and quercetin were used as reference standards at different concentration (0.5 – 30 µg/mL). Each value represents mean ± SEM of three replicates.

The IC_{50} values corresponding to radical scavenging activity are represented in **Table 1**. IC_{50} of F03, F05 and F06 were not significantly different among them, although it was higher for the F06 fraction. Similar results in DPPH were observed in hydroalcoholic extracts of defatted seeds of different cultivars: Futura (IC_{50} 0.27 ± 0.03 mg/mL) and Felina (IC_{50} 0.10 ± 0.02 mg/mL).[5] Other studies have reported lower IC_{50} in DPPH and ABTS assays (0.012 – 5.63 mg/mL), using different solvents for the extraction and showing the highest antioxidant activity in the hull than in the kernel of hempseed extracts.[14] Furthermore, other data, from using the by-products generated during processing mechanical hemp oil (whole hemp meal and fractions with different particles size) as source of biocompounds, showed IC_{50} of 5.29 -17.18 mg/mL in the DPPH assay.[15]

3.2 Quantification of total phenols and flavonoids content

Total phenolic content of the main fractions ranged from 0.88 to 5.67 mg GAE/100 mg and total flavonoids ranged from 0.45 to 1.76 mg QE/100mg respectively **(Table 1).** The result in phenolic content showed by F05 (ethyl acetate fraction),obtained from subsequent separation procedure of ethanol 75% extract of defatted seeds, was higher than when other systems of solvents used. Other studies showed higher values in phenols recovery from hull and kernel in defatted hempseed with aqueous solvents[14], as well as total phenolic content in ranging between 4.57 - 8.04 mg/100mg and flavonoids 5.39 - 10.90 mg/100mg, from different hemp cultivars, using methanol-water (8:2).[5] Although a significant increase in phenolic content has been reported in acetone mixtures using a specific solvent ratio, temperature and ultrasonic treatment, chlorophylls extracted from hemp seed with acetone can influence phenols content analyses, as well as a prolonged ultrasonic treatment and high temperatures decrease flavonoids content.[16,17] Furthermore, aqueous solutions of cyclodextrins, an eco-friendly process to recover polyphenols have showed a total polyphenol of 4.51 mg GAE/g dw,[18] and high pressure extraction techniques have resulted efficient to recover polar compounds from hemp residues.[19] Therefore, the analysis of individual compounds in the extract is important for proper comparison of results and optimization of conditions to improve the extraction of bioactive compounds.

The antioxidant fractions: F05 and F03 with the highest phenolic and flavonoid total contents (1.90 - 5.67mg GAE/100 mg extract and 0.45 - 1.76 mg QE/100 mg extract) were selected for their phytochemical analysis.

3.3 Phytochemical characterization and quantification by UHPLC HRMS/MS

Retention time (R_t), accurate mass (m/z), predicted molecular formula, fragment ion, in negative ion mode $[M-H]^-$ of the compounds identified are shown in **Table 2**. The attribution of the different compound was achieved by comparison with the respective standards, literature and free database. In all cases the maximum allowed difference between the expected and the measure mass did not exceed 5 ppm.

Most of the compounds identified in both fractions (F03 and F05) were of phenolic structure. Among those: phenolic acids (gallic, protocatechuic, gentisic, 4-OH benzoic, chlorogenic, caffeic, vanillic, p-coumaric, salicylic, ferulic and sinapic acids), flavonoids (vitexin, isovitexin, rutin, quercetin, naringenin, genistin, apigenin and diosmetin) and phenolic amides. The last compounds were detected in higher concentrations than others in the hempseed profile and were present in a more quantity in fraction F05 than in F03 (**Table 2**), highlighting *N-trans* caffeoyltyramine with 1.272 ± 0.10 mg/g and 6.362 ±

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0.892 mg/g in the fractions F03 and F05 respectively.

Its identification was based on the comparison with fragments ion, elemental composition (EC), accurate mass, databases. *N*-caffeoyloctopamine, m/z 314.104, $[C_{17}H_{16}O_5N]^-$ and its isomers at R_t 4.81, 5.25 shown similar fragments at m/z 122, 135, 152, 161, 178. The ion at m/z 298.108 $[C_{17}H_{16}O_4N]^-$ and R_t 5.69 was assigned to *N-trans* caffeoyltyramine **(supplementary Fig. 1)** and the ion at m/z 282.114 $[C_{17}H_{16}O_3N]^-$ and R_t 6.11 to *N-trans* coumaroyltyramine in comparison with standards.

Table 1 Phenol, Flavonoid contents expresses as mg GAE/100mg and mg QE/100mg extract and IC_{50} values DPPH, ABTS radical scavenging activity with TEAC values (Trolox Equivalent Antioxidant Capacity) of defatted hempseed fractions and reference standards.

Sample	Phenol and Flavonoid contents (mg/100 mg extract)		IC_{50} µg/mL		µM TE/g extract	
	total phenolic content	total flavonoid content	DPPH	ABTS	DPPH	ABTS
F01	nd	nd	>500 b	>500 b	32.17 ± 2.04 a	24.18 ± 1.15 a
F02	nd	nd	>500 c	428.11 ± 23.03 c	47.30 ± 3.49 a	42.16 ± 2.29 ab
F03	1.90 ± 0.01 a	0.45 ± 0.20 ac	237.80 ± 6.72 a	46.07 ± 9.24 a	247.77 ± 7.76 b	409.61 ± 60.90 c
F04	0.88 ± 0.01 c	0.71 ± 0.19 ac	>500 d	192.38 ± 6.67 d	76.07 ± 4.18 c	92.99 ± 3.57 b
F05	5.67 ± 0.02 b	1.76 ± 0.39 bc	211.74 ± 3.55 a	34.15 ± 3.01 a	308.80 ± 5.29 d	539.98 ± 17.39 d
F06	1.68 ± 0.02 d	0.77 ± 0.16 c	380.69 ± 16.03 a	70.19 ± 1.08 a	170.25 ± 5.45 e	253.41 ± 4.49 e
quercetin [1]	-	-	10.79 ± 1.14	0.68 ± 0.10	-	-
caffeic acid [1]	-	-	6.25 ± 0.63	2.18 ± 0.04	-	-

nd: no detected; each value represent mean ± SD of three independent experiments (phenol and flavonoid contents). Values IC_{50}, $TE_{ABTS,DPPH}$ are expressed as means ± SEM; [1]: used as positive control. Values with different letters in the same column are significantly different at ($p < 0.05$)

N-feruloyltyramine m/z 312.124 $[C_{18}H_{18}O_4N]^-$ and R_t 6.25 was identified by comparison with data found in literature.[20] Recently, different researchers have shown that hempseed extracts have high antioxidant properties, attributed to the significant content of bioactive compounds, mainly phenols[21,22] and lignanamides[23], obtained after defatted process of seeds hull and kernel[14], processing the byproduct acquired after hemp oil extraction[5,15] or from hemp threshing residues.[19] Furthermore, anti-neuroinflammatory[24], anti-inflammatory[25,26,27] and tyrosinase inhibitory activities[28] by phenolic amide compounds have been described.

Some lignanamides were also identified in the analyzed fractions, among them, Cannabisin A, B and C. These were assigned by matching molecular ion formulas with error <5ppm, at m/z 593.193, $[C_{34}H_{29}O_8N_2]^-$ and R_t 6.05; m/z 595.208, $[C_{34}H_{31}O_8N_2]^-$ and R_t 6.01 and m/z 609.224, $[C_{35}H_{33}O_8N_2]^-$ and R_t 6.29. Cannabisin A, B are products of the polymerization of *N-trans* caffeoyltyramine[29], the latter compound was used to estimate the quantification of both compounds in the fractions analyzed, where the concentration of cannabisin B was higher than cannabisin A. Cannabisin B has been reported as the predominant compound in hempseed hull and possess a high antioxidant activities[14,23] as well as antiproliferative activity in human hepatocarcinoma HepG2 cells.[30]

Furthermore, ions m/z 357.207, R_t 10.05 and 11.02 (error <5ppm) were assigned to Cannabidiolic acid (CDBA) and Cannabichromenic acid (CBCA) and ion m/z 359.2232, $[C_{22}H_{31}O_4]^-$, R_t 10.21 to Cannabigerolic acid (CBGA). The area of CDBA was predominant over those of CDBA and CBGA. In other reports, non-psychoactive cannabinoids CDBA and CBGA were the main compounds present in hemp with their respective decarboxylated products cannabidiol and cannabigerol.[31] The last compounds were no detected in our analysis.

Finally, other ions were assigned to L-glutamic, L-malic, quinic, citric acid compared to their respective standards. Ion am / z 187.097 Rt 6.33 was assigned to azelaic acid compared to literature data, its presence could be due to the defatting method used in hempseed or also to the oxidation of oleic acid[32,33], because sometimes traces of this acid remain, even after degreasing process.

While there are numerous studies regarding to chemical hempseed oil composition, the ones about the remaining compounds in the seed after a defatted process are limited. The utilization of modern and combined analytical techniques such as Ultra High Performance Liquid chromatography (UHPLC) and High Resolution MS (HRMS) increase considerably the detection of hundreds compounds at complex mix, in a short time of analysis.[34] Related studies have identified some main polyphenols (caffeoyltyramine, cannabisin A, B, C) present in hempseed and sprouts using high resolution mass spectrometry in negative ion.[13] Similar analyses by UPLC/ESI-QTOF of polar extract from hemp residues (leaves, flowers, floral bracts, immature seeds), using pressure liquid extraction (PLE EtOH/H2O 4:1 v/v) was reported a high yield of flavonoids in fractions, characterizing some individual compounds.[19]

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Table 2. Retention time (R_t), accurate mass (*m/z*), molecular formula, MS^2 fragment ions, identification and quantification of the compounds in the F03 and F05 fractions of defatted hempseed (*Cannabis sativa* L).

R_t (min)	$[M-H]^-$ (*m/z*)	Formula	MS/MS fragments	Attribution	Concentration mg/g extract	
					F03	F05
0,51	146,046	$C_5H_9NO_4$	128 (35), 102 (100), 85 (1)	L-Glutamic acid	-	-
0,54	191,056	$C_7H_{12}O_6$	117 (6), 93 (18), 85 (38),	Quinic acid [j]	0.007 ± 0.000	0.003 ± 0.000
0,55	133,014	$C_4H_6O_5$	115 (100), 71 (90)	L- Malic acid	-	-
0,79	191,019	$C_6H_8O_7$	129 (6), 111 (100), 87 (62), 85 (33)	Citric acid	-	-
1,11	169,014	$C_7H_6O_5$	69 (14), 97 (11), 125 (100)	Gallic acid [c]	0.003 ± 0.001	0.002 ± 0.000
2,24	153,019	$C_7H_6O_4$	109 (100), 91 (5)	Protocatechuic acid [c]	0.041 ± 0.007	0.099 ± 0.040
3,33	153,019	$C_7H_6O_4$	109 (52), 108 (100), 81 (4)	Gentisic acid [c]	0.009 ± 0.001	0.009 ± 0.003
3,39	137,024	$C_7H_6O_3$	93 (100), 65 (4)	4-hydroxibenzoic acid [b]	0.020 ± 0.003	0.099 ± 0.038
3,62	289,072	$C_{15}H_{14}O_6$	245 (37), 203 (24), 151 (19), 125 (35), 123 (70), 109 (100) 97 (27)	Catechin [f]	Tr	nd
3,90	353,087	$C_{16}H_{18}O_9$	191 (100), 127(4), 85 (24), 71 (2)	Chlorogenic Acid [d]	Tr	nd
4,07	179,035	$C_9H_9O_4$	135 (100), 134 (22), 107 (5)	Caffeic acid [d]	0.008 ± 0.001	0.049 ± 0.021
4,09	167,035	$C_8H_8O_4$	152 (100), 123 (16), 108 (94)	Vanillic acid [c]	0.002 ± 0.000	0.004 ± 0.001
4,37	289,072	$C_{15}H_{14}O_6$	245 (46), 205 (20), 179(11), 151 (22), 125 (34), 123 (72), 109 (100)	Epicatechin [f]	Tr	nd
4,81	314,104	$C_{17}H_{17}O_5N$	178(9), 161 (30), 152 (30), 135 (100), 122 (20)	*N*-caffeoyloctopamine 1*[,h]	0.102 ± 0.013	0.311 ± 0.058
4,85	163,040	$C_9H_8O_3$	119 (100), 93 (8),	p-Coumaric acid [e]	0.018 ± 0.002	0.124 ± 0.045
5,05	303,051	$C_{15}H_{12}O_7$	285 (28), 175 (18),125 (100), 123 (16), 109 (5)	Taxifolin [f]	Tr	0.001
5,16	193,051	$C_{10}H_{10}O_4$	178 (37), 149 (13), 134 (100)	Ferulic acid [d]	0.002 ± 0.000	0.015 ± 0.005
5,24	223,061	$C_{11}H_{12}O_5$	121 (80), 93 (100)	Sinapic acid [d]	nd	Tr
5,25	314,104	$C_{17}H_{17}O_5N$	178 (12), 161 (30), 152 (40), 135 (100), 122 (22)	*N*-caffeoyloctopamine 2*[,h]	0.136 ± 0.014	0.629 ± 0.136
5,36	431,098	$C_{21}H_{20}O_{10}$	311 (100), 283 (42)	Vitexin [g]	0.005 ± 0.000	0.010 ± 0.002
5,55	431,098	$C_{21}H_{20}O_{10}$	311 (100), 283 (68)	Isovitexin [g]	Tr	Tr
5,65	609,146	$C_{27}H_{30}O_{16}$		Rutin [g]	nd	0.005 ± 0.000
5,69	298,108	$C_{17}H_{17}O_4N$	178 (8), 135 (100), 107 (7)	*N- trans* caffeoyltyramine [h]	1.279 ± 0.110	6.362 ± 0.892
5,86	137,024	$C_7H_6O_3$	93 (100), 65 (5)	Salicylic acid [b]	0.413 ± 0.034	1.006 ± 0.186
6,01	595,208	$C_{34}H_{32}O_8N_2$		Cannabisin B*[,h]	0.059 ± 0.008	0.573 ± 0.112
6,05	593,193	$C_{34}H_{30}O_8N_2$		Cannabisin A*[,h]	0.017 ± 0.003	0.192 ± 0.036
6,11	282,114	$C_{17}H_{17}O_3N$	162 (9), 132 (7), 119 (100), 93 (5)	*N-trans* coumaroyltyramine[i]	0.040 ± 0.005	0.447 ± 0.067
6,25	312,124	$C_{18}H_{19}O_4N$	297 (22), 178 (52), 148 (100), 135 (48)	*N*-feruloyltyramine*[,h]	0.161 ± 0.021	1.757 ± 0.333
6,29	609,224	$C_{35}H_{34}O_8N_2$		Cannabisin C*	-	-
6,33	187,097	$C_9H_{16}O_4$	125 (100), 169 (2), 97 (16)	Azelaic acid *	-	-
6,69	301,035	$C_{15}H_{10}O_7$	179 (35), 151 (100), 121 (40), 107 (44)	Quercetin [f]	nd	0.001 ± 0.000
6,84	271,061	$C_{15}H_{12}O_5$	177 (6), 151 (54),119 (100), 107 (25)	Naringenin [f]	nd	Tr
6,96	431,098	$C_{21}H_{20}O_5$		Genistin [g]	nd	Tr
7,34	269,045	$C_{15}H_{10}O_5$	117 (94)	Apigenin [f]	nd	0.104 ± 0.036
7,36	299,056	$C_{16}H_{12}O_6$	227 (8), 183 (4), 151 (7), 107 (7)	Diosmetin [f]	Tr	0.015 ± 0.006
10,05	357,207	$C_{22}H_{30}O_4$	339 (92), 313 (37), 311 (34), 245 (68), 170 (32), 137 (14), 107 (100)	Cannabidiolic acid *	-	-
10,21	359,223	$C_{22}H_{32}O_4$	341 (100), 315 (42), 191 (20), 136 (12)	Cannabigerolic acid *	-	-
11,02	357,207	$C_{22}H_{30}O_4$	313 (68), 245 (24), 191 (22), 136 (8), 107 (16)	Cannabichromenic acid *	-	-

nd: no detected; Tr: traces; *: attribution compound, were compared with bibliography available and free databases (mzCloud, Pubchem); Calibration curve employed to quantification (mg/g extract) [b]: salicylic acid, [c]: gallic acid, [d]: caffeic acid, [e]: p-coumaric acid, [f]: naringenin, [g]: isovitexin, [h]: *N-trans* caffeoyltyramine, [i]: *N-trans* coumaroyltyramine, [j]: quinic acid.

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3.4 Anti-inflammatory activity in primary human monocytes

After 24 h of treatment, F03 and F05 fractions at concentrations up to 200 µg/mL had no significant effects, by means of Trypan Blue exclusion test, on viability of human primary monocytes (data not shown). Various evidence indicates that blood monocytes consist on several subpopulations of cells, which differ, in size, nuclear morphology, granularity, and functionality.[35] Our study also undertook to explore whether F03 and F05 can affect to monocyte subset distribution. We determinate three different subsets of human monocytes: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$). After 24 h of treatment, F05 induced a decrease of CD14 and an increase of CD16 surface expression in LPS-treated human primary monocytes (**Fig. 3**). These effects resulted in a decrease of the relative proportion of the classical (**Fig. 3A**) monocyte subsets and an increase of the relative proportion of the nonclassical monocyte subset (**Fig. 3C**). The classical and intermediate monocyte subsets have a pro-inflammatory response that actively produces TNF-α (in response to LPS) and IL-6, and can contributed to the progression of chronic diseases such as atherosclerosis.[35,36] Thus, an appropriate balance among the different monocyte subsets play a pivotal role to prevent inflammation and controlled repair.

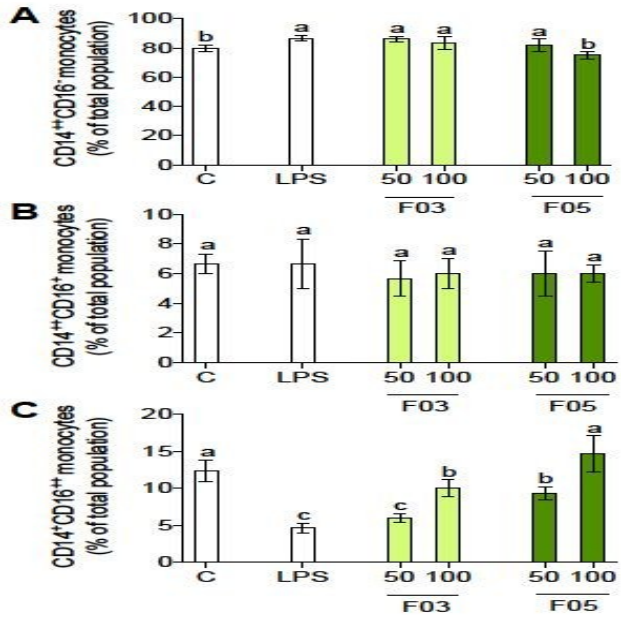

Fig. 3 Effect of F03 and F05 at 50 – 100 µg/mL for 24 h in LPS-treated human monocytes: (A) Classical monocytes, (B) intermediate monocytes and (C) non-classical monocytes. Values shown are the mean ± SD (n=3) and those marked with different lowercase letters are significantly different ($P < 0.05$).

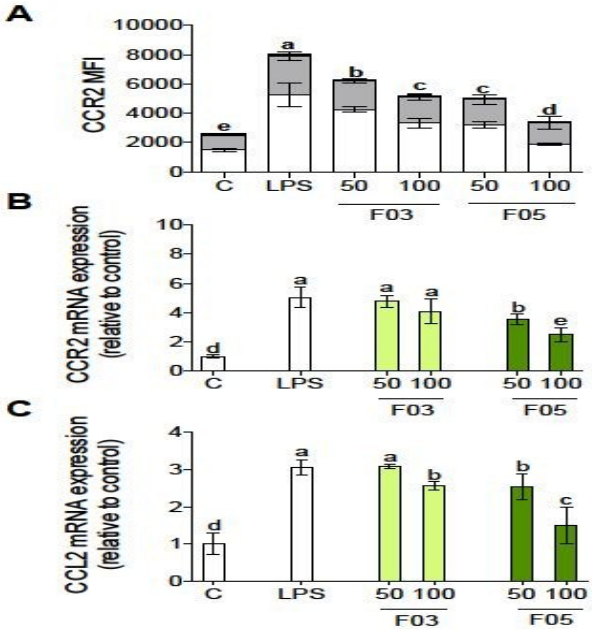

Fig. 4 Effect of F03 and F05 fraction at 50 - 100 µg/mL for 24 h in LPS-treated human monocytes (A) CCR2 protein expression on monocytes subset (classical monocytes). The gene expression of (B) CCR2, (C) CCL2 was measured by qPCR. Values shown are the mean ± SD (n=3) and those marked with different lowercase letters are significantly different ($P < 0.05$).

CCR2/CCL2 axis were studied (**Fig. 4**). CCR2 protein expression was analyzed in monocyte subsets by FACS analysis (**Fig. 4A**). F03 and F05 decreased significantly CCR2 expression primarily on classical monocyte subsets compared to LPS, although no significantly differences were found on CCR2 MFI in non-classical or intermediate monocytes, there was a reduction tendency. In addition, mRNA expression of CCL2 and its receptor, CCR2 was analyzed. LPS-treated monocytes showed a CCR2 up-regulation (**Fig. 4B**). However, those that were treated with F05 had a lower increased in CCR2 mRNA levels than those that were treated with LPS. Regarding to CCL2 mRNA levels, LPS treatment up-regulated its expression, whereas that F03 and F05 at 100 µg/mL down-regulated CCL2 expression compared to these treated with LPS **(Fig. 4C)**.

To gain insight into the effects of F03 and F05 fractions in human primary monocytes upon LPS treatment, we analysed secretion and gene expression of both pro-inflammatory (TNF-α and IL-6) cytokines. F03 and F05 down-regulated IL6 and TNFα mRNA levels and diminished LPS-induced release of these cytokines (**Fig. 5**). These observations reinforce the notion that F03 and F05 may have a role in reducing the inflammatory response in activated human monocytes.

Monocytes are innate blood cells involved in the early inflammatory response, as the first defence line to recognize

and eliminated pathogens involved in acute infections.[35,37] This is the first studies on anti-inflammatory activity of defatted hempseed fractions on primary human monocytes. Individually phenol amide compounds present in hempseed has been evaluated on pro-inflammatory cytokines (TNF-α) in BV2 microglia cells and anti-neuroinflammatory effects of total extract containing phenylpropionamides principally, using lipopolysaccharide (LPS)-induced mouse model.[24,27] Contrasting with our research several studies on primary human monocytes, using fractions obtained from different sources such as phenolic fraction from virgin olive oil, unsaponifiable fraction from grape seed oil has showed beneficial effects on different inflammatory diseases.[37,38,39] In the present study, we observed that with the correct extraction interesting compounds can obtained in different fractions, for it wide and low quantity of compounds present in hempseed after a defatted process, the vegetal material consumption and reagents are extensive for compound isolation and use of the fractions can be a viable application.
However, establishing a correct doses is necessary, by the possible adverse effect with the use of high doses in assays reported in anti-neuroinflammatory activities at cell level using a hempseed extract with high amount of phenylpropionamides.[24]

This study provides data about hempseed properties such as a healthy food and indicates that with an appropriate extraction, it is possible to obtain fractions with these groups of compounds and to use them as a potential source of therapeutic agents for treatment of inflammatory diseases.

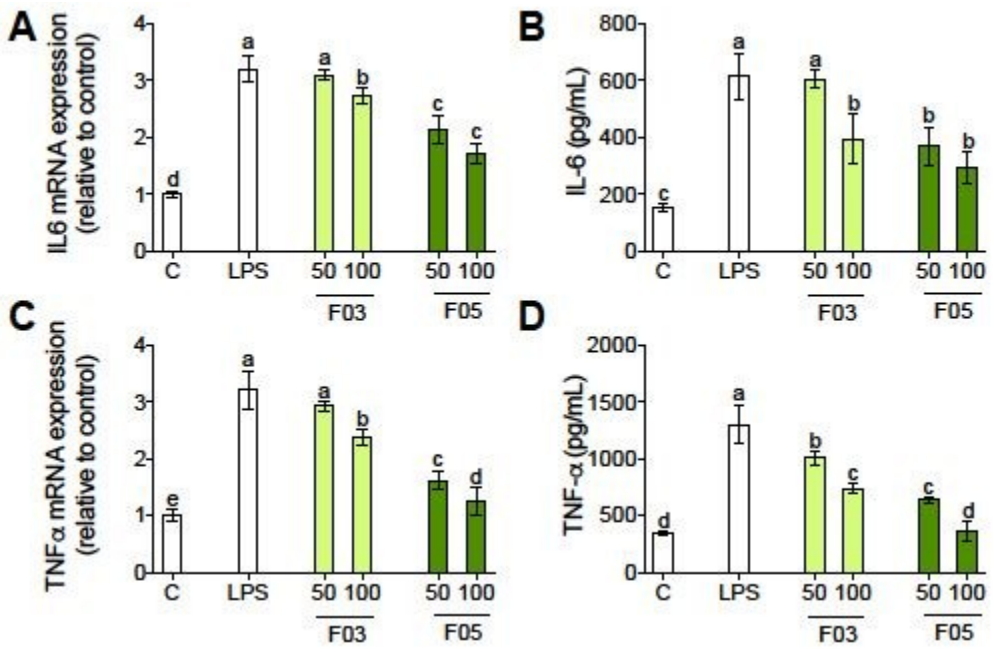

Fig. 5 Effect of F03 and F05 fraction at 50 - 100 µg/mL on secretion and gene expression of pro-inflammatory cytokines in LPS-treated human monocytes. Cells were untreated (Control) or treated with LPS (100 ng/mL) in the absence (LPS) or presence of F03 and F05 for 24 h. The expression of IL-6 (A) and TNF-α (C) gene was measured by RT-qPCR, whereas the concentration of IL-6 (B) and TNF-α (D), in culture supernatants was measured by ELISA. Values shown are the mean ± SD (n=3) and those marked with different lowercase letters are significantly different ($P < 0.05$).

4. Conclusion

In sum, high amount compounds were identified, highlighting phenolic amides present in fractions besides acid phenols, flavonoids and terpenphenols cannabinoids precursors. However, the psychotropic cannabinoids were not detected. In this context, the high amount of phenolic compounds justified the great antioxidant activity and anti-inflammatory effects in monocytes cells, especially, in the ethyl acetate fraction. This gives the hempseed a hopeful future as a rich source of

bioactive compounds for food and pharmaceutical applications.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgements

We thank Mass Spectrometry Service from the CITIUS Facility of the University of Seville for assistance in the samples analyzed. Authors thank Cell Biology Unit of Instituto de la Grasa for its assistance during the fulfilment of this work. SMP acknowledges financial support from "V Own Research Plan" (University of Seville).

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DOI: 10.1039/D0FO00066C

Characterization of bioactive compounds in defatted hempseed (*Cannabis sativa* L.) by UHPLC-HRMS/MS and anti-inflammatory activity in primary human monocytes

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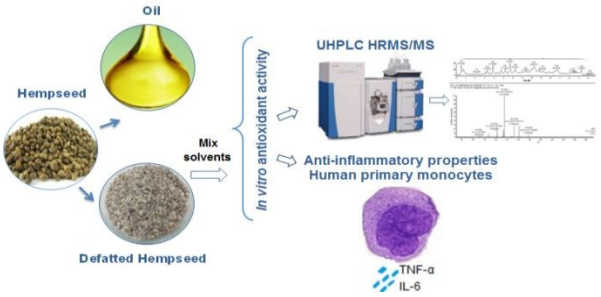

Anti-inflammatory effects on human monocytes by phenolic amides present in defatted hempseed fractions (*Cannabis sativa* L.)