COMPUESTOS BIOACTIVOS DE LA SEMILLA DE CÁÑAMO

(*Cannabis sativa* L.) EN NEUROINFLAMACIÓN

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COMPUESTOS BIOACTIVOS DE LA SEMILLA DE CÁÑAMO (*Cannabis sativa* L) EN NEUROINFLAMACIÓN

DEPARTAMENTO DE FARMACOLOGÍA

Memoria presentada por D. Julio Rea Martinez para

optar al grado de Doctor por la Universidad de Sevilla

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CERTIFICAN:

Que la Tesis Doctoral titulada "**COMPUESTOS BIOACTIVOS DE LA SEMILLA DE CAÑAMO (*Cannabis sativa* L.) EN NEUROINFLAMACION**" ha sido llevada a cabo bajo nuestra dirección.

V.º B.º

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V.º B.º

La Tutora

Dra. Mª Dolores García Giménez

“Se el cambio que quieres ver en el mundo”

Mahatma Gandhi

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ABREVIATURAS

ABTS	Acido 2,2'- azino-bis(3-etilbenzotiazolina) - 6-sulfonico
AD	Enfermedad de Alzheimer
ARE	Elemento de respuesta antioxidante
BACE 1	Enzima 1 de escisión de la proteína precursora de amiloide del sitio beta
CAE	Equivalentes de ácido cafeico
CAFT	*N-trans* Caffeoyltyramine
BBB	Barrera hematoencefálica
CB_1	Receptor cannabinoide tipo 1
CB_2	Receptor cannabinoide tipo 2
CBC	Cannabicromeno
CBD	Cannabidiol
CBG	Cannabigerol
COX	Ciclooxigenasas
CRP	Proteína C reactiva
CUMT	*N-trans* Coumaroyltyramine
DA	Dopamina
DOPAC	Acido 3, 4-dihidroxifenilacético
DAMPs	Patrones moleculares asociados a daños/peligro
DPPH	2,2-difenil-1-picrilhidrazilo
EAF	Fracción de acetato de etilo
GAE	Equivalentes de ácido gálico
GM-CSF	Factor estimulante de colonias de granulocitos y macrófagos

HDL	Lipoproteína de alta densidad
HVA	Ácido Homovanillico
HIV	Virus de inmunodeficiencia humana
IFN-γ	Interferón *gamma*
iNOS	Óxido nítrico sintasa inducible
LDL	Lipoproteína de baja densidad
LPS	Lipopolisacárido de pared bacteriana
MAE	Extracción asistida por microondas
MAOs	Monoaminaoxidasas
MAPK	Proteín quinasas activadas por mitógenos
MTT	Bromuro de 3-(4,5-dimetiliazol-2 il)-2,5-difenil tetrazolio
NF-kB	Factor nuclear de transcripción kappa
NFT	Ovillos neurofibrilares
NRF2	Factor 2 relacionado con el factor nuclear eritroide 2
PAMPA	Ensayo de permeabilidad de membrana artificial paralelo de alto rendimiento
PAMPs	Patrones moléculas asociados a patógenos
PBMCs	Células mononucleares de sangre periférica
PBS	Tampón fosfato salino
PCG-1α	PPARγ-coactivator-1α
PD	Enfermedad de Parkinson
PPARγ	Receptor gamma activado por proliferador de peroxisomas
PRRs	Receptores de reconocimiento de patrones
PUFA´s	Ácidos grasos poliinsaturados
QE	Equivalentes de quercetina

ROS	Especies reactivas de oxigeno
SIRT1	Regulador de información silencioso transcript-1
SNC	Sistema nervioso central
TGFs	Factores transformadores de crecimiento
TLRs	Receptores tipo Toll
TNF-α	Factor de necrosis tumoral *alfa*
UAE	Extracción asistida por ultrasonido
Δ^8-THC	Delta 8, tetrahidrocannabinol
Δ^9-THC	Delta 9, tetrahidrocannabinol

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RESUMEN

Cannabis sativa L. es considerada una de las especies más importantes dentro de la Familia Cannabaceae. Ha sido cultivada desde la antigüedad en diferentes regiones del mundo, utilizándose tanto en medicina tradicional, como también en la industria textil, farmacéutica, alimentaria y en la construcción. Actualmente más de 565 fitocompuestos han sido reportados o aislados en el género Cannabis, convirtiéndose en una potencial fuente de compuestos con diferentes aplicaciones biológicas. Generalmente se describen 3 fenotipos: Fenotipo I (Δ9-THC > 0.3%), Fenotipo II (CNB % > Δ9-THC%) y Fenotipo III del tipo fibra (Δ9-THC < 0.3%). De este último, también conocido como tipo fibra, se ha incrementado su producción y cultivo a nivel mundial durante los últimos años para el aprovechamiento de sus compuestos no psicoactivos en la industria farmacéutica y alimentaria.

En el cáñamo, la semilla también es utilizada a nivel industrial, entre otros fines, para la obtención de aceite por su alto contenido de ácidos grasos poliinsaturados. Además de su fracción lipídica, la semilla presenta una alta concentración de proteínas, carbohidratos, fibra y vitaminas. Durante muchos años la fracción lipídica de las semillas ha sido ampliamente investigada, con numerosas publicaciones sobre su composición y propiedades beneficiosas, dejando en un segundo plano a los componentes no lipídicos que también se encuentran formado parte de su compleja matriz.

Por todo ello, en la presente Tesis Doctoral se describen la obtención de diferentes extractos y fracciones procedentes de semillas de cáñamo desengrasadas, para la recuperación de fitocompuestos con solventes polares. Comenzamos con la evaluación de la capacidad captadora de radicales, contenido de fenoles totales y flavonoides de las fracciones obtenidas a partir de los diferentes extractos. Estas técnicas nos sirvieron para seleccionar aquellas que eran más prometedoras, siendo posteriormente analizadas mediante UHPLC-HRMS/MS para conocer su concentración e identificar los compuestos que permanecen en la matriz de la semilla después de un proceso de desengrasado. Se aislaron e identificaron una amplia cantidad de compuestos, tales como: ácidos fenólicos, flavonoides, terpenofenoles, lignanamidas, destacando una alta concentración de amidas fenólicas como: cafeoiltiramina, feruloiltiramina, cafeoiloctopamina y

cumaroiltiramina, principalmente. En este trabajo se ha detallado que la obtención de fracciones ricas en este tipo de compuestos es una opción viable y económica.

La evaluación de las fracciones obtenidas sobre monocitos humanos de sangre periférica, mostraron efectos positivos reduciendo la respuesta inflamatoria al ejercer una reducción de la expresión génica y sobre la liberación de citoquinas pro-inflamatorias IL-6 y TNF-α, inducidas por LPS. Por otra parte, en esta Tesis se ensayaron, *in vitro*, los posibles efectos, de la fracción rica en amidas fenólicas, y de dos de sus compuestos aislados, sobre diferentes enzimas implicadas en procesos neuronales. Enzimas que son dianas en la búsqueda de nuevos compuestos para el tratamiento de enfermedades neurodegenerativas como Parkinson y Alzheimer. Complementamos la investigación con su evaluación sobre un modelo *in vivo*, donde se pudo apreciar un efecto protector de la degradación de la dopamina estriatal una vez administrada la fracción y la posterior cuantificación de las aminas biógenas, hechos que no había sido descritos anteriormente.

Por otra parte, se realizaron ensayos de la fracción y sus principales compuestos aislados sobre células N2a-APP, y su influencia sobre BACE 1, implicada en la acumulación de placas amiloides, característica principal en la enfermedad de Alzheimer, como también de dos de sus reguladores PGC-1α y PPARγ. Los resultados en la expresión génica mostraron que la fracción como el compuesto *N-trans* cafeoiltiramina, poseen un efecto dual: agonista/inhibidor, aumentando la expresión génica de sus reguladores e inhibiendo la expresión de BACE 1.

Con estos resultados se puede concluir que los diferentes compuestos obtenidos de la semilla de cáñamo, entre los cuales destacan las hidroxicinamoil amidas, son moléculas con múltiples actividades biológicas: antioxidante, anti-inflamatoria, inhibidora de enzimas involucradas en el SNC, inhibidoras de BACE 1 y agonistas naturales de reguladores master PGC-1α y PPARγ. Todo ello indica que el aprovechamiento de las semillas de cáñamo o de los subproductos después del proceso de obtención del aceite, nos ofrece un nuevo campo de búsqueda, para el posible desarrollo de fármacos efectivos en el tratamiento de enfermedades neurodegenerativas.

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ABSTRACT

Cannabis sativa L. is considered one of the most important species within the family Cannabaceae. It has been cultivated since ancient times in different regions of the world and being used both in traditional medicine as well as in textile, pharmaceutical, food and construction industries. Currently more than 565 phytocompounds have been reported or isolated from the Cannabis genus, making it a potential source of compounds with different biological applications. Generally, 3 phenotypes are described: Phenotype I (Δ9-THC> 0.3%), Phenotype II (CNB%> Δ9-THC%) and Phenotype III of fiber type (Δ9-THC <0.3%). The latter, also known as fiber type, has increased its production and cultivation worldwide in recent years for the use of its non-psychoactive compounds in the pharmaceutical and food industries

In hemp, the seed is also used industrially for oil extraction, among other purposes, because of its high content of polyunsaturated fatty acids. In addition to its lipid fraction, the seed has a high concentration of proteins, carbohydrates, fiber and vitamins. For many years, the lipid fraction of seeds has been extensively investigated, with numerous publications on its composition and beneficial properties, leaving in the background the non-lipid components that, are also part of its complex matrix. Therefore, this Doctoral Thesis describes the obtaining of different extracts and fractions from defatted hempseed, for the recovery of phytocompounds using polar solvents. We begin with the evaluation of the radical scavenging capacity, total phenols and flavonoids content of the fractions obtained from the different extracts. These techniques helped us to select the most promising ones, which were subsequently analyzed by UHPLC-HRMS / MS to know their concentration and identify the compounds that remain in the seed matrix after a defatted process. A large number of compounds were isolated and identified such as: phenolic acids, flavonoids, terpenophenols, lignanamides, highlighting a high amount of phenolic amides such as: caffeoyltyramine, feruloyltyramine, caffeoyloctopamine and coumaroyltyramine, mainly. In this work, it has been detailed that the recovery of fractions rich in these types of compounds is a viable and economical option.

The evaluation of the obtained fractions on human peripheral blood monocytes showed positive effects on the reduction of inflammatory response through a

decrease in gene expression and on the release of pro-inflammatory cytokines IL-6 and TNF-α, induced by LPS. On the other hand, in this thesis, the possible effects of the fraction rich in phenolic amides and of two of their isolated compounds on different enzymes involved in neuronal processes were tested *in vitro*. Enzymes that are targets in the search for new compounds for the treatment of neurodegenerative diseases such as Parkinson`s and Alzheimer´s. We complemented the research with the evaluation on *in vivo* model, where a protective effect of striatal dopamine degradation was seen after administration of the fraction and the subsequent quantification of biogenic amines, facts that had not been previously published.

On the other hand, the fraction and its main isolated compounds were tested on N2a-APP cells and their influence on BACE 1, implicated in the accumulation of amyloid plaques, the main characteristic in Alzheimer's disease, and on two of its regulators PGC-1α and PPARγ. The results in gene expression showed that the fraction as the compound *N-trans* caffeoyltyramine, have a dual effect: agonist / inhibitor, increasing the gene expression of its regulators and inhibiting the expression of BACE 1.

From these results it can be concluded that the different compounds obtained from the hemp seed, among which the hydroxycinnamoyl amides stand out, are molecules with multiple biological activities: antioxidant, anti-inflammatory, inhibitor of enzymes involved in the CNS, inhibitors of BACE 1 and natural agonists of master regulators PGC-1α and PPARγ. All this indicates that the use of hempseed or by-products after the oil extraction process offers us a new field of search for the possible development of effective drugs in the treatment of neurodegenerative diseases.

SAŽETAK

Cannabis sativa L. smatra se jednom od najvažnijih vrsta u obitelji *Cannabaceae*. Uzgaja se od davnina u različitim regijama svijeta i koristi se kako u tradicionalnoj medicini, tako i u tekstilnoj, farmaceutskoj, prehrambenoj i građevinskoj industriji. Trenutno je izolirano više od 565 fitospojeva iz roda *Cannabis*, što ga čini potencijalnim izvorom spojeva s različitim biološkim primjenama. Općenito su opisana 3 fenotipa: Fenotip I (Δ9-THC> 0,3%), Fenotip II (CNB%> Δ9-THC%) i Fenotip III tipa vlakana (Δ9-THC <0,3%). Posljednjem, koji je poznat i kao vrsta vlakana, zadnjih je godina povećana proizvodnja i uzgoj u svijetu zbog upotrebe njegovih nepsihoaktivnih spojeva u farmaceutskoj i prehrambenoj industriji.

Sjeme konoplje se također industrijski koristi za ekstrakciju ulja, između ostalog i zbog visokog sadržaja polinezasićenih masnih kiselina. Uz frakciju lipida, sjeme ima visoku koncentraciju bjelančevina, ugljikohidrata, vlakana i vitamina. Već dugi niz godina lipidni udio sjemena opsežno se istražuje, s brojnim publikacijama o njegovom sastavu i korisnim svojstvima, ostavljajući u pozadini nelipidne komponente koje su također dio njegove složene matrice. Stoga, ovaj doktorski rad opisuje dobivanje različitih ekstrakata i frakcija iz odmašćenih sjemenki konoplje za dobivanje fitospojeva pomoću polarnih otapala. Započinjemo s procjenom kapaciteta uklanjanja radikala, ukupnog sadržaja fenola i flavonoida u frakcijama dobivenim iz različitih ekstrakata. Navedene metode su nam pomogle odabrati pojedine frakcije koje su naknadno analizirane UHPLC-HRMS / MS metodom kako bi se saznala njihova koncentracija i identificirali spojevi koji ostaju u matrici sjemena konoplje nakon postupka odmašćivanja. Izoliran je i identificiran veliki broj spojeva kao što su: fenolne kiseline, flavonoidi, terpenofenoli, lignanamidi, ističući veliku količinu fenolnih amida kao što su: kafeoiltiramin, feruloiltiramin, kafeoiloktopamin i kumaroiltiramin. U ovom radu detaljno je utvrđeno da je dobivanje frakcija bogatih ovim vrstama spojeva održiva i ekonomična opcija.

Procjena dobivenih frakcija na monocitima ljudske periferne krvi pokazala je pozitivne učinke na smanjenje upalnog odgovora smanjenjem ekspresije gena i na oslobađanje proupalnih citokina IL-6 i TNF-α, inducirano LPS-om. S druge strane, u ovoj doktorskoj disertaciji ispitani su *in vitro* mogući učinci frakcije

bogate fenolnim amidima i dva njihova izolirana spoja na različite enzime koji sudjeluju u neuronskim procesima. Ciljani enzimi u potrazi za novim spojevima za liječenje neurodegenerativnih bolesti poput Parkinsonove i Alzheimerove bolesti. Dopunili smo istraživanje evaluacijom na *in vivo* modelu, gdje se pokazao zaštitni učinak razgradnje striatalnog dopamina nakon davanja frakcije i naknadne kvantifikacije biogenih amina. Ove činjenice do sada nisu objavljene u znanstvenim časopisima.

S druge strane, frakcija i njezini glavni izolirani spojevi testirani su na stanicama N2a-APP kao i njihov utjecaj na BACE 1, uključen u nakupljanje amiloidnih plakova, što je glavna karakteristika Alzheimerove bolesti, te na dva regulatora PGC-1α i PPARγ. Rezultati u ekspresiji gena pokazali su da frakcija kao spoj N-trans kafeoiltiramina ima dvostruki učinak: agonist / inhibitor, povećavajući ekspresiju gena svojih regulatora i inhibirajući ekspresiju BACE 1.

Iz ovih rezultata može se zaključiti da različiti spojevi dobiveni iz sjemena konoplje, među kojima se posebno ističu hidroksicinamoil amidi, predstavljaju molekule s višestrukim biološkim djelovanjem: antioksidativno, protuupalno, inhibitorno za enzime uključene u CNS, inhibitori BACE 1 i prirodni agonisti glavnih regulatora PGC-1α i PPARγ. Sve to ukazuje da nam upotreba konoplje ili nusproizvoda nakon postupka ekstrakcije ulja nudi novo polje istraživanja za mogući razvoj učinkovitih lijekova u liječenju neurodegenerativnih bolesti.

CAPITULO I
INTRODUCCION GENERAL

OBJETIVOS

I.1.- INTRODUCCION GENERAL

I. 1.1. Interés de la especie *Cannabis sativa*

I. 1.1.1. *Cannabis sativa* L.: Generalidades botánicas y fitoquímicas de la especie

Es una de las especies más importantes dentro de la familia Cannabaceae, con un único género (*Cannabis*), y única especie (*C. sativa*), presentando una extensa variabilidad. Es una planta dioica anual, originaria de Asia central, y que ha sido ampliamente cultivada por el hombre desde la antigüedad, en casi todos los lugares del mundo, desde regiones tropicales a andinas. Posee flores masculinas y femeninas que se desarrollan por separado, propagándose a partir de semillas, con un buen crecimiento en espacios abiertos. Con buena iluminación, drenaje, nutrientes y agua, puede llegar a alcanzar hasta los 6 m de altura dependiendo de la variedad y las condiciones de crecimiento [1,2]. Ha sido empleada desde la antigüedad tanto en medicina tradicional como en la industria textil, siendo una importante fuente de fibras. Por su rápido crecimiento, su uso se ha incrementado durante los últimos años, al tratarse de un cultivo donde se puede aprovechar diferentes partes de la planta o su totalidad. Distintos sectores como el farmacéutico, la industria alimentaria o la construcción, han unido esfuerzos para obtener el máximo aprovechamiento de esta especie [3,4].

Con respecto a la clasificación taxonómica de esta planta puede describirse como [1,2]:

Reino	Plantae
Subreino	Tracheobionta (plantas vasculares)
Superdivision	Sphermatophyta (plantas con semillas)
División	Magnoliophyta (flores)
Clase	Magnoliopsida (Dicotiledóneas)
Subclase	Hamamelididae
Orden	Urticales
Familia	Cannabaceae
Genero	*Cannabis*
Especie	*Cannabis sativa* L.

En relación a su composición química, sus principales metabolitos, los canabinoides, han sido ampliamente investigados desde el último siglo, con el descubrimiento de su componente más importante Δ9-tetrahidrocannabinol (Δ9-THC) responsable del efecto psicoactivo; además del Δ8-tetrahidrocannabinol (Δ8-THC), y otros canabinoides no psicoactivos (canabidiol- CBD, canabigerol-CBG, canabicromeno-CBC etc.), Todos ellos actúan sobre receptores canabinoides endógenos: CB1 localizados principalmente en el cerebro y CB2 mayormente en el sistema inmunitario [5,6]. El género Cannabis puede dividirse principalmente en tres fenotipos o

quimiotipos, dependiendo de la concentración de estos canabinoides. Según este criterio, se reconocen 3 fenotipos: Fenotipo I (Δ9-THC > 0.3%), Fenotipo II (CNB % > Δ9-THC%), Fenotipo III del tipo fibra (Δ9-THC < 0.3%), teniendo este último altas concentraciones de canabinoides no psicoactivos.

Actualmente han sido detectados más de 565 fitocompuestos en *Cannabis sativa* y hoy día, continúan aislándose e identificándose más compuestos por diferentes investigadores [5,7,8].

Los cannabinoides proceden de la condensación de pirofosfato de geranilo con un fenol, el olivetol,. Son metabolitos secundarios y actúan como mecanismo de defensa o reguladores del desarrollo de la planta [9] (Figura 1). De todos los compuestos del cáñamo, aproximadamente 120 son cannabinoides teniendo en común una estructura conformacional terpenfenólica con un esqueleto (C21) en sus formas neutras y (C22) en formas carboxiladas, clasificados en 7 subtipos diferentes. (Figura 2). Se encuentran en diferentes partes de la planta, con una mayor concentración en flores, en etapas previas a la floración, disminuyendo su concentración en hojas, tallos, semillas y raíces [10]. Se ha confirmado en algún caso, la presencia de estos compuestos en el aceite de cáñamo. Hoy se sabe que las semillas no los contienen; por lo tanto, su presencia,en estos casos, se debe a la contaminación con la resina producida por los tricomas glandulares de las flores femeninas, en etapas de floración y fructificación, pues la mayor concentración de estos fitocannabinoides se encuentra en las brácteas, que rodean a la semilla [11,12].

En algunas ocasiones, en aceites de semilla comercializados, han sido detectados hasta 32 de estos compuestos, mediante cromatografía líquida

acoplada a espectrofotometría de masas de alta resolución (UHPLC-HRMS/MS) [13].

En los últimos años la producción de cáñamo se ha incrementado en países como Canadá, USA, China y Europa, principalmente por su fibra, aceite, además de su versatilidad y fácil cultivo. Por otro lado, muchos estudios también se centran en el aprovechamiento y la obtención de compuestos bioactivos que pueden tener aplicaciones en la industria farmacéutica y alimentaria [14].

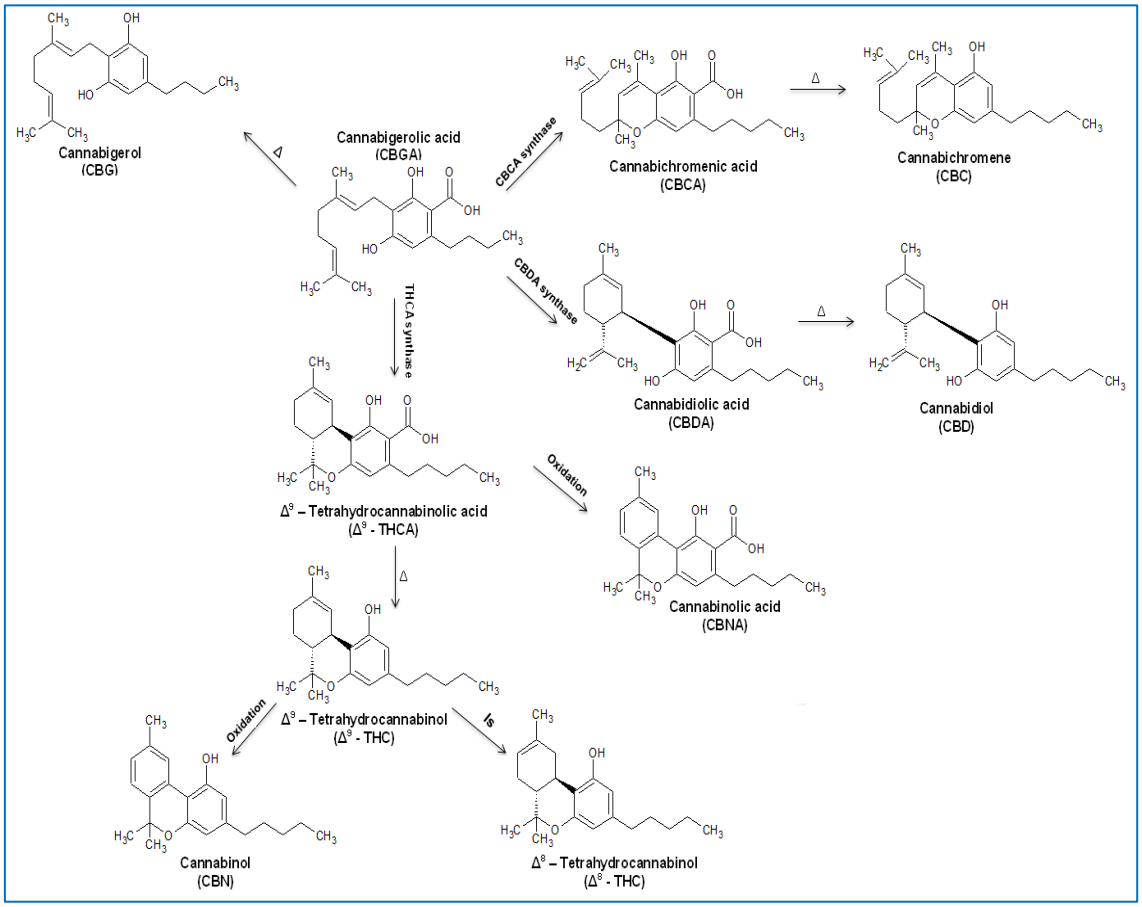
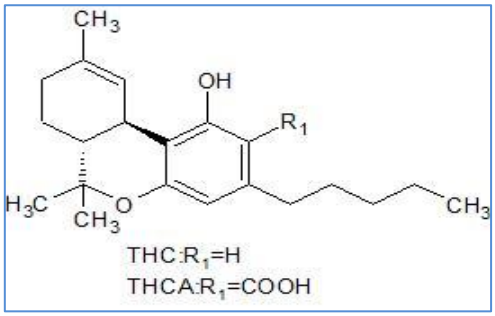
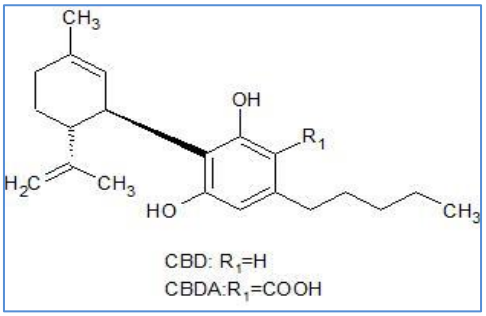


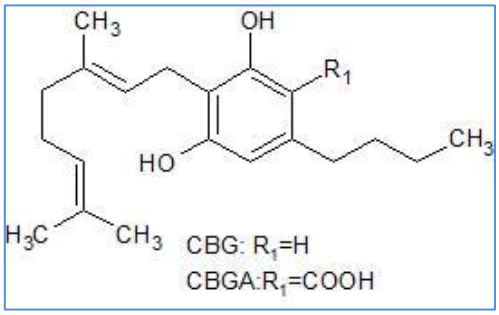
Figura 1. Biosíntesis de los principales cannabinoides presentes en *Cannabis sativa* L.



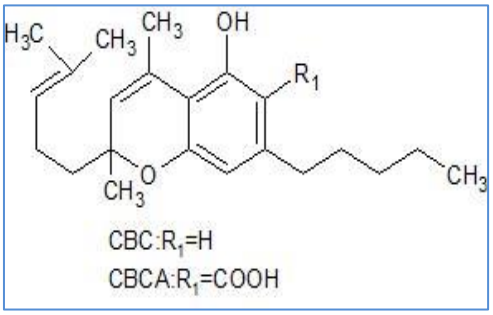
THC:R_1=H
THCA:R_1=COOH



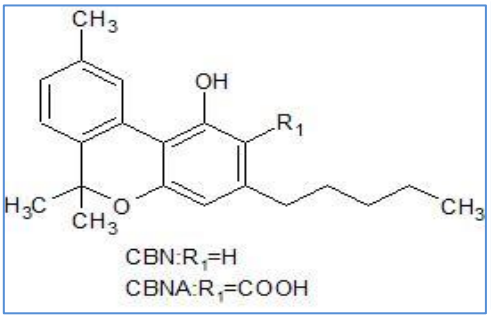
CBD: R_1=H
CBDA:R_1=COOH



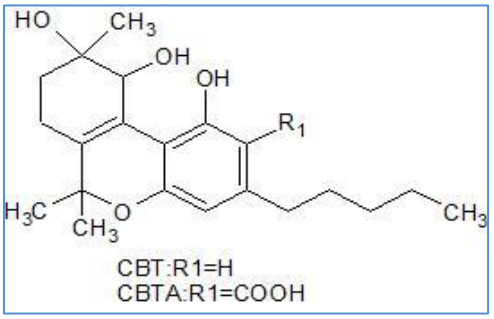
CBG: R_1=H
CBGA:R_1=COOH



CBC:R_1=H
CBCA:R_1=COOH



CBN:R_1=H
CBNA:R_1=COOH



CBT:R1=H
CBTA:R1=COOH

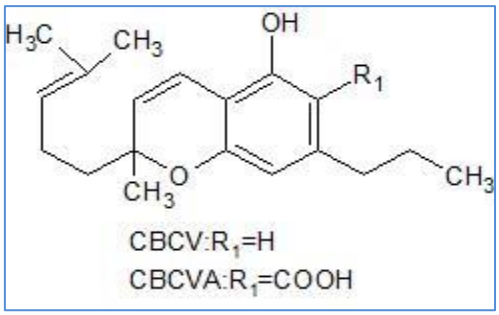


Figura 2. Estructura de cannabinoides neutros y formas carboxílicas presentes en el aceite de cáñamo.

El aceite proveniente del cáñamo industrial se caracteriza por una significativa cantidad de CBD y una baja cantidad de THC, como también de su producto de degradación CBN. Para considerar legal su cultivo para obtención de fibra y semillas, en países europeos su límite es de 0.2% en THC,pero en diferentes estudios relacionados con la composición de algunos tipos de aceites comercializados, han aparecido valores superiores a lo legalmente permitido. La determinación en composición de estos cannabinoides permite distinguir el tipo de variedad del que fue obtenido [15].

CBDA es el compuesto en mayor concentración que se ha encontrado en el aceite de semillas de cáñamo, considerándose la proporción CBDA/CBD como un marcador para conocer las condiciones de procesamiento y almacenamiento, debido a que las formas acidas (CBGA, CBDA, THCA) sufren un proceso de descarboxilación a sus correspondientes formas neutras (CBG, CBD, THC), mediada principalmente por la exposición a altas temperaturas y también a temperatura ambiente, aunque en menor grado [16].

Con respecto a la actividad farmacológica, los cannabinoides son el grupo donde mayoritariamente se centran las investigaciones sobre sus usos y posibles aplicaciones: antiémesis en tratamientos oncológicos, depresión, glaucoma, esclerosis múltiple, migraña, Alzheimer, cáncer y HIV, entre otras.

Se ha publicado recientemente, que Canabidiol (CBD), constituyente no psicoactivo, puede ser efectivo para paliar ataques de pánico, y como ansiolítico [17]. En los últimos años su compleja composición química y gran variedad de otros compuestos minoritarios como terpenos, fenoles, flavonoides, ha despertado el interés de sus metabolitos para posibles aplicaciones terapéuticas [4,5,18].

I.1.1.2. Semillas de Cáñamo: Interés y composición química

Las semillas han sido utilizadas desde la antigüedad como un valioso producto para la alimentación del hombre, extendiéndose su uso tanto para tratar problemas de salud como desde el punto de vista nutricional. Las semillas de cáñamo contienen aproximadamente un 30% de aceite, 20-25% de proteínas fácilmente digeribles, 30% de carbohidratos y cantidades apreciables de fibra, vitaminas y minerales [19]. Su composición se detalla en la **(Tabla 1)**.

Tabla 1. Composición típica de semilla de cáñamo - (Finola cultivar) [19].

Macrocomposición nutricional de Semilla de Cáñamo (%)		
	Semilla	Harina
Aceite	35.5	11.1
Proteínas	24.8	33.5
Carbohidratos	27.6	42.6
Humedad	6.5	5.6
Ceniza	5.6	7.2
Fibra total	27.6	42.6

El aceite es uno de los productos de la semilla de cáñamo que más se ha investigado. Su composición en ácidos grasos poliinsaturados (PUFA´s) con una proporción nutricional óptima (n-6/n-3, 3:1), la convierte en un alimento equilibrado para el consumo **(Tabla 2)**. Su composición particularmente varía de acuerdo al tipo de cultivar, almacenamiento, tiempo de cosecha, y método de procesamiento para la obtención de aceite [11].

Tabla 2. Composición lipídica de Aceite semilla de Cáñamo

Componentes	% (w/w)
Ácidos grasos	
a. linoleico (18:2, *ω*-6)	50-70
a. α-linolenico (18:2, *ω*-3)	15-25
a. oleico (18:1)	10-16
a. palmítico (16:0)	6-9
a. palmitoleico (16:1)	0.31*
a. esteárico (18:0)	2-3
a. γ-linolenico (18:3, *ω*-6)	1-6
a. eicosanoico (20:0)	2.50*
a. eicosenoico (20:1)	1.44*
a. eicosadenoico (20:2)	0.00-0.09
a. docosanoico (22:0)	0.40*

***Montserrat *et al*. (2014)**

Además de compuestos mayoritarios, presentes en el aceite de cáñamo, otros importantes componentes minoritarios han sido caracterizados en la fracción insaponificable, correspondiendo a un porcentaje de entre 1,5 – 2 % del aceite. Entre sus componentes, cabe destacar la presencia de esteroles (campesterol, β-sitosterol, estigmasterol), alcoholes alifáticos lineales (fitol, geranylgeraniol), triterpenos (cicloartenol, β-amirin), tocoferoles, mayoritariamente γ-tocoferol y sus análogos en menor proporción (α,β,δ). Estos componentes minoritarios presentan importantes funciones biológicas, entre las que destacan actividades antioxidantes y antiinflamatorias [20].

Por lo tanto, la gran versatilidad de compuestos que forman parte de la composición del aceite de semilla de cáñamo, muestran propiedades

Constituyentes no lipídicos en semilla de cáñamo: Estructuras y actividad

La composición química de la semilla es bastante compleja, además de los ácidos grasos del aceite anteriormente descritos, se ha demostrado la presencia de: fenoles ácidos, flavonoides, terpenos, alcaloides, lignamidas.

La producción y presencia de diferentes compuestos obedecen a mecanismos intrínsecos, desarrollados para defensa de la planta frente a patógenos, como de protección frente a radiación UV, incrementando o regulando su producción de ser necesaria. Durante muchos años la presencia de estos componentes ha estado relegada a un segundo plano debido a la rica composición en ácidos grasos poliinsaturados y proteínas en la semilla [9,21], que le han conferido tradicionalmente un mayor interés en el campo de la nutrición.

Pero en los últimos años, un gran número de investigaciones se han centrado en la caracterización, aislamiento y evaluación de diferentes compuestos polifenólicos presentes en la semilla cáñamo (aceite y residuos), donde principalmente su actividad antioxidante ha sido descrita en numerosos estudios [22,23]. También el aislamiento de diferentes lignamidas denominadas Cannabisin A, B, C, D, E, F, G, Grosamida etc., así como amidas fenólicas: *N-trans* caffeoyltyramine, *N-trans* coumaroyltyramine, *N-trans* feruoyltyramine [24,25,26] **(Figura 3)**, han sido de gran interés para los investigadores [11].

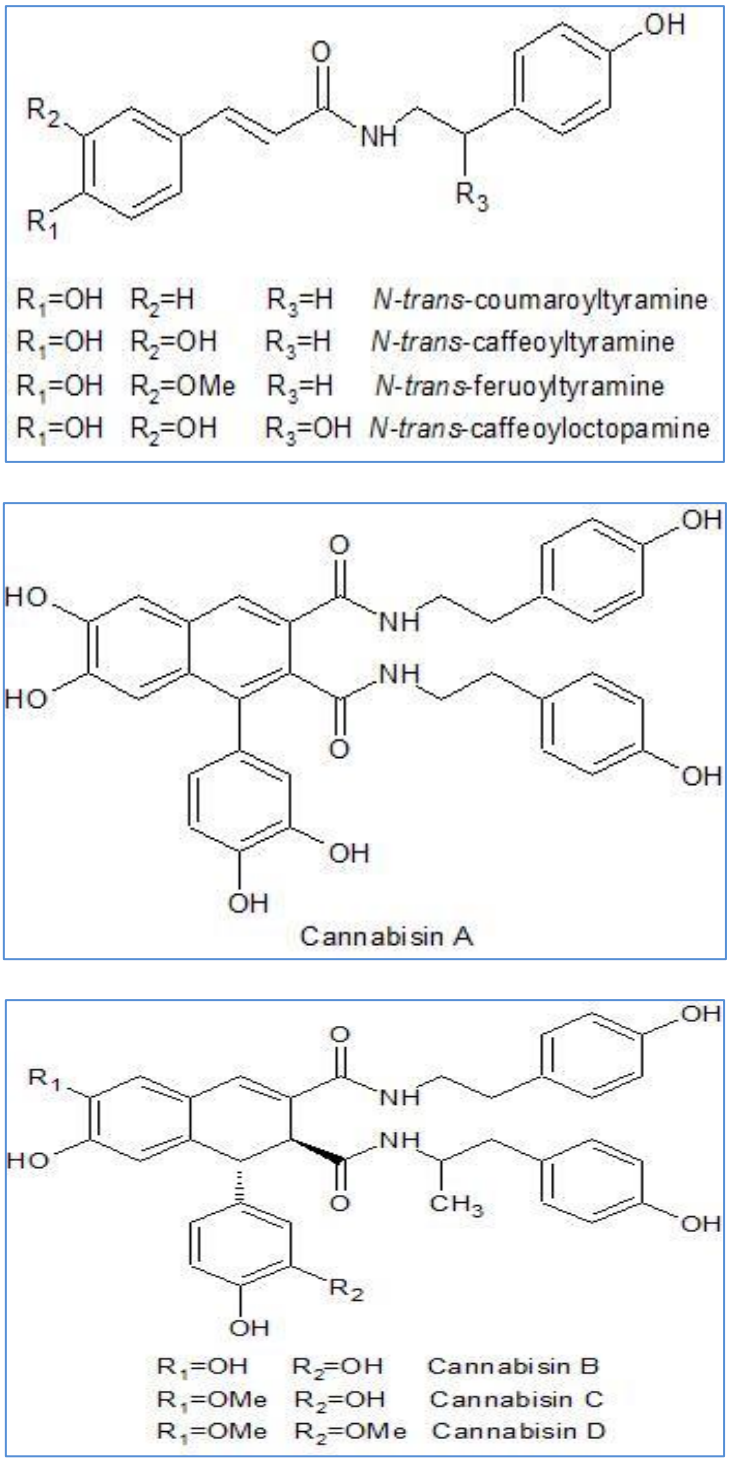


Figura 3. Estructuras de principales amidas fenólicas y lignamidas en semillas de cáñamo

En los últimos años existe un creciente interés para el aprovechamiento de estos compuestos presentes en la semilla, que abarcan desde fenoles simples a complejas estructuras como las lignamidas, haciendo que esta semilla sea una fuente de compuestos bioactivos para aplicaciones en la industria farmacéutica y alimentaria [4].

Muchas de las actividades farmacológicas de estos compuestos están íntimamente relacionadas con su efecto antioxidante y han quedado resumidas en la **Tabla 3**.

Tabla 3. Actividades de compuestos aislados de semilla de Cáñamo (*Cannabis sativa* L.)

Compuesto	Actividad/Inhibición	Referencia
Cannabisin A	Antioxidante, AChE[a], anti-neuroinflamatoria[b]	Yan *et al*. (2015), Zhou *et al*. (2018)
Cannabisin B	Antiradicalaria, arginasa, antioxidante, antiproliferativa (celulas HepG2), antineuroinflamatoria[b]	Chen *et al*. (2012), Bourjot *et al.* (2016), Chen *et al* (2013), Zhou *et al*. (2018)
Cannabisin C	Antioxidante, AChE[a]	Yan *et al*. (2015)
Cannabisin D	Antioxidante, AChE[a]	Yan *et al*. (2015)
Cannabisin E	Antioxidant, AChE[a]	Yan *et al.* (2015)
Cannabisin F	Antioxidante, AChE[a], anti-neuroinflamatoria (BV2 cells)	Bourjot *et al*. (2016), Yan *et al*. (2015) Wang *et al*.(2019)
Cannabisin G	Anti-neuroinflamatoria	Zhou *et al*. (2018)
Cannabisin H	-	-
Cannabisin I	Antioxidante, Arginasa[a]	Bourjot *et al*. (2016)
Cannabisin M	Antioxidante, Arginasa[a], antineuroinflamatoria	Yan *et al*. (2015), Bourjot *et al*. (2016), Zhou *et al*. (2018)

Cannabisin N	Antioxidante[a], AChE[a]	Yan *et al*. (2015)
Cannabisin O	Antioxidante[a]	Yan *et al*. (2015)
Grossamide	Antioxidante[a], AChE[a], anti-neuroinflamatoria (células BV2)	Yan *et al*. (2015), Lou *et al* (2017)
3,3´-Demethylgrossamide	Antioxidante, AChE, Arginasa, anti-neuroinflamatoria	Yan *et al.* (2015), Bourjot *et al*. (2016), Zhou *et al*. (2015)
N-trans-coumaroyltyramine	MAOs inhibición	Rea *et al*. (2020)
N-trans-caffeoyltyramine	Antiradicalaria, arginasa, antioxidante, AChE, anti-neuroinflamatoria, MAOs inhibición	Chen *et al*. (2012), Bourjot *et al.* (2016) Yan *et al.* (2015), Zhou *et al*. (2018)
N-trans-feruoyltyramine	Antioxidante, AChE[a], anti-neuroinflamatoria	Yan *et al*. (2015), Zhou *et al*. (2018)
N-trans-caffeoyloctopamide	Anti-neuroinflamatoria	Zhou *et al*. (2018)
(±) – Sativamides A y B	Reducción citotoxidad inducida por estrés del retículo endoplasmatico en células de neuroblastoma	Zhu *et al.* (2018)

[a]: Baja Inhibición en concentraciones evaluadas; [b]: Sin diferencias significativas vs control; -: Sin estudios reportados

En las últimas investigaciones se ha demostrado que ácidos fenólicos, flavonoides, terpenos, canabinoides, lignamidas y amidas fenólicas derivadas de ácido cafeico, ferúlico, *p*-cumárico existentes en las semillas, presentan interesantes actividades biológicas por lo cual pueden ser útiles para el desarrollo de nuevas moléculas y su posterior aplicación para el tratamiento de diferentes patologías [30].

Aunque los recientes estudios han establecido una base para la investigación de las propiedades de la semilla de cáñamo en enfermedades neurodegenerativas, es necesario conocer el aporte de cada uno de los compuestos frente a estas patologías. El gran número de compuestos presentes y sus cantidades relativamente bajas de la mayoría (mg/kg), requieren una gran cantidad de material vegetal, solventes y equipamiento para el aislamiento y purificación [28,36]. Durante los últimos años la utilización de extractos y/o fracciones que concentren gran cantidad de estos compuestos están siendo de gran interés para posibles aplicaciones, como: la neuroprotección, evitando el daño neuronal inducido por LPS en ratones, disminuyendo la liberación de citoquinas pro-inflamatorias (IL-1β, IL-6, TNF-α) a nivel cerebral [36] y reduciendo la secreción y expresión génica de IL-6, TNF-α en monocitos primarios humanos estimulados con LPS entre otros [37].

Métodos utilizados para obtener diferentes extractos a partir de la semilla de cáñamo

El uso de extractos o diferentes fracciones con actividad biológica, obtenidas secuencialmente con disolventes de diferente polaridad a partir de la semilla, es una opción interesante para una posible aplicación clínica. Se pueden obtener fácilmente del material vegetal desengrasado, aplicando diferentes

métodos de extracción tanto convencionales (maceración, percolación, infusión, decocción o soxhlet), como no convencionales (Green methods: extracción asistida por microondas (MAE), extracción asistida por ultrasonido (UAE), extracción asistida por enzimas (EAE), extracción de líquido a presión (PLE), extracción de fluidos supercríticos (SFE) entre otros [38].

Las técnicas no-convencionales son más respetuosas con el medio ambiente y han mostrado ser efectivas y adecuadas para la extracción de compuestos antioxidantes de plantas y alimentos con posterior aplicación en la industria farmacéutica o alimentaria (aditivos alimentarios). Entre las diferentes ventajas, figuran: la reducción del tiempo de extracción y del uso de disolventes orgánicos, menor consumo de energía y un mayor porcentaje de rendimiento. Sin embargo, el alto coste económico de los equipos y establecer los diferentes parámetros para una adecuada extracción son las principales desventajas de los métodos no convencionales [39,40].

Por otro lado, los métodos convencionales se continúan usando ampliamente por su facilidad de ejecución, y no demandan la utilización de equipamiento complejo, pero requieren la utilización de grandes volúmenes de disolventes, aumento del tiempo y los rendimientos son inferiores a los métodos asistidos [38].

Aplicaciones extractos obtenidos de semillas de cáñamo

Durante los últimos años existe un creciente interés por conocer los beneficios de las semillas de cáñamo y están aumentando las investigaciones sobre las acciones farmacológicas que presentan los diferentes extractos y fracciones obtenidas de las semillas de Cannabis. A modo de resumen, lo representamos en la **Tabla 4**.

Tabla 4. Diferentes actividades de extractos obtenidos de semillas de cáñamo (*Cannabis sativa* L.)

Tipo extracto/fracción	Actividad Evaluada	Referencia
Acuoso	Reducción de colesterol plasmático total, LDL, actividad anti-aterosclerótica en ratones ApoE KO	Seo *et al.* (2012)
Ethanol 95% Fluidos supercríticos (CO2), semillas descascaradas	Protección celular mediante la estimulación génica de enzimas antioxidantes (SOD, GPx, CAT) en células HepG2	Hong *et al.* (2015)
Etanólico (80%) semillas y brotes	Antioxidante celular en eritrocitos humanos, anti-hemolítica	Frassinetti *et al.* (2018)
Metanol (80%) aceite Metanol (80%) semillas	Sin actividad anti-proliferativa en células CaCo2 y HT-29	Moccia *et al*. (2019
Etanol (70%)	Anti-neuroinflamatoria en daño neuronal inducido por LPS	Zhou *et al*. (2018)
Fracción-Acetato de etilo	Anti-inflamatoria vía reducción de secreción y expresión génica de citoquinas pro-inflamatorias in monocitos primarios humanos MAOs inhibición, incremento de aminas biogénicas (DA) en modelo animal	Rea *et al.(*2020) Rea *et al*.(2020)
Fracción-Metanol	Inhibición de la proliferación de células cancerosas U-87	Nigro *et al.* (2020)
Etanol (80%) Semillas	Acción inhibitoria selectiva frente a cepas bacterianas patógenas, actividad anti-biofilm sobre *S. aureus*	Frassinetti *et al*. (2020)

Con respecto a su seguridad, algunos estudios recomiendan evitar el consumo de suplementos a base de semillas de cáñamo, en mujeres embarazadas y madres lactantes, por los efectos nocivos mostrados sobre la función reproductiva y neuroconductual observada en un estudio en animales (ratas en gestación y lactancia) que fueron sometidas a dietas con un contenido 50% semillas de cáñamo y otra dieta completamente a base de semillas de cáñamo, por la pérdida de peso en madres y crías [46].

I.1.2. Inflamación, generalidades

La inflamación incumbe a la respuesta biológica del sistema inmune, activándose por la presencia de una variedad de factores, como pueden ser patógenos, células deterioradas, o la exposición a compuestos tóxicos, los mismos que desatan respuestas inflamatorias agudas o crónicas en diferentes órganos como: cerebro, corazón, tracto digestivo, pulmones, hígado, riñones, aparato reproductivo, provocando daños y enfermedades en los tejidos. Los agentes infecciosos, como también el daño celular, activan las células inflamatorias, desencadenando la respuesta inflamatoria a través de tres principales vías NF-kB, MAPK and JAK-STAT, que cumplen un rol fundamental en la inflamación [47]. Independientemente del tipo de estímulo y la localización del tejido, la respuesta inflamatoria responde a mecanismos comunes: I) Respuesta de la superficie celular a agentes extraños, II) Activación de las vías inflamatorias, III) Generación de marcadores inflamatorios y IV) Acumulación de células inflamatorias.

I.1.2.1. Activación del receptor de reconocimiento de patrones microbianos

La respuesta inflamatoria se inicia por la detección temprana de diferentes patrones moleculares asociados a patógenos (PAMP), patrones moleculares asociados a daños (DAMP), desencadenando la respuesta inflamatoria por medio de los receptores de reconocimiento de patrones (PRR) del huésped, que son los principales receptores para PAMP y DAMP. Los receptores expresados en células inmunes y no inmunes detectan los productos microbianos o endógenos liberados por parte de las células dañadas, desencadenando la respuesta y activando las vías de transducción de señales intracelulares. PRR actúan como receptores innatos además de unir y formar respuestas innatas y adaptativas [48,49]. Han sido ampliamente investigados y documentados en enfermedades humanas. PRR se clasifican en tres familias: I) Receptores endociticos - superficies celulares (receptor CD14 del lipopolisacárido anclado al glucosilfosfatidilinositol - GPI, receptor captador – SR, receptor de lectina de tipo C – CLR); II) Receptores de señalización, involucradas en la activación como respuesta a restos microbianos, proteínas, glicanos, ácidos nucleicos, (receptores tipo Toll – TLRs, receptores de tipo NOD - NLR, helicasas similares RIG1 - RLH); (III) Moléculas puente solubles (opsoninas) ejecutan el reconocimiento y eliminan sus ligandos por medio de fagocitos [50].

I.1.2.2. Mediadores inflamatorios

La inflamación es la respuesta por parte del cuerpo a estímulos externos o agentes infecciosos, químicos o fisiológicos, liberando y produciendo citoquinas proinflamatorias como, IL-1β, IL-6, TNF-α, quimiocinas, factores de crecimiento,

proteínas y enzimas inflamatorias, para contrarrestar el daño en los diferentes tejidos a través de células inmunes como neutrófilos, macrófagos y linfocitos. Durante los últimos años se han estudiado muchos marcadores inflamatorios y su asociación a enfermedades cardiovasculares, cáncer, HIV, diabetes, Parkinson, Alzheimer, entre otras [51,52].

I.1.2.3. Citoquinas Inflamatorias

Las citoquinas son sustancias liberadas por parte del sistema inmune, favoreciendo la cicatrización y reparación de tejidos, siendo esenciales en la defensa del huésped contra patógenos. Aunque pueden ser secretadas por células inmunes donde se incluyen monocitos, macrófagos o linfocitos, también pueden producirse por una amplia variedad de células como con los interferones en respuesta a la inflamación. Las mismas que actúan a través de varias vías de señalización que modulan el metabolismo celular y programación transcripcional. Las citoquinas, controlan el crecimiento, desarrollo, supervivencia y la programación de células en diferentes tejidos para ejecutar respuestas específicas. Su papel de respuesta a infecciones de forma controlada es beneficioso para evitar daños celulares; por otra parte, su producción exagerada puede conducir a cambiar la estabilidad hemodinámica, daños en tejidos, órganos, incluso la muerte. Las citoquinas pueden ser pro-inflamatorias (IL-1, IL-2, IL6, IL-7), anti-inflamatorias (IL-4, IL-10, IL-13), factores estimulantes de colonias (CSF), interferones (IFNs), factores de crecimiento (TGFs), necrosis tumoral (TNFs) y quimiocinas [47,53,54] (**Tabla 5).**

Tabla 5. Citoquinas y sus funciones

Citoquina	Fuentes principales	Función
IL-1β	Macrófagos, monocitos	Pro-inflamatoria, apoptosis, diferenciación
IL-2	Células T-CD4	Estimulación crecimiento, proliferación linfocitos T y células B
IL-3	Células T, macrófagos, mastocitos, eosinofilos	Crecimiento, diferenciación, supervivencia
IL-4	Células T- CD4	Anti-inflamatoria, proliferación células T y B, diferenciación células B
IL-6	Macrófagos, monocitos, hepatocitos, fibroblastos	Pro-inflamatoria, fase aguda proliferación, diferenciación, madurez de neutrófilos, macrófagos
IL-8	Macrófagos, células epiteliales y endoteliales	Pro-inflamatorias, angiogénesis, quimiotaxis
IL-10	Células T, B, otras	Disminución expresión MHC clase II, disminución de la presentación del antígeno, Inhibición citoquinas pro-inflamatorias
IL-11	Fibroblastos, células epiteliales, neuronas, osteoblastos	Anti-inflamatoria, proliferación, inducción de proteínas de fase aguda
IL-12	Macrófagos, células dendríticas, células B	Pro-inflamación, diferenciación celular, proliferación, citotoxicidad
IL-13	Células T- CD4, mastocitos, basófilos	Anti-inflamatoria, inhibición NO y citoquinas, proliferación, incremento IgE
IL-15	Varias células	Proliferación, supervivencia, activación
IL-17A	Linfocitos T- CD4, células T CD8	Pro-inflamación, formación quimiocinas
TNF-α	macrófagos, monocitos células NK, linfocitos	Pro-inflamación, producción citoquinas, inductor metabolismo muscular, coagulación, apoptosis

IFN-γ	células T, NK, NKT	Activación, incremento expresión MHC clase II, inmunidad adaptativa antiviral
TGF-β	células T, macrófagos	inhibición producción citoquinas pro-inflamatorias, NO, anti-inflamatoria
GM-CSF	macrófagos, fibroblastos, células T, endotelio	Crecimiento, supervivencia, activación macrófagos, incrementa la función de monocitos y neutrófilos

Adaptado de Barros de Oliveira, C. *et al* (2011); Chen, L. *et al* (2018); O´Shea, J. *et al* (2019).

I.1.2.4. Proteínas y enzimas antiinflamatorias

El efecto de las interleucinas sobre células hepáticas estimula la producción de ciertas proteínas inflamatorias en sangre, donde en condiciones normales su concentración en suero son basales. De acuerdo a su grado de elevación como respuesta inflamatoria tenemos: I) proteínas que sus niveles incrementan entre 1.5 - 5 veces, como fibrinógeno, haptoglobina, complemento componente C3, proteína de unión a manosa (MBP); II) proteínas con incrementos que oscilan incrementos entre 100 - 1000 veces, como proteína C reactiva (CRP), suero amiloide A (SAA). Estas proteínas inflamatorias agudas aumentas sus niveles en los sitios de infección o inflamación dentro de 24 – 72 H del daño tisular, ayudando a fagocitos a la identificación de células dañadas o patógenos para su eliminación [55,56]. También hay una activación de enzimas antioxidantes como: Superoxido dismutasas (SOD), Glutation peroxidasa (GPx), Glutation reductasa (GSR), tiorredoxina reductasa (TXNRD), peroxiredoxina (PRDX), hemo oxigenasa (HO), NADPH oxidase (NOX), óxido nítrico sintasa inducible (iNOS)y ciclooxigenasa (COX)-2 que se activan como mecanismos de respuesta inmune innata, frente a estímulos biológicos, químicos o físicos. Varios componentes de alimentos y productos obtenidos de plantas, han

mostrado incrementar los niveles de expresión de ciertas enzimas, interrumpiendo la traducción de señales en los procesos inflamatorios, manteniendo el balance redox y protegiendo del stress oxidativo, la inducción de la expresión de ciertas enzimas antioxidantes se activan vía diferentes factores de transcripción como Nrf2 [57,58,59].

I.1.3. Neuroinflamación

Aunque la inflamación se resalta como un proceso beneficioso para la eliminación de patógenos, respuesta a daños y compuestos tóxicos, el prolongado proceso inflamatorio conlleva a enfermedades crónicas que se traducen en un daño o fallo crónico del órgano afectado (corazón, páncreas, hígado, riñón, cerebro, tracto intestinal, etc.). El proceso inflamatorio resulta un elemento clave para conocer la progresión de la enfermedad, por lo tanto,el control del proceso inflamatorio, mediante el uso de agentes antiinflamatorios, resultan esenciales para controlar y limitar su progresión [47,55]. En el SNC la respuesta inflamatoria ocurre en muchas enfermedades neurodegenerativas y autoinmunes, la respuesta inflamatoria a nivel cerebral aumentan la excitabilidad neuronal, daño celular y aumentan la permeabilidad de la barrera hematoencefálica permitiendo el paso de sustancias tóxicas a su interior. La neuroinflamación involucra a todas las células presentes en el SNC (neuronas, macroglía, microglía). La activación de las células microgliales cumplen un rol importante en la respuesta inmune e inflamación, activando vías pro-apoptóticas mediadas por moléculas de señalización. Las más comunes en neuroinflamación NF-κB, MAPK y JAk-STAT inician y regulan los procesos inflamatorios en diferentes enfermedades neurodegenerativas [47,60].

En enfermedades del SNC asociadas a la inflamación como Parkinson (PD), Alzheimer (AD), Esclerosis múltiple (MS), Huntington (HD), Esclerosis lateral amiotrófica (ALS), desordenes psiquiátricos como estrés, depresión, esquizofrenia y metabólicos, la activación de células residentes del cerebro y microglia, producen la activación de varios genes y proteínas: iNOS, citoquinas proinflamatorias (IL-1β, TNF-α, COXs, ROS) y compuestos neurotóxicos que provocan el malfuncionamiento y muerte celular, en procesos crónicos la liberación de citoquinas y compuestos neurotóxicos permanecen activas durante largos periodos, afectando a las neuronas [60]. **(Figura 4)**

Si bien las citoquinas proporcionan la capacidad para que las células puedan comunicarse entre sí, y desempeñen un papel importante en procesos de homeostasis, como primer paso para controlar el desequilibrio o reparar el daño del tejido, la evidencia apunta a que la liberación descontrolada llega a afectar y asociarse con condiciones patológicas del SNC, llegando a atrofiar y agotar las células propias del cerebro [61].

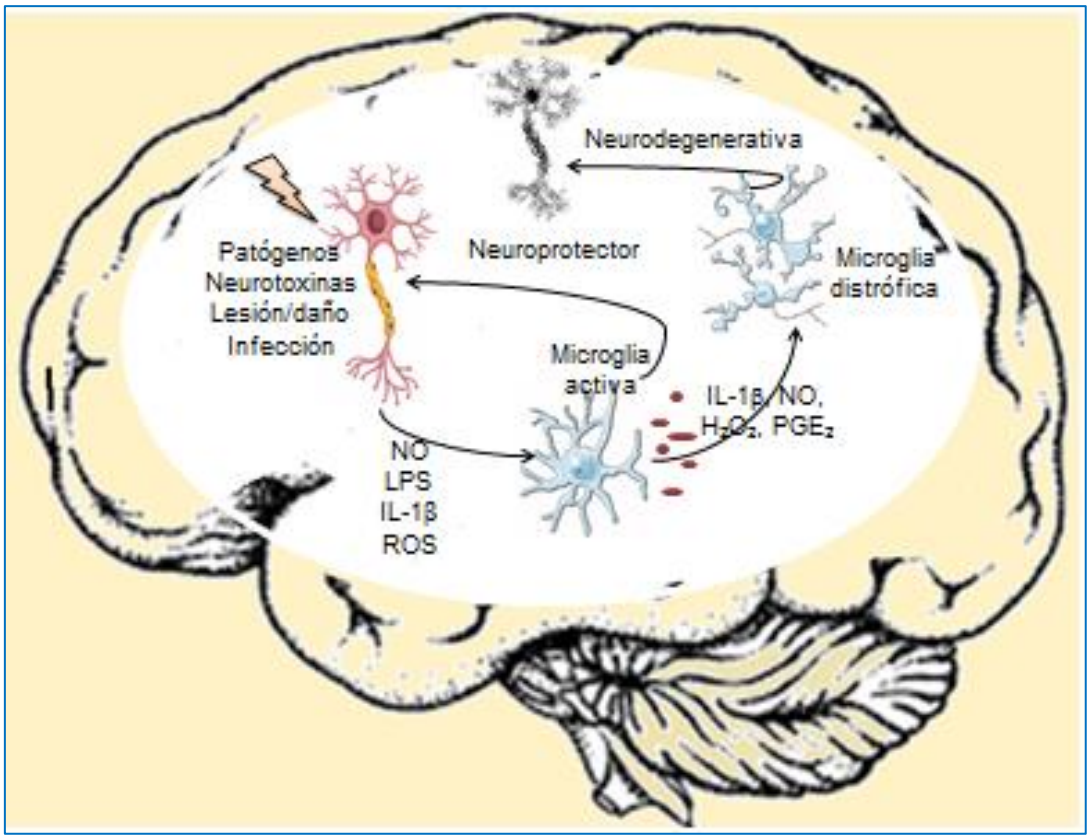


Figura 4. Activación de microglía y muerte neuronal. Microglia responde a patógenos, toxinas activándose y cambiando su morfología, fagocitando y secretando citoquinas y proteínas: en un proceso óptimo ejerce su función neuroprotectora eliminando patógenos, toxinas restableciendo la homeostasis. En daños crónicos la liberación prolongada ejerce un papel neurodegenerativo, destruyendo las células neuronales

Como se menciona, las células residentes del SNC (microglía), en un papel descontrolado pueden matar las células neuronales provocando la neurodegeneración, por lo que células microgliales cumplen el papel de macrófagos en el SNC, al encontrarse muertas o dañadas no pueden cumplir su rol como neuroprotectoras facilitando el proceso de fagocitosis, liberando sustancias dañinas y patógenos, por lo que un proceso activo y balanceado es necesario para evitar mantener la homeostasis y liberar el exceso de proteínas dañinas en el cerebro, previniendo el posterior desarrollo de la neurodegeneración [60].

Citoquinas y TLRs son los mayores mediadores inflamatorios. En el SNC la respuesta inflamatoria también puede desencadenarse por ligandos exógenos o endógenos que son reconocidos por los TLRs capaces de reconocer una amplia gama de patrones y moléculas derivadas de patógenos, iniciando el proceso inflamatorio, activando moléculas como NF-kB y otros factores de transcripción, promoviendo la fagocitosis microglial, liberando citoquinas y moléculas co-estimuladoras para generar la respuesta inmune adaptativa. TLRs son receptores de reconocimiento de patrones (PRR) claves en el reconocimiento de patrones moleculares asociados a patógenos (PAMPs) como también en el reconocimiento de patrones moleculares asociados a daños (DAMPs). Son expresados por varias células presentes en el SNC como neuronas. 13 TLRs han sido descritos en mamíferos mientras unos se expresan en células cerebrales: microglía, astrocitos, neuronas, otras están ausentes. TLR1, TLR2 y TLR4 se expresan en la superficie celular de la microglía, mientras TLR3, TLR7 y TLR 8 se expresan intracelularmente en microglía (endosomas), si bien la activación de TLR3 frente a un estímulo viral y otros TLRs producen la liberación de citoquinas, quimiocinas e IFN pro-inflamatorias. Su liberación al espacio extracelular resulta letal para las neuronas y oligodendrocitos por su papel en la síntesis de mielina. Otros factores de crecimiento liberados y moléculas antiinflamatorias como IL-10 liberadas por la microglía resultan beneficiosas para la supervivencia de las neuronas. Por otro lado la activación de TLR4 por β-amiloide produce apoptosis en neuronas por activación de quinasas JNK y caspasa 3 (CASP3) promoviendo la AD [60,62].

I.1.3.1. Neuroinflamación y neurodegeneración

Por definición propia, la neurodegeneración altera las propiedades del SNC, afectando la función neuronal, estructura y supervivencia de las neuronas, dando inicio a su degeneración que termina con la reducción del tamaño cerebral, como ocurre en AD. Al contrario de células pertenecientes a otras partes del cuerpo, estas no pueden regenerarse, después del daño producido por patógenos, isquemia o lesiones, sin que hasta la fecha ningún tipo de tratamiento existente pueda mejorar el curso natural de la enfermedad [60,63]. En enfermedades neurodegenerativas como PD, AD, MS y otros trastornos psiquiátricos, un anormal funcionamiento de las células de microglía puede desencadenarlas y el envejecimiento de las células microgliales que ocurre por la edad, acelera más aún el proceso. Junto con la inflamación cerebral que provocan algunas patologías como meningitis o traumas cerebrales, en etapas iniciales se presenta una pérdida de la población de células neuronales, encargadas de la comunicación a través de señales (sinapsis).

Entre las mayores enfermedades neurodegenerativas se incluyen: Alzheimer (AD), demencia lobular frontotemporal (FTLD), Parkinson (PD) y esclerosis lateral amiotrófica (ALS), aunque individuos que padecen esclerosis múltiple (MS) en las fases agudas, presentan el riesgo de desarrollar el curso neurodegenerativo [63]. Por tanto neuroinflamación y neurodegeneración comparten la misma respuesta inmediata mediada por las células gliales (astrocitos y microglía) que desencadenan una elevada liberación de citoquinas, pero la principal diferencia radica que en enfermedades infecciosas agudas como, encefalitis (viral o bacteriana) y esclerosis múltiple, además de las citoquinas liberadas por las células naturales del cerebro, existe una

invasión de leucocitos del parénquima del SNC y perdida de la integridad de la barrera hematoencefálica. Los leucocitos que invaden el SNC son los principales liberadores de mediadores inflamatorios y citoquinas, con cambios tisulares más drásticos, opuestos a los cambios progresivos de la función tisular y homeostasis observados en neurodegeneración, donde la producción de citoquinas para para restaurar el desequilibrio homeostático puede ser por una proteína plegada anormal, como en caso de AD y ALS [61]. **(Figura 5)**

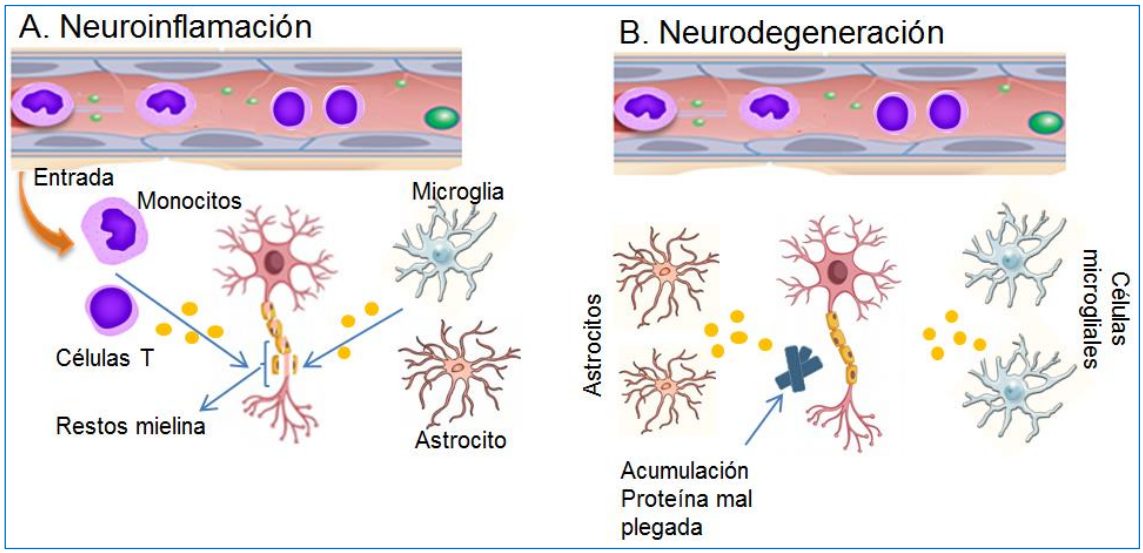


Figura 5. Neuroinflamación y Neurodegeneración. A) Neuroinflamación.- En enfermedades desmielinizantes como MS e infecciones virales o bacterianas, se caracteriza por la invasión de leucocitos del parénquima del SNC perdiendo integridad la Barrera hematoencefálica permitiendo su ingreso, donde linfocitos y células mieloides responden al daño tisular liberando citoquinas como IL-1β, IL-6 TNF, IL-23, IFNγ, GM-CSF. B) Neurodegeneración.- El desequilibrio homeostático y agregados proteínas anormales generan la activación de células estromales y microglía produciendo la liberación de citoquinas en respuesta al desequilibrio homeostático, existe una muy limitada intervención de leucocitos provenientes de los vasos sanguíneos.

I.1.3.2. Enfermedades Neurodegenerativas

Las enfermedades neurodegenerativas comparten varios aspectos comunes como: 1) La pérdida progresiva de la función neuronal 2) Plegamiento

incorrecto y acumulación de metaloproteinas 3) La inexistencia de una terapia que mejore o detenga el curso de la enfermedad [64].

Enfermedad de Alzheimer

La enfermedad de Alzheimer se caracteriza por una anormal acumulación de placas β-amiloide (extracelular) que produce la disminución de la sinapsis, generando una respuesta inflamatoria y posterior perdida del número de neuronas por desbalance homeostático. Las proteínas tau (intraneuronales) lleva forman ovillos neurofibrilares en regiones intraneuronales (NFT), daño de los micro túbulos, células dendríticas y degeneración de axones con posterior perdida de neuronas [65].

La acumulación extracelular de placas β-amiloide e intracelular NFT en diferentes regiones del cerebro en individuos de edad avanzada, conduce a la pérdida de memoria, cambios de humor, dificultad para manejarse en su entorno y confusión que continua con la pérdida progresiva de capacidades en periodos estimados de entre 10-20 años. Condiciones más tardías y graves presentan alteraciones cognitivas, perdida de respuesta al entorno, dificulta para hablar, y coordinar movimientos, afectando la calidad de vida de los pacientes con AD. Análisis estadísticos estiman que en pacientes diagnosticados con AD su esperanza de vida es alrededor de 8-12 años [64,65].

Enfermedad de Parkinson

La enfermedad de Parkinson se caracteriza por la pérdida progresiva de neuronas dopaminérgicas de la sustancia negra pars compacta siendo la segunda enfermedad neurodegenerativa más común, detrás del Alzheimer. Si

bien se desconocen los mecanismos patológicos de la enfermedad, es conocido que factores genéticos y ambientales contribuyen a la aparición de la PD. Una característica patológica es la presencia de inclusiones citoplasmáticas proteínicas intraneuronales llamadas cuerpos de Lewy, que incluyen α-sinucleína, ubiquitina y neurofilamentos. Si bien la mayoría de casos se presentan de forma esporádica (95%), solo un 5% demuestran una herencia mendeliana involucrando mutaciones en varias proteínas: α-sinucleína, LRRK2, PINK1, Parkin, DJ-1, VPS35, GBA1 [66]. Los síntomas característicos de la enfermedad son: la dificultad de movimiento (bradiquinesia), temblores en maños y antebrazos, dificultad para tragar y perdida de coordinación. La mayoría de pacientes también desarrollan depresión y demencia. La exposición a ciertos factores ambientales como pesticidas, químicos y metales incrementan el riesgo de desarrollar PD [66,67].

Aunque la investigación no cesa y diferentes terapias están siendo probadas en pacientes, es una característica de las enfermedades neurodegenerativas el no contar, en muchos casos, con una terapia que mejore los síntomas de la enfermedad. En PD el tratamiento está enfocado a paliar los síntomas. La levodopa es el fármaco más utilizado, permitiendo a las neurona producir dopamina [64].También se utilizan inhibidores de MAO-B en etapas tempranas de la enfermedad, donde todavía las neuronas son funcionales. La terapia puede ser sola o combinada con inhibidores MAO-B y agonistas dopaminérgicos [68].

Esclerosis Lateral Amiotrófica

La esclerosis lateral amiotrófica (ALS), es una enfermedad devastadora degenerativa que afecta el sistema de neuronas motoras, con pérdida progresiva de las mismas en la corteza, tronco encefálico y medula espinal, empezando de forma focal, pero se disemina causando parálisis y muerte. Se estima una mortalidad de alrededor de 30000 mil pacientes al año [69], con un tiempo de supervivencia desde su diagnóstico estimado entre 24 - 48 meses. Sólo un 10 - 20% de los pacientes tienen un periodo de supervivencia superiores a 10 años [70]. Alrededor del 10% de casos se relacionan con la mutación de diferentes genes. Alrededor de más de 40 genes están relacionas con ALS, desencadenando una alteración de la funcionalidad de mitocondria, excitotoxicidad, estrés del retículo endoplasmatico, transporte lipídico o transporte axonal [71].

Si bien no existen todavía marcadores biológicos para conocer la progresión de la enfermedad, como la mayoría de enfermedades neurodegenerativas no existen un amplio número de fármacos disponibles para su tratamiento, siendo el Riluzole el fármaco utilizado desde su aprobación en USA (1995) y Europa (1996). Aunque durante los últimos años un número superior a 60 moléculas se han ensayado como posibles tratamientos para ALS, la mayoría fracasa en los ensayos clínicos, sin embargo en los últimos años dos posibles tratamientos se perfilan como potencialmente eficaces (Mastinib y Edaravone) para su uso en ALS [69].

I.1.3.3. Antiinflamatorios naturales

Las enfermedades neurodegenerativas representan uno de los principales problemas de salud pública a nivel mundial, con una creciente causa de mortalidad y morbilidad, especialmente en personas de edad avanzada. Con

contadas moléculas aprobadas para su uso en enfermedades neurodegenerativas, el uso de productos naturales y sus metabolitos se ha incrementado a lo largo de los años, como posibles terapias y tratamientos para diferentes patologías del SNC, aunque no está exenta de preocupaciones, por la falta de apoyo científico o evidencia que respalde su uso y seguridad en el paciente [72]. Sin embargo el uso de formulaciones de plantas o fitocompuestos, pueden ser utilizados como una alternativa a los tratamientos convencionales que se utilizan en la terapia de las enfermedades neurodegenerativas, ya que muchos cumplen con las condiciones exigidas de eficacia y seguridad comparados con otros fármacos investigados, mostrando resultados prometedores en estudios *in vitro* e *in vivo*, al intervenir de forma positiva en varias vías de señalización que conllevan a un efecto neuroprotector. Estos fitofármacos resultarían efectivos por sus propiedades antioxidantes, antiinflamatorias o anticolinesterasicas. Compuestos como: terpenoides, derivados fenólicos, alcaloides, glucósidos y saponinas esteroidales han mostrado ser fitocompuestos con potencial terapéutico para su aplicación en enfermedades neurodegenerativas [73].

Aunque hasta el momento, diferentes terapias para contrarrestar el avance y progresión de enfermedades neurodegenerativas han fracasado, es una nueva vía de investigación el posible uso de productos naturales, como los fenilpropanoides, que por sus múltiples efectos positivos a nivel biológico son propuestos para su aplicación en AD [74].

Al igual que el uso de inhibidores de MAO y COMT son sugeridos como terapia para neurodegeneración crónica por la elevación de aminas biogénicas en la hendidura sináptica [75], diferentes productos de origen natural: los flavonoides

Quercetina [76] y Naringenina [77], otros fenoles de etiología diversa, alcaloides o antraquinonas [78], así como también diferentes extractos procedentes de plantas están siendo evaluados como inhibidores de MAOs [79].

Aunque los productos naturales son una fuente abundante de metabolitos que presentan diferentes actividades biológicas y aplicaciones para enfermedades neurodegenerativas, el principal obstáculo importante para el desarrollo y uso de fármacos es la barrera hematoencefálica, que mediante mecanismos anatómicos, fisicoquímicos y bioquímicos controlan el paso de diferentes moléculas entre el cerebro y la sangre, siendo prácticamente impermeable a diferentes compuestos que podrían aplicarse en este tipo de patologías del SNC [64], por lo que queda todavía mucho por investigar. Es fundamental aprender a caracterizar los diferentes compuestos que podrían tener un importante potencial, si lograsen alcanzar diferentes áreas del cerebro donde ejercerían su acción terapéutica.

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I.2.- OBJETIVOS

Cannabis sativa L. es una de las especies más conocida dentro de la familia Cannabaceae, por el enorme interés que despiertan sus principios activos, presentes en diferentes partes de la planta. Clasicamente han sido los cannabinoides los compuestos más estudiados, pero recientemente se han identificado nuevas moléculas no psicoactivas que tienen un alto potencial farmacológico.

Las semillas, cuyo interés radica principalmente en su aceite rico en ácidos grasos poliinsaturados, es también fuente de otros compuestos no grasos que constituyen el principal objetivo de esta Tesis Doctoral.

Por esta razón, los objetivos planteados han sido:

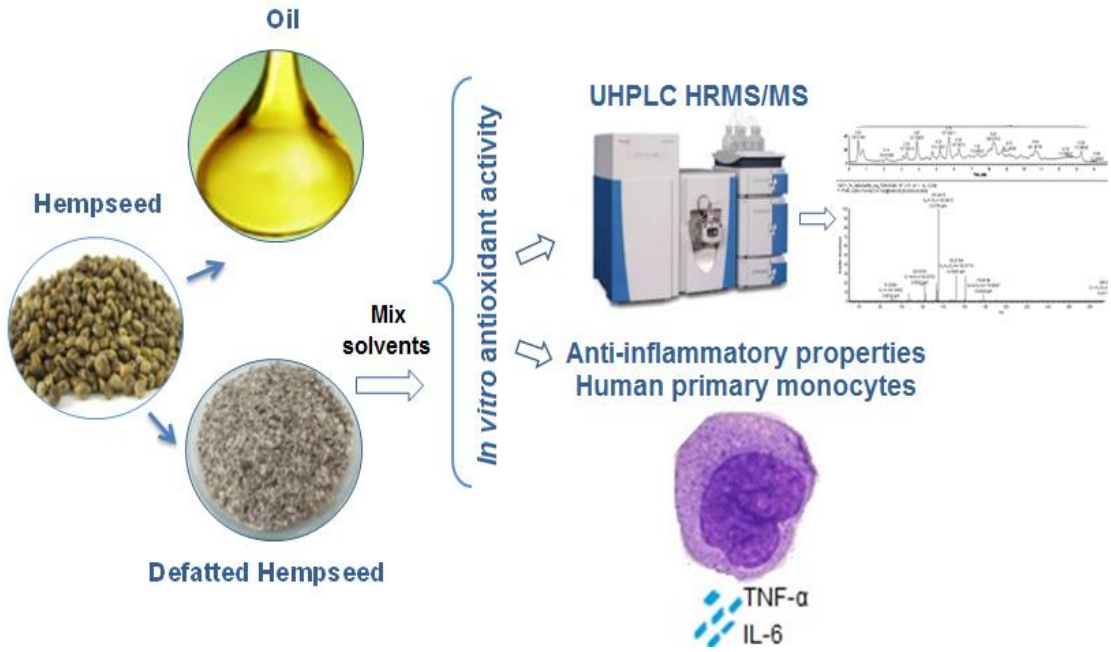
- Aislar nuevas moléculas con interés farmacológico utilizando diferentes técnicas de extracción, caracterización y cuantificación fitoquímica.

- Investigar el efecto de fracciones y moléculas aisladas sobre mecanismos implicados en el proceso inflamatorio.

- Estudiar la implicación sobre enzimas del sitema nervioso central, así como en aminas biógenas, relacionadas con los procesos neurodegenerativos.

CAPITULO II

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



Abstract

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.

II.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.

II.2.-MATERIALS AND METHODS

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, 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).

Plant Material and preparation of extracts

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

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 4°C prior to analysis.

***In vitro* antioxidant activity**

DPPH$^{\bullet}$ 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 4°C. 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.

$ABTS^{\bullet+}$ 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.

Quantification of total phenols and flavonoids content

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 1 mL adding 450 µL of distilled water and maintenance in a room temperature for 60 minutes. Finally, the absorbance was measured at 750 nm. 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.

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 at 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.

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 1 mL 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.

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.

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.

Immunostaining of human monocytes by FACS

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.

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.

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.

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.

II.3.- RESULTS AND DISCUSSION.

In vitro antioxidant activity

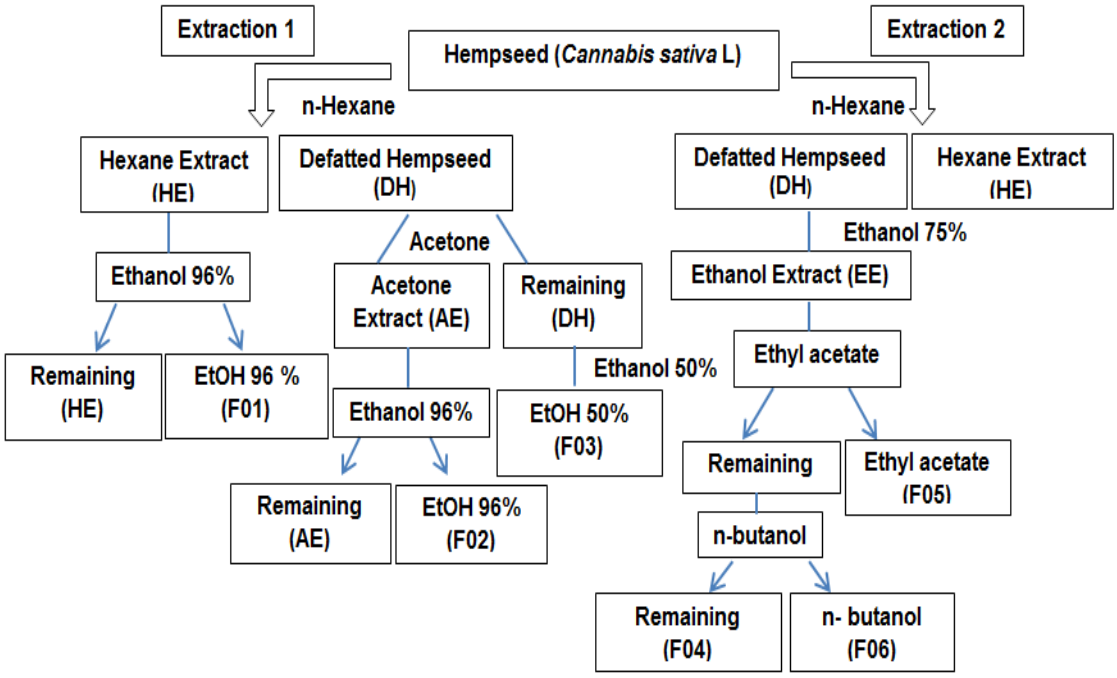


Fig. 1. Extraction procedures to obtain the different fractions from hempseed

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 have 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].

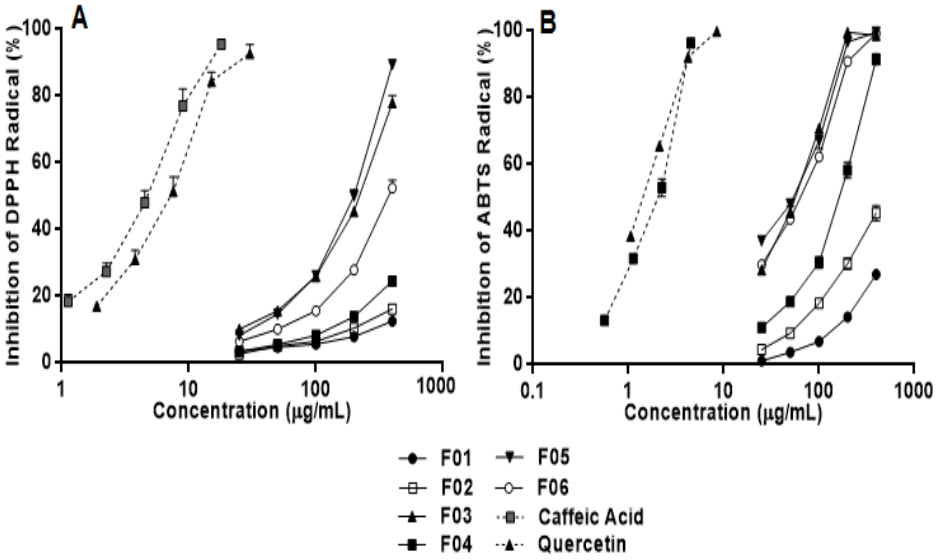


Fig. 2. DPPH and ABTS radical scavenging activity of Hempseed fractions (25 – 400 µg/mL): **(A)** inhibition of DPPH radical and **(B)** inhibition of ABTS radical. Caffeic acid and quercetin were used as reference standards at different concentration (0.5 – 30 µg/mL). Each value represents mean ± SEM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

Quantification of total phenols and flavonoids content

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 ($p < 0.05$**)**

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 have 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.

Phytochemical characterization and quantification by UHPLC HRMS/MS

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	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.

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 ± 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.

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 at m/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) has reported a high yield of flavonoids in fractions, characterizing some individual compounds [19].

Anti-inflammatory activity in primary human monocytes

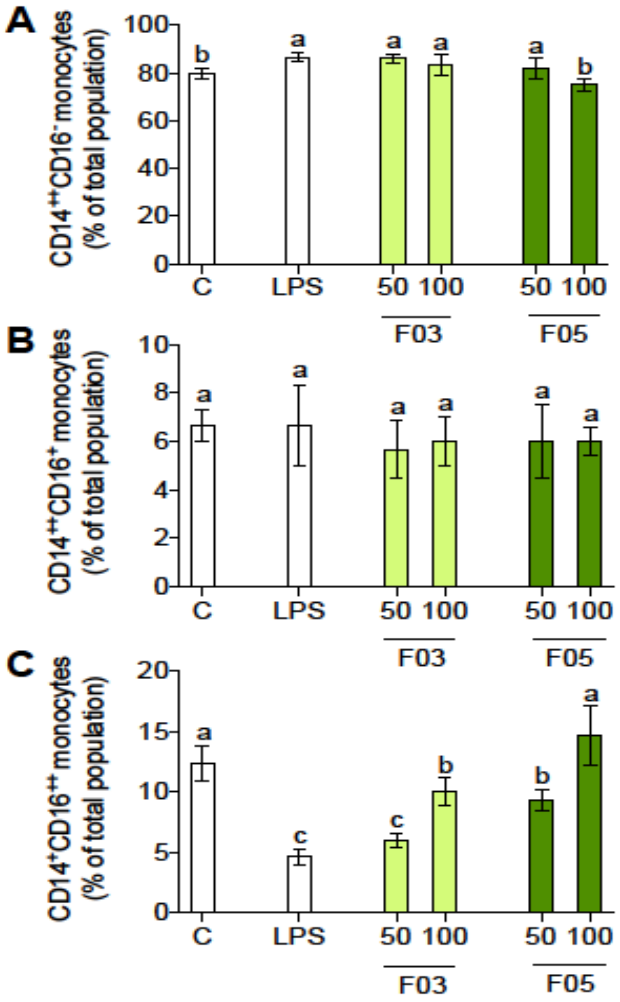


Fig. 3. Effect of F03 and F05 at 50 – 100 µg/mL for 24 h in LPS-treated human monocytes: (A) Classical monocytes, (B) intermediate monocytes and (C) non-classical monocytes. Values shown are the mean ± SD (n=3) and those marked with different lowercase letters are significantly different ($P < 0.05$)

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.

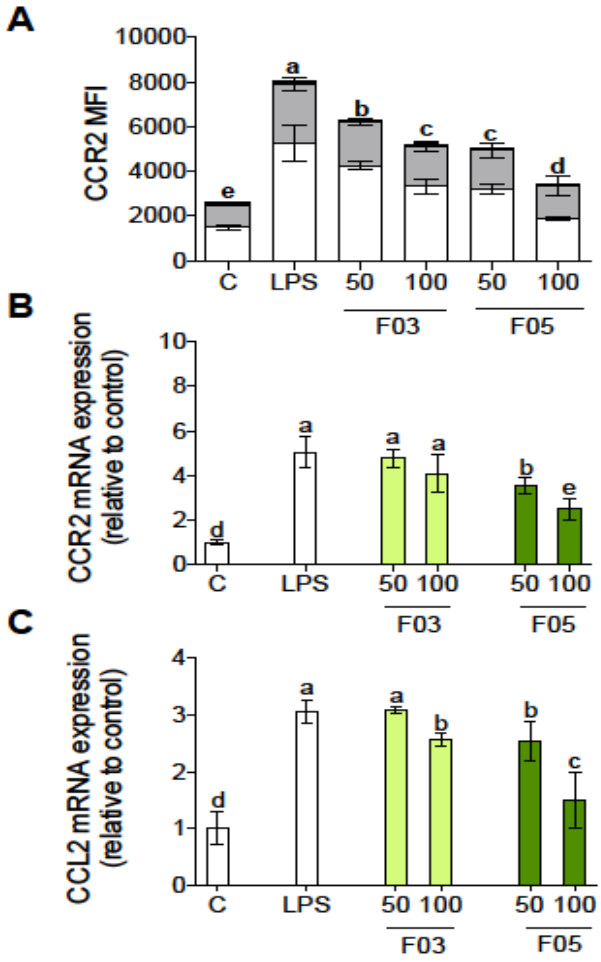


Fig. 1 Effect of F03 and F05 fraction at 50 - 100 µg/mL for 24 h in LPS-treated human monocytes (A) CCR2 protein expression on monocytes subset (classical monocytes). The gene expression of (B) CCR2, (C) CCL2 was measured by qPCR. Values shown are the mean ± SD (n=3) and those marked with different lowercase 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)**.

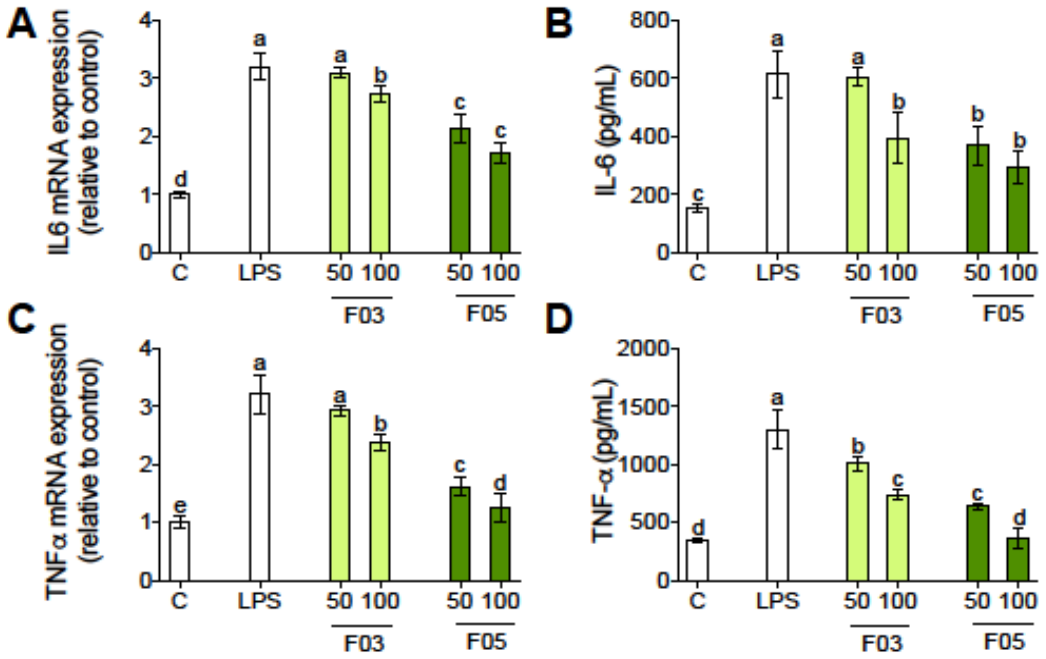


Fig. 2 Effect of F03 and F05 fraction at 50 - 100 µg/mL on secretion and gene expression of pro-inflammatory cytokines in LPS-treated human monocytes. Cells were untreated (Control) or treated with LPS (100 ng/mL) in the absence (LPS) or presence of F03 and F05 for 24 h. The expression of IL-6 (A) and 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 ($P < 0.05$).

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 the use of fractions can be a viable application.

However, establishing a correct dose 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.

II.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.

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II.6.-SUPPLEMENTARY MATERIAL

Table 1. Sequences of RT-qPCR primers for gene expression analysis.

Target	**GenBank accession number**	**Direction**	**Sequence (5'→3')**
TNFa	NM_000594	Forward	TCCTTCAGACACCCTCAACC
		Reverse	AGGCCCCAGTTTGAATTCTT
IL6	NM_000600	Forward	TACCCCCAGGAGAAGATTCC
		Reverse	TTTTCTGCCAGTGCCTCTTT
CCR2	NM_001123396.1	Forward	TGCCTGACTCACACTCAAGG
		Reverse	GGCTTCTCAGCAACTGAACC
CCL2	NM_002982.3	Forward	CCCCAGTCACCTGCTGTTAT
		Reverse	ACGAAGCCATTTGGTAAACG
GAPDH	NM_001289746	Forward	CACATGGCCTCCAAGGAGTAAG
		Reverse	CCAGCAGTGAGGGTCTCTCT
HPRT	NM_000194	Forward	ACCCCACGAAGTGTTGGATA
		Reverse	AAGCAGATGGCCACAGAACT

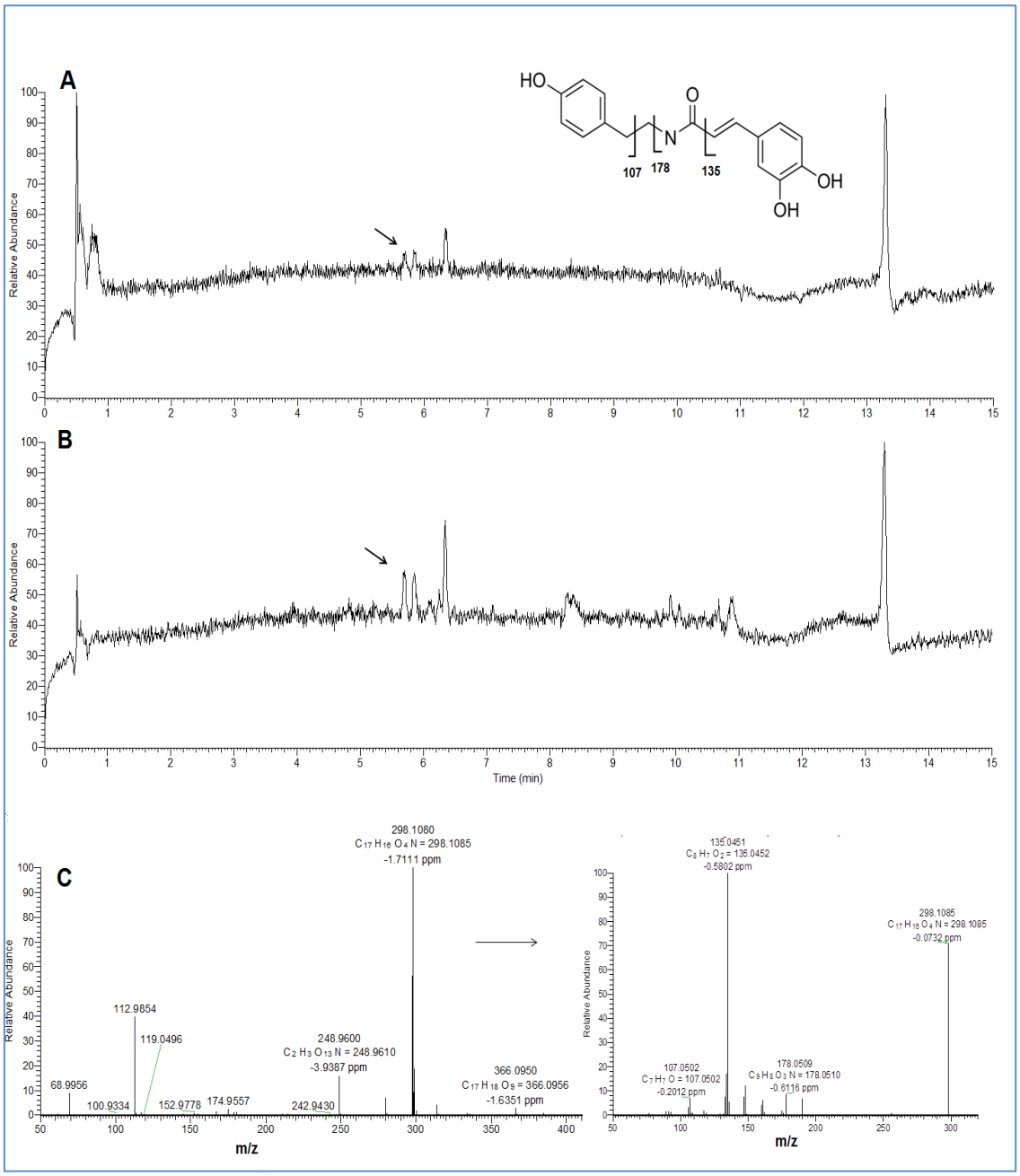


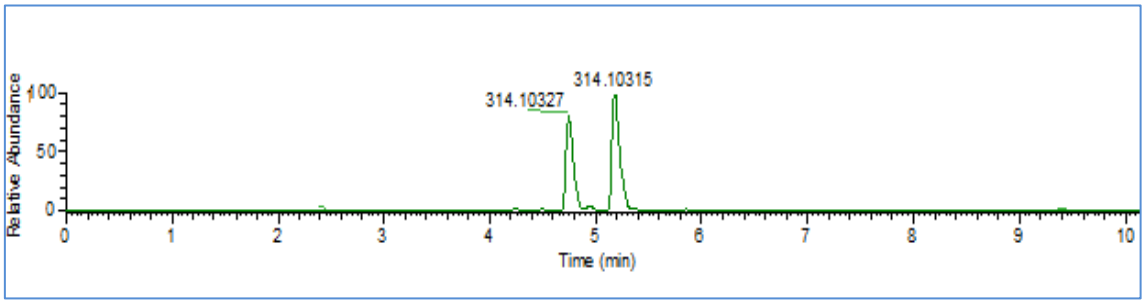
Fig 1. UHPLC HRMS/MS chromatograms (negative ion mode): (A) F03 fraction (B) F05 fraction; with arrow the peaks corresponding to *N-trans*-caffeoyltyramine and its MS and MS/MS chromatogram (C).

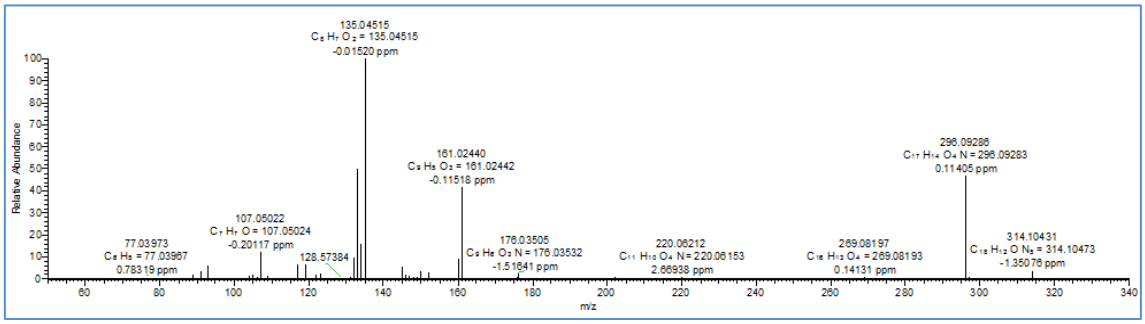
UHPLC HRMS/MS de fenilamidas, lignanamidas y compuestos mayoritarios identificadas tentativamente en la fracción de semillas de cáñamo investigada.

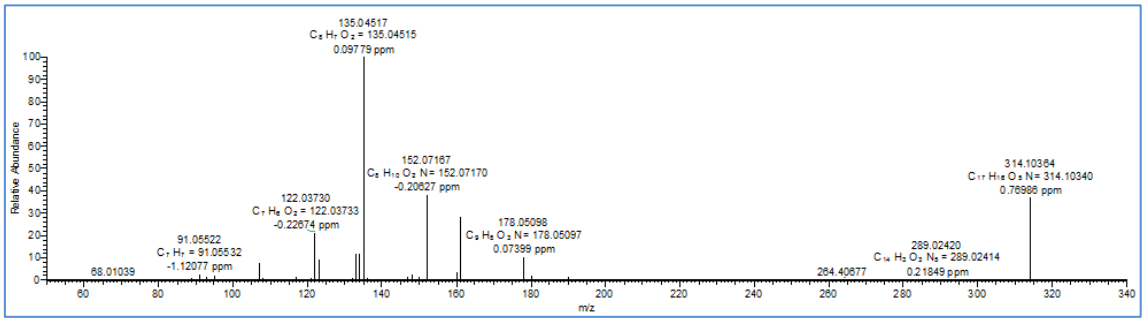
Caffeoyloctopamine isómeros

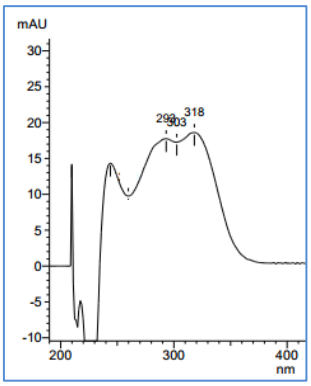
Rt: 4.81 min

Rt: 5.25 min



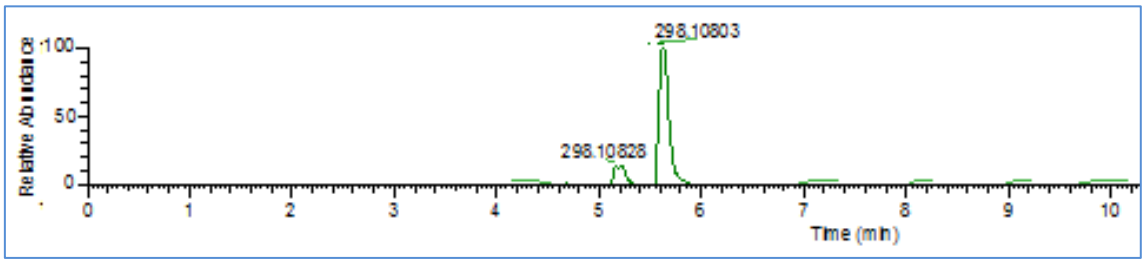


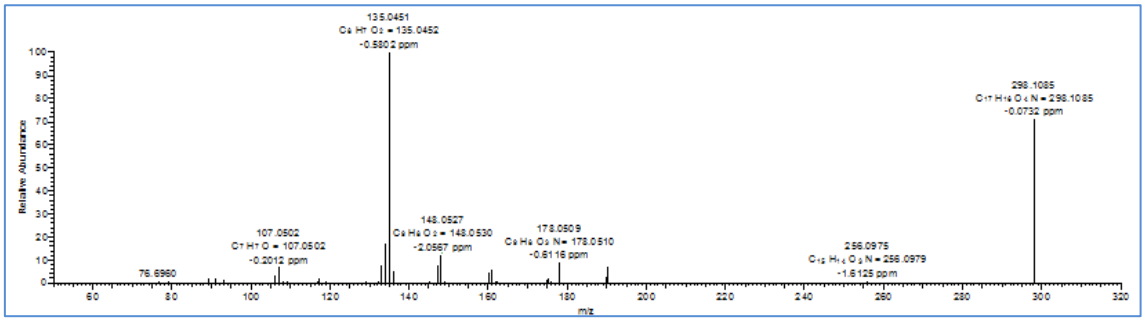


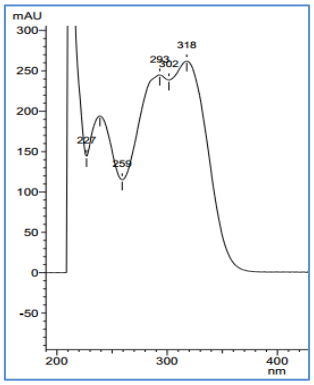


***N-trans* caffeoyltyramine**

Rt: 5.69 min

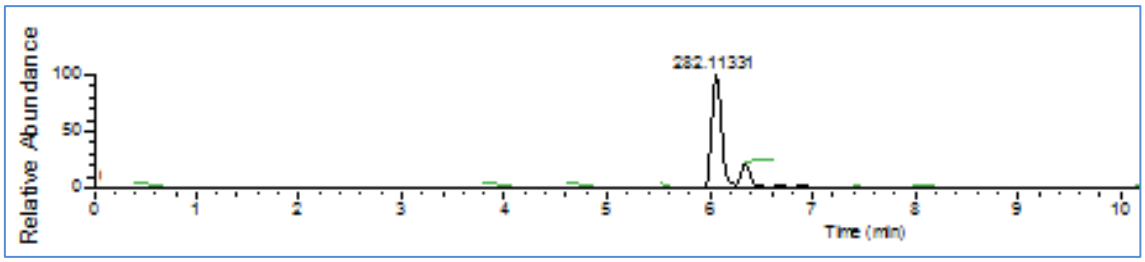


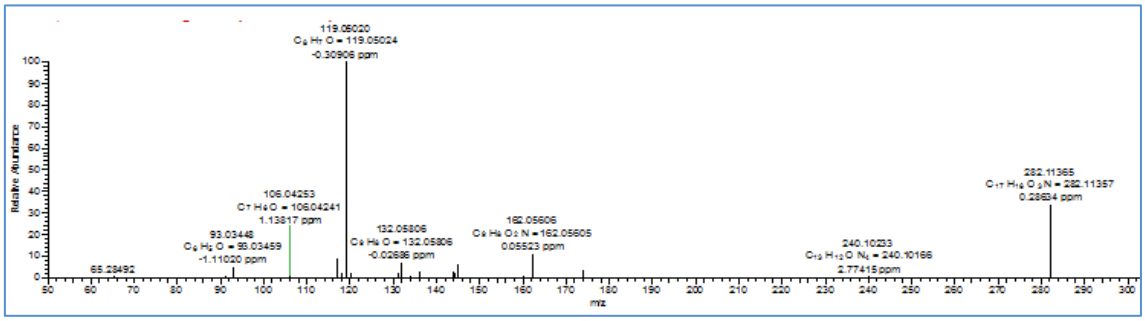


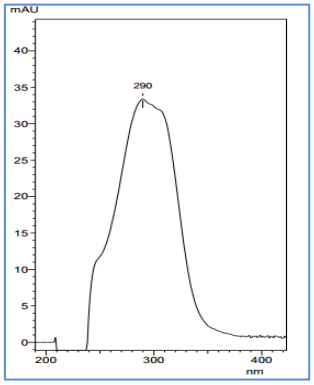


***N-trans* coumaroyltyramine**

Rt: 6.11

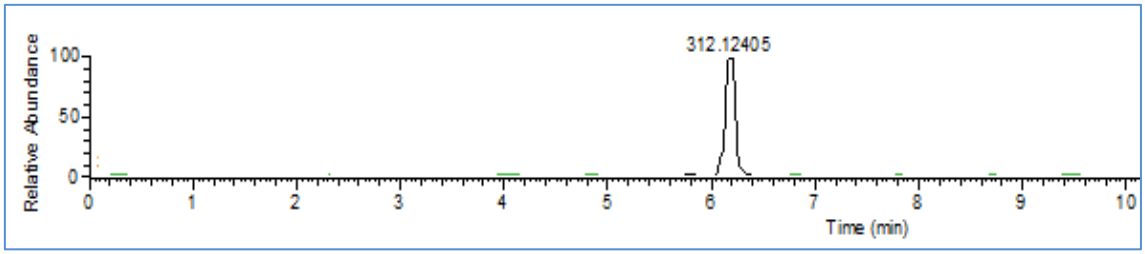


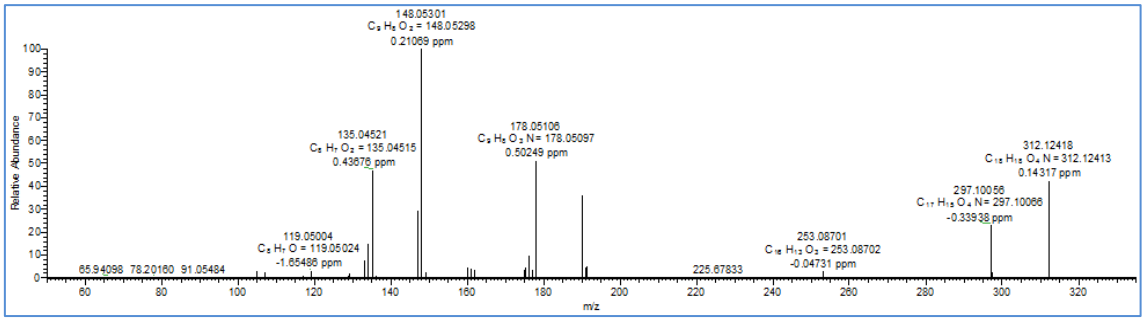


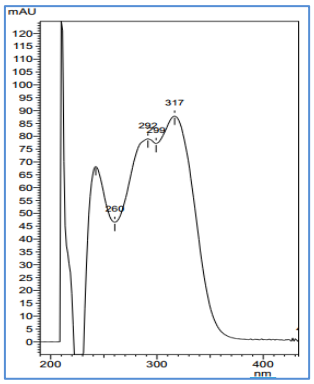


N-feruoyltyramine

Rt: 6.25 min

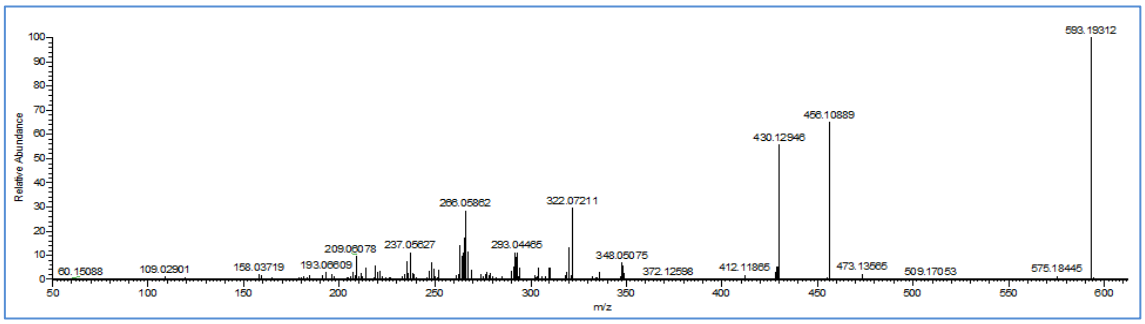


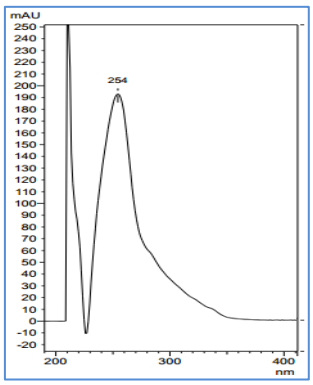




Cannabisin A

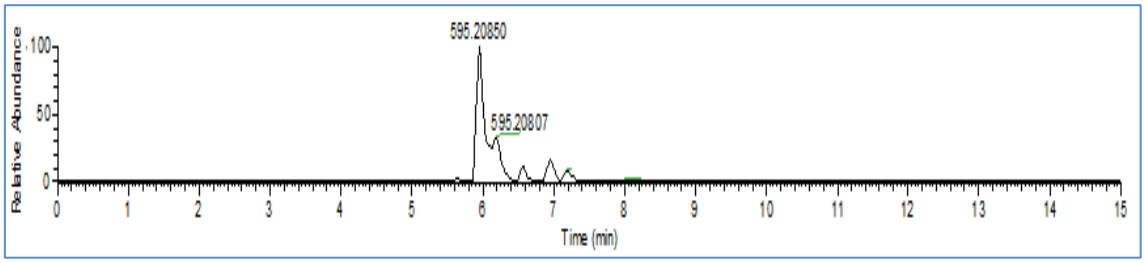
Rt: 6.05 min

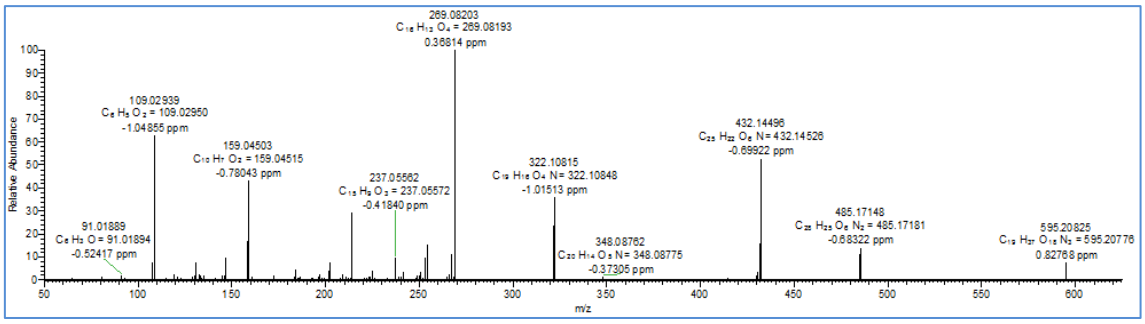


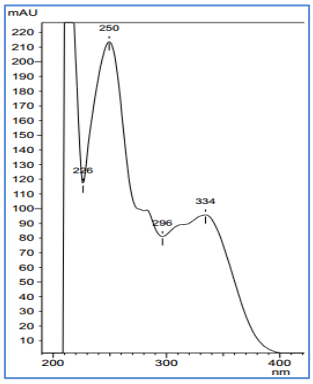


Cannabisin B

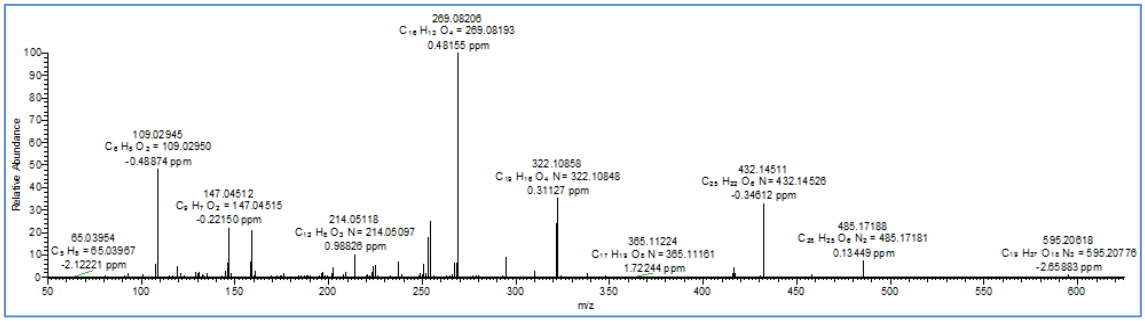
Rt: 6.01 min ; Isomero Rt: 6.18 min





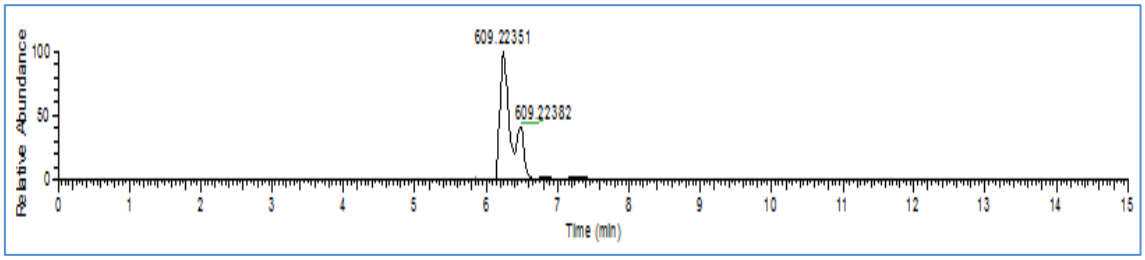


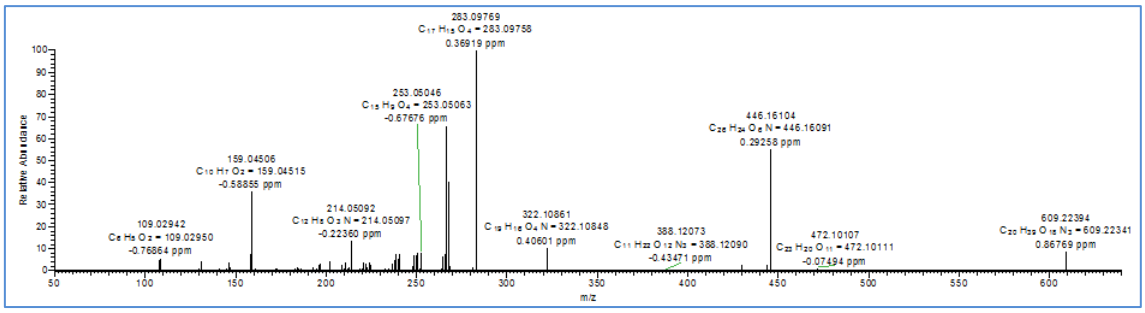
Cannabisin B isomer

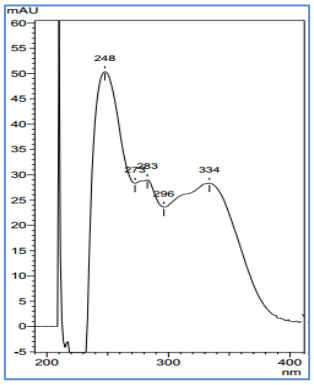


Cannabisin C

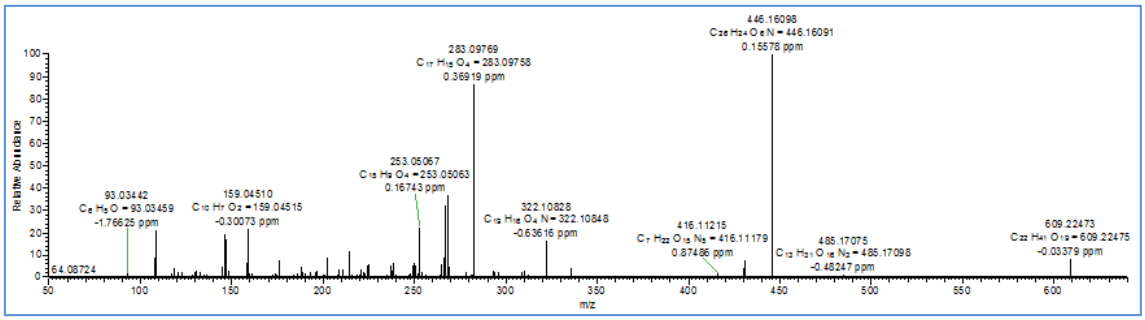
Rt: 6.29 min; Isomer Rt: 6.51 min





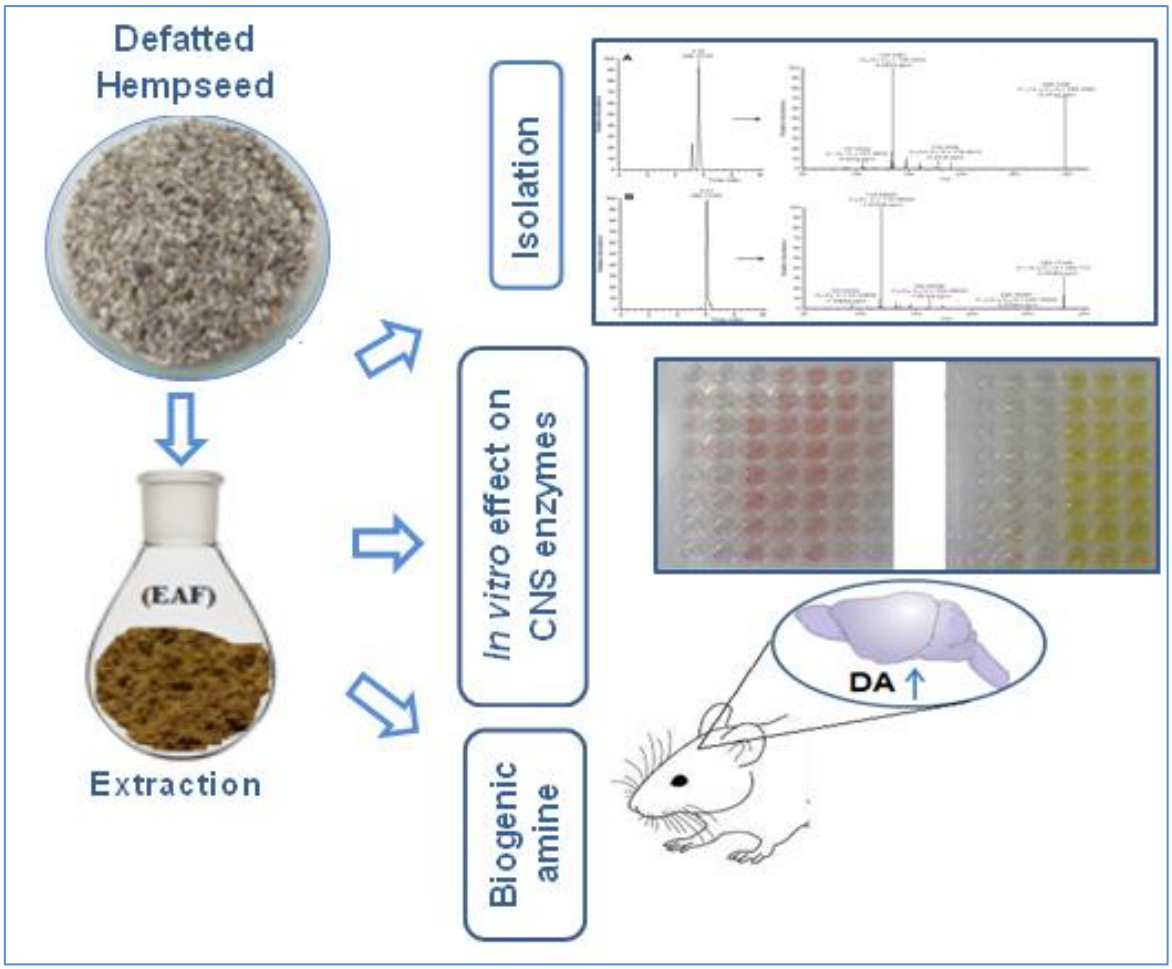


Cannabisin C Isomer



CAPITULO III

Hydroxycinnamic acid derivatives isolated from hempseed and their effects on central nervous system enzymes



Abstract

New neuroprotective treatments of natural origin are being investigated. Both, plant extracts and isolated compounds have shown bioactive effects. Hempseed is known for its composition of fatty acids, proteins, fiber, vitamins, as well as a large number of phytochemical compounds. After a defatting process of the seeds, hydroxycinnamic acids and its amine derivatives are the majoritarian compounds in an ethyl acetate fraction (EAF). In the present study, we investigated *in vitro* effect on neuronal enzymes: MAO-A, MAO-B, tyrosinase and acetylcholinesterase. Besides, the effect of EAF on striatal biogenic amines in mice was evaluated. Both, EAF and isolated compounds (*N-trans*-caffeoyltyramine and *N-trans*-coumaroyltyramine), showed inhibitory action on MAO-A, MAO-B and tyrosinase. Furthermore, an increasing of biogenic amines was observed in the corpus striatum of the mice, after administration of EAF. These findings show that EAF and the hydroxycinnamic acid derivatives may represent a potential treatment in degenerative neuronal diseases.

III.1.-INTRODUCTION

The incidence of neurodegenerative diseases has suffered a dramatic growth, mainly due to the increase in life expectancy. Neurodegenerative disorders are a common cause of cognitive impairment in elderly, affecting behavior and interfering in their activities of daily living. Diseases such as Parkinson's or Alzheimer's affect millions of people global. These chronic and progressive diseases lead to loss of neurons and therefore neurotransmitters in the central nervous system, damaging the brain function. Although its etiology is not fully known, it is recognized that there are common cellular and molecular mechanisms that are involved in to the progression of the disease. These include oxidative stress, mitochondrial dysfunction, protein folding (amyloid beta - Aβ, alpha synuclein - αS), excitotoxicity, dysregulation of calcium, homeostasis and inflammation [1,2,3]. Oxidative stress has been related with many chronic diseases, although moderate concentration of free radicals and oxidants species are involved in normal signaling process and defense mechanism, an imbalance produces cell damage, changes ADN, lipids and proteins and leads to the development of cancer, neurodegeneration, diabetes, cardiovascular, inflammatory and kidney diseases [4,5].

New neuroprotective treatments of natural origin are being investigated. Both, extracts of plant species and isolated compounds, have shown to possess bioactive effects, *in vitro* and in different animal model tests. The use of natural products may offer great opportunities in the prevention and therapy of neurodegenerative diseases [6]. Among the natural compounds, some phenolic compounds have demonstrated to reduce the risk of neurodegenerative diseases, due to their scavenging free radical capacity. It could be thought that

these compounds do not cross the membrane of the brain due to their polarity. In fact, their effect will depend on the degree of lipophilicity, polarity and molecular weight of each compound and less polar and lower molecular weight lipophilic compounds are easily permeable [7]. Although, studies have shown that ability to across blood brain barrier (BBB) will also depend on the interaction with specific transporters expressed in the BBB, such as P-glycoprotein, as seen in the flux of quercetin and naringenin into the brain [8]. These studies could demonstrate the ability of some phenolic compounds to reach brain areas, involved in neurodegenerative diseases.

Hempseed (*Cannabis sativa* L.) traditionally has been an important source of nutrition, for the high concentrations of PUFA´s (polyunsaturated fatty acids), protein, fiber , vitamins and minerals [9,10]. In the last years, the studies to identify the phytochemicals that remain in the waste after hempseed oil extraction process have increased significantly. The application and modification of different parameters: time, temperature, solvent ratio, mix solvents, ultrasonic treatment, microwave, pulse electric field (PEF) and use of cyclodextrins to improve the recovery of compounds specially phenols type in the extraction have been applied [11-14]. In addition, the evaluation of antioxidant activities [12,15,16], acetylcholinesterase inhibition [17], anti-neuroinflammatory activity [18,19,20], protection against cytotoxicity [21], arginase inhibition [22] and antiproliferative activity in HepG2 cells [23] have been investigated.

After a defatted process in hempseed, a high number of phytochemical compounds remain in the waste, these compounds could be recovered and evaluated in different biological activities. The aim of this work was to

investigate the effect of an ethyl acetate fraction (EAF) and two isolated phytochemical compounds on neuronal enzymes inhibition, which may lead to a neuroprotective activity. Monoamine oxidase A/B, tyrosinase and acetylcholinesterase inhibition were assayed, all of them are involved in metabolism and degradation of monoamines. Furthermore, the content of striatal biogenic amines in mice, after an i.p administration of EAF was measured. Inhibitors of these enzymes are proposed as a pharmacological treatment for neurodegenerative disorders such as Alzheimer´s (AD) and Parkinson´s diseases (PD).

III.2.-METHODS AND MATERIALS

Chemical and reagents

Folin-Ciocalteu´s phenol reagent, gallic acid, caffeic acid, vanillic acid, tyramine, 4-Aminoantipyrine, R-(-)- deprenyl hydrochloride, peroxidase from horseradish, clorgyline, L-Dopa- (phenyl-d3), tyrosinase from mushroom, kojic acid, Acetylcholinesterase (AChE) from electric eel, 5,5′-dithiobis(2-nitro- benzoic acid) (DTNB), and acetylthiocholine iodide (ATCI) were purchased from Sigma-Aldrich (St. Louis, MO, USA), methanol, formic acid LC-MS grade, rutin (MERCK), bovine serum albumin (BSA) were from PAA laboratories GmbH, physostigmine free base was from Tokyo Chemical Industry, protein assay from Bio-Rad (Munich-Germany).

Plant Material

Hempseed (Jarad-Seeds; batch: 250816) was commercially acquired in the province of Seville, Spain.

Extract Preparation

Hempseed (3kg) were crushed and defatted with n-hexane (3 times x 24 h). After filtration and evaporation of n-hexane, defatted seeds (2226 g) were extracted with 75% aqueous ethanol solution (1:3 ratio) in an ultrasonic bath (Ultrasons HD, JP Selecta) with a fixed power (180W) for twenty minutes at 45ºC with periodical stirring. The filtrate was concentrated under vacuum, until reduce the volume approx. 500 mL and was stored in 100 mL tubes at 4ºC for 48 hours. Then, the hydroalcoholic extract was extracted with ethyl acetate (four times), concentrate under vacuum, freeze dried and stored in a dark glass bottles at 4ºC prior to analysis. The residue obtained from the ethyl acetate fraction (EAF) was 14.7 g (0.66%).

Total phenolic content

Total phenolic assay was determined by the Folin-Ciocalteu´s method [24] with slight modifications for a microplate reader [25]. Briefly, 50 µL of extract was mixed with 1.25 mL Folin-Ciocalteu´s reagent (1:10) and 1 mL of sodium carbonate saturated solution (7.5%). After 30 minutes, the absorbance was measured at 750 nm. A standard curve was prepared using gallic acid. The results were expressed as mg gallic acid equivalent (GAE) per 100 mg extract.

Total Flavonoids

Total flavonoids content was determined according to Gouveia and Castillo (2011) [25]. In brief, to 500 µL of sample 4.3 mL of water was added, followed by 0.1 mL of sodium acetate (1 M) and 0.1 mL of aluminum chloride solution (10%) and incubated for 30 min at room temperature. Absorbance was read at 405 nm in a microplate reader. Rutin was used as a standard. The results were expressed as mg rutin equivalents (RE) per 100 mg of extract.

Free Hydroxycinnamic acids

The total free hydroxycinnamic acids were quantified following the method described by Matkowski et al. (2008) [26], with slight modifications to reduce reagents volume. 0.2 mL of sample (5 mg/mL) was mixed with 0.4 mL HCl (0.5 M), followed of 0.4 mL Arnow reagent ($NaNO_2$ and Na_2Mo_4 aqueous solution 10%, w/v) and 0.4 mL NaOH (1 M) followed by addition of distilled water until complete 2 mL. The absorbance was read at 492 nm, a standard curve was prepared with the reference caffeic acid. The results were expressed as mg caffeic acid equivalents (CAE) per 100 mg of extract.

Isolation, identification and quantification by UHPLC-HRMS/MS.

The ethyl acetate fraction was analyzed by UHPLC-HRMS/MS on a Hybrid Mass Spectrometer Orbitrap Quadropole Q Exactive® (Thermo Scientific). Samples were diluted using methanol 50%, containing 0.1 % formic acid (v/v) and filtered by 0.2 µm, UHPLC (Dionex Ultimate 3000) conditions were as follow: ACQUITY UPLC BEH C18 Column (130Å, 1.7 µm, 2.1 mm X 100 mm), mobile phase A: water containing 0.1% formic acid (v/v); mobile phase B: methanol containing 0.1% formic acid (v/v), flow rate 0.5 mL/min, column temperature at 40ºC and injection volume 5µL. The gradient elution system was operated as follow: 0 - 5% B (0-1 minutes), 5- 100% B (1-12 minutes), 100 - 5% B (12.1-15 minutes). The MS data was acquired in negative mode over a scan range 50-750 m/z, Full MS/AIF (Full MS /All ion fragmented scan mode) scan time = 1 micro scans and maximum inject time (IT) 200 ms, TIC (Total Ion Current) chromatogram with a resolution of 70000 were the equipment conditions. MS/MS (AIF) fragmentation spectra were generated with (NCE) normalized collision energy of 60, at resolution 35000, maximum IT 100 ms.

The ESI conditions were capillary temperature 320ºC, spray voltage 3.0 kV, sheath gas (nitrogen) 60 Au and auxiliary gas 25 Au. The raw data were acquired and processed with Xcalibur 4.0 software from Thermo Scientific, the peaks were identified by comparison their retention times, exact mass (deviation not exceed 5ppm), fragment ions of the corresponding standards and data provided by the bibliography.

The quantification of compounds 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: caffeic acid (y = 100380x + 341813; R^2=0.999), *p*-cumaric 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). The results were expressed in mg compound/g extract, as mean ± SD. The analysis was done by triplicate.

To proceed with the isolation of the majoritarian compounds, ethyl acetate fraction (4.3 g) was fractioned by column chromatography using silica gel (1:20 ratio), using the following solvents mixture: hexane: ethyl acetate (80:20 – 0:100) and ethyl acetate: methanol (80:20 – 0:100). Tubes were pooled together according their similarity on thin layer chromatography. The precipitate compounds from hexane: ethyl acetate (20:80 – 0:100) fractions were recovered and analyzed by UHPLC HRMS/MS method and compared with UV spectrum, retention time, MS data, fragmentation with the respective standards and data provided by the bibliography.

Animals and Treatment

Male Swiss albino mice (45-50 g) were used for this study. They were housed in four groups, at constant room temperature of 22 ± 1°C and relative humidity (60%), with a 12-h light-dark cycle and free access to food and water until the sacrifice day. Two hours before sacrifice, mice were i.p administered and divided in the following groups: Group 1 (Control) - 1 mL saline, Group 2 (positive control) a therapeutic dose of R-(-)-Deprenyl hydrochloride (10 mg/70kg), Group 3 (EAF 50) – a dose of 50 mg dry extract/kg body weight of mice and Group 4 (EAF 100) – a dose of 100 mg dry extract/kg body weight of mice (mg dry extract/kg bw). Experiments were carried out in accordance with the Guidelines of the European Union Directive (2010/63/EU) and Spanish regulations (BOE 34/11370-421, 2013) for the use of laboratory animals; the study was approved by the Scientific Committee of the University of Seville.

***In-vitro* nervous system enzyme inhibition.**

Preparation mitochondrial fraction

As a source of MAO, enzyme rat liver homogenates was used, following the process described by Holt et al. (1997) [27], with some variations. Male Wistar rat (280 – 300 g) was euthanatized by isoflurane. Immediately organs were removed and washed in ice cold sodium phosphate buffer (0.2 M pH 7.6) to retire blood excess. Portions of 1 g liver tissue were homogenized using Polytron (TissueRuptor-QIAGEN) - 3 cycles 10-15 seconds with sucrose 0.3 M, ratio 1:5 (w/v) and centrifuged at 1000xg for 10 minutes, the supernatants were separated and preserve, the pellet was re-suspended again with sucrose (5 mL) and centrifuged at 1000xg for 10 minutes. The supernatants were joined and centrifuged at 8000xg for 20 minutes. Then, the pellet resulting was re-suspended in 10 mL of sucrose and centrifuged at 12500xg for 30 minutes.

Finally the mitochondrial fraction was washed once with buffer and re-suspended in phosphate buffer. Total protein concentration was measured, as previously reported [28] and adjusted with buffer phosphate (0.2 M pH 7.6) until 0.5 mg protein/mL. Fractions were stored in eppendorf tubes at -80ºC until the analysis. All procedure was realized at 4ºC.

Inhibition of Monoamine oxidase (MAO-A/B)

Previous to test specific MAO-A and MAO-B, the rat liver mitochondrial fraction (0.5 mg protein/mL) were thawed and pre-incubated at 37ºC during 30 minutes with clorgyline and deprenyl (15 µg/mL) (selective MAO-A / MAO-B inhibitors) diluted 1:100 (v/v). The assay was performed following some described and modified protocols [27,29]. Briefly, 50 µL of sample or reference inhibitor at different concentrations, 50 µL chromogenic solution (1 mM vanillic acid, 2.5 mM 4-Aminoantipyrine and 4 U/mL horseradish peroxidase in sodium phosphate buffer), 50 µL MAO-A/B (0.5 mg protein/mL) and 100 µL 2.5 mM tyramine were added in 96-well microplate, in the blanks, 100 µL of buffer replace tyramine. Absorbance was measured at 490 nm for 5, 10, 20, 30, 40 minutes. The percent of MAO-A/B inhibition was calculated by slopes of the evaluated samples, compared with the slope of control. Clorgyline and deprenyl were used as reference inhibitors.

Inhibition of Tyrosinase

Tyrosinase inhibitory assay with slightly modifications was assessed following the dopachrome method with L-dopa as substrate, as previously reported [30,31]. 20 µL of sample (the percentage of EtOH was not higher than 0.5% in the final concentration to not affect the tyrosinase activity), 100 µL of 0.06 M

phosphate buffer (pH 6.8), 40 µL of tyrosinase, 100 U/mL (EC 1.14.1.8.1; mushroom tyrosinase diluted in buffer), were added in 96 well microplate. The samples were incubated at 23ºC for 10 minutes. Then 40 µL L-dopa 2.5 mM in buffer phosphate was added. After incubation at 23ºC for 10 min, the absorbance was measured at 475 nm. Kojic acid was used as reference inhibitor. The percent inhibition of tyrosinase was calculated using the equation: Inhibition (%) = [(1 - (A - A´)/A^0)] x 100, where A is the absorbance of sample, A´ is the absorbance of blank sample without enzyme and A^0 is the absorbance of control.

Inhibition of acetylcholinesterase (AChE)

AChE inhibitory activity was performed spectrophotometrically, as previously described [32], with minor modifications described in literature [17,33]. Firstly, 100 µL of 0.1 M phosphate buffer (pH 7.8), 20 µL of an acetylcholinesterase solution (0.5 U/mL in 0.1 M phosphate buffer, pH 7.8) and 20 µL of sample test were mixed and incubated at 37ºC per 15 minutes.

Secondly, 40 µL 0.75 mM DNTB (diluted in phosphate buffer, pH 7.8) and 20 µL ATCI solution in demineralized water were added to initiate the reaction. Finally, the hydrolysis was measured using a microplate reader at 405 nm after six minutes. Physostigmine free base an inhibitor of AChE was used as control positive. The percent of inhibition (I%) was calculated with the formula previously detailed.

Measurement of biogenic amines and metabolites by High-Performance Liquid Chromatography (HPLC)

Analysis of striatal biogenic amines: dopamine (DA), 3, 4 dihydroxyphenylacetic

acid (DOPAC), 5-hydroxyindolacetic acid (5-HIAA), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT) was performed by HPLC with electrochemical detection. A HPLC ELITE LaChrom Pump L–2130 HITACHI was used in conjunction with a glassy carbon electrode set at +700 mV (DECADE II, ANTEC, Netherlands). A Merck Lichrocart cartridge (125 mm × 4 mm) column filled with Lichrospher reverse-phase C_{18} 5 µm material was used. The mobile phase contained 0.05 M sodium acetate, 0.4 mM 1-octanesulfonic acid, 0.3 mM Na_2EDTA and 70 ml methanol/L (pH was adjusted to 4.1 with acetic acid). All reactive agents and water were HPLC grade. The flow rate was 1.0 ml/min. Measurement of biogenic amides (DA, 5-HT) and their metabolites (DOPAC, 5-HIAA, HVA) in fresh tissue was performed according to the method described by Ismaiel et al. (2016) [34]. Concentrations of striatal amines in samples were calculated with the aid of the eDAQ PowerChrom 280 software.

Statistical analysis

Data were processed by GraphPad Prism 6.01 software and expressed as means ± SEM. According to the type of experiments, one and two-way analysis of variance (ANOVA) was applied to analyze the differences in data, followed by Tukey`s and Sidak`s post hoc for pairwise multiple comparison. The IC_{50} values of fraction and isolated compounds were calculated by non-linear regression log (inhibitor) vs. response.

III.3.-RESULTS AND DISCUSSION

Phytochemical analysis, identification and quantification of EAF

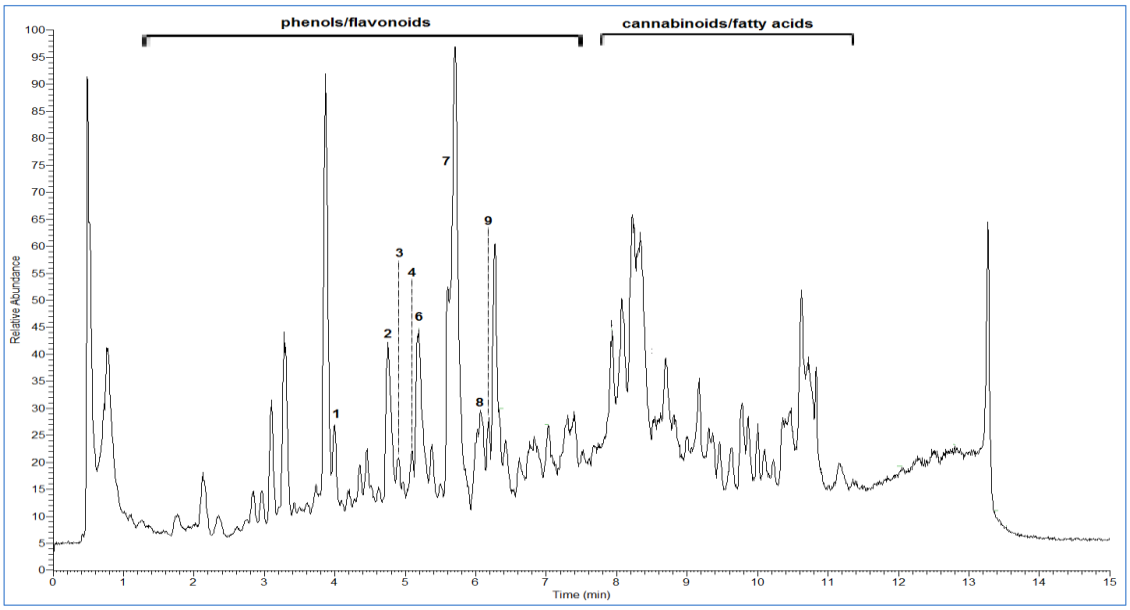


Figure 1. UHPLC chromatogram of EAF with 15 min gradient: 1) caffeic acid; 2, 6) N-caffeoyloctopamine isomer; 3) *p*-coumaric acid; 4) ferulic acid; 7) *N- trans* caffeoyltyramine; 8) *N- trans* coumaroyltyramine; 9) N- feruloyltyramine.

From ethyl acetate fraction, two compounds were isolated and analyzed by UHPLC HRMS/MS, their identification were done by comparison with UV spectra, retention time, MS data and fragmentation with respective standards, resulting: *N-trans*-coumaroyltyramine (9 mg) isolated from hexane: ethyl acetate fractions (20:80) and *N-trans*-caffeoyltyramine (38 mg) isolated from hexane: ethyl acetate fractions (0:100).

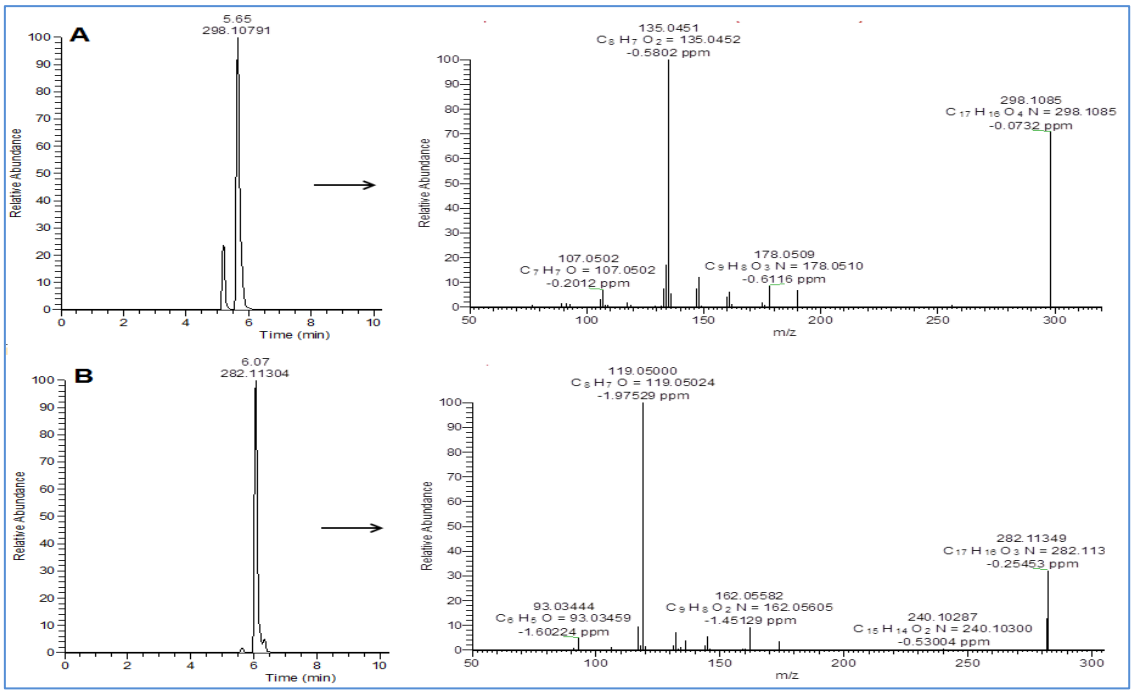


Figure 2. UHPLC – Base peak chromatogram and MS/MS fragments of isolated compounds A) *N-trans*-caffeoyltyramine B) *N-trans*-coumaroyltyramine.

Previous the quantification, phenols, flavonoids and hydroxycinnamic acid content in ethyl acetate fraction were estimated by colorimetric reactions, EAF was comprised by: total phenols 10.35 ± 0.7 mg GAE/100 mg extract; flavonoids 3.09 ± 0.64 mg RE/100 mg extract and hydroxycinnamic acid 6.25 ± 0.44 mg CAE/100 mg extract. The phytochemical analysis was performed by UHPLC-HRMS/MS, negative ion mode and the chromatogram of EAF is represented in **Figure 1**. Representative hydroxycinnamic acids and main compounds quantified are shown in **Table 1**. Indeed, in a previous work we have characterized other compounds in this extract from the defatted seed, such as phenol acids, flavonoids and traces of cannabinoid precursors [35].

Chromatogram peaks corresponding to the isolated compounds are shown in **Figure 2.** At Rt 5.65 min, *N-trans*-caffeoyltyramine m/z 298.1079 $[C_{17}H_{16}O_4N]^-$ and Rt 6.07 min *N-trans*-coumaroyltyramine m/z 282.1130 $[C_{17}H_{16}O_3N]^-$.

Table 1. Quantification of hydroxycinnamic acids and hydroxycinnamic acid amides in ethyl acetate fraction.

Rt (min)	$[M-H]^-$ experimental	Error (ppm)	Formule [M-H]-	Theoretical m/z	MS/MS fragments	Attribution[a]	Content mg/g extract
3,99	179,0347	-1,40	$C_9H_6O_4$	180,0422	89, 107, 134, 135	caffeic acid	0.118 ± 0.003
4,70	314,1033	0,77	$C_{17}H_{16}O_5N$	315,1107	178, 161, 152, 135, 122	*N*-caffeoyloctopamine isomer 1[b]	0.277 ± 0.003
4,78	163,0399	-1,31	$C_9H_6O_3$	164,0473	93, 119	*p*-coumaric acid	0.465 ± 0.017
5,10	193,0504	-1,27	$C_{10}H_8O_4$	194,0579	134, 149, 178	ferulic acid	0.037 ± 0.004
5,17	223,0609	-1,29	$C_{11}H_{10}O_5$	224,0684	93, 121, 149	sinapic acid	0.001 ± 0.000
5,25	314,1033	0,77	$C_{17}H_{16}O_5N$	315,1107	178, 161, 152, 135, 122	*N*-caffeoyloctopamine isomer 2[b]	0.582 ± 0.005
5,65	298,1079	-0,07	$C_{17}H_{16}O_4N$	299,1158	107, 135, 178	*N- trans* caffeoyltyramine	8.869 ± 0.446
6,07	282,1130	-0,25	$C_{17}H_{15}O_3N$	283,1208	93, 119, 132, 162	*N- trans* coumaroyltyramine	1.130 ± 0.036
6,25	312,1240	0,14	$C_{18}H_{18}O_4N$	313,1314	297, 178, 148, 135	*N*- feruloyltyramine [b]	0.766 ± 0.004

[a] Compound identification was verified with standard. [b] Attribution compound, were compared with bibliography and free databases available (mzCloud, Pubchem)

Table 2. IC_{50} values for the different nervous system enzymes with reference inhibitor

sample	nervous system enzyme inhibition (IC_{50} µg/mL)				
	MAOs	MAO-A	MAO-B	TYR	AChE
EAF	50.12 ± 1.20	55.59 ± 1.06 [a]	46.34 ± 1.16	60.39 ± 1.05	> 400
N-*trans*-coumaroyltyramine	-	22.86 ± 1.56	23.77 ± 1.06	11.78 ± 1.24 [b]	85.56 ± 0.15
N-*trans*-caffeoyltyramine	-	18.24 ± 1.64	17.06 ± 1.94	8.13 ± 1.03 [a]	157.56 ± 21.04
Clorgyline[c]	-	0.004 ± 0.001	-	-	-
Deprenyl[c]	-	-	0.008 ± 0.001	-	-
kojic acid[c]	-	-	-	4.47 ± 1.03 [a]	-
Physostigmine[1]	-	-	-	-	0.018 ± 0.001

IC_{50} values were calculated by non- linear regression log (inhibitor) vs response. TYR: significant differences were calculated using one way ANOVA following Tukey´s post hoc test, between compounds and reference standard: [b]($p < 0.05$); MAO-A/B: significance differences between two enzymes were determined using two-way ANOVA following Sidak´s multiple comparison test: [a] ($p<0.01$).EAF and compounds IC_{50} values are expressed as: µg dry extract/mL, w/v; and µg compound/mL, w/v, respectively.

[c] Used as reference inhibitor; -, not tested.

Nervous system enzyme inhibition.

The values IC_{50} for the different nervous system enzymes are shown in **Table 2.**

Inhibition of Monoamine Oxidase A/B

Biogenic amines have an important role in neurotransmission. The inhibition of monoamine oxidases A/B increases their levels in the synaptic space. Although therapeutic strategies are oriented to increase the levels of biogenic amines, their degradation produce high amounts of neurotoxic compounds, which can influence in the neurodegeneration course. Inhibitors of MAO-B are applied to symptomatic treatment in PD, while inhibitors of MAO-A are used by their antidepressant effect [36].

Active phytochemical compounds, such as coumarins, terpenes, flavonoids, alkaloids are abundant in plants and could be a source of monoamine oxidase inhibitors (MAOIs) [37]. Previously, our group has identified phenol acids, flavonoids, phenolic amides, and cannabinoids in different fractions from hempseed [35]. MAOs inhibition by ethyl acetate fraction showed considerable inhibition, IC_{50} values ranging 46.34 – 55.59 µg/mL (**Table 2**), with significant differences in selectivity toward MAO-B isoform and may be beneficial to prevent Parkinson`s and Alzheimer`s diseases by MAO-B inhibition. Although, isolated compounds did not show a specific selectivity for MAO-A or B, inhibiting both isoforms equally.

Inhibition of Tyrosinase

Tyrosinase plays an important role in melanin synthesis, it has protection again ultraviolet damages on skin and its overproduction cause esthetic problems by accumulation. In human brain, alteration on Dopamine (DA) neurons produce

an excess of DA or L- Dopa and these are quickly oxidized by tyrosinase, producing highly reactive quinone-species, linked with oxidative stress, inflammation and neurodegenerative diseases. Although the neuromelanin accumulates with the age and is an indicator in PD, the overproduction of tyrosinase could contribute to the neuromelanin formation and could be associated with PD [38,39].

IC_{50} value of ethyl acetate fraction showed a significant tyrosinase inhibition (60.39 ± 1.05 µg/mL). Related to isolated compounds *N-trans*-coumaroyltyramine and *N-trans*-caffeoyltyramine, the IC_{50} values were 11.78 and 8.13 µg/mL respectively, the latter one not exhibited significantly differences in comparison with the reference inhibitor (kojic acid) (**Table 2**). Similar studies have reported a complete inhibition by *N-trans*-caffeoyltyramine in concentration of 0.1 mM [40], a strong tyrosinase inhibition by phenylethylamide derivatives comparable with reference standard hydroquinone [41], as well as potent tyrosinase inhibition by phenylpropanoids amides of octopamide derivate [42]. In addition, other studies detail potential anti-inflammatory and antioxidant activities of hydroxycinnamic acid amides [43,44].

Inhibition of Acetylcholinesterase

In Alzheimer`s disease, the acetylcholinesterase enzyme inhibitors (AChEIs) are the principal drugs used for it management, however, is necessary the search of new compounds, capable to reduced AChE activity [45]. The results with ethyl acetate fraction showed low AChE activity (11.62 ± 2.95 inhibition % at 400 µg/mL). IC_{50} values corresponding to *N-trans*-coumaroyltyramine was 85.56 ± 0.15 µg/mL and for *N-trans*-caffeoyltyramine 157.56 ± 21.04 µg/mL (**Table 2**). Related studies has reported an IC_{50} 64.58 µg/mL (216 µM) in AChE

inhibition by *N-trans*-caffeoyltyramine [17], while other shown IC_{50} values of 25.21 µg/mL (84.3 µM) and 29.4 µg/mL (98.3 µM) for *N-trans*-caffeoyltyramine and *N-trans*-coumaroyltyramine, respectively [46].

Although, IC_{50} values were lowest in other studies, the results can not always be compared, as the assay can be performed under different conditions. These differences may be due to variations in the course of the assay. Besides, solvent concentration % (v/v) used to dilute samples, prior to acetylcholinesterase assays (DMSO > acetonitrile > acetone > methanol) affect the AChE activity [47].

Effects of Ethyl acetate fraction on the biogenic amine levels in mice striatum.

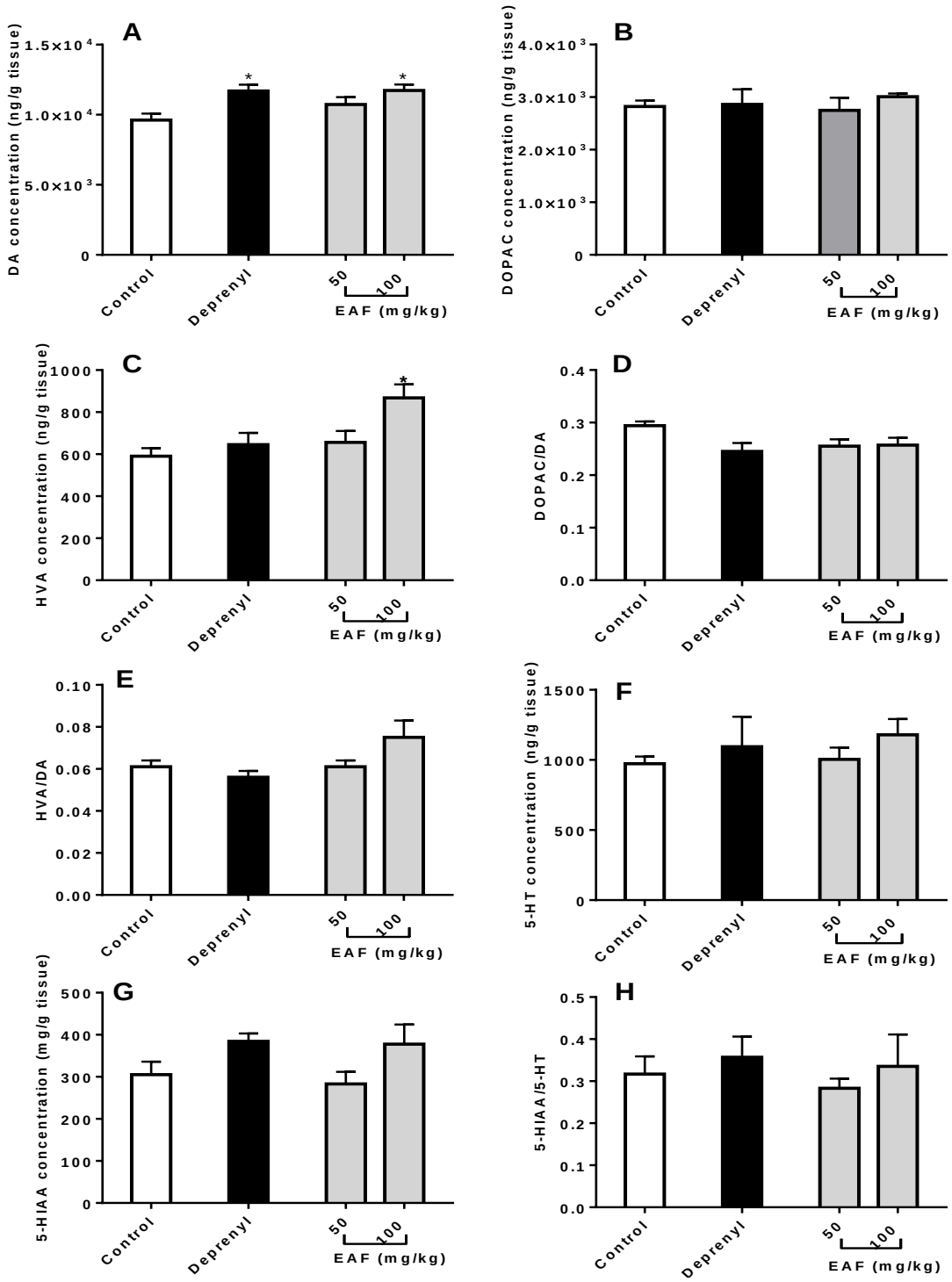


Figure 3. Measure of intracellular levels of A) dopamine (DA), B) 3,4-dihydroxyphenylacetic acid (DOPAC), C) homovanillic acid (HVA), F) 5-hydroxytriptamina (5-HT) and G) 5-hydroxyindolacetic acid (5-HIAA) in mice striatal tissues, as well as the ratios of D) DOPAC/DA, E) HVA/DA and H) 5-HT/5-HIAA in control (white box), deprenyl (black box) and EAF, 50 and 100 mg dry extract/kg bw (grey boxes). Values shown are the means (ng/g wet tissue) ± SEM (n=5). Marked with asterisk are significantly different ($p < 0.05$) vs control. Deprenyl was used as a selective and irreversible MAO-B inhibitor in therapeutic doses (10 mg/70kg).

In the present study we examined the inhibition effect *in vivo* on MAOs of the ethyl acetate fraction from hempseed on mice striatum, using four experimental groups of animals: first group was given saline solution (control animals), the second group was subjected to a therapeutic dose of deprenyl (10 mg/70 kg), third group with a dose of EAF (50 mg dry extract/kg bw) and fourth group with a dose of EAF (100 mg dry extract/kg bw). The results showed an increase in the level of striatal dopamine and its metabolites DOPAC and HVA by EAF, with significance differences respect to the control at 100 mg dry extract/kg dw for DA and HVA (**Figure 3A, C**), as well the drug used as positive control (deprenyl). While DOPAC/DA and HVA/DA ratio remained without a significantly change (**Figure 3D, E**). Finally, 5-HT content and it metabolite 5-HIAA shown a slightly increase at 100 mg dry extract/kg bw in EAF dose, did not show significance differences, and 5-HIAA/5-HT ratios, remained unchanged (**Figure 3F-H**).

Deprenyl is a selective and irreversible inhibitor of MAO B enzyme, avoiding DA degradation and increasing their levels in central nervous system. Since it shows neuroprotective activity by the reduction of oxidative species, it can be chosen as a remedy for treatment of Parkinson´s disease [48,49]. Till date, no study has revealed the effect of ethyl acetate fraction from defatted hempseed on the levels of biogenic amines neurotransmitters, such as DA, 5-HT and their metabolites in mice striatum. The utilization of components that remain in hempseed after the oil extraction has gained popularity by the possible use and applications of these compounds. Extracts with high concentration of phenylamides have shown neuroprotective effects [20], and anti-inflammatory activities in human primary monocytes [35]. Besides, phenylpropanoids have

been suggest in neurodegenerative diseases by their multiple biological functions, as well as a base for the drugs development [50].

After i.p administration of deprenyl and EAF (100 mg dry extract/kg bw) the striatal DA level was higher in comparison with the control (saline). Pharmacokinetic studies of deprenyl are related with the administration type. Data have shown plasmatic concentrations after i.p administration, reached C_{max} at first 5 minute, decreasing over time (6 hours). It also showed an inhibition of over 90% MAO-B at brain and liver in rats treated subcutaneous [51]. Together with *in vitro* MAO-A and B inhibition, these results provide data about beneficial properties of hempseed and the use as a potential source of phytocompounds, displaying therapeutic role in the ameliorate and prevention of neurodegenerative diseases.

III.4.-CONCLUSIONS

In summary, we have evaluated monoamine oxidase A, B, tyrosinase and acetylcholinesterase inhibitory activities of the ethyl acetate fraction from hempseed (*Cannabis sativa* L.) together with two isolated phenolic amides derivate from *p*-coumaric and caffeic acid. The results revealed that the fraction and the compounds have inhibitory activities on both monoamine oxidases and tyrosinase enzymes, which are targets in neurodegenerative diseases and may influence positively in the treatment of Parkinson`s and Alzheimer`s diseases. In addition, these data demonstrate that treatment with an ethyl acetate fraction is capable of protecting striatal dopamine degradation in an animal model.

However, despite the valuable results obtained in this study we are aware of the

limits of our research. Larger preclinical studies would be necessary, in order to know the real contribution of EAF components and their applications in neurodegenerative diseases. In addition, clinical evaluations of the biological functions of phenylpropanoid amides and their derivatives present in hemp seed would be desirable for the development of effective treatments against central nervous system pathologies.

Declaration of Competing Interest

The authors declare that they have no conflicts interest.

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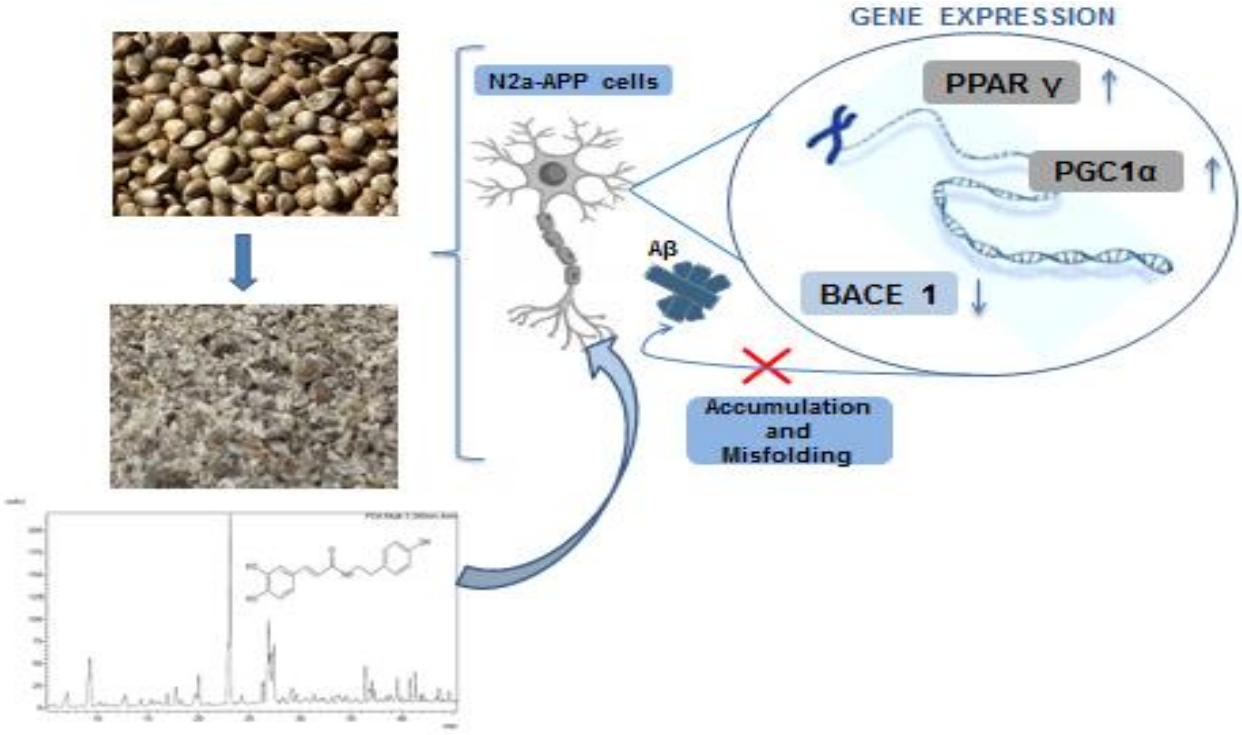
CAPITULO IV

Dual role of phenyl amides from hempseed on BACE 1, PPARγ and PGC-1α in N2a-APP cells



ABSTRACT

In Alzheimer´s disease (AD) the accumulation of amyloid β (Aβ) plaques in the brain lead to neuroinflammation, neuronal cell dysfunction, and progressive memory loss. Therefore, blocking the formation of Aβ plaques has emerged as one of the most promising strategies to develop AD treatments. Hempseed is widely used as a food, and recently their compounds have shown beneficial effects on neuroinflammation. The objective of this study was to investigate whether a fraction rich in phenyl amide compounds, N-trans caffeoyltyramine (CAFT) and N-trans coumaroyltyramine (CUMT) can affect gene expression: β-site amyloid-precursor- protein-cleaving enzyme 1 (BACE 1), Peroxisome proliferator-activated receptor gamma (PPAR γ) and PPARγ-coactivator-1α (PGC-1α) in N2a-APP cells. The mRNA levels were measured using RT-qPCR. Ethyl acetate fraction (EAF) and CAFT were found that reduce BACE1 gene expression and are promissory PPARγ and PGC-1α natural agonists. The results show that hempseed compounds can inhibit the expression of BACE 1, which is involved in the accumulation of Aβ plaques and positively affect transcription factors involved in complex and diverse biological functions.

Keywords: *Cannabis sativa* L., phenyl amides, BACE 1, PPARγ, PGC-1α, Alzheimer´s disease.

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IV.1.-INTRODUCTION

Alzheimer´s disease is a neurodegenerative pathology characterised by the extracellular accumulation of amyloid β (Aβ) plaques, generated from amyloid-β precursor protein (APP) via amyloidogenic pathways by β-secretase and γ-secretase and intracellular neurofibrillary tangles, and principally affects the elderly in terms progressive memory loss, cognitive damage, and deteriorating bodily functions. Although the etiology remains unclear, certain possible causes have been proposed, such as: protein deposition (misfolding) disorders and aggregation of amyloid β (Aβ) and tau proteins, activation of the innate immune system, mitochondrial dysfunction, and oxidative stress [1,2]. With no possible treatment to control, prevent or cure the devastating effects of this disease, therapy has focused only on treating the symptoms rather than understanding the pathology or other possible hallmarks [3]. According to the latest report in 2019, it is estimated that over 50 million people worldwide suffer from dementia and this number could increase to 152 million by 2050 [4]. In recent years, bioactive compounds from plants have shown promising effects in neurodegenerative diseases and appear to present an interesting source of alternative medicine for their evaluation regarding Alzheimer's disease [5,6]. Depending on their applicability, compounds should reach different areas of the central nervous system (CNS). The first step is to cross the Blood-Brain Barrier (BBB), which uses anatomical, biochemical, and physicochemical mechanisms to control the exchange of different molecules between blood and brain [7]. To solve this problem Parallel Artificial Membrane Permeation Assay (PAMPA-BBB) has been established as a predictive tool for the early stages of the

discovery drugs, which filters the possible compounds from natural sources or plant extracts that can penetrate the BBB [8].

Hempseed from Cannabis sativa L. or similar is a well-known seed that has traditionally been used as both a food and a medicine, and provides a source of high concentrations of polyunsaturated fatty acids, proteins, and vitamins [9,10]. Recently, positive effects of its compounds on neuroinflammation and memory dysfunction have been reported [11,12,13]. Previously, acetylcholinesterase inhibitory activities and beneficial effects from hempseed compounds were described in degenerative processes associated with inflammation and oxidative stress. However, whether the ethyl acetate fraction and isolated compounds from hempseed plays a role on inhibition of BACE 1, which is involved in Aβ formation, a neuropathological feature associated with the early stages of Alzheimer's disease [14], and two negative regulators of BACE 1 in the form of PGC-1α, which regulate the transcription of BACE 1, and further Aβ formation in AD [3], and in the form of PPAR γ, which is involved in the regulation of the transcription of genes anti-inflammation, redox homeostasis, glucose and lipids metabolism, tissue recovery of acute brain injuries among others [15], have yet to be studied and are an attractive target in numerous therapies for neurological disorders. In the search for natural BACE 1 inhibitors, we focus on a promising fraction, the ethyl acetate fraction (EAF), which has been obtained from defatted hempseed with a high content of phenyl amides, and include two isolated compounds (caffeoyltyramine and coumaroyltyramine). In order to evaluate the possible inhibitory effects on BACE 1 and post-

production APP, the fraction and compounds were assessed *in vitro*, using mutant APP overexpressed N2a cells.

IV.2.-MATERIAL AND METHODS

Chemical and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-iphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), n-dodecane, cholesterol, TRI reagent, pioglitazone, *N-trans* caffeoyltyramine and *N-trans* coumaroyltyramine were purchased from Sigma-Aldrich, iScript cDNA Synthesis Kit from BIO-RAD, methanol, formic acid LC-MS grade from VWR chemicals, DMEM medium from Biochrom AG, brain polar lipid extract (BPL) from Avanti Polar Lipids.

Plant material

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

Preparation of the fraction tested

An ethyl acetate fraction (EAF) and two phenolic amides isolated from it were used for our tests, and the procedure is described below: Hempseed (3kg) were crushed and defatted three times with n-hexane (each for 24 h). After filtration and evaporation of the n-hexane, the defatted seeds were extracted with 75% aqueous ethanol, solvent in a ratio 1:3, twice (each for 24 h), followed by ultrasonic bath extraction (Ultrasons HD, JP Selecta) (solvent in a ratio 1:1) with a fixed power (180W) for twenty minutes at 45°C with periodical stirring. The filtrates were concentrated under vacuum until the volume was reduce to about to 500 mL and were stored in 100 mL tubes at 4°C for 48 hours. Then, this aqueous solution was liquid-liquid extracted with ethyl acetate (4 x 500mL),

and the ethyl acetate solution was subsequently evaporated under vacuum, freeze-dried and stored in a dark glass bottle at 4°C prior to analysis, resulting in EAF.

Compounds isolation

Isolation was performed as previously reported [16] from the ethyl acetate fraction (4.3 g) was fractionated by column chromatography with approximately 85.0 g of silica gel (1:20 ratio). The following solvent mixtures were used, in a volume of 500 mL each: hexane: ethyl acetate (80:20 – 0:100) and ethyl acetate: methanol (80:20 – 0:100). The tubes were pooled together according to their similarity in thin-layer chromatography. The compounds from the hexane: ethyl acetate (20:80 - 0:100) fractions were recovered and purified with Sephadex LH-20 using methanol. The isolated compounds were analyzed and confirmed by UHPLC HRMS/MS method [17], retention time, MS data, fragmentation and UV spectrum were compared with the corresponding standards, 9 mg of *N-trans* coumaroyltyramine and 38 mg of *N-trans* caffeoyltyramine were obtained.

PAMPA- BBB Procedure.

PAMPA was used as a high-throughput assay to predict the BBB permeation of the isolated compounds and the total fraction, following the process detailed in the bibliography with slight modifications [18]. Stock solution of the isolated compounds (2 mg/mL) and EAF (20 mg/mL) were diluted in ETOH 50%, filtered with 0.45 µm pore size and mixed with phosphate buffered saline (0.01 M PBS, pH 7.4) to obtain a donor start solution with a final concentration of 200 µg/mL for the compounds and 2 mg/mL for EAF, respectively. The filter membrane of

the donor (top) plate (96-well polycarbonate-based filter plate, Multiscreen-IP, MAIPN4510, pore size 0.45 µm, Millipore) was coated with 5 µL of BBB specific lipid solution (16 mg PBL and 8 mg cholesterol dissolved in 600 µL n-dodecane) and the well acceptor plate (bottom) was filled with 300µL of PBS buffer. Then, a 150 µL aliquot of the samples were applied to a donor well and carefully placed on the acceptor plate to form a ¨sandwich¨ and left undisturbed for 4 h at 37 °C. After incubation, the acceptor plate was separated from the donor plate. EAF compounds in the donor starting solution, and in both donor and acceptor wells after the incubation period were performed in triplicate by UHPLC (Nexera XR, Shimadzu, Japan) with UV detection from 200 to 400nm according to the described method [19]. Chromatograms were extracted at the appropriate wavelengths

Cells culture and treatment.

Mutant APP overexpressed N2a (N2a-APP) cells were used for the study. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containig 10% fetal bovine serum (FBS) and L-glutamine in 5% CO_2 at 37°C. Cells were plated in six well plates at density 5 x 10^5 /mL for 24 h and 5 x 10^5 /2mL for 48 h treatment with ethyl acetate fraction at 25 – 100 µg/mL, compounds at 0.03 – 0.08 µM and PPARγ agonist pioglitazone (14 µM). Control cells were incubated with medium alone.

Cell viability assay (MTT)

N2a-APP cells were incubated in a 96-well plate (1x10^4 cells/well) for 24 h with various concentrations of 25-100 µg/mL of ethyl acetate fraction and of 0.03 – 0.08 µM of *N-trans* caffeoyltyramine and coumaroyltyramine. Cells control were

incubated with medium alone. Afterwards, cells were incubated with MTT (1 mg/mL) for 2 h at 37°C until a purple precipitate was visible. MTT-formazan crystals were solubilized with DMSO (200 µL) and then measured with a microplate reader at 570 nm corrected to 650 nm. Cell survival was expressed as a percentage of absorbance compared with to non-treated cells.

RNA isolation and RT-qPCR analysis

RNA was extracted using TRI Reagent (Sigma) as indicated by the manufacturer. The A260/A280 ratio in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Madrid, Spain) was used to determine RNA quality. Momentarily, RNA (1µg) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain) according to the manufacturer's protocol. An amount of 20 ng of the resulting cDNA was used as template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a MX3000P system (Stratagene). For each PCR reaction, the cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad), which contained primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and m18s as housekeeping genes (**Table 1**). All amplification reactions were performed in triplicates and the average threshold cycle (Ct) counts of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change in mRNA expression for the candidate genes was calculated using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to the levels of the endogenous reference genes (GAPDH and 18s) and expressed as a percentage of controls.

Table 1. Sequences of RT-qPCR primers for gene expression analysis

Target	GenBank accession number	Direction	Sequence (5´→ 3´)
mBACE1	AF190726.2	Forward	AGAGGCAGCTTTGTGGAGAT
		Reverse	CTGGTAGTAGCGATGCAGGA
mPGC1	NM_008904.2	Forward	AGCCTCTTTGCCCAGATCTT
		Reverse	GGCAATCCGTCTTCATCCAC
mPPARg	NM_001127330.2	Forward	AGGGCGATCTTGACAGGAAA
		Reverse	CGAAACTGGCACCCTTGAAA
mGAPDH	NM_008084.3	Forward	CAACTCCCACTCTTCCACCT
		Reverse	GAGTTGGGATAGGGCCTCTC
m18s	NM_008084.3	Forward	AGAAACGGCTACCACATCCA
		Reverse	CCCTCCAATGGATCCTCGTT

Statistical analysis

All values are expressed as arithmetic means ± standard deviations (SD). Data were evaluated using Graph Pad Prism Version 6.01 software (San Diego, CA, USA). Statistical significance of differences between each parameter in the groups was evaluated using one-way analysis of variance (ANOVA), followed by Dunnett´s multiple comparisons test as a post hoc test. P values less than 0.05 were considered statistically significant.

IV.3.-RESULTS AND DISCUSION

Phytochemical results

The chromatographic profile of the ethyl acetate fraction and two phenyl amides is shown in **Fig. 1**. Analysis was performed by ultra-high liquid performance chromatography (UHPLC) as described by Bucić-Kojić et al. [19]. The majority of the ethyl acetate fraction consists of phenyl amide compounds, and in smaller amounts, contains acid phenols, flavonoids, and terpenphenols. Studies have shown that caffeoyltyramine is the major compound in the fraction with concentration above 6.36 mg/g extract [17].

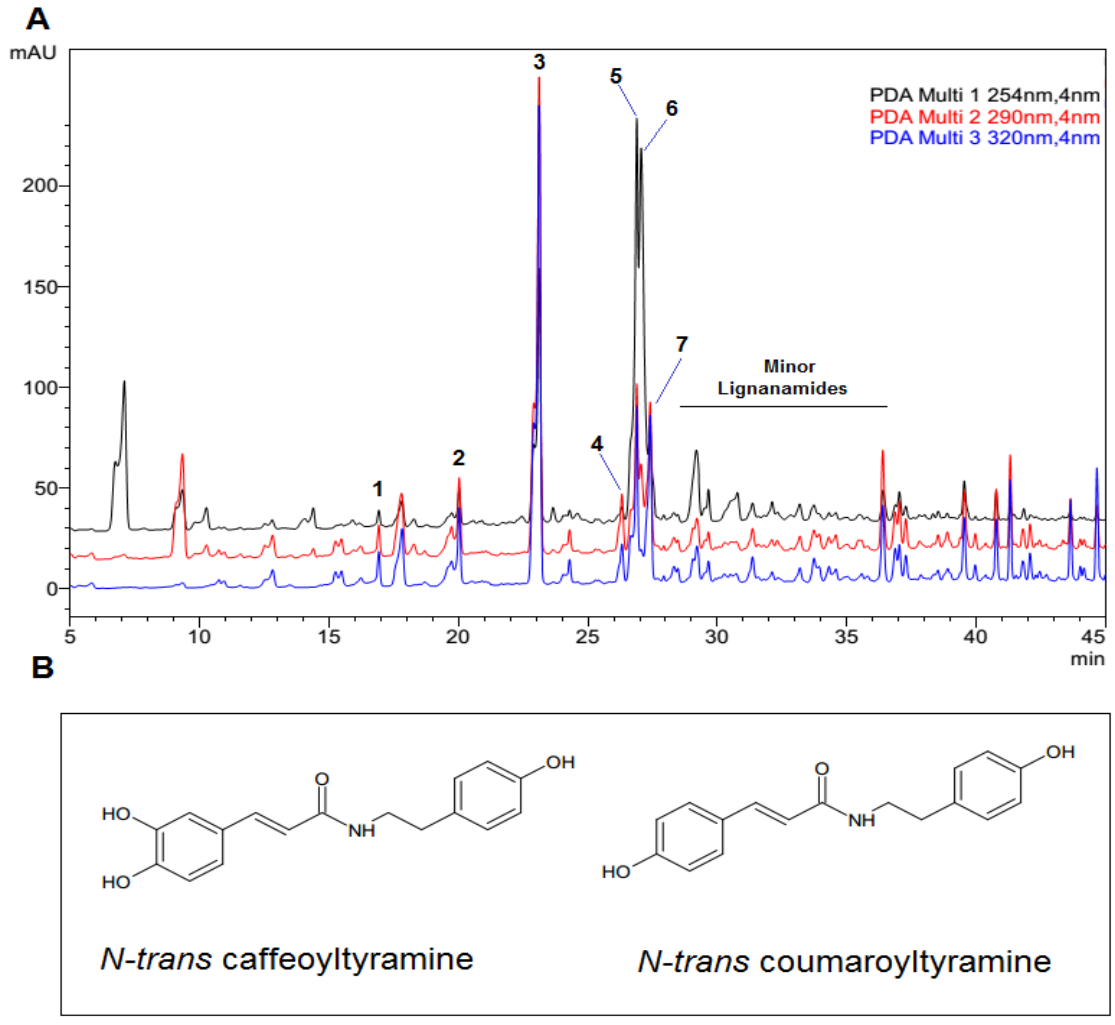

Fig. 1. A) UHPLC profile of the ethyl acetate fraction from hempseed *Cannabis sativa* L. **B)** Structures of *N-trans* caffeoyltyramine and *N-trans* coumaroyltyramine.

Qualitative PAMPA-BBB assay of EAF

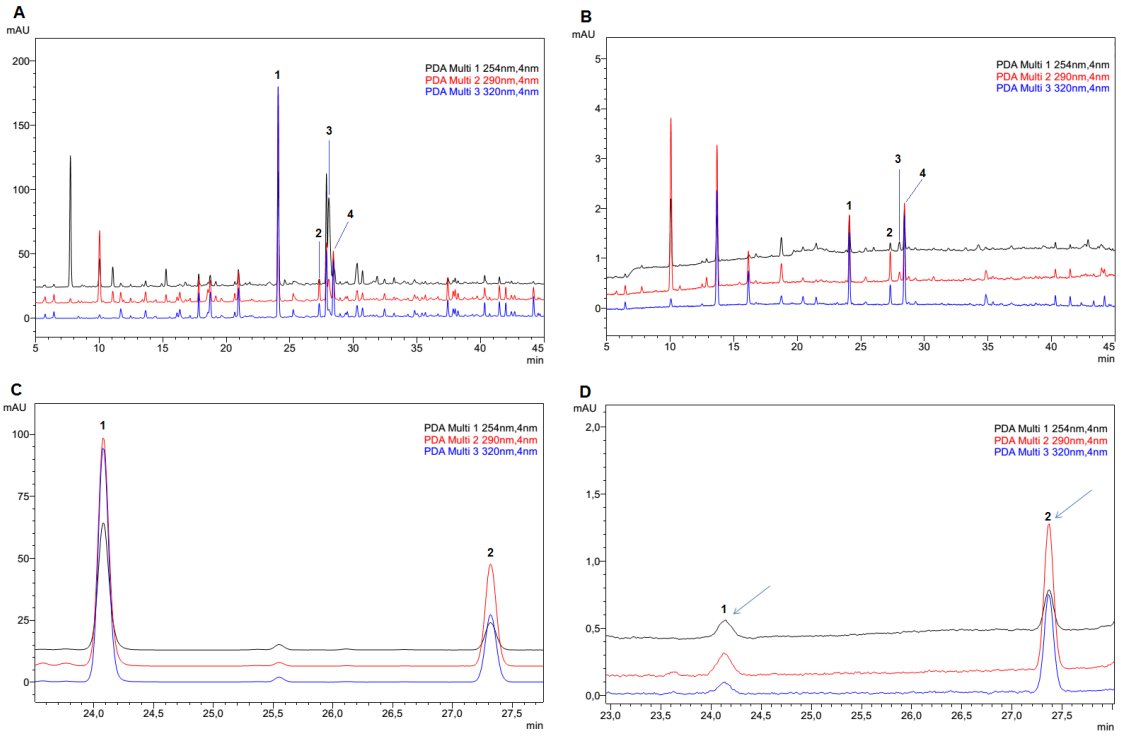

Fig.2. Chromatograms of EAF **A)** stock solution in donor solution before and **B)** acceptor solution after the PAMPA-BBB assay. Isolated compounds **C)** Stock solution isolated compounds in donor solution before and **D)** acceptor solution after assay. Phenyl amides 1) *N-trans* caffeoyltyramine, Rt 24.08 min; 2) *N-trans* coumaroyltyramine, Rt 27.32 min; 4) feruloyltyramine, Rt 28.42 min and lignanamide 3) Cannabisin A, Rt 28.07 min; were identified in acceptor after assays.

PAMPA-BBB assays constitute one of the most widely used models for the prediction of transcellular passive absorption *in vitro*, by filtering out potential compounds with applications in the CNS early in their development. In order to ascertain the applicability of the PAMPA-BBB system, the EAF was screened. The initial solution (donor) containing possible brain-permeable compounds was screened through a model of a lipid-infused artificial membrane on a solid filter support. This was analysed by means of the UHPLC method descripted [19], as well as the acceptor solution compartment after permeability assay, whose solution contains certain constituents of the donor (**Figure 2**). Although

fractions/extracts rich in phenyl amides from hempseed have shown anti-neuroinflammatory [13] effects, increased biogenic amine levels in mice striatum [16] in animal models, and inhibition of U-87 cancer cells proliferation *in vitro* [20], it has also been shown that isolated compounds exert anti-neuroinflammatory effects on LPS-induced BV2 microglia cells [11]. However, no data on hempseed compounds indicates that it can cross BBB and reach different areas of the brain. After EAF assessment, phenyl amides such as *N-trans* caffeoyltyramine, *N-trans* coumaroyltyramine **(Fig. 2 D)**, feruloyltyramine, and the lignanamide Cannabisin A were detected in the acceptor solution (**Fig. 2 B**). These compounds derive from the products of conjugation between phenolic acids and arylmonoamines, such as tyramine and octopamine, which are present in hempseed in large proportions [20,21] and in high quantities [17]. Hempseed can be a source of potential candidates with application in neurodegenerative diseases. It has also been reported that N-methylated tyramine derivatives of Ginkgo biloba can cross the BBB [22]. Although the permeability assay is a filtering tool for the selection of potential brain-permeable compounds from plant extracts, *in vivo* assays and specific studies, are required for the identification of compounds that can cross the BBB.

Effect of EAF and phenyl amides on BACE 1 gene expression in N2a-APP cells.

Results have shown that polar fractions from defatted hempseed present a strong radical scavenging activity and anti-inflammatory effect in human primary monocytes. Moreover, quantification shows a high content of phenyl amide compounds in the fraction composition in comparison with other phenols and flavonoids [17]. The addition of ultrasonic treatment and temperature (45ºC) in

the extraction process has increased the content of phenyl amide compounds. The data is similar to that reported in the literature where the application of heat during ultrasonic extraction treatment improved the yield of polyphenol content in extracts from seed cake powder [23]. It has been reported that an extract rich in phenyl amides improves cognitive functions and reduces the expression of pro-inflammatory cytokines in the brain of LPS-induced mice at a concentration of 1 g/kg [13] and significantly reduces TNF-α expression in BV2 microglial cells [11]. However, for N2a-APP cells, the protection and cytotoxicity of the ethyl acetate fraction, *N-trans* caffeoyltyramine and *N-trans* coumaroyl tyramine derivatives have yet to be evaluated. In this study, EAF with a high content of phenyl amide compounds at concentrations of 25 – 100 µg/mL and the isolated compounds (CAFT and CUMT) at 0.03 – 0.08 µM, were selected to treat N2a-APP cells for 24 and 48 h, respectively. The MTT assay showed that only the EAF fraction at high concentrations affected cell viability. The compounds had no effect on cell viability at the concentrations tested (**Fig. 3**).

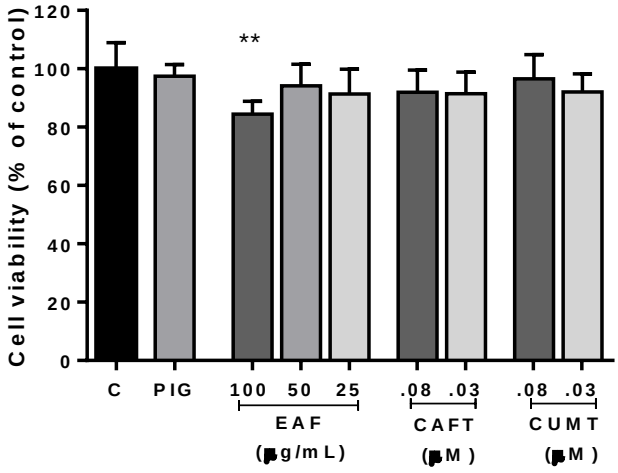

Fig. 3. N2a-APP cells were treated with ethyl acetate fraction (EAF) at 100, 50, 25 µg/mL, compounds *N-trans* caffeoyltyramine (CAFT), *N-trans* coumaroyltyramine (CUMT) at 0.03 – 0.08 µM and PIG (14 µM) as positive control for 24 h, respectively. Cell viability was detected by MTT assay. The results showed that EAF at high dose had a significant effect on cell viability. All data are presented as means ± SD. The p values were calculated using one-way ANOVA, **p<0.01 vs. control group.

Using this culture system, we first analysed whether EAF treatment and the two phenyl amide compounds could inhibit the BACE 1 expression. After 24 h of treatment, our results showed a strong inhibition of the BACE 1 expression, with significant differences in EAF (µg/mL) and caffeoyltyramine (µM) at all tested concentrations, compared to the control cells (**Fig. 4**). However, after 48 h of treatment, EAF was less effective in reducing gene expression, while CAFT was shown to remain active and to suppress BACE 1 gene expression. Pioglitazone (PIG), a special pharmacological PPARγ agonists used in studies to elucidate the neuroprotective mechanism, was used as the positive control [24].

The enzyme BACE 1 is necessary for the formation of all monomeric forms of Aβ peptides. Its subsequent accumulation in vulnerable brain parts is linked to as the main cause of Alzheimer´s disease pathogenesis [25,26]. In recent years, and with evidence supporting the amyloid hypothesis as the main factor responsible for the initiation of AD, large amounts of resources have been devoted to the search for potential drug candidates that can act as BACE 1 inhibitors [27]. Furthermore, more and more research is focusing on the use of medicinal plants as a promising source of molecules against AD [28]. These results show that EAF rich in phenyl amides and the main compound present in the fraction (caffeoyltyramine) have an inhibitory effect on the expression of BACE 1. The inhibitory result may help to interrupt Aβ generation and accumulation, which play a role the development of the pathology of Alzheimer´s disease. Studies consider metabolites of plants to be a useful platform in the discovery and development of drugs for the treatment of AD and phenylpropanoid metabolites have emerged as prime candidates due their diverse biological functions [2].

Caffeoyltyramine has shown protective effects against H_2O_2-induced neurotoxicity in PC12 cells in other studies, and anti-neuroinflammatory activity by down-regulating TNF-α released by LPS-induced BV2 cells [11,29]. This data indicates that *N-trans* caffeoyltyramine may suppress the production and the secretion of Aβ by means of the inhibition of the enzyme involved in its generation.

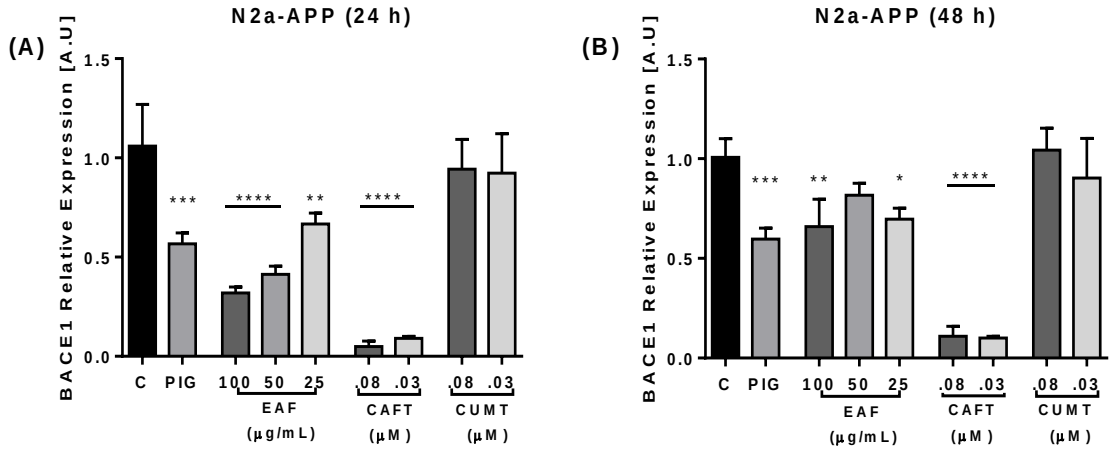

Fig. 4. Effects of EAF, CAFT and CUMT on BACE1 gene expression. N2a-APP cells were treated with EAF at 25-100 µg/mL, CAFT and CUMT at 0.03 – 0.08 µM for 24 h **(A)** and 48 h **(B).** PIG (14 µM) was used as positive control. The result showed significant reduction in BACE 1 gene expression of EAF and CAFT after 24 h **(A)** and after 48 h treatment EAF decreased activity on gene expression and CAFT showed stable activity suppressing gene expression **(B)**, CUMT showed no significant changes. EAF, CAFT and CUMT-treated cells were compared with control cells. Data are expressed as mean ± SD. n = 3. The p values were calculated using one-way ANOVA, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Effect of EAF and phenyl amides on PPARγ gene expression in N2a-APP cells.

In order to further confirm whether defatted hempseed compounds up-regulate the PPARγ expression in N2a-APP cells, the gene expression was measured of cells treated with the EAF and its metabolites for 24 h and 48 h. The result showed a significant increase in PPARγ gene expression by EAF in cells treated for 24 h at all concentrations in the evaluation. Although the expression

decreased after 48 h of treatment, differences with the control cells remained only in high EAF concentrations (**Fig.5**). However, caffeoyltyramine showed a substantially higher PPARγ expression compared to that of total EAF with a slight decrease after 48 h of treatment (**Fig.5 B**). As described above (BACE 1), CAFT retained its activity over time and induced PPARγ gene expression. Coumaroyltyramine showed no effect on the PPARγ expression. Studies have shown that PPARγ regulates the transcription of genes involved in lipid and glucose metabolism, inflammation, and redox equilibrium, among others. The overexpression of BACE 1 in the brain has been observed under inflammatory conditions, activated by pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α) released by microglial cells. PPARγ is also affected by the secretion of inflammatory cytokines, thereby strongly reducing their expression, an effect that can be suppressed with the use of PPARγ agonist drugs, which have emerged as a new therapy in the treatment of AD. The regulation of the transcription of the enzyme BACE 1 responsible for the production of neurotoxic amyloid β oligomers, seems to be the principal therapeutic target of AD drugs production, reports show how PPARγ regulates their transcription [15,30,31]. Results show that EAF and CAFT treatment could reduce Aβ deposition in the brain by means of decreased levels of BACE 1 gene expression through the activation of the PPAR γ pathway, which is a well-known regulator of BACE 1.

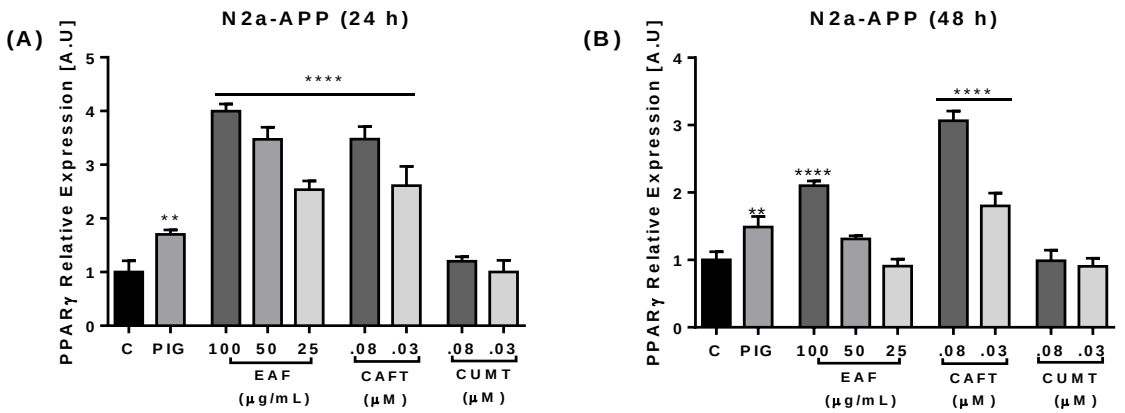

Fig. 5. EAF, CAFT and CUMT promoted PPARγ gene expression. N2a-APP cells were treated with EAF at 25-100 µg/mL, CAFT and CUMT at 0.03 – 0.08 µM for 24 h (**A**) and 48 h (**B**). PIG (14 µM) was used as positive control. The result showed significant changes in PPARγ gene expression of EAF and CAFT after 24 h at all concentrations test (**A**) and after 48 h treatment EAF and CAFT showed decreasing activity (**B**), CUMT showed no significant changes. EAF, CAFT and CUMT- treated cells were compared with control cells. Data are expressed as mean ± SD. n = 3. The p values were calculated using one-way ANOVA, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Effect of hempseed EAF and phenyl amides on PPARγ Co-Activator-1α (PGC-1α) gene expression.

EAF treatment for 24 h significantly increased PGC-1α expression. On the other hand, treatment for 48 h has a tendency to decrease said expression and shows no differences with control cells in gene expression. Similar are found for caffeoyltyramine in both 24 h and 48 h treatments with significant differences in relation with control, while coumaroyltyramine shows no effects on PGC-1α expression (**Fig. 6**). Our present study demonstrates that incubation with CAFT for 24 h and 48 h results in an apparent reduction of PGC-1α gene expression in N2a-APP cells. PGC-1α is a coactivator involved in the transcription of PPARγ, the regulation of mitochondrial biogenesis, fatty acids, respiratory capacity, and oxidative metabolism [3,32,33]. Studies point to its participation in neurodegenerative diseases, where decreased mRNA expression in the brain with Alzheimer's pathology has been found [34]. Overall, caffeoyltyramine may have negatively regulated BACE 1 activity (**Fig. 4**). Various inflammation-related

transcription factors, such as PPARγ, NF-κB and PGC-1α are involved in the process of BACE 1 regulation. Given that PGC-1α play a role in PPARγ transcription and that both are associated with neurodegenerative disorders, the positive effect of EAF and caffeoyltyramine on the expression of these two genes could lead to a positive impact in the prevention of mental decline and could work as neuroprotective agents. However, further studies are needed to determine the pathways related to the transcription of these negative regulators of BACE 1.

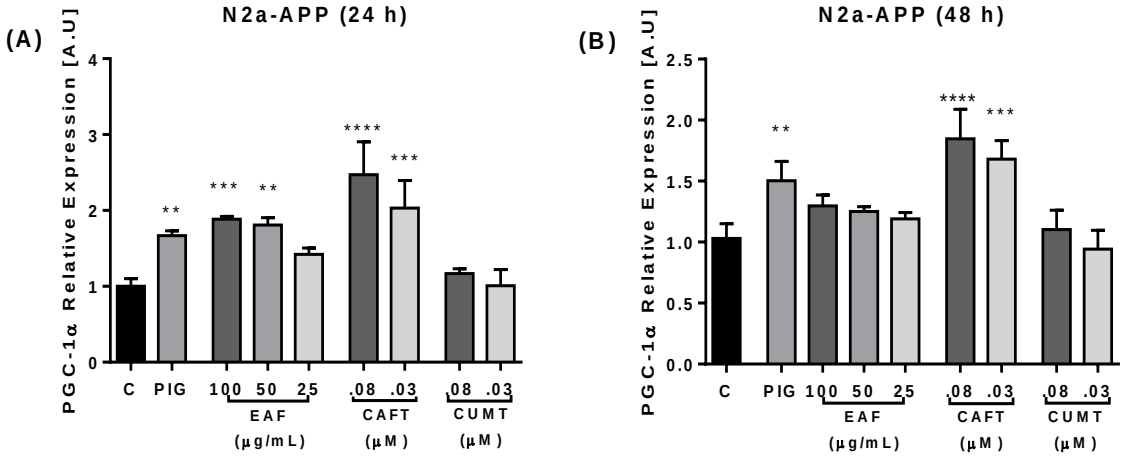

Fig. 6. EAF, CAFT and CUMT promoted PGC-1α gene expression. N2a APP cells were treated with EAF at 25-100 µg/mL, CAFT and CUMT at 0.03 – 0.08 µM for 24 h (**A**) and 48 h (**B**). PIG (14 µM) was used as positive control. Gene expression levels showed significant changes of PGC-1α in EAF and CAFT after 24 h (**A**) and after 48 h treatment, only the expression levels of CAFT were markedly increased in treated-cells (**B**), CUMT showed no significant changes. EAF, CAFT and CUMT-treated cells were compared with control cells. Data are expressed as mean ± SD. n = 3. The p values were calculated using one-way ANOVA, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

IV.4.-CONCLUSIONS

This study reveals the beneficial effects of the ethyl acetate fraction and main compound *N-trans* caffeoyltyramine from hempseed in the prevention as well as in the potential treatment of neurodegenerative diseases, through the positive effect on hallmarks involved in the development of Alzheimer´s diseases by reducing BACE 1 and increasing PGC-1α and PPARγ gene expression. In this context, hempseed constitutes a rich source of various bioactive compounds. Consumption of the ethyl acetate fraction and its bioactive metabolites has shown a wide range of promising activities with various human health benefits and this report indicates the neuroprotective properties of this vegetal. In the future, additional molecular studies together with clinical trials are required to establish the therapeutic safety and efficacy of EAF and CAFT.

Conflict of interest

The authors declare no competing financial interests.

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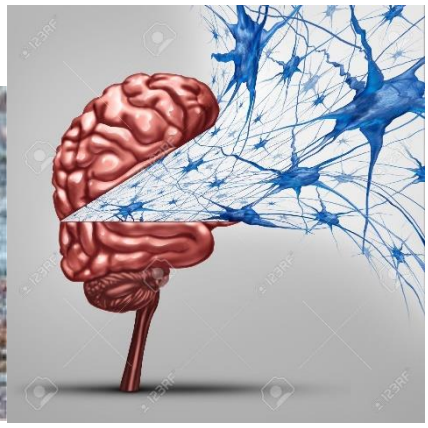
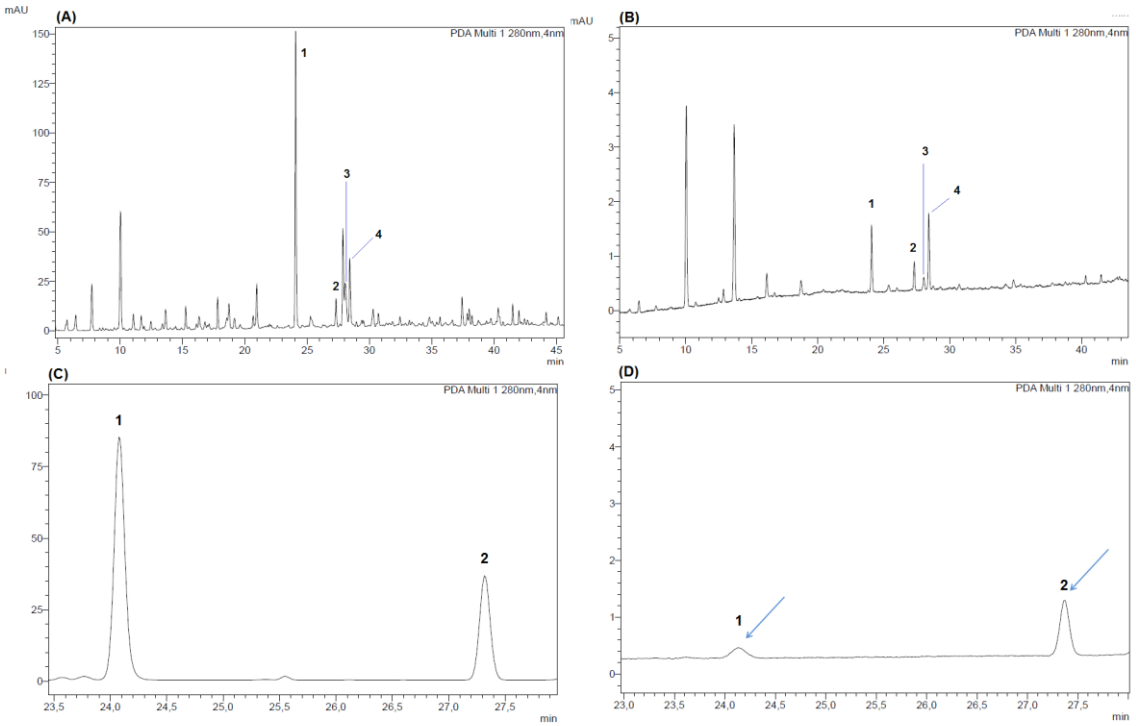
CAPITULO V

Discusión General de resultados





Cannabis sativa L. es una especie herbácea, originaria de Asia central, que ha sido empleada desde la antigüedad, tanto en medicina tradicional como en el ámbito industrial. Su resurgimiento durante los últimos años, es debido a su versatilidad, fácil cultivo y rápido crecimiento. Sus múltiples aplicaciones y la gran variedad de compuestos fitoquímicos que contiene, hacen que esté considerada como la planta de las mil y una moléculas, teniendo aplicaciones en el sector farmacéutico, alimentario, textil y de la construcción [1]. Entre los más de 565 compuestos publicados/aislados de *Cannabis sativa* L. durante los últimos años, 120 de ellos, poseen esqueleto terpenofenólico C_{21} a los que se les denomina “cannabinoides” [2]. Si bien en el género *Cannabis* la atención se ha centrado, principalmente, en sus compuestos psicoactivos, en especial el tetrahidrocannabinol (THC), como agentes terapéuticos, particularmente como analgésicos [3].Sin embargo, también hay un gran número de investigaciones enfocadas hacia otros componentes no cannabinoides (fenoles, flavonoides, esteroides, alcaloides, lignanamidas, espiroidanos, estilbenos y dihidrofenantrenos) [4], que han mostrado una gran variedad de actividades biológicas.

Más de 70 tipos de cultivares aprobados de fibra de cáñamo, se encuentran destinados para la producción agrícola en Europa, donde su contenido en THC dentro del límite legalmente permitido se restringe a valores < 2% (% p/p de inflorescencias maduras secas) [5,6].

Por otro lado, el aceite obtenido de las semillas de cáñamo mediante prensado en frio, sin el uso de disolventes orgánicos y calor, es uno de los principales productos aprovechados en la industria y contiene diferentes compuestos

bioactivos como ácidos grasos, fenoles, flavonoides y tocoferoles principalmente [7].

Los residuos o subproductos restantes de la semilla, después de la obtención del aceite, han mostrado tener un alto contenido de compuestos fenólicos con propiedades antioxidantes [8,9]. Estos compuestos, pueden extraerse con diferentes disolventes como agua, metanol, etanol, acetona, acetato de etilo, butanol o sus mezclas. De acuerdo a nuestros resultados presentados (**Capitulo 2**), después de un tratamiento para desengrasar las semillas de cáñamo triturada y sometida a maceración, utilizando un disolvente no polar (n-hexano) y la posterior obtención de diferentes fracciones/extractos con la ayuda de disolventes polares o sus mezclas (etanol, butanol, acetato de etilo, agua), en diferentes proporciones, el contenido de fenoles y flavonoides totales varían en un rango de 0.88 - 5.67 mg GAE/100 mg en fenoles y de 0.45 - 1.76 mg QE/100mg en flavonoides, donde las mezclas acuosas resultan ser aptas para una mejor extracción, resultados que se pueden contrastar con estudios similares descritos en bibliografía [9].

La determinación de la capacidad de captación de radicales libres, mediante ensayos de DPPH y ABTS, resultan viables como ensayos preliminares para estimar el potencial de los extractos, ya que la actividad antioxidante es proporcional a la cantidad de compuestos bioactivos presentes. La semilla de cáñamo al ser una matriz muy compleja que posee una amplia variedad de compuestos, requiere de técnicas y equipos analíticos modernos con alta sensibilidad para su análisis, como es el caso de Cromatografía liquida de alta resolución, acoplada a espectrometría de masas (UHPLC-HRMS/MS), que se va abriendo paso durante los últimos años como una técnica eficaz que permite

el análisis, determinación e identificación de cientos de compuestos en mezclas complejas como los extractos vegetales, en periodos de tiempo relativamente cortos [10,11]. Después de desarrollar un protocolo para determinación y cuantificación de los compuestos que permanecen en la semilla de cáñamo tras la eliminación del aceite, se obtuvieron diferentes fracciones, siendo las más prometedoras las llamadas F03 y F05, por su alto contenido en compuestos fenólicos totales y en flavonoides en particular, y por su mayor capacidad antioxidante. Dichas fracciones fueron analizadas por UHPLC-HRMS/MS en modo negativo, mostrando la presencia de aminoácidos, ácidos fenólicos, flavonoides, amidas fenólicas, lignamidas, cannabinoides, destacando la alta concentración de *N-trans* caffeoyltyramina (6.362 ± 0.892 mg/g extracto), que en comparación con otros estudios es uno de los principales metabolitos encontrados en las semillas, junto con lignanamida Cannabisin B [8,9].

Después de la caracterización y cuantificación fitoquímica, procedimos a investigar la actividad biológica de dichas fracciones, iniciando el estudio con la evaluación de su acción anti-inflamatoria en monocitos humanos primarios. Los monocitos desempeñan un papel fundamental en la reparación de lesiones, homeostasis y desarrollo de tejido. Al formar parte del sistema inmunitario innato, participan activamente regulando el inicio, desarrollo y resolución de los procesos inflamatorios, mediante la liberación de citoquinas pro-inflamatorias y anti-inflamatorias [12,13]. En nuestro estudio los monocitos fueron aislados de células mononucleares de sangre periférica de donadores sanos, si bien los monocitos humanos son una población heterogénea con tres subconjuntos bien definidos por su expresión de antígenos en superficie CD14

y CD16: clásicos ($CD14^{++}CD16^{-}$), intermedios ($CD14^{++}CD16^{+}$) y no-clásicos ($CD14^{+}CD16^{++}$). Mediante el tratamiento con las fracciones, se exploró si las mismas afectarían a la distribución de los diferentes subconjuntos de monocitos, encontrándose un incremento de la expresión en superficie de $CD16^{+}$ (no – clásicos) en los monocitos humanos primarios estimulados con LPS, para la fracción F05 (100 µg/mL). Estudios *in vitro,* muestran a este subconjunto de monocitos como los más pro-inflamatorios en respuesta a la estimulación de TLR [14], como también tienen efecto protector de la homeostasis, con un periodo de vida de aproximadamente 7 días [15].Los monocitos clásicos e intermedios tienen una respuesta inflamatoria inmediata en lesiones alcanzando concentraciones máximas en tiempos cercanos a las 48 h, donde liberan activamente citoquinas pro-inflamatorias que sin el respectivo balance, conducen a la progresión de diferentes enfermedades, razón por la cual los diferentes subconjuntos de monocitos deben circular manteniendo un equilibrio dinámico.

Podemos resaltar que las fracciones obtenidas, tienen efectos positivos, controlando la secreción y expresión génica de citoquinas pro-inflamatorias evaluadas (TNF-α y IL-6) en monocitos humanos primarios estimulados con LPS. La fracción F05 muestra una mayor reducción de la respuesta inflamatoria y se corresponde con la mayor concentración de compuestos presentes. Los resultados reflejan, que obtener fracciones ricas en compuestos como amidas fenólicas, presentes en las semillas de cáñamo, es una opción viable, además de poder evaluarse diferentes bio-actividades para conocer su papel como posibles agentes terapéuticos en enfermedades inflamatorias.

Los productos naturales han sido utilizados desde la antigüedad, para tratar diferentes enfermedades y basándonos en el conocimiento producido por nuestros antepasados, forman la base para el descubrimiento de nuevas moléculas con aplicaciones terapéuticas. El número de investigaciones que busca dilucidar las propiedades de diferentes especies vegetales es muy bajo, debido principalmente a la extensa biodiversidad de plantas existentes. Sin embargo, desde hace décadas han aportado nuevos medicamentos a la medicina moderna, por la amplia variedad de estructuras químicas y bioactividades atribuidas [16,17].Un ejemplo importante es cuando en 1805 se investigó la bio-actividad del primer alcaloide aislado del opio, la morfina. Respecto a las semillas de cáñamo, un gran número de investigaciones han surgido durante los últimos años, donde se han evaluado fracciones/extractos así como, principios activos aislados (**CAPITULO 1**). El aprovechamiento de este tipo de compuestos ha sido hasta el momento muy escaso, pues el interés hasta ahora de las semillas se ha debido a su aceite, al que se atribuyen propiedades beneficiosas para la salud por su aporte de altos niveles de ácidos grasos esenciales [18].

Continuando con la evaluación de posibles actividades biológicas de los compuestos presentes en las semillas de cáñamo desengrasadas, la fracción de acetato de etilo obtenido mediante maceración y UAE, con la posterior extracción secuencial (líquido - líquido), presentó una alta concentración en el contenido de fenoles (10.35 ± 0.7 mg GAE/100 mg extracto), flavonoides (3.09 ± 0.64 mg RE/100 mg extracto) y ácidos hidroxicinámicos (6.25 ± 0.44 mg CAE/100 mg extracto), determinados por métodos espectrofotométricos, que posteriormente se confirmaron con técnicas más específicas (UHPLC-

HRMS/MS) que muestran una gran proporción de ácidos hidroxicinámico y sus derivados (**CAPITULO 3**). La utilización de técnicas no convencionales o también conocidas como “Green Methods” como UAE o MAE, aplicados en la extracción de principios activos de semillas de cáñamo, han mostrado ser más eficientes, aumentando el porcentaje de obtención de polifenoles [19,20], aunque se deben considerar diferentes parámetros como temperatura, tiempo de extracción y volumen de disolvente, debido a que diferentes compuestos pueden verse afectados por una sobreexposición a los distintos tratamientos para su extracción, por lo que los estudios sugieren que deben optimizarse diferentes parámetros para maximizar la extracción de compuestos bioactivos [21]. En nuestro estudio mediante la combinación de métodos clásicos y asistidos (UAE), se reflejó un aumento de la cantidad compuestos extraídos (fenoles, flavonoides, ácidos hidroxicinámicos) comparados con los valores de cuando fueron extraídos sólo mediante maceración (1.90 - 5.67mg GAE/100 mg extracto y 0.45 - 1.76 mg QE/100 mg extracto), al igual que la cantidad del componente mayoritario *N-trans* cafeoiltiramina 8.869 ± 0.446 mg/g extracto y ácidos hidroxicinámicos representativos. Para conocer la influencia de los diferentes tratamientos en la extracción de compuestos bioactivos, es necesaria la cuantificación individual de los compuestos presentes por HPLC o LC-MS, ya que la cuantificación solo por métodos espectrofotométricos puede verse afectada por la presencia de azucares reductores, ciertas proteínas y clorofila que se encuentra en gran proporción en la semilla [7].

N-trans cafeoiltiramina y *N-trans* cumaroiltiramina fueron aislados de la fracción de acetato de etilo, mediante cromatografía en columna utilizando diferentes mezclas de disolventes (n-hexano: acetato etilo: metanol). Posteriormente al

aislamiento, los compuestos fueron identificados por UHPLC-HRMS/MS, en base a: tiempo de retención, masa molecular exacta, fragmentación y absorción UV. Siguiendo con los ensayos farmacológicos, se procedió a evaluar, *in vitro,* la actividad inhibitoria sobre varias enzimas implicadas en los procesos de la neurotransmisión como: Acetilcolinesterasa, Monoamino oxidasa A/B y Tirosinasa. Inhibidores de estas enzimas son propuestos como agentes terapéuticos en diferentes etapas de enfermedades neurodegenerativas, ya que existe un limitado número de fármacos aprobados para estas patologías y no están exentos de efectos adversos [22]. Por todo ello, la utilización de productos naturales surge como una gran promesa para el desarrollo de terapias efectivas [23]. Las enfermedades neurodegenerativas más comunes como Alzheimer y Parkinson comparten características similares, entre las que se encuentran: la pérdida progresiva de función neuronal, acoplamiento y agregación de proteínas (Aβ, αS) e iones metálicos (Cu^{2+}, Zn^{2+}, Fe^{3+}), y de momento las terapias existentes solo pueden mejorar o retrasar el transcurso de estas enfermedades moderadamente [24].

Como resultado de la evaluación de la actividad inhibitoria *in vitro,* EAF muestra valores de IC_{50} de 50.12 ± 1.20 µg/mL en la inhibición no especifica de MAOs, IC_{50} 55.59 ± 1.06 µg/mL en la inhibición especifica de MAO –A y IC_{50} 46.34 ± 1.16 µg/mL para MAO –B. Según estos datos, presenta inhibición de las dos enzimas, con diferencias significativas entre ellas, destacándose que posee mayor tendencia a inhibir MAO-B. Del mismo modo, los compuestos ensayados, presentan inhibición de las dos enzimas, sin diferencias significativas entre las dos MAOs. La inhibición de estas enzimas tiene un importante papel en enfermedades como PD, AD así como en trastornos

depresivos, ya que la inhibición de MAOs eleva la disponibilidad fisiológica de aminas biogénicas (noradrenalina, dopamina, serotonina) en el espacio sináptico, mejorando la función cerebral debido al papel que desempeñan en la neurotransmisión [25]. Inhibidores MAO-B son aplicados en diferentes tratamientos en la enfermedad de Parkinson, ya que en modelos experimentales, se ha observado que disminuyen la progresión de la muerte neuronal, reducen la degradación de dopamina, disminuye el estrés oxidativo provocado por el recambio de aminas biogénicas por la acción de la MAO-B, influyendo positivamente en la progresión de la enfermedad [26].

La Tirosinasa es otra de las enzimas que ha sido investigada ampliamente, por su papel en la degradación de monoaminas, si bien la dopamina es la catecolamina más importante dentro del cerebro por su papel como neurotransmisor y como precursora de otras aminas adrenérgicas. La enfermedad de Parkinson se caracteriza por la muerte prematura de neuronas dopaminérgicas de la sustancia negra pars compacta, de ahí la influencia de este neurotransmisor en esta patología. Aunque en condiciones normales las pequeñas cantidades de dopamina extracelular o citosólica llegan a convertirse en neuromelanina, su producción excesiva genera la oxidación de ésta a especies altamente reactivas DA-quinonas [27]. Entre éstos, el aminocromo, el más estable de los productos de la degradación de dopamina, está involucrado en reacciones neurotóxicas produciendo una neurotoxicidad crónica en neuronas dopaminérgicas [28]. Se ha observado que pacientes con PD también padecen AD, por lo cual la búsqueda de moléculas con múltiples actividades biológicas como inhibidores de tirosinasa o de AChE, son enfoques bastante idóneos para encontrar, posibles nuevos tratamientos [29]. Teniendo

en cuenta esta perspectiva, los resultados obtenidos por la fracciones y compuestos aislados de las semillas de cáñamo muestran una marcada actividad en la inhibición de Tirosinasa: EAF (IC_{50} µg/mL) 60.39 ± 1.05; CUMT 11.78 ± 1.24; CAFT 8.13 ± 1.03, donde este último no mostro diferencias significativas con el control positivo (Ácido Kojico). En estudios similares se han destacado las propiedades inhibitorias de las fenilamidas (amidas derivadas del acoplamiento de ácido cafeico, cumárico con tiramina) como potentes inhibidores de Tirosinasa [30]. Por otro lado, la fracción evaluada, presentó una moderada inhibición de AChE como también los compuestos ensayados: (IC_{50} µg/mL) para: CUMT 85.56 ± 0.15 y CAFT 157.56 ± 21.04, aunque estudios similares han revelado valores más bajos de inhibición (IC_{50} µg/mL) 25.21 µg/mL para CAFT y 29.4 µg/mL para CUMT catalogando a estos compuestos como inhibidores moderados de AChE [31], así también se han publicado valores más altos para CAFT (IC_{50} µg/mL) 64.58 µg/mL [32]. La diferencia de valores entre estudios, se debería en gran medida al tipo de disolventes utilizados para ejecutar los ensayos y la influencia de la concentración de los mismos [33].

Tras los resultados positivos obtenidos en los ensayos *in vitro*, se evaluó la influencia en los niveles de aminas biogénicas en un modelo *in vivo*. Como se menciona anteriormente, la enfermedad de Parkinson es compleja, está relacionada con la edad y asociada con la deficiencia de dopamina, como a su vez la combinación de factores ambientales, genéticos, exposición a químicos tóxicos o lesiones a nivel cerebral [34].

Como se detalla en el **Capítulo 3,** procedimos a la cuantificación de aminas biogénicas en el cuerpo estriado de los diferentes grupos de animales,

mostrándose un incremento en los niveles de aminas biogénicas. DA y su metabolito no reactivo HVA, presentan diferencias significativas con el control utilizado ($p < 0.05$) cuando se utiliza la concentración de EAF de 100 mg de Extracto seco/Kg. Lo mismo ocurre con el fármaco utilizado como control positivo Deprenyl, un inhibidor selectivo de MAO-B, ampliamente utilizado para el tratamiento de PD, AD, trastornos depresivos y también como fármaco antienvejecimiento que fue registrado en más de 60 países desde su descubrimiento (Josep Knoll, 1960s) [35]. Además del incremento de dopamina a nivel estriatal, la fracción EAF, a la dosis más alta ensayada, mostró niveles superiores al control del neurotransmisor cuantificado 5-HT y su metabolito de degradación 5-HIAA, aunque sin diferencias significativas. Los resultados obtenidos en el análisis *in vivo,* son similares a los obtenidos *in vitro,* ya que el ensayo de la fracción total sobre la inhibición de MAOs no específica, mostró valores de IC_{50} de 50.12 ± 1.20 µg/mL, en fracciones mitocondriales que fueron la fuente de MAOs, por lo que se puede asumir que ejerce una marcada actividad inhibitoria sobre las dos isoformas de MAO (A y B).Con la creciente popularidad de los beneficios de la semilla de cáñamo en la nutrición humana, un gran número de investigaciones han comenzado a surgir para conocer su papel en enfermedades neurodegenerativas. En estudios *in vivo,* donde se han administrado diferentes dosis de una fracción rica en fenilpropionamidas han mostrado tener efectos positivos sobre el daño inducido por LPS en modelos animales, ejerciendo un efecto anti-neuroinflamatorio a dosis de 1 g/ Kg [36]. También produce la inhibición de la proliferación de células cancerígenas en líneas celulares de glioblastoma U-87 [37]. A su vez, el compuesto aislado Cannabisin F, se ha evaluado en células microgliales BV2

estimuladas con LPS, mostrando supresión de la expresión génica de mediadores pro-inflamatorios, además de mejorar la expresión de SIRT1, el mismo que juega un papel importante en procesos antioxidantes y anti-inflamatorios [38]. También se inhibe el TNF-α producido por células microgliales por parte de las diferentes lignanamidas y fenilamidas, resaltándose el potente efecto inhibidor producido por un nuevo derivado cumaroilamino glucósido aislado [39].

Las investigaciones relacionadas con el efecto sobre diferentes áreas del SNC, tienen el problema de si estas moléculas pueden o no atravesar la barrera biológica que permite el paso de diferentes fármacos hacia el cerebro. Así es como esta barrera semipermeable (BBB) evita el ingreso de químicos tóxicos, sustancias nocivas y regula el paso de nutrientes desde el torrente sanguíneo al cerebro. Esta barrera fisiológica se ha convertido en uno de los grandes desafíos en lo que corresponde al desarrollo de fármacos con aplicaciones en enfermedades neurodegenerativas, que si bien presentan potentes actividades *in vitro,* muy rara vez pueden atravesar esta barrera biológica y alcanzar regiones del cerebro donde está destinada su acción farmacológica [40]. En etapas tempranas de desarrollo de fármacos, los modelos PAMPA son los más utilizados para predecir la absorción pasiva intracelular en modelos *in vitro.* En este caso los modelos PAMPA-BBB están orientados al descubrimiento de fármacos para SNC, aunque son menos representativos biológicamente, ofrecen un mejor rendimiento, consistencia y bajos costes por lo cual son más adecuados para preseleccionar moléculas candidatas a fármacos [41].

En este sentido, diferentes compuestos presentes en la compleja matriz de la semilla de cáñamo, fueron investigados sobre un modelo *in vitro* con vistas a

predecir si atraviesan o no esta barrera biológica (**CAPITULO IV**). Las fenilamidas como: *N-trans* cafeoiltiramina, *N-trans* cumaroiltiramina y feruloiltiramina se detectaron en los pocillos aceptores después del análisis (PAMPA-BBB), además de la ligananamida Cannabisin A. La fracción rica en este conjunto de compuestos resulta de la conjugación de ácidos hidroxicinámicos (cafeico, ferúlico, cumárico) con arilmonoaminas, principalmente la tiramina y octopamina, siendo las hidroxicinamoil amidas, las principales y las que se encuentran en mayor proporción en las semillas de cáñamo [37], por lo que estos compuestos pueden ser prometedores para su utilización en enfermedades del SNC. Por otro lado, se ha observado en diferentes estudios que la introducción de amidas en moléculas antineoplásicas, mejora la permeabilidad a través de BBB [42], así como también derivados de tiramida metilados se identifican como compuestos capaces de atravesarla [43]. Hidroxicinamoil amidas, como también sus productos de biosíntesis mediante acoplamiento aleatorio (lignanamidas) cuentan con grupos amida (Cannabisin A y B).Las lignanamidas derivadas del acoplamiento de ácido ferúlico (Cannabisin C - G, grossamida) poseen también grupos metilados en su estructura lo cual podría mejorar su permeabilidad y atravesar la BBB. Actualmente, diferentes estudios sugieren que estos compuestos podrían tener efectos positivos en enfermedades neurodegenerativas [44,45,46].

Los diferentes estudios de permeabilidad de extractos de plantas deben ser evaluados en mezclas, como también de forma individual, ya que diferentes compuestos podrían actuar como co-solventes mejorando la solubilidad y permeabilidad de otros, en los ensayos PAMPA [47].

Durante las últimas décadas, las enfermedades neurodegenerativas han emergido como un serio problema global de salud. AD es la principal enfermedad neurodegenerativa con datos que afirman que alrededor de 50 millones de personas padecen la enfermedad y se piensa que más de 152 millones padecerán demencia en 2050. Los costes anuales se estiman en 1 billón de dólares, los mismos que se duplicaran a 2030 [48]. Al considerarse un problema global, diferentes investigaciones se han enfocado en la búsqueda de nuevos compuestos para su tratamiento, sin que hasta la fecha los diferentes fármacos aprobados puedan mejorar el transcurso de la enfermedad.

Nuevas evidencias e informaciones aportadas por laboratorios y clínicas corrobora que un factor de iniciación del Alzheimer, resulta del desequilibrio en la producción y aclaramiento de placas Aβ 42 y péptidos relacionados [49]. En nuestro estudio para conocer si la fracción rica en fenilamidas y los compuestos aislados ejercen efectos positivos sobre AD, se consideró evaluar sus efectos sobre los niveles de ARNm de BACE1, responsable de la acumulación anormal de Aβ, que es la característica más representativa de la enfermedad. También se evaluó el efecto sobre los conocidos reguladores de BACE1: PCG-1α y PPARγ en células N2a-APP.

El tratamiento de las células N2a-APP con la fracción rica en fenilamidas y las dos hidroxicinamoil amidas (cafeoiltiramina y cumaroiltiramina) en diferentes concentraciones (25 -100 µg/mL EAF; 0.03 – 0.08 µM CAFT y CUMT) mostraron efectos positivos, regulando la expresión génica de BACE1, que es la enzima encargada de la escisión APP y que inicia la formación de Aβ. Al inhibir su acción se elimina el primer paso en la acumulación y acoplamiento de placas amiloides a nivel cerebral [50]. EAF y CAFT muestran ser prometedores

en la inhibición de BACE 1. Al evaluarse su efecto en diferentes tiempos de tratamiento (24 y 48 h), se observa que solo CAFT se mantiene activo sin variaciones marcadas de su efecto tras 48 h de tratamiento, convirtiendo a este compuesto como un firme candidato para futuros ensayos, dado su potente efecto inhibidor de BACE1 en el orden de nM. Diferentes compuestos de origen natural se han evaluado como inhibidores de BACE1, trans-cinamaldehido el principal componente de la corteza de canela (*Cynamomum ceylanicum*) [51], miyabenol C aislado de una uva silvestre originaria de Taiwan [52], Catalpol un iridoide glucósido, aislado de *Rehmannia glutinosa* [53], como también diferentes flavonoides han mostrado tener efectos beneficiosos en el deterioro cognitivo y podrían desempeñar un efecto positivo, reduciendo el riesgo de demencia [54]. Durante los últimos años los productos de origen natural se presentan como una fuente de moléculas para ser ensayadas con enfoques terapéuticos en enfermedades neurodegenerativas.

Además de la búsqueda de compuestos que inhiban la acción de BACE1, para bloquear la generación y agregación de péptidos beta amiloides en el cerebro, como estrategia de tratamiento de la AD, también hay un gran interés en la investigación de moléculas con otras actividades biológicas, como inhibir la expresión génica de ARNm de BACE1, o si estas poseen un efecto doble y son agonistas de diferentes reguladores de BACE1 como PPARγ y PGC-1α.

PPARγ está involucrado en el control de genes relacionados con metabolismo de glucosa, lípidos, inflamación, equilibrio redox y sensibilidad a la insulina.La importancia de los agonistas de PPARγ radica en que se han indicado como agentes neuroprotectores, mejoran la plasticidad sináptica, actúan mejorando la respuesta al estrés, supervivencia celular y recuperando el equilibrio en

lesiones cerebrales. En lesiones agudas, evita el daño tisular por la inhibición de NF-kB, reduciendo la inflamación y estimulando Nrf2/ARE, bloqueando el estrés oxidativo generado [55,56].

Los productos naturales continúan siendo una fuente de fármacos, habiéndose demostrado que diferentes agonistas de PPARγ de origen natural, son capaces de desempeñar efectos y funciones vitales además de ejercer efectos antioxidantes, anti-inflamatorios, antitumorales o reguladores del metabolismo. Estos efectos positivos se deben a que PPARγ se encuentra involucrada en diferentes procesos fisiopatológicos [57].

El co-activador transcripcional PGC-1α es un inductor de la biogénesis mitocondrial, que controla el metabolismo oxidativo, funcionando como un poderoso regulador en la desintoxicación de ROS, al incrementar los niveles de expresión de diferentes enzimas involucradas en la eliminación de estas especies reactivas, minimizando el impacto fisiológico de las diferentes ROS a nivel celular y manteniendo la homeostasis, por lo que es de importancia en trastornos neurodegenerativos como el envejecimiento, donde las funciones mitocondriales se encuentran deterioradas [58]. Estudios sobre este regulador de BACE1, han mostrado que sus niveles de expresión se encuentran disminuidos en el cerebro de personas con Alzheimer. Conocer diferentes compuestos que modulen la sobreexpresión de PGC-1α y disminuyan los niveles de Aβ secretada, tendrían potenciales efectos positivos sobre dicha enfermedad [59,60].

En este estudio hemos demostrado que tanto EAF como la cafeoiltiramina disminuyen los niveles de expresión génica de BACE1 e incrementan la

expresión de PPARγ y PGC-1α, dos de sus principales reguladores, ejerciendo un efecto doble: Inhibidor / Agonista.

A pesar de las diferentes propiedades ensayadas y publicadas de esta fracción rica en fenilamidas, (antioxidante, anti-inflamatoria, inhibidora de enzimas cerebrales involucradas en diferentes patologías de SNC) y por otro lado, la gran riqueza de compuestos que posee la semilla de cáñamo, todavía se está lejos de haber completado su estudio **(CAPITULO I).**

Hasta el momento, las diferentes fenilamidas y lignanamidas de la semilla de Cáñamo, solo se habían aislado en pequeñas cantidades, debido a sus concentraciones relativamente bajas (µg/g extracto), sin embargo en la actualidad, están emergiendo nuevos métodos de extracción, que permiten obtener estos compuestos con un mayor rendimiento.

Esto hace que el estudio fitoquímico y farmacológico de estas moléculas, sea una opción más viable y económica [36,37,61] además de ampliar el campo de posibilidades, en la investigación sobre el tratamiento de enfermedades neurodegenerativas.

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CAPITULO VI

Conclusiones / Conclusions / Zaključci

1. Las diferentes fracciones y extractos obtenidos de las semillas de cáñamo (*Cannabis sativa* L.), presentaron una alta riqueza en compuestos fenólicos. El contenido total de los mismos, varía en función del tipo de disolvente utilizado para la extracción, siendo las mezclas de etanol: agua las que produjeron un mayor rendimiento.

Different fractions and extracts obtained from hemp seeds (*Cannabis sativa* L.), have a high richness of phenolic compounds. Their total content varies according to the type of solvent used for extraction, ethanol: water mixtures giving the highest yield.

Različite frakcije i ekstrakti dobiveni iz sjemenki konoplje (*Cannabis sativa* L.) bogati su fenolnim spojevima. Njihov ukupni sadržaj varira ovisno o vrsti otapala koje se koristi za ekstrakciju, a smjesa otopina etanola : voda daju najveći prinos.

2. Todos los extractos/fracciones obtenidos a partir de la semilla de cáñamo, tras ser sometida a un proceso de desengrasado, poseen actividad antioxidante, siendo la fracción hidroalcohólica denominada F05, la que mostró una capacidad antioxidante más significativa.

All extracts / fractions obtained from hemp seed, after undergoing a degreasing process, have antioxidant activity, with the hydroalcoholic fraction, called F05, being the one that has a most significant antioxidant capacity.

Svi ekstrakti / frakcije dobiveni iz sjemenki konoplje, nakon procesa odmašćivanja, imaju antioksidativno djelovanje, a hidroalkoholna frakcija, nazvana F05, ima značajniji antioksidativni kapacitet.

3. Entre los compuestos aislados e identificados en la semilla de cáñamo, destacan las hidroxicinamoil amidas (cafeoiltiramina, feruloiltiramina, cafeoiloctopamina, cumaroiltiramina) con un porcentaje superior, a otros grupos de compuestos también presentes como son: lignanamidas, ácidos fenólicos, flavonoides, terpen-fenoles y ácidos hidroxicinámicos.

Among the compounds isolated and identified in hemp seed, the hydroxycinamoyl amides (caffeoyltyramine, feruloyltyramine, caffeoyloctopamine, coumaroyltyramine) stand out with a higher percentage than other groups of compounds also present, such as: lignanamides, phenolic acids, flavonoids, terpene-phenols and hydroxycinnamic acids.

Među spojevima izoliranim i identificiranim u sjemenkama konoplje, hidroksicinamoilamidi (kafeoiltiramin, feruloiltiramin, kafeoiloktopamin, kumaroiltiramin) zastupljeni su u većem postotku od ostalih skupina spojeva također prisutnih, kao što su: lignanamidi, fenolne kiseline, flavonoidi, terpenen-fenoli i hidroksicimetne kiseline.

4. En los ensayos de actividad anti-inflamatoria, realizados in vitro, sobre monocitos humanos primarios, las fracciones con mayor capacidad antioxidante (F03 y F05), fueron las que mostraron resultados más significativos, ejerciendo una reducción en la expresión génica y

liberación de citoquinas pro-inflamatorias IL-6 y TNF-α inducidas por LPS.

In the anti-inflammatory activity tests, performed *in vitro* on primary human monocytes, the fractions with the highest antioxidant capacity (F03 and F05) showed the most significant results, exerting a reduction in gene expression and release of the pro-inflammatory cytokines IL-6 and TNF-α induced by LPS.

U testovima protuupalne aktivnosti provedenim *in vitro*, na primarnim ljudskim monocitima, frakcije s najvećim antioksidativnim kapacitetom (F03 i F05) pokazale su najznačajnije rezultate, što je rezultiralo smanjenjem ekspresije gena i oslobađanjem proupalnih citokina IL-6 i TNF-α induciranih LPS-om.

5. Tanto las hidroxicinamoil amidas, derivadas de ácido cafeico y cumárico, como su fracción de procedencia, mostraron una marcada actividad inhibitoria *in vitro*, de las enzimas Monoamino oxidasa A y B, y de la Tirosinasa, así como una moderada inhibición de la Acetil-colinesterasa. *In vivo,* dicha fracción también indujo un incremento de las aminas biogénas, especialmente Dopamina, siendo cuantificadas en el núcleo estriado de los animales tratados.

Both hydroxycinnamoyl amides, derived from caffeic acid and coumaric acid, and their fraction of origin, showed a marked inhibitory effect on the enzymes Monoamine oxidase A and B, and on Tyrosinase *in vitro*, as well as a moderate inhibition of Acetylcholinesterase. *In vivo*, this fraction

also induced an increase in biogenic amines, particularly dopamine, which was quantified in the striatum of treated animals.

Oba hidroksicinamoilamidi, dobivena iz kofeinske kiseline i kumarinske kiseline, i njihova frakcija podrijetla, pokazali su značajan inhibitorni učinak na enzime monoaminooksidaze A i B i na tirozinazu *in vitro*, kao i umjerenu inhibiciju acetil-kolinesteraze. *In vivo*, ova frakcija također je inducirala porast biogenih amina, posebno dopamina, koji je kvantificiran u striatumu tretiranih životinja.

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6. Tras los ensayos de permeabilidad de membrana (PAMPA-BBB), se detectaron en la solución aceptora, los compuestos cafeoiltiramina, cumaroiltiramina, feruloiltiramina, y la lignanamida Cannabisina A. Esto indica que este grupo de compuestos, debido a su naturaleza más lipófila, podrían ser objeto de futuras investigaciones en la búsqueda de nuevos fármacos activos a nivel de SNC.

According to the membrane permeability tests (PAMPA-BBB), the compounds caffeoyltyramine, coumaroyltyramine, feruloyltyramine and the lignanamide cannabisin A were detected in the acceptor solution. This suggests that this group of compounds, due to their rather lipophilic nature, could be the subject of future research in the search for new active drugs at the CNS level.

Nakon testova propusnosti membrane (PAMPA-BBB), u akceptorskoj otopini detektirani su spojevi kafeoiltiramin, kumaroiltiramin, feruloiltiramin i lignanamid kanabisin A. To ukazuje na to da bi ova

skupina spojeva zbog svoje lipofilne prirode mogla biti predmet budućih istraživanja u potrazi za novim aktivnim lijekovima na razini CNS-a.

7. La fracción rica en fenilaminas y cafeoiltiramina, disminuye la expresión génica de BACE1 e incrementa la expresión génica de dos de sus principales reguladores PPARγ y PGC-1α mostrando un efecto positivo, actuando como agonistas de estos dos reguladores. Caeoiltiramina presente mayoritariamente en esta fracción, muestra ser responsable de un efecto doble: antagonista/agonista que permanece estable a diferentes tiempos, en los ensayos realizados en células N2a-APP.

The fraction rich in phenylamines and caffeoyltyramine decreases gene expression of BACE1 and increases gene expression of two of its main regulators PPARγ and PGC-1α showing a beneficial effect by acting as agonists of these two regulators. Caffeoyltyramine, mainly present in this fraction, has been shown to be responsible of a dual effect: antagonist / agonist, remaining stable at different time intervals, in the tests performed on N2a-APP cells.

Frakcija bogata fenilaminima i kofeoiltiraminom smanjuje ekspresiju gena BACE1 i povećava ekspresiju gena dvaju svojih glavnih regulatora PPARγ i PGC-1α pokazujući pozitivan učinak djelujući kao agonisti ova dva regulatora. Pokazalo se da je kofeoiltiramin, uglavnom prisutan u ovoj frakciji, odgovoran za dvostruki učinak: antagonist / agonist, koji ostaje stabilan pri različitim vremenskim intervalima u testovima provedenim na stanicama N2a-APP.

8. La conclusión final a la que hemos llegado en este trabajo de Tesis doctoral, sugiere que los diferentes compuestos obtenidos de la semilla de cáñamo, entre los cuales destacan las hidroxicinamoil amidas, son moléculas con múltiples actividades biológicas: antioxidante, anti-inflamatoria, inhibidora de enzimas involucradas en el SNC, inhibidoras de BACE 1 y agonistas naturales de reguladores master PPARγ y PGC-1α. Todo ello indica que el aprovechamiento de las semillas de cáñamo o de los subproductos después del proceso de obtención del aceite, nos ofrece un nuevo campo de búsqueda, para el posible desarrollo de nuevos fármacos efectivos en el tratamiento de enfermedades neurodegenerativas.

The final conclusion we have reached in this doctoral thesis, suggests that the various compounds obtained from hemp seed, among which the hydroxycinamoyl amides stand out, are molecules with multiple biological activities: antioxidant, anti-inflammatory, enzyme inhibitors involved in the CNS, BACE 1 inhibitors and natural agonists of the master regulators PPARγ and PGC-1α. All this indicates that the use of hemp seeds or by-products after the oil production process offers us a new field of search for the possible development of new effective drugs in the treatment of neurodegenerative diseases.

Konačni zaključak do kojeg smo došli tijekom ove doktorske disertacije sugerira da su različiti spojevi dobiveni iz sjemenki konoplje, među kojima se posebno ističu hidroksicinamoil amidi, molekule s višestrukim biološkim djelovanjem: antioksidativnim, protuupalnim, inhibitori enzima, uključeni u CNS, inhibitori BACE 1 i prirodni agonisti glavnih regulatora

PPARγ i PGC-1α. Sve to ukazuje da nam upotreba sjemenki konoplje ili nusproizvoda nakon postupka proizvodnje ulja nudi novo polje istraživanja za mogući razvoj novih učinkovitih lijekova u liječenju neurodegenerativnih bolesti.