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DOCTORADO EN BIOLOGÍA EXPERIMENTAL

EVALUACIÓN DEL EFECTO NEUROPROTECTOR DE LA SILIMARINA EN UN MODELO MURINO DE LA ENFERMEDAD DE PARKINSON

T E S I S

QUE PARA OBTENER EL GRADO DE

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P r e s e n t a:

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Resumen

La enfermedad de Parkinson (EP) se caracteriza por un déficit progresivo en la función motora como resultado de la pérdida de neuronas dopaminérgicas en la sustancia nigra. Un pequeño porcentaje de la incidencia de la EP presenta un componente hereditario, pero la mayoría de los casos son esporádicos o relacionados con algún factor químico y/o ambiental. La 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) induce características histopatológicas similares a las de la EP en ratones, primates y humanos. En la actualidad, el tratamiento para la EP consiste en la administración de levodopa, sin embargo, esta terapia sólo es efectiva durante los primeros años del tratamiento, por lo que es necesario encontrar nuevas alternativas terapéuticas. La silimarina, un extracto estandarizado de las semillas y frutas de la planta *Silybum marianum*, contiene una mezcla de flavonoides con propiedades anti-oxidantes, anti-inflamatorias, citoprotectoras, anti-carcinogénicas y antivirales. En este trabajo se evaluó el efecto neuroprotector de la silimarina en un modelo murino de la EP con MPTP, para lo cual se determinó la concentración de dopamina en el estriado de ratones machos adultos intoxicados con MPTP y ratones intoxicados con MPTP tratados con la silimarina. Con estos resultados se estableció la dosis de 100 mg de silimarina /kg/día como la más efectiva. Por otra parte, la evaluación de la apoptosis así como la expresión de la enzima tirosina hidroxilasa (TH) mostró que la silimarina no tiene efecto alguno por sí sola, y brinda un grado de protección en los animales intoxicados con MPTP, ya que previene la muerte celular y preserva las neuronas presentes en el mesencéfalo que expresan TH, así como sus terminaciones nerviosas en el estriado. Además, se observó que la administración

de silimarina eleva las concentraciones de malonaldialdehído (MDA) y que sólo ejerce su efecto antioxidante en los animales intoxicados con la MPTP.

Nuestros datos muestran que la silimarina conserva los niveles de dopamina estriatal al disminuir la apoptosis previniendo la pérdida neuronal y reduciendo el estrés oxidante asociado a la intoxicación con MPTP.

Abstract

Parkinson's disease (PD) is characterized by a progressive deficit in motor function as a result of the loss of dopaminergic neurons in the substantia nigra. A small percentage of the PD incidence presents a hereditary component, but most cases are sporadic or associated with some chemical and/or environmental factor. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a chemical compound that induces PD-like histopathological features in mice, primates and humans. At present, PD treatment consists in the administration of levodopa, however, this therapy is only effective during the first years of treatment, so it is necessary to find new therapeutic alternatives. Silymarin, a standarized extrac from seeds and fruit of plant *Silybum marianum*, is a mixture of flavonoids with, mainly, anti-oxidant, anti-inflammatory, anti-carcinogenic, cytoprotective and antiviral activities. In this study the neuroprotective effect of silymarin was evaluated in a murine model of PD intoxicated with MPTP, for which dopamine concentration was determined in the striatum of adult male mice either treated with MPTP or MPTP and silymarin. According to these results, the best sylimarin effective dose was established as 100 mg/Kg. Furthermore, the assessment of apoptosis and the expression of the enzyme tyrosine hydroxylase (TH) showed that silymarin had no effect by itself, and provided a degree of protection in animals intoxicated with MPTP, by preventing cell death and preserving neurons in the substantia nigra and nerve endings expressing TH in the striatum. Additionally, we observed that the administration of silymarin, raised the levels of malondialdehyde (MDA) and exerts its antioxidant effect only in animals intoxicated with MPTP.

Our data show that silymarin preserves striatal dopamine levels by diminishing apoptosis, preventing neuron loss and lowering oxidative stress associated with MPTP intoxication.

ABREVIATURAS

EP= Enfermedad de Parkinson

UCHLI= Ubiquitin hidrolasa carboxilo –terminal esterasa

ROS= Especies reactivas de oxígeno (de sus siglas en inglés)

MPTP= 1-metil-4-fenil-1,2,3,6-tetrahidropiridina

IL1β= Interleucina 1 Beta

IL6= Interleucina 6

TNFα= Factor de necrosis tumoral alpha

NGF= Factor de crecimiento nervioso

BDNF= Factor neurotrófico derivado del cerebro

iNOS= Óxido nítrico sintasa inducible

GSH= Glutatión reducido

GSSG= Glutatión oxidado

GPi= Globo pálido interno

GPe= Globo pálido porción externa

LRRK2= Quinasa rica en repeticiones de leucina 2

PINK1= Quinasa putativa 1 inducida por PTEN

6-OHDA= 6-Hidroxidopamina

DAT= Transportador de dopamina,

LAT-1= Transportador de aminoácidos 1 tipo L

BHE= Barrera hematoencefalica

SNC= Sistema nervioso central

$MPDP^+$= 1-metil-4-fenil-2,3-dihidropiridinio

MAO-B= Monoamino oxidasa B

MPP^+= 1-metil-4-fenilpiridinio

GNDF= Factor neurotrófico derivado de células gliales

TGFβ= Factor de crecimiento transformante beta

IL10= Interleucina 10

LPS= Lipopolisacárido

OMS= Organización Mundial de la Salud

ICAM-1= Molécula de adhesión intercelular 1

VCAM-1= Molécula de adhesión vascular 1

IFNγ= Interferón gamma

IFNα= Interferón alfa

IL2= Interleucina 2

IL-12= Interleucina 12

SOD= Superóxido dismutasa

TH= Tirosina hidroxilasa

BSA= Albúmina sérica de bovino

SIL= Silimarina

MDA= Malondialdehído

Aβ= Péptido Beta-Amiloide

Reβ= Receptor de estrógenos β

NO= Óxido nítrico

$O2^-$= Radical superóxido

NF-κβ= Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas

ÍNDICE

1. INTRODUCCIÓN

La enfermedad de Parkinson (EP) es la segunda enfermedad neurodegenerativa más frecuente a nivel mundial. En México es un problema creciente de salud pública, ya que es una de las primeras causas de atención en consulta externa del tercer nivel y presenta un número elevado de consultas anuales debido a la cronicidad del padecimiento y al manejo específico que requiere. En las próximas décadas se espera que el número de pacientes con EP se incremente debido al aumento en la esperanza de vida (SSA, 2006).

La EP es un padecimiento crónico, progresivo e incapacitante causado por la pérdida de neuronas dopaminérgicas, principalmente de la pars compacta de la sustancia nigra y por lo tanto por un déficit de dopamina (Fahn y Przedborski, 2000). La EP presenta una edad media de inicio entre 43 y 66 años (Diederich et al., 2003), afecta aproximadamente del 1-2% de las personas mayores de 65 años y es más frecuente en hombres que en mujeres (Mouradian, 2002; Pachana et al., 2013).

En la actualidad el tratamiento de la EP se centra principalmente en la restitución de los niveles de dopamina a través de la administración de levodopa (L-3,4-dihidroxifenilalanina, L-dopa), sin embargo, la mayoría de los pacientes después de unos años de haber iniciado el tratamiento presenta resistencia a la levodopa (Thanvi y Lo, 2004; Wenzel et al., 2014), por lo que es de suma importancia la búsqueda de nuevos fármacos que modulen la inflamación y el estrés oxidante, poco agresivos y con bajos efectos secundarios y colaterales como lo puede ser la silimarina.

2. ANTECEDENTES

2.1. Enfermedad de Parkinson

La enfermedad de Parkinson es la segunda enfermedad neurodegenerativa más común después de la enfermedad de Alzheimer. Estudios realizados en poblaciones europeas en edades de 55 o 65 años mostraron una tasa de incidencia de 410 y 529 casos nuevos por cada 100, 000 habitantes por año (Baldereschi et al., 2000; Benito-León et al., 2004; De Lau et al., 2004), y recientemente se reportó que en Norte América existe una tasa de incidencia de 224 casos nuevos por cada 100, 000 habitantes por año en individuos de 65 años de edad o más (Driver et al., 2009). Una revisión basada en 12 estudios de alta calidad en Estados Unidos y Europa revela que la prevalencia de la EP aumenta en personas mayores de 65 años de edad, alcanzando cifras de 950 casos por cada 100,000 habitantes, lo que equivale a 349,000 individuos afectados para el caso de Estados Unidos (Hirtz et al., 2007). Actualmente en Europa occidental hay 4.1 a 4.6 millones de personas mayores de 50 añose afectados con la enfermedad de Parkinson y se espera que para el año 2030 esta cifra se duplique (Dorsey, 2007), lo que conlleva un problema de salud pública. Es pertinente mencionar que en México la prevalencia se ha estimado entre 40-50 casos por cada 100,000 habitantes/año (Secretaria de Salud, 2008).

2.2. Etiología

La etiología de la EP está asociada tanto a factores genéticos como ambientales. Sólo un pequeño porcentaje (5%) de incidencia de la EP tiene un componente hereditario (Parkinson familiar), mientras que la mayoría de los casos son esporádicos (Parkinson idiopático) o bien relacionados con algún factor como la exposición a ciertas toxinas, pero todos están relacionados con el envejecimiento (Nagatsu y Sawada, 2006).

2.2.1. Estructuras motoras afectadas en la enfermedad de Parkinson

La EP es una enfermedad neurodegenerativa frecuente, caracterizada por déficits progresivos de la función motora que generan rigidez muscular, bradicinesia, temblor en reposo e inestabilidad postural (Fahn y Przedborski, 2000; Przedborski et al., 2001). Los síntomas cardinales de la EP son resultado de la pérdida específica de las neuronas catecolaminérgicas A9 de la pars compacta de la sustancia nigra y por lo tanto de un déficit de dopamina (Fahn y Przedborski, 2000; Smeyne y Jackson-Lewis, 2005; Nagatsu et al., 2006).

2.2.1.1 Afectación de la vía indirecta y la vía directa en la EP

La sustancia nigra inerva al estriado (vía mesoestriatal) y está involucrada en el control del movimiento voluntario. El descenso de los niveles de dopamina en el estriado causa una pérdida en la regulación del circuito motor de los ganglios basales. En el circuito motor, las áreas corticales motoras inervan al putamen de este se originan dos vías distintas que se denominan “indirecta” y “directa”,

ambas confluyen en los núcleos de salida de los ganglios de la base, el globo pálido interno (GPi) y la sustancia nigra pars reticulata. Las neuronas del GPi y de la sustancia nigra pars reticulata inervan al tálamo donde inhiben a las neuronas de proyección tálamo-corticales. En la vía "directa" el putamen envía proyecciones inhibidoras directamente al GPi y a la sustancia nigra pars reticulata, que dejan de inhibir al tálamo favoreciendo la realización de movimientos voluntarios. En la "vía indirecta" las neuronas inhibidoras del putamen inervan la porción externa del globo pálido (GPe) que conecta con el núcleo subtálamico que es quien proyecta al GPi y a la sustancia nigra pars reticulata. La vía directa se ve activada por la inervación dopaminérgica procedente de las neuronas de la sustancia nigra pars compacta en el putamen a través de receptores tipo D1, mientras que las neuronas de la "vía indirecta" poseen receptores D2 que inhiben su descarga (ver Figura 1A). De esta manera la "vía directa" favorece la realización de movimientos voluntarios mientras que la "vía indirecta" evita los movimientos involuntarios (De Long y Wichmann, 2007; Obeso et al., 2002).

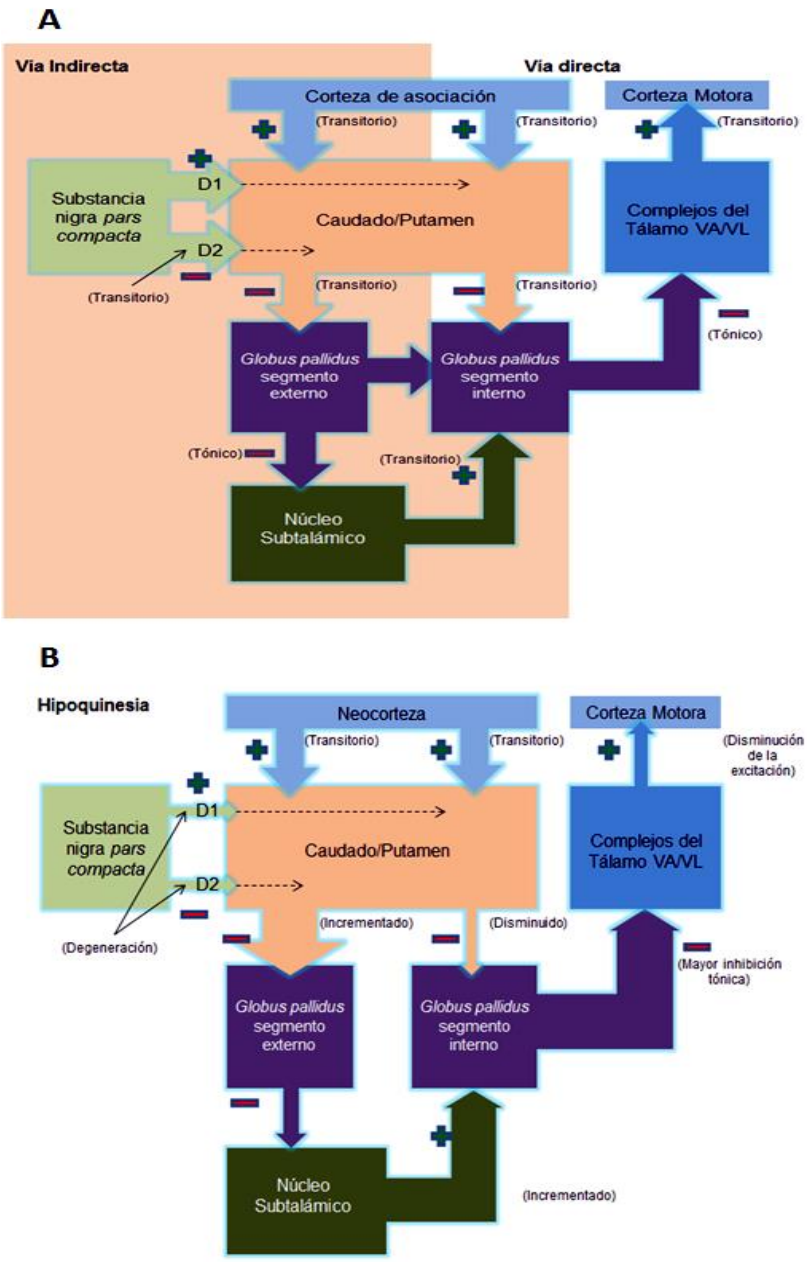

Figura 1. ***Circuito motor de la vía nigroestriatal en la enfermedad de Parkinson***. A) Desinhibición de la vía directa y la indirecta a través de los ganglios basales. B) Alteraciones en las señales inhibitorias de la vía directa e indirecta en la enfermedad de Parkinson (Imagen adaptada de Purves et al., 2004).

2.2.2 Otros neurotransmisores involucrados en la EP

Además de la pérdida neuronal, la EP es caracterizada por la presencia de cuerpos de Lewy y neurofibrillas conformadas principalmente por α-sinucleina y ubiquitina en múltiples regiones del cerebro, no sólo en la sustancia nigra pars compacta sino también en el núcleo motor dorsal del vago, locus coeruleus, núcleo dorsal de Rafe, formación reticular, tálamo, amígdala, núcleo olfatorio, núcleo pedunculopontino y corteza cerebral, entre otros (Braak et al., 2003; Marsden 1983). Esto indica que la EP es más que un síndrome relacionado a la deficiencia de dopamina y que involucra a más sistemas de neurotransmisores; ya que la EP asociada con el deterioro de otro tipo de funciones no motoras como pueden ser: déficit cognitivo, cambios emocionales como la depresión y la ansiedad, trastornos del sueño, disfunción autonómica y trastornos gastrointestinales (Chaudhuri y Schapira, 2009; Langston, 2006). La levodopa que es el fármaco de elección en casos clínicos de la EP, incrementa los niveles de dopamina pero no de manera específica y, aunque reduce considerablemente los síntomas motores de la EP (Brichta et al., 2013) no tiene un efecto positivo en cuanto a los síntomas no-motores; es por eso que en algunos casos se usan otros agonistas a receptores de dopamina como el pramipexol, que es un agonista con alta afinidad al receptor a dopamina tipo 3 (D3). Este receptor es expresado predominantemente por neuronas de la región límbica como el estriado ventral. En pacientes con la EP la expresión de D3 está disminuida en esta zona (Boileau et al., 2009), por lo que la administración de pramipexol alivia la depresión asociada a la EP (Barone et al., 2010). Otro neurotransmisor que se ve afectado en la EP es la serotonina. Durante la enfermedad ésta disminuye en el estriado dorsal,

mesencéfalo (Guttman et al., 2007; Albin et al., 2008) y recientemente se reporto que existe una baja tasa de unión entre serotonina y su transportador en el giro cingulado y el núcleo caudado en pasientes con EP (Güzey et al., 2012). Estos cambios están relacionados con los cuadros depresivos que preceden a los trastornos motores (Kish et al., 2008; Aarsland et al., 2011). La degeneración de neuronas noradrenérgicas en el locus coeruleus con su consecuente déficit de norepinefrina son hallazgos constantes en la EP y se han relacionado con la dificultad para caminar y la inestabilidad postural (Grimbergen et al., 2009), características del padecimiento que son resistentes al tratamiento con levodopa. También se ha documentado que el metilfendiato, un compuesto que inhibe la recaptura de dopamina y norepinefrina, es capaz de mejorar la postura congelada y la dificultad para caminar en pacientes con EP avanzada que recibieron electroestimulación cerebral profunda (Moreau et al., 2012). Las evidencias citadas nos muestran que esta enfermedad es una entidad compleja y difícil de tratar con monoterapia, lo que exige realizar estudios más profundos que incluyan las características no motoras de la EP.

2.2.3. Genes involucrados en la EP

Recientemente se han descrito genes mutados que se asocian a la frecuencia del padecimiento, algunos de ellos son: PARK1 (alfa sinucleína, principal componente de los cuerpos de Lewy) (Feany, 2004); PARK4 (triplicación de alfa sinucleína) (Forman et al., 2004); PARK2 (parkina, una E3 ubiquitin ligasa) (Selkoe, 2004) y PARK 5 (UCHLI, una ubiquitin hidrolasa carboxilo –terminal) (Cookson, 2005),

ambas involucrados en la función del proteosoma; PARK 6 (PINK 1, una cinasa mitocondrial) (Grandhi y Wood, 2005); PARK 7 (DJ-1 que participa en la regulación del estrés oxidante) (Lozano y Kalia, 2005) y PARK 8 (LRRK 2, un complejo proteínico involucrado en cascadas de señalización intracelular) (Mizuno, 2006). En la Tabla 1 se describen estos genes y proteínas, patrones de dominancia, prevalencia y patología que desencadenan en el humano.

Tabla 1. Genes relacionados con la EP

Gen/proteína	Patrón	Frecuencia	Características
PARK1 y 4 /α-sinucleina	AD	Muy raro	Demencia temprana relacionada con el tipo de mutación. Con presencia de cuerpos de Lewy.
PARK2/Parkina	AR (principalmente)	18%	Aparición temprana de la enfermedad con progresión lenta con escasos cuerpos de Lewy. La mayoría con antecedentes familiares.
PARK7/DJ-1	AR	<1%	Aparición temprana de la enfermedad con progresión lenta.

PARK6/Pink 1	AR (portadores están en mayor riesgo)	2-3% de inicio temprano.	Aparición temprana de la enfermedad con progresión lenta
PARK8/LRRK2	AD	Muy variable	Típica EP con cuerpos de Lewy

AR: Autosómico recesivo, AD: Autosómico dominante. La aparición temprana ocurre generalmente antes de los 50 años de edad. Tabla modificada de Savitt et al., 2006.

2.2.4. Defectos en la mitocondria y estrés oxidante

La disfunción mitocondrial ha sido reportada en la mayoría de las enfermedades neurodegenerativas, la cual incluye defectos bioenergético, estrés oxidante, defectos de la dinámica mitocondrial, aumento de la apoptosis, y la acumulación de mitocondrias dañadas con ADN mitocondrial inestable (Procaccio et al., 2014). La mitocondria juega un papel importante, particularmente en las neuronas dopaminérgicas, ya que controla las concentraciones de calcio intracelular y provee energía para efectuar el transporte vesicular de la dopamina, evento necesario para la liberación y recaptura de este neurotransmisor (Van Laar y Berman, 2009). Estudios *post mortem* han mostrado que los pacientes con EP exhiben una actividad reducida del complejo I mitocondrial en la sustancia nigra (Parker et al., 1989; Schapira et al., 1990), esta condición hace que se incremente la producción de radicales libres de oxígeno y por lo tanto estas neuronas sean más susceptibles a toxinas (Swerdlow et al., 1996). La sustancia nigra está

expuesta a una alta concentración de especies reactivas de oxígeno (ROS, por sus siglas en inglés), esto es debido en gran medida a las concentraciones abundantes de dopamina en el citoplasma ya que ésta es propensa a la auto-oxidación además de que estas neuronas contienen altas cantidades de hierro en su núcleo (Jenner y Olanow, 1996). Este ambiente oxidante puede afectar la respiración mitocondrial así como la permeabilidad de su membrana (Berman y Hastings, 1999; Premkumar y Simantov, 2002). También las proteínas mutadas asociadas a la EP afectan de manera indirecta la función mitocondrial. Las fallas mitocondriales y el estrés oxidante están estrictamente relacionados, por lo tanto no es de sorprender que numerosos estudios *post mortem* muestran incremento en la oxidación de proteínas, lípidos y DNA en pacientes con la EP (Alam et al., 1997; Jenner et al., 1992; Jenner y Olanow, 1996). Por otra parte, todas las neurotoxinas que se utilizan para reproducir las características patológicas de la EP en animales, tales como la MPTP/MPP^+, la 6-hidroxidopamina, la rotenona o el paraquat, son toxinas que afectan la función mitocondrial y causan daño oxidativo masivo en el tracto nigroestriatal.

2.2.5. Factores ambientales

Estudios epidemiológicos han identificado tanto riesgos como compuestos que están involucrados en la patogénesis de la EP como por ejemplo vivir en comunidades rurales, consumir agua de pozo artesanal, exposición a metales, solventes y pesticidas (Di Monte, 2001). De éstos, los pesticidas, herbicidas y fungicidas han recibido la mayor atención como factores de riesgo (Cannon y Greenamyre, 2011). Estudios recientes donde se examinaron poblaciones grandes

y pesticidas específicos valiéndose de técnicas epidemiológicas avanzadas validaron que la exposición a paracuat y rotenona es un factor de riesgo significante para padecer EP (Tanner et al., 2011).

En 1982 la 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) fue reconocida como una toxina selectiva para la vía nigroestriatal al generar cuadros parkinsonianos descritos en jóvenes consumidores de drogas del norte de California, Estados Unidos. Estos cuadros se desencadenaron unos días después de la inyección intravenosa de una preparación casera de heroína sintética (1-metil-4-fenil-4-propionoxipiperidina), compuesto análogo a la meperidina que contenía cerca del 3% en peso de MPTP como subproducto (Langston et al., 1983). En la actualidad, estos compuestos son utilizados como modelos animales para el estudio de la EP, punto que discutiremos más adelante.

2.2.6. Respuesta inflamatoria

El proceso de neuroinflamación en pacientes con la EP se ha descrito ampliamente, reportando niveles elevados de las citocinas pro-inflamatorias IL1β, IL6 y TNFα en el líquido cefalorraquídeo (LCR) y en células gliales de las regiones nigro-estriales (Boka et al., 1994; Mogi et al., 1999; Nagatsu et al., 2000). Esta observación aunada a la presencia de ROS en cerebros de pacientes con la EP sugiere la activación de las células microgliales y su participación en la patogenia de la EP (Mount et al., 2007). La sustancia nigra es particularmente rica en células microgliales, y se ha observado que en los cerebros de pacientes con la EP hay un mayor número de éstas en estado activado (Lawson et al., 1990; Czlonkowska

et al., 1996; Gao et al., 2002; Imamura et al., 2003; Barcia et al., 2004; Block et al., 2007).

La microglia activada puede ser fuente de citocinas pro-inflamatorias y este evento, sumado al incremento en la expresión del receptor de TNFα en las neuronas de la sustancia nigra, pudiera facilitar la degeneración neuronal (Mogi et al., 1999). Asimismo, se ha observado una disminución de los factores neuroprotectores/neurotróficos como NGF, BDNF, neurotrofina-3 y neurotrofina-4/5 (Mogi et al., 1999). La disminución de estos factores neuroprotectores-neurotróficos y el incremento importante de citocinas pro-inflamatorias pueden ser fundamentales en iniciar y acelerar la muerte apoptótica de las neuronas dopaminérgicas de la sustancia nigra.

Adicionalmente, el proceso inflamatorio en la EP está asociado a un aumento en la actividad de enzimas tales como la óxido nítrico sintasa (iNOS) y la ciclooxigenasa 2 (Long-Smith et al., 2009); así como el estrés oxidante con una disminución importante del glutatión reducido (GSH), que es la mayor defensa celular antioxidante (Sofic et al., 1992; Sian et al., 1994; Jenner, 2003). La disminución de los niveles de GSH también se ha visto asociada con la aparición de cuerpos de Lewy (Sian et al., 1994). El glutatión protege a la célula contra toxinas endógenas y exógenas, así como contra ROS y especies reactivas de nitrógeno, tales radicales son removidos por una vía no enzimática, a través de la oxidación del GSH a glutatión oxidado (GSSG) (Dickinson y Forman, 2002).

2.3. Modelos experimentales de la EP

Los modelos animales son una herramienta esencial, para comprender los mecanismos moleculares asociados a una patología, evaluar la eficacia de intervenciones terapéuticas y establecer estimaciones iniciales de dosificación para nuevos fármacos. Aunque existen numerosas limitaciones en estos, son indispensables para evaluar los procesos patológicos en el tiempo que son pobremente entendidos y que por razones éticas no se pueden estudiar directamente en humanos. El estudio de las enfermedades en el sistema nervioso central (SNC) es particularmente difícil por la escases de información sobre los mecanismos epigenéntico y moleculares que las originan, la naturaleza heterogénea de estas condiciones y los subjetivos y a veces contradictorios puntos de vista para describir los síntomas y severidad de los mismos. En el caso del Parkinson los modelos animales más usados para inducir síndromes parkinsónicos son los roedores (ratón y rata), los primates y recientemente se agregó a la lista el uso de especies como el pez cebra.

2.3.1. Modelos genéticos

El principio subyacente para el estudio de mutaciones genéticas de una enfermedad es la evidencia de que las similitudes clínicas entre las formas de la enfermedad hereditaria y esporádica comparten un mecanismo común que puede conducir a la identificación de vías moleculares y bioquímicas implicadas en la patogénesis de la enfermedad. En el caso de la EP es necesario conocer qué

consecuencias tienen las mutaciones relacionadas con la enfermedad a través de modelos experimentales, ya que representan un potencial de blancos terapéuticos. La α-sinucleína normalmente participa en el reciclaje de las vesículas sinápticas, pero se ha observado que dos mutaciones en el gen que la codifica (A30P y A53T) causan una forma dominante de EP hereditario (Krüger et al., 1998). Estudios con ratones transgénicos han demostrado que la mutación en A53T puede resultar en un fenotipo de EP con afecciones motoras severas llegando eventualmente a la parálisis y la muerte (Giasson et al., 2002); sin embargo, esta mutación no induce la formación de cuerpos de Lewy, éstos sólo se encuentran en los ratones transgénicos con la mutación A30P (Masliah et al., 2000). Algunos ratones transgénicos para α-sinucleína desarrollan problemas no motores relacionados con la enfermedad como fallas olfatorias y disfunción en el colon (Wang et al., 2008). A pesar de estos modelos en los que se prueban ambas mutaciones del gen de la α-sinucleína podemos decir que la función fisiologica de la α-sinucleína todavía tiene que ser resuelta.

Mutaciones en el gen de la quinasa rica en repeticiones de leucina 2 (LRRK2) se han relacionado con formas dominantes de la EP (Zimprich et al., 2004), ratones knock out para LRRK2 no despliegan ningún déficit en el desarrollo de neuronas dopaminérgicas ni en el mantenimiento de las mismas (Wang et al., 2008). Por otro lado, mutaciones en los genes de Parkina, DJ1, y PINK1 causan una forma recesiva de la EP. No obstante, los modelos en roedores knock out para estos genes no mostraron degeneración nigroestriatal, inclusiones intracelulares o algún problema en neuronas dopaminérgicas que se relacione con el Parkinson idiopático o hereditario; excepto el knock out para PINK1, el cual despliega una

reducción limitada de los niveles de dopamina en el estriado (Moore y Dawson 2008). Por lo que estas mutaciones no resultan ser un buen modelo para el estudio de esta enfermedad en animales.

2.3.2. Modelos neurotóxicos

Aunque la tendencia actual en el modelado de la EP gira en torno al estudio de factores genéticos que producen la enfermedad, los que pueden llamarse "Modelos etiológicos", el uso de modelos neurotóxicos sigue siendo una herramienta potente para producir muerte neuronal selectiva tanto en sistemas *in vitro* como *in vivo*. Estos modelos han sido comúnmente conocidos como "modelos patógenos". Los principales modelos patógenos de la EP incluyen los generados por la 6-OHDA (6-Hidroxidopamina) comúnmente utilizada en ratas y la MPTP (1-metil-4-fenil-1,2,3,6-tetrahidropiridina) principalmente usado en ratones y en primates no humanos, en las últimas décadas se ha incursionado en establecer nuevos modelos neurotóxicos para el estudio de la EP en base a investigaciones epidemiológicas y toxicológicas generando sistemas experimentales en los cuales se recurre al uso de sustancias como lo son: la rotenona, paraquat, derivados de isoquinolina, y la metanfetamina. En los siguientes apartados ahondaremos en la descripción de estos y sus mecanismos de acción que se resumen de manera grafica en la Figura 2.

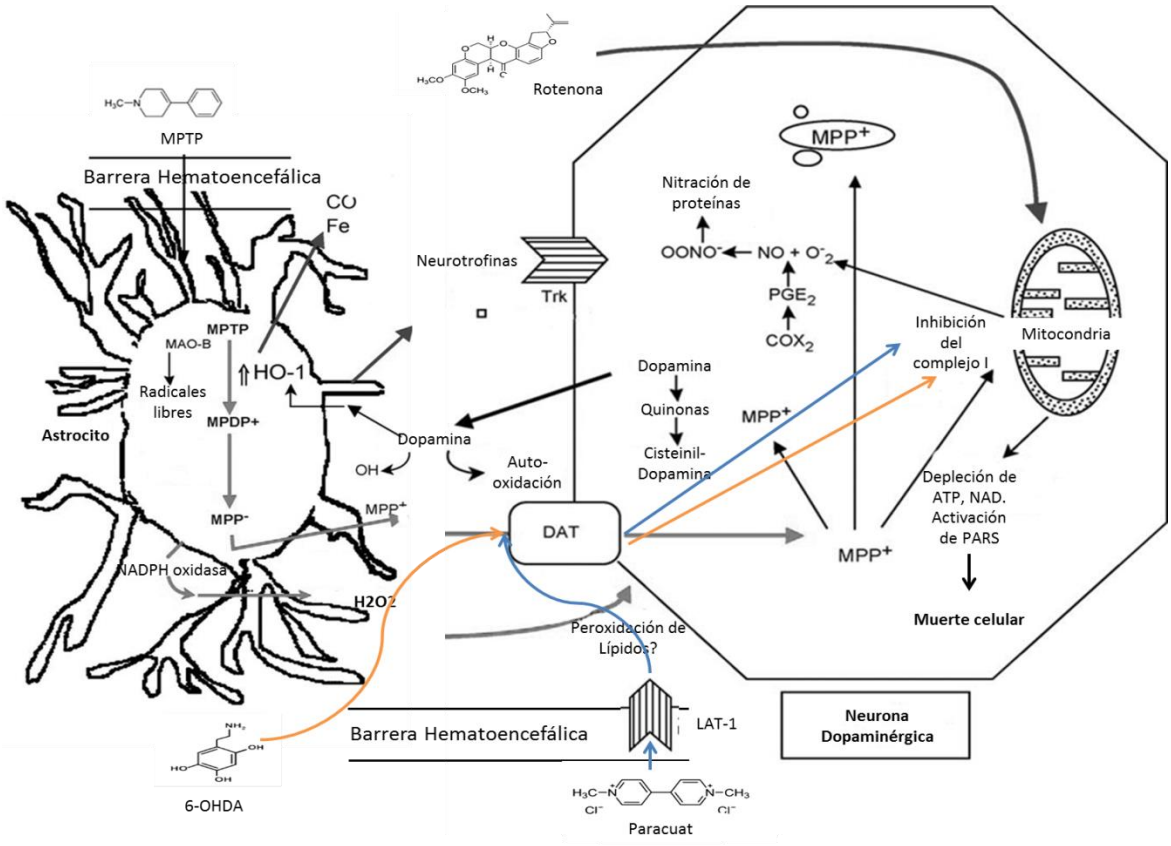

Figura 2. Neurotoxinas empleadas en modelos de la enfermedad de Parkinson. Rrepresentación esquemática de la estructura química, y sitios de acción de las diferentes toxinas que se emplean en los modelos de la enfermedad de Parkinson. MPTP: 1-metil-4-fenil-1,2,3,6-tetrahidropiridina, 6-OHDA: 6 hidroxidopamina, DAT: Transportador de dopamina, LAT-1: Transportador de aminoácidos 1 tipo L. (imagen adaptada de Smeyne y Jackson-Lewis, 2005).

2.3.2.1 Modelos inducidos con pesticidas

Paraquat, Dicloruro de 1,1'-dimetil-4-4'bipiridilo, es un herbicida ampliamente usado en la agricultura que comparte similitudes estructurales con el 1-metil-4-

fenilpiridinio (MPP+, el metabolito tóxico del MPTP). El daño con paraquat es dado por el estrés oxidante que genera, ya que produce ROS (radical superóxido, peróxido de hidrógeno y radicales hidróxilo) que llevan a la peroxidación de lípidos, proteínas, ADN y ARN (Day et al., 1999; Przedborski y Ischiropoulos, 2005). Sin embargo, los efectos del paraquat sobre el sistema nigroestriatal son ambiguos; algunos investigadores reportan que después de la administración de este herbicida los ratones muestran reducción en la actividad motora, reducción de fibras estriatales y neuronas tirosina hidroxilasa positivas (TH+) de la sustancia nigra de manera dosis dependiente (Brooks et al., 1999; McCormack et al., 2002). Otros investigadores proponen que el paraquat no induce cambios en el sistema dopaminérgico nigroestriatal (Thiffault et al., 2000). El maneb (etilenbisditiocarbonato de manganeso), un fungicida que disminuye la actividad locomotora en ratones, potencializa el efecto tanto del paraquat como del MPTP (Thiruchelvam et al., 2000; Takahashi et al., 1989), por lo que el modelo más utilizado es la combinación de paraquat con maneb. Sin embargo, es necesario realizar experimentos más detallados para disipar las dudas respecto a este modelo de la EP.

Rotenona, es un herbicida e insecticida con un tiempo de vida media de 3 a 5 días, dependiendo de la exposición al sol (Inden et al., 2007). La rotenona es altamente lipofíilica y cruza la barrera hematoencefalica rápidamente (BHE). La exposición crónica a este compuesto resulta en la inhibición de la cadena de transporte de electrones de la mitocondria. La administración oral tiene un efecto neurotóxico limitado (Inden et al., 2011), por lo que la administración crónica es a través de bombas miniosmóticas, siendo el método más empleado en ratas Lewis

que son muy sensibles a este químico (Betarbet et al., 2000). La administración intravenosa es capaz de causar daño a neuronas dopaminérgicas, formar cuerpos de Lewy, estrés oxidante y problemas gastrointestinales (Cannon et al., 2009), debido a que la rotenona no es específica para neuronas dopaminérgicas, si no que también afecta a otras poblaciones neuronales de naturaleza serotoninérgica, noradrenérgica y colinérgica no resulta ser un buen modelo para el estudio de la EP (Höglinger et al., 2003).

2.3.2.2 Modelos inducidos con neurotoxinas

Un modelo animal clásico de la EP es el que se genera con 6-hidroxidopamina (6-OHDA de sus siglas en inglés), toxina aislada por primera vez en 1959 (Schwarting y Huston, 1996), que ha brindado una gran información sobre los efectos conductuales, bioquímicos y fisiológicos de la dopamina en el sistema nervioso central (SNC).

Aunque varios animales (ratones, gatos, perros y monos) son susceptibles a la 6-OHDA, es más utilizada en ratas (Ruffy y Leonard, 1997). La 6-OHDA tiene mucha afinidad por transportadores catecolaminérgicos como el transportador de dopamina y el de norepinefrina (Sachs y Jonsson, 1975) de tal manera que al usarse de manera conjunta con un inhibidor de la recaptura de noradrenalina como la desipramina se protegen las neuronas noradrenérgicas, afectando exclusivamente a las neuronas dopaminérgicas (Martin et al., 1976). Ya que este compuesto no cruza la BHE es necesario inyectarlo directamente en la sustancia nigra pars compacta o en el estriado (Perese et al., 1989; Przedborski et al.,

1995). Cabe resaltar que este modelo no mimetiza al 100% la EP; la 6-OHDA es frecuentemente usada de manera unilateral ya que la inyección bilateral en el estriado produce adipsia, afagia y muerte (Ungerstedt, 1971).

La 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), descubierta en 1980, produce una sintomatología similar a la de la EP en ratones, primates y también en humanos (Langston et al., 1983). Es un compuesto análogo de la mepiridina (heroína sintética) y es un precursor neurotóxico altamente lipofílico. Después de la administración sistémica, rápidamente cruza la BHE y entra al SNC. Una vez dentro la MPTP se metaboliza al ión 1-metil-4-fenil-2,3-dihidropiridinio ($MPDP^+$) con la formación del ion superóxido (O_2*) por la enzima monoamino oxidasa B (MAO-B), la cual está localizada en la membrana externa de las mitocondrias de las células no dopaminérgicas, tales como las células gliales y las neuronas serotoninérgicas. El $MPDP^+$ se auto-oxida a 1-metil-4-fenilpiridinio (MPP^+) el cual es transportado a través de la membrana celular al interior de las neuronas dopaminérgicas nigro-estriatales A9, mediante los transportadores de dopamina (DAT, por sus siglas en inglés), los cuales están localizados principalmente en las terminales nerviosas del estriado. El MPP^+ es transportado al citoplasma de las neuronas y se acumula en la membrana interna de las mitocondrias inhibiendo el complejo I, interrumpiendo el transporte de electrones, favoreciendo la generación de ROS y disminuyendo la síntesis de ATP. Estas condiciones propician la liberación del citocromo c desde la mitocondria, lo cual activa una serie de señales intracelulares teniendo como punto final la muerte apoptótica de las neuronas dopaminérgicas (Fornai et al., 2005a).

El tratamiento con MPTP induce una pérdida selectiva de las neuronas dopaminérgicas de la sustancia nigra con una disminución importante de la dopamina del estriado y alteraciones del sistema de otros neurotransmisores monoamínicos (Smeyne y Jackson-Lewis, 2005). Los monos intoxicados con MPTP responden positivamente al tratamiento con levodopa o apomorfina, incluso algunos estudios recientes incursionan en el uso de este modelo para analizar la sintomatología no motora (Pessiglione et al., 2004; Vezoli et al., 2011).

Por razones financieras, este modelo es más utilizado en ratones que en primates y existen básicamente tres esquemas de tratamiento que pueden variar según las inquietudes de cada investigador. *i)* Esquema agudo, que consiste en cuatro inyecciones diarias de MPTP (10 mg/kg) vía intraperitoneal, distribuida en dos días con intervalos de una hora entre cada administración; *ii)* esquema subcrónico, que consiste en 5 inyecciones intraperitoneales de MPTP (30 mg/kg) distribuidas una diaria durante cinco días consecutivos y *iii)* esquema crónico, que consiste en una inyección diaria de MPTP (15 mg/kg) durante catorce días consecutivos. En todos los esquemas de administración se observan cambios histológicos, pérdida de neuronas dopaminérgicas y reactividad glial; sin embargo, las características clínicas varían de tal manera que sólo con el tratamiento agudo se pueden observar cambios sensibles en la conducta de los animales. En cuanto al estrés oxidante, las afectaciones son más destacables en los esquemas subcrónicos y crónicos (Kurosaki et al., 2004). La principal limitación de la MPTP, y en general de los modelos con toxinas, es que no se asocian claramente a la formación de cuerpos de Lewy, marcador histopatológico característico de la EP. Varios investigadores se han dado a la tarea de resolver este problema, encontrando que

el tratamiento crónico administrado a través de una bomba miniosmótica induce la formación de inclusiones celulares inmunoreactivas a ubiquitina y a α-sinucleina (Fornai et al., 2005b). Este modelo presenta características histopatológicas similares a la EP, por lo que es frecuentemente usado para estudiar tanto el desarrollo de la enfermedad así como en nuevas terapias para su tratamiento (Smeyne y Jackson-Lewis, 2005).

2.4. Regulación de la respuesta inflamatoria y la enfermedad de Parkinson

Una característica común de la EP y los modelos de estudio de esta enfermedad es el componente de neuroinflamación, en el cual participan citocinas, factores neurotróficos y de manera importante la microglia (Nagatsu et al., 2006). En el 2007, Reynols y colaboradores demostraron que la respuesta microglial en ratones intoxicados con MPTP puede ser modulada con la transferencia de células T provenientes de ratones inmunizados con el copolímero-1, protegiendo las neuronas dopaminérgicas A9 y que ésta respuesta neuroprotectora involucra linfocitos T CD4+CD25+, linfocitos T reguladores, los cuales se caracterizan por regular la respuesta inmune y participar en el mantenimiento de la homeostasis y tolerancia inmunológica en el SNC (Reynolds et al., 2007). También se demostró que la transferencia de linfocitos T reguladores activados contra MPTP protegen al sistema nigroestriatal en un 90%, esta respuesta protectora es dependiente de la modulación de la respuesta microglial y de la sobre-expresión del factor neurotrófico derivado de células gliales (GNDF), del TGFβ y de IL10 (Reynolds et al., 2007). También se ha observado que la administración de IL10 de forma

intracraneal protege a las neuronas dopaminérgicas de la muerte celular inducida por lipopolisacárido (LPS) y está relacionada con el número disminuido de células microgliales activadas (Arimoto et al., 2007).

Estos datos muestran que la modulación de la inflamación previene la pérdida neural, por lo que resulta de gran interés buscar terapias que no sólo restituyan los niveles de dopamina, como el tratamiento clásico con levodopa, si no que prevengan la pérdida de neuronas dopaminérgicas.

2.5. Fitomedicina

El principal problema a vencer en el diseño de estas terapias es que sólo moléculas con determinada lipofilicidad, peso molecular y carga apropiada son capaces de atravesar la BHE. Se han estudiado métodos para que los fármacos sean aptos para traspasar la BHE, siendo más aceptados los que tienen un enfoque fisiológico que aproveche la capacidad de transcitosis de algunos receptores. Otras estrategias incluyen la manipulación de drogas, disrupción de la BHE y la búsqueda de rutas alternativas para la administración de fármacos (Misra et al., 2003). Todas estas propuestas tienen un aumento intrínseco en el valor monetario del posible fármaco en estudio, por lo que es pertinente revisar las fuentes naturales de sustancias que no requieran modificaciones o procesos de extracción mayor y que sean de relevancia en el tratamiento de las enfermedades neurodegenerativas como la EP. En este sentido la fitomedicina resulta de vital relevancia para encontrar nuevas alternativas para el control de dichas enfermedades.

La Organización Mundial de la Salud (OMS) estima que alrededor del 65% de la población utiliza las plantas (fitoterapia) como primer recurso en el tratamiento de diversas enfermedades y su uso ha estado siempre muy arraigado a los usos y costumbres de cada región en donde son empleados (Fabricant y Farnsworth, 2001). Desde hace más de treinta años, a nivel mundial, se ha vuelto a considerar a la fitoterapia para tratar distintas enfermedades como el resfriado común, la hipertensión, la diabetes, el cáncer, manejo del dolor y alteraciones del sistema nervioso central como la epilepsia, depresión, ansiedad, estrés, enfermedades neurodegenerativas, entre otras (Morales y Morales, 2009).

La OMS ha definido a la fitomedicina como la aplicación de principios activos de origen vegetal en terapéutica, basado en el conocimiento científico moderno (Morales y Morales, 2009). Entre estos principios activos los más estudiados y que han mostrado efectos biológicos son los polifenoles, grupo de compuestos químicos presentes en hierbas y vegetales consumidos por el hombre (Joseph et al., 2007; Ramos, 2007). Sus principales funciones son la protección contra las ROS, producidas de manera natural durante la fotosíntesis y generar un mal sabor en las plantas para evitar ser consumidas por los herbívoros. Su bioactividad contra la neurodegeneración ha estado relacionada principalmente por su efecto anti-oxidante (Rice-Evans et al., 1996); sin embargo, recientemente se está obteniendo información con respecto a su efecto neuroprotector ya que intervienen en cascadas de señalización, modulación de mediadores y enzimas relacionadas con la síntesis de acetilcolina como la acetil-colinesterasa, inhibición de la neurotoxicidad vía receptores de N-metil-D-aspartato, efectos anti-amiloidogénicos, y efectos anti-inflamatorios en distintos modelos de

enfermedades neurodegenerativas (Pérez et al., 2014 enviado a publicacion). Entre los compuestos que han suscitado interés como potencial agente terapéutico por presentar estas propiedas está la silimarina, la cual ha sido empleada apliamente en patologías del hígado (Al-Harbi et al., 2014)

2.6. *Silimarina*

La silimarina es un extracto obtenido de las semillas y frutos de la planta *Silybum marianum* (con sinonimia científica de *Cardus marianus*), que contiene en un 80% una mezcla de cuatro flavonolignanos y aproximadamente un 20% de una fracción química de compuestos polifenólicos no identificados. Los flavonolignanos están conformados por un 70% de silibina que es el principal componente activo, seguido de un 20% de silicristina, 10% de silidianina y 5% de isosilibina (Wellington y Jarvis, 2001; Kren y Walterová, 2005), (ver Figura 3).

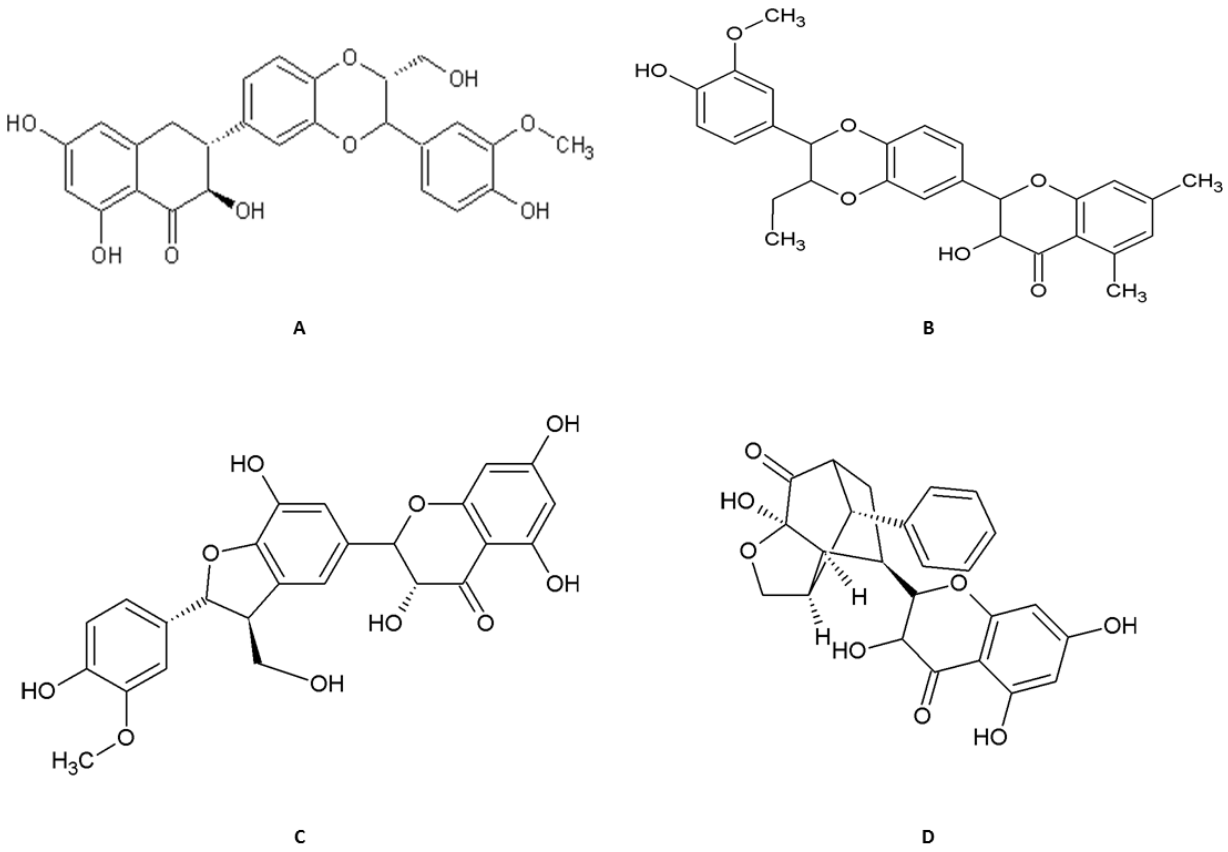

Figura 3. ***Estructura química de los principales componentes del extracto de estandarizado de la Silimarina****.* A) Silibina. B) Isosilibina. C) Silicristina. D) Silidianina.

Los efectos farmacológicos de la silimarina han sido estudiados ampliamente *in vitro* e *in vivo* tanto en animales como en humanos, presentando principalmente efectos anti-oxidantes, anti-inflamatorios, citoprotectores, anti-carcinogénicos, anti-virales y neuroprotectores (Borah et al., 2013)

2.6.1 Efecto anti-inflamatorio de la silimarina

Las propiedades moduladoras sobre la respuesta inmunológica que induce la silimarina han sido descritas principalmente en la prevención del daño hepático, en la fibrosis hepática y en la respuesta linfocitaria inducida por diferentes agentes tóxicos como tetracloruro de carbono, palloidina y concanavalina A (Shi et al., 1997; Schümann et al., 2003). El tratamiento *in vitro* con la silimarina incrementa la proliferación de los linfocitos T murinos, de linfocitos T de pacientes con cirrosis hepática de tipo alcohólica y de linfocitos T de rata (Láng et al., 1988; Agoston et al., 2001). Se ha observado que la administración de silimarina en ratones BALB/c incrementó la proliferación tanto de linfocitos T CD4+ como CD8+ obtenidos de timo y de bazo (Johnson et al., 2002). Adicionalmente, el tratamiento con la silimarina inhibe la migración de los linfocitos T en diferentes modelos de inflamación aguda (De La Puerta et al., 1996).

Por otro lado, se ha observado que dosis bajas de silimarina tienen un efecto inhibidor sobre la proliferación y la función de los linfocitos T en ratones BALB/c normales (Johnson et al., 2002). También se ha documentado que la administración de la silimarina inhibe tanto *in vitro* como *in vivo* el proceso de maduración de las células dendríticas y su capacidad de inducir linfocitos Th1 en ratones normales (Lee et al., 2007). De igual manera puede inhibir la producción y la expresión de moléculas de adhesión como ICAM-1, VCAM-1 y E-selectina inducidas por TNFα en células endoteliales de humano (Kang et al., 2003). Asimismo, modula la producción y expresión de citocinas inflamatorias y anti-inflamatorias *in vitro* e *in vivo*. El tratamiento con silimarina incrementa la

producción de citocinas como IL4, IL10 e IFNγ en linfocitos T *in vitro* en el daño hepático inducido por concanavalina A (Schümann et al., 2003). Se ha descrito que la silimarina inhibe la producción de citocinas inflamatorias (IL1β, IFNγ e IFNα) inducidas por LPS en macrófagos y linfocitos T (Kang et al., 2004). Igualmente se ha reportado que el tratamiento con la silibina inhibe la expresión de citocinas pro-inflamatorias (IFNγ, TNFα e IL2) y anti-inflamatorias como IL4 en el daño hepático inducido por concanavalina A o por fumonicina B1 (Schümann et al., 2003).

Los datos presentados demuestran el efecto anti-inflamatorio que tiene la silimarina a nivel sistémico.

2.7. Silimarina y neuroprotección

Aunado al efecto anti-inflamatorio se ha reportado que la silimarina tiene un efecto neuroprotector. La administración de silimarina inhibe la activación de la microglia, disminuyendo el TNFα y óxido nítrico, reduciendo el daño sobre las neuronas dopaminérgicas por lo que protege de la neurotoxicidad inducida por LPS en cultivos celulares mixtos de neuronas y células gliales provenientes del mesencéfalo (Wang et al., 2002).

También se ha observado que la silimarina protege del daño por estrés oxidante en el SNC después de la administración oral de acetaminofén, fármaco que induce una disminución de la concentración de glutatión redusido (GSH) y de glutatión oxidado (GSSG), de ácido ascórbico y reduce la actividad enzimática de la superóxido dismutasa (SOD). El tratamiento con silimarina aumentó significativamente los niveles de GSH, de ácido ascórbico, y la actividad de la

SOD después de la administración del acetaminofén previniendo la peroxidación de lípidos (Nencini et al., 2007).

Asimismo, la silimarina modula el estrés oxidante en cerebros de ratas viejas, previniendo la oxidación de proteínas. Estos datos sugieren que la silimarina pudiera prevenir procesos neurodegenerativos relacionados con la edad (Galhardi et al., 2009). La silimarina también se probó en un modelo experimental de esclerosis múltiple y de encefalomielitis autoinmune experimental, reduciendo significativamente los signos histológicos de desmielinización e inflamación en la encefalomielitis autoinmune experimental al inhibir la producción de citocinas como IL-12, IL-2 e IL-4 de manera dosis dependiente *ex vivo* (Min et al., 2007). Igualmente, en un modelo de la enfermedad de Alzheimer, la silimarina redujo el deterioro de la memoria y el daño oxidativo inducido por el péptido β-amiloide administrado intraventricularmente (Lu et al., 2009).

Estos antecedentes muestran que la silimarina posee un potencial terapéutico para la prevención y tratamiento de enfermedades neurodegenerativas.

3. JUSTIFICACIÓN

La EP es un trastorno neurológico crónico, degenerativo, progresivo e incapacitante que resulta de la pérdida selectiva de las neuronas dopaminérgicas de la sustancia nigra del mesencéfalo. Es la segunda enfermedad neurodegenerativa más frecuente después de la enfermedad de Alzheimer y en México se estima que existen alrededor de 500,000 pacientes con esta enfermedad y en los próximos años se espera un incremento en el número de

personas afectadas por la EP, por el aumento en la esperanza de vida de la población mexicana (Secretaria de Salud, 2006).

A pesar del avance de las investigaciones médicas, el tratamiento de la EP se basa principalmente en la sustitución de los niveles de dopamina a través de la administración de levodopa. Sin embargo, aún no se ha logrado curar la enfermedad, restablecer la función de las neuronas dopaminérgicas o detener la progresión de la enfermedad, por lo que los estudios destinados a modular la respuesta inflamatoria asociada a la EP y/o restablecer la función dopaminérgica adquieren una gran importancia. La búsqueda de nuevos fármacos que modulen la inflamación y el estrés oxidante durante el desarrollo y la progresión de la EP son de gran relevancia, ya que esta modulación podría evitar la muerte de las neuronas dopaminérgicas. Entre los fármacos en estudio que muestran características anti-oxidantes, anti-inflamatorias, citoprotectoras y neuroprotectoras está la silimarina, que es una mezcla de flavonolignanos obtenidos de las semillas y frutos de la planta *S. marianum*. Por sus características la silimarina pudiera tener un impacto en el manejo terapéutico de la EP, reduciendo las complicaciones asociadas a la enfermedad, no sólo disminuyendo los costos para el sistema de salud sino mejorando la calidad de vida de los pacientes con EP.

4. HIPÓTESIS

Si la silimarina tiene un efecto neuroprotector en el SNC su administración en un modelo murino de la enfermedad de parkinson inducido con MPTP protegerá a las neuronas dopaminérgicas de la sustancia nigra al disminuir el estrés oxidante.

5. OBJETIVOS

5.1. Objetivo general

Evaluar el efecto neuroprotector y anti-oxidante de la silimarina en un modelo murino de la EP.

5.2. Objetivos específicos

1.- Determinar los niveles de dopamina estriatal en ratones intoxicados con MPTP y tratados con 25, 50, 100, 200, 250, 300 o 400 mg de silimarina mediante HPLC, a los 7 días de evolución.

2.- Determinar el número de neuronas dopaminérgicas en la sustancia nigra de ratones intoxicados con MPTP y tratados con 100 mg/kg de silimarina mediante técnicas de inmunohistoquímica, a los 7 días de evolución.

3.- Determinar la apoptosis en la sustancia nigra de ratones intoxicados con MPTP y tratados con 100 mg/kg de silimarina mediante la técnica de TUNEL, a los 3 días de evolución.

4.- Determinar la peroxidación de lípidos en el estriado de ratones intoxicados con MPTP y tratados con 100 mg/kg de silimarina mediante espectrofotometría de fluorescencia, a los 7 días de evolución.

5.- Determinar la actividad de la superóxido dismutasa en el mesencéfalo de ratones intoxicados con MPTP y tratados con 100 mg/kg de silimarina mediante espectrofotometría de absorción, a los 4 días de evolución.

6. METODOLOGÍA

6.1. Animales

Se usaron ratones machos de la cepa C57BL/6J de 25-30g de peso, los cuales se mantuvieron en un ciclo luz-oscuridad 14:10 horas, con agua y comida *ad libitum*. Se dividieron en 16 grupos con una *n* de cinco animales por grupo, como se muestra en la Tabla 2. Los ratones fueron proporcionados por el Instituto Nacional de Neurología y Neurocirugía, el Departamento de Medicina Experimental de la Facultad de Medicina, de la Universidad Nacional Autónoma de México. Además, una parte de los ratones se compraron a la empresa Harlan Laboratories, Inc., Ciudad de México, gracias al Programa de Apoyo al Doctorado de Biología Experimental de la Universidad Autónoma Metropolitana-Iztapalapa, otorgado por el Consejo Nacional de Ciencia y Tecnología.

6.2. Tratamiento con MPTP

Para iniciar el cuadro histopatológico de la EP en los animales, se les administró, vía intraperitoneal, una dosis de 30 mg de clorhidrato de MPTP (disueltos en 250 µL de agua destilada) por cada kg de peso corporal, durante 5 días consecutivos. La dosis acumulativa fue de 150 mg/kg, la cual es menor a la que produce muerte celular necrótica (Tatton & Kish, 1997).

6.3. Tratamiento con silimarina

Se probaron 7 dosis de silimarina (ver la Tabla 2) administradas intraperitonealmente durante 5 días consecutivos, 30 minutos posteriores a la administración de la MPTP con la finalidad de determinar el efecto de la silimarina durante la patogenia de la EP derivada de la administración de la MPTP. Al grupo control se le administró el vehículo en el que se disolvió la silimarina (PBS), de igual manera se administraron dosis de silimarina de 25, 50, 100, 200, 250, 300 o 400 mg durante 5 dias consecutivos a los animales pertenecientes a los grupos testigo. El sacrificio de los sujetos experimentales se realizó a los 3, 4 o 7 días posteriores al término del tratamiento.

Tabla 2. Grupos, tratamientos y dosis.

Grupo	Tratamiento	
1	PBS	Control
2	Silimarina	25 mg/kg/día
3		50 mg/kg/día
4		100 mg/kg/día
5		200 mg/kg/día
6		250 mg/kg/día
7		300 mg/kg/día
8		400 mg/kg/día
9	MPTP	30 mg/kg/día

10	MPTP + silimarina 25 mg/kg/día
11	50 mg/kg/día
12	100 mg/kg/día
13	200 mg/kg/día
14	250 mg/kg/día
15	300 mg/kg/día
16	400 mg/kg/día

Nota: Se eligió la dosis de silimarina de 100 mg/kg/día ya que fue la que mostró un efecto positivo en la recuperación de los niveles de dopamina.

6.4. Determinación del contenido de dopamina en el estriado

El contenido de dopamina se analizó por cromatografía líquida de alta resolución (HPLC, por sus siglas en inglés). Los animales se sacrificaron por dislocación cervical, sus cerebros se removieron rápidamente y se colocaron en una placa fría para la disección del estriado (Leng et al., 2005). Inmediatamente después se homogenizaron en 300 µL de una solución de ácido perclórico 0.4 N que contenía 0.1% (p/v) de metabisulfito de sodio, posteriormente las muestras se centrifugaron a 4000 × *g* a 4 ºC durante 15 minutos. El sobrenadante se filtró y se preservó a −70 ºC hasta su análisis cromatográfico. La cuantificaión de dopamina se realizó en un aparato de HPLC (Perkin Elmer LC-4C) acoplado a un detector electroquímico BAS CC-5 (Perkin Elmer LC-4C). Las curvas de calibración se

construyeron inyectando concentraciones conocidas de una disolución estándar de dopamina.

6.5. Determinación de apoptosis con la técnica de TUNEL

Una vez determinada la dosis de silimarina a emplear, de acuerdo con la curva dosis respuesta, se procedió a determinar la muerte celular con la técnica de TUNEL, utilizando el kit de detección de apoptosis *in situ* Apoptag (Chemicon,). Los ratones se sacrificaron a los 3 días post-tratamiento con una sobredosis de pentobarbital sódico (20 mg/kg de peso corporal), se perfundieron con 50 mL de solución salina isotónica seguido de 100 mL paraformaldehído al 4% (v/v en 0.1M, y con un pH de 7.4), se decapitaron y sus cerebros se removieron cuidadosamente para evitar dañarlos. Los cerebros se fijaron durante 2 h adicionales en paraformaldehído al 4%, se congelaron por inmersión en 2-metilbutano pre-enfriado con hielo seco y se almacenaron a −80 °C. Se realizaron cortes coronales seriados del mecencéfalo (20 µm de espesor), a una temperatura de −20 °C utilizando un criostato (Leica CM 18UV). Las secciones se incubaron en una solución de etanol:ácido acético (2:1) durante 5 minutos a −20 °C. Después, las laminillas se colocaron en una solución comercial de citratos (Immuno DNA Retriver BioSB) durante 60 minutos a 90 °C. Al término de esta incubación, los cortes se incubaron en el amortiguador de equilibrio durante 10 minutos y posteriormente con la enzima transferasa deoxinucleotidil terminal (TdT) y su sustrato durante una hora a 37 °C en una cámara húmeda. La reacción se detuvo con el buffer de parado, incubando las secciones durante 10 minutos a temperatura ambiente, los cortes se lavaron 3 veces con PBS (1 minuto por

lavado) y se incubaron con el anticuerpo anti-digoxigenina acoplado a rodamina, posteriormente se montaron con un medio de montaje el cual contenía 4',6-diamino-2-fenilindol (DAPI) para marcar los núcleos celulares.

6.6. Determinación de las neuronas dopaminérgicas por inmunohistoquímica

Para determinar la expresión de la enzima tirosina hidroxilasa se sacrificaron los ratones con una sobredosis de pentobarbital sódico (20 mg/kg de peso corporal), se perfundieron con 50 mL de solución salina isotónica seguido de 100 mL paraformaldehído al 4%, se decapitaron y sus cerebros se removieron cuidadosamente para evitar dañarlos. Los cerebros se fijaron 2 h adicionales en paraformaldehído al 4%, se congelaron por inmersión en 2-metilbutano pre-enfriado con hielo seco y se almacenaron a −80 °C. Se realizaron cortes coronales seriados de los cerebros (25 µm de espesor), a una temperatura de −20 °C utilizando un criostato (Leica CM 18UV). Los cortes se recuperaron y se colocaron en pozos con una solución crioprotectora de etilenglicol y de glicerol en una solución amortiguadora de fosfatos de sodio (PB) 0.1M, pH de 7.4.

Se hicieron tres lavados de 10 minutos con PBT (Tritón X-100 al 0.3% en PB), en seguida se inactivó la actividad de la peroxidasa endógena en el tejido con una solución de peróxido de hidrógeno al 0.3% en PB durante 10 minutos y se lavaron 3 veces más con PBT (10 minutos por lavado). Los cortes se incubaron con una solución comercial de citratos (Immuno DNA Retriver BioSB) durante 60 minutos a 70 ºC. Posteriormente se dejaron a temperatura ambiente durante 15 minutos y se realizaron 3 lavados con PBT (10 minutos por lavado), luego se les agregó el anticuerpo primario anti-tirosina hidroxilasa diluido en una solución de bloqueo

(1:1000, de Chemicon), la cual contenía albúmina bovina sérica al 3%, azida de sodio al 0.0125% y tritón X-100 al 0.3% disueltos en PB y se mantuvieron en agitación constante a temperatura ambiente durante toda la noche. Al día siguiente los cortes se lavaron 3 veces con PBT (10 minutos por lavado), se incubaron con el anticuerpo secundario biotinilado dirigidos contra IgGs de ratón (1:500, Chemicon) diluido en PB, por 90 minutos a temperatura ambiente con agitación constante. Al tiempo indicado se retiró el exceso del anticuerpo y los cortes se lavaron 3 veces con PBT (10 minutos por lavado). Los cortes se incubaron con el complejo avidina-peroxidasa (Elite AB Kit, Vector Laboratories, Burlingame, CA, USA) por una hora a temperatura ambiente con agitación constante, se lavaron 3 veces con PB (10 minutos por lavado), y la actividad de la peroxidasa se reveló con un kit de 3,3-diaminobencidina y peróxido de hidrógeno de acuerdo al protocolo recomendado por el proveedor. Al final del procedimiento, los cortes se montaron sobre laminillas cubiertas de gelatina y se dejaron secar hasta el día siguiente, en un ambiente libre de polvo, después se montaron en resina de fijación (Pertmount FisherChemicals, New Jersey) y se observaron en un microscopio de campo claro.

6.7. Cuantificación celular

Las imágenes se capturaron y se procesaron con el software Image J para la cuantificación de células, se tomaron en promedio ocho campos por mesencéfalo de ratón, cuntificando células en la sustancia nigra, haciéndose el reporte como neuronas TH positivas o células apoptóticas por mm^2.

6.8. Determinación de la peroxidación de lípidos

Se utilizó el kit para peroxidación de lípidos TBARS Assay Kit (Cayman Chemical, USA) siguiendo el protocolo recomendado por el proveedor. Los animales se sacrificaron por dislocación cervical, sus cerebros se removieron rápidamente y se colocaron en una placa fría para la disección del estriado (Leng et al., 2005). Se dispusieron aproximadamente 20 mg de tejido en tubos de centrifuga de 1.5 mL y se les agregó 250 µL de amortiguador RIPA (Cayman Chemical, USA), las muestras se homogenizaron durante 15 segundos cada una a 40 V en frío, después se centrifugaron a 1600 × *g* durante 10 minutos a 4 °C y se tomó el sobrenadante para el análisis. Se colocaron 25 µL de cada muestra o de cada uno de los estándares en un vial de 1.5 mL identificados apropiadamente, se les adicionó 25 µL de una solución de SDS para ser mezclados, posteriormente se les agregó 1 mL de la solución colorante (ácido tiobarbitúrico, NaOH, ácido acético) y se incubaron durante una hora en baño María a punto de ebullición, posteriormente los viales se colocaron en hielo durante 10 minutos para detener la reacción, después se centrifugaron de nuevo a 1600 × *g* durante 10 minutos a 4 °C. 150 µL de cada una de las muestras y estándares se colocaron por duplicado en una placa de ELISA de fondo obscuro y se determinó la fluorescencia intrínseca en detector multimodo (Beckman Coulter 880), excitaando las muestras a 540 nm y detectando su emisión a 590 nm.

6.9. Cuantificación de proteínas.

Para la determinación de las proteínas en las muestras a las cuales se les determinaría la actividad de la SOD, se empleó el método de Bradford (www.uco.es) modificado por nuestro grupo de trabajo. La curva patrón se construyó usando concentraciónes crecientes de albúmina sérica de bovino (BSA), en un intervalo de 2.5–12.5 µg de proteína. En cada pozo se adicionarón 250 µL del reactivo de Bradford más la cantidad pertinente de BSA o de muestra, para alcanzar un volumen final de 275 µL. La absorbancia se leyó a 595 nm en lector de placas (Biotek ELx800). Se construyó la curva de calibración y se obtuvo la ecuacion de la recta, misma que se empleó para obtener la concentración de cada una de las muestras.

6.10. Determinación de la actividad de la superóxido dismutasa (SOD)

Se utilizó el kit Superoxide Dismutase Assay Kit (Cayman Chemical, USA) siguiendo el protocolo recomendado por el proveedor. Los ratones se sacrificaron con una sobredosis de pentobarbital sódico (20 mg/kg de peso corporal) a los cuatro días de haber concluido el tratamiento con MPTP y/o silimarina, se perfundieron con 50 mL de solución salina amortiguadora de fosfatos (PBS 0.1M, pH de 7.4), sus cerebros se removieron y se disecaron los mesencéfalos. Los tejidos se homogenizaron en 200 µL de una solución amortiguadora de HEPES 20 mM, pH 7.2 que contenía ácido tetraacético etilenglicol (EGTA) 1mM, manitol 210 mM y sacarosa 70 mM. Las muestras se centrifugaron a 1500 × *g* durante 5 minutos a 4 °C y se colectaron los sobrenadantes. Posteriormente se colocaron

200 µL del detector de radicales (50 µL de la solución de sal de tetrasolio proveniente del stock más 19.95 mL de un amortiguador tris-HCl 50 mM, pH 8 que contenía EGTA 0.1 mM e hipoxantina 0.1 mM) por pozo más 10 µL de los estándares o muestras, se inició la reacción agregando 20 µL de la xantina oxidasa (50 µL del stock diluidos en un vial con 1.95 mL de un amortiguador tris-HCl 50 mM, pH 8) la placa se incubó durante 20 minutos en agitación suave para después leer las absorbancias a 450 nm en un lector de placas (Biotek ELx800).

6.11. Análisis estadístico

Los resultados se expresaron como la media +/- la desviación estándar. Las diferencias estadísticas entre los grupos experimentales se determinaron mediante el uso de una prueba de análisis de varianza (ANOVA), para la comparación múltiple de las medias entre los distintos grupos se utilizó la prueba de Tukey´s post hoc. Los datos fueron procesados con la ayuda del paquete estadístico SPSS 17.0 para Windows.

7. RESULTADOS

7.1. Efecto de la silimarina sobre la concentración de dopamina en el estriado de ratones adultos intoxicados con MPTP

Nuestros datos mostraron que el tratamiento con MPTP depleta los niveles de dopamina y se recupera parcialmente con el tratamiento con silimarina. El tratamiento con silimarina en los animales tratados con MPTP mostró una curva no monotónica y sólo conservó significativamente los niveles de dopamina con las

dosis de 50 mg/kg ($p \leq 0.006$) y 100 mg/kg ($p \leq 0.002$) (Figura 4). La silimarina sola no mostró cambios en los niveles de dopamina (Figura 4).

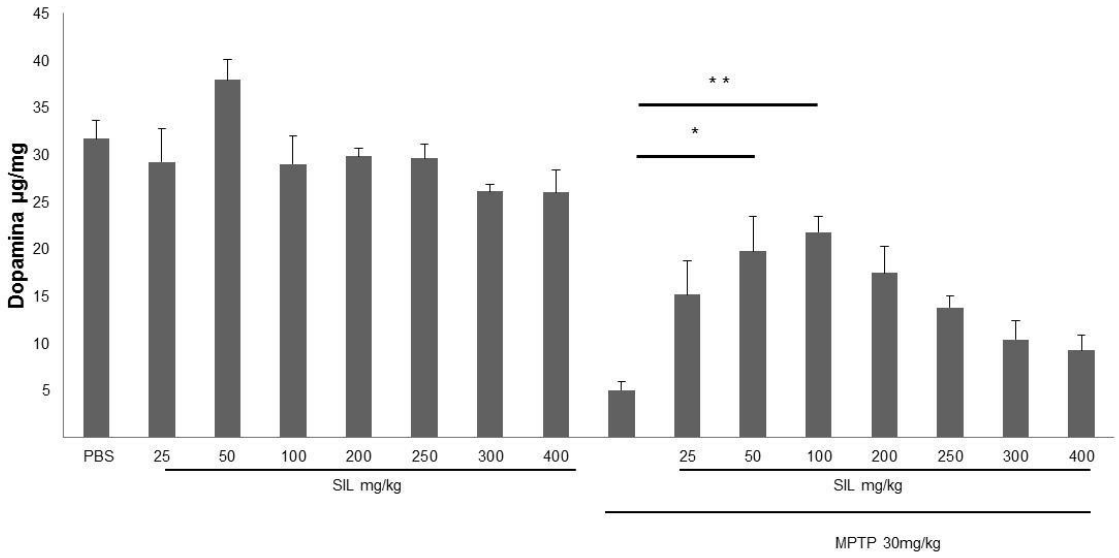

Figura 4. Efecto de la silimarina sobre las concentraciones de dopamina en el estriado de ratones en condiciones fisiológicas o intoxicados con MPTP. Las concentraciones de dopamina fueron determinadas mediante HPLC. Los datos representan los valores de las medias +/- SEM. *p=0.006, **p=0.002.

7.2. Efecto de la silimarina sobre la muerte celular por apoptosis en la sustancia nigra de ratones adultos intoxicados con MPTP

Los animales tratados con la dosis de 100 mg/kg/día de silimarina no mostraron diferencia alguna con respecto a la cantidad de células apoptóticas observadas en los animales pertenecientes al grupo control, los cuáles prácticamente no tuvieron células positivas (Figura 4), mientras que la intoxicación con MPTP aumentó significativamente el número de células apoptóticas ($p \leq 0.001$) (Figuras 5 y 6). En

los animales intoxicados con MPTP y tratados con silimarina la cantidad de células apoptóticas fue significativamente menor ($p \leq 0.001$) con respecto a los animales intoxicados con MPTP (Figuras 5 y 6).

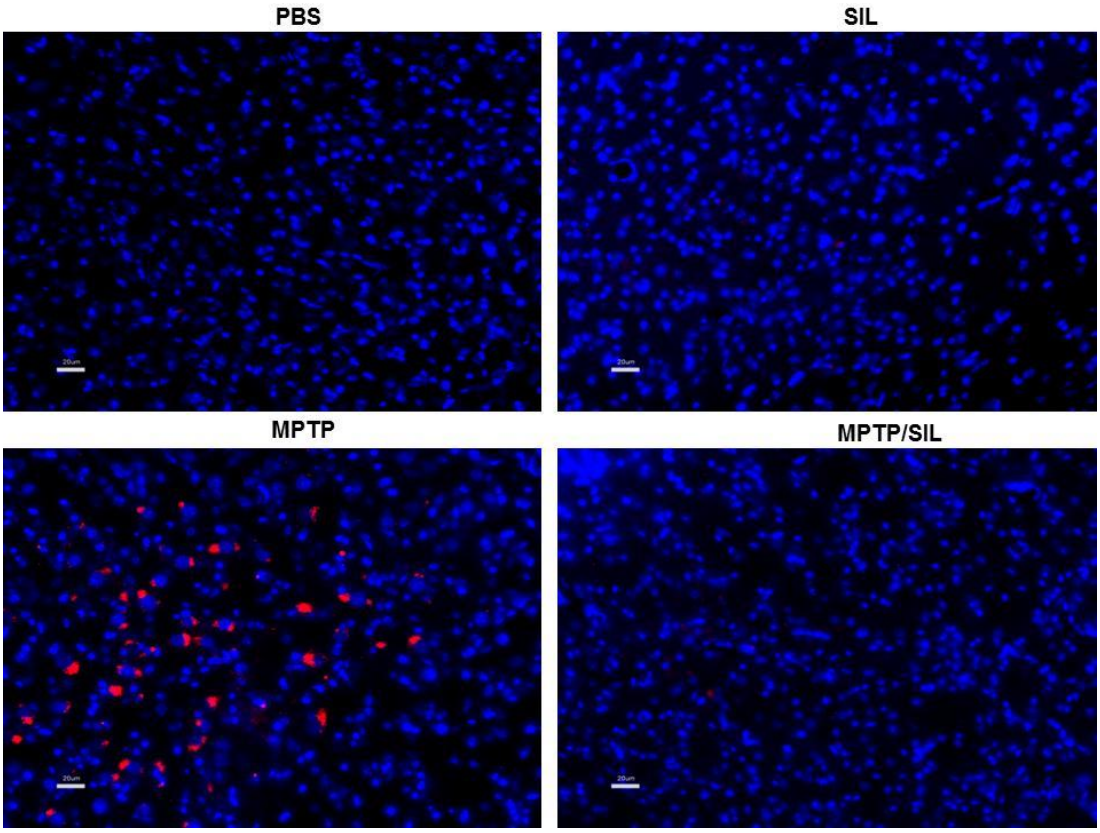

Figura 5. Efecto de la silimarina sobre la apoptosis en la sustancia nigra del ratón adulto intoxicado con MPTP. La detección de la apoptosis se determinó por la técnica de TUNEL *in situ*. Micrografías representativas de células positivas a apoptosis de los distintos grupos, dimenscion de la barra de escala 20 µm, células apoptóticas marcadas en rojo, en azul nucleos celulares.

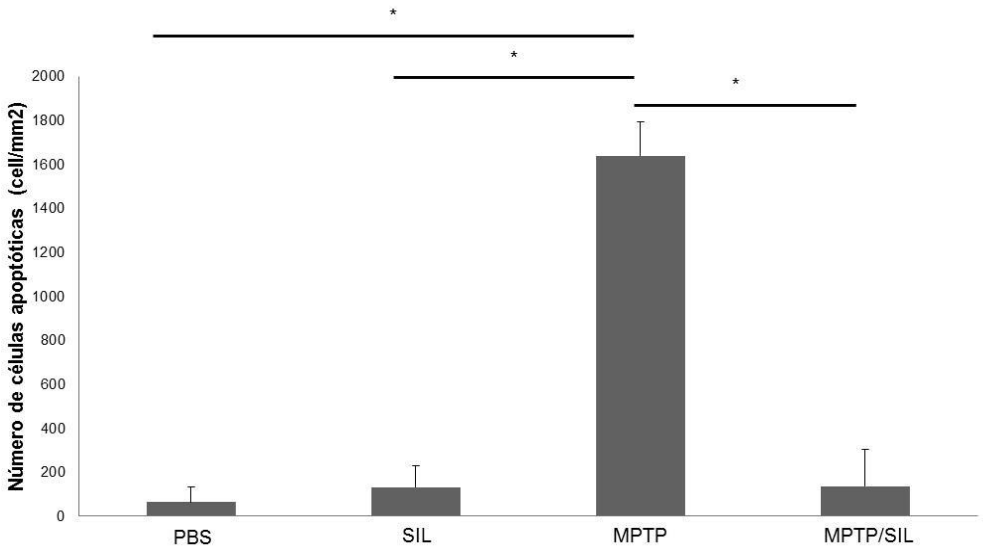

Figura 6. Efecto de la silimarina sobre el número de células apoptóticas en la sustancia nigra del ratón adulto intoxicado con MPTP. Número de células apoptóticas en los diferentes grupos. Los valores están expresados como las medias +/- SEM. * p ≤ 0.001.

7.3. Efecto de la silimarina sobre las neuronas dopaminérgicas en la sustancia nigra de ratones adultos intoxicados con MPTP

El tratamiento con silimarina por sí sola no modifica la cantidad de neuronas TH+ presentes en la sustancia nigra con respecto al grupo control (Figura 7 SIL y PBS respectivamente, Figura 8), pero sí conserva significativamente las neuronas (p≤0.002) tras la administración del MPTP (Figura 7 MPTP/SIL, Figura 8).

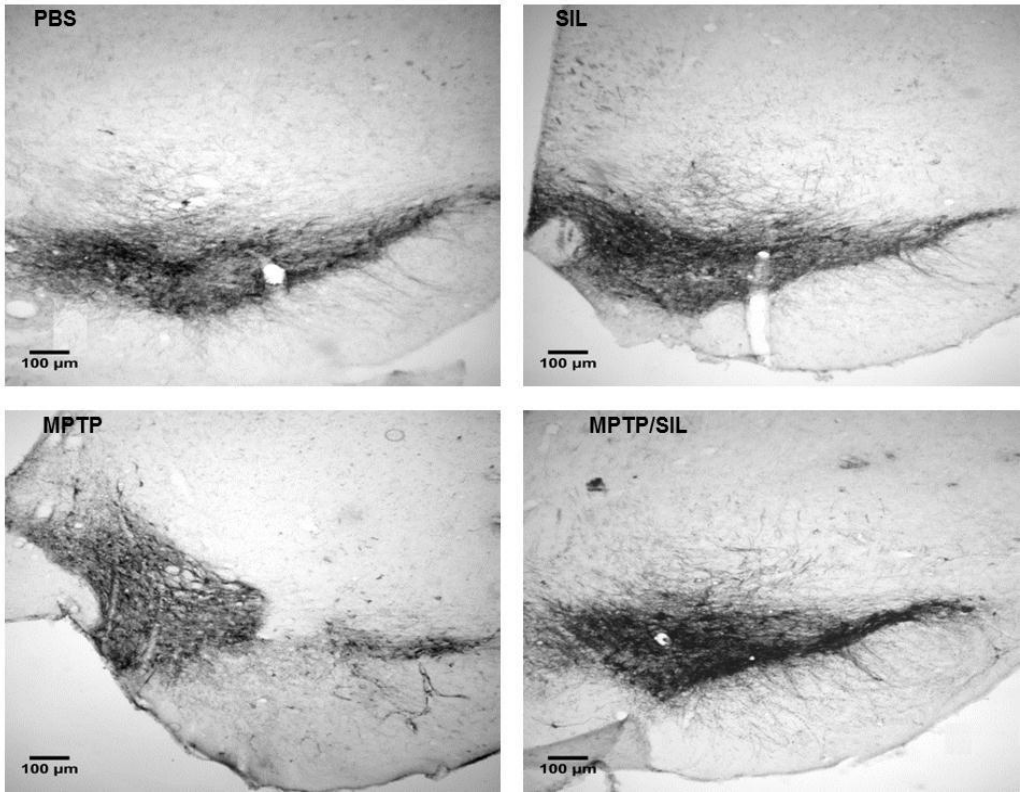

Figura 7. Efecto de la silimarina sobre el número de neuronas dopaminérgicas en la sustancia nigra de ratones adultos intoxicados con MPTP. La expresión de tirosina hidroxilasa fue determinada mediante tinción inmunohistoquímica.

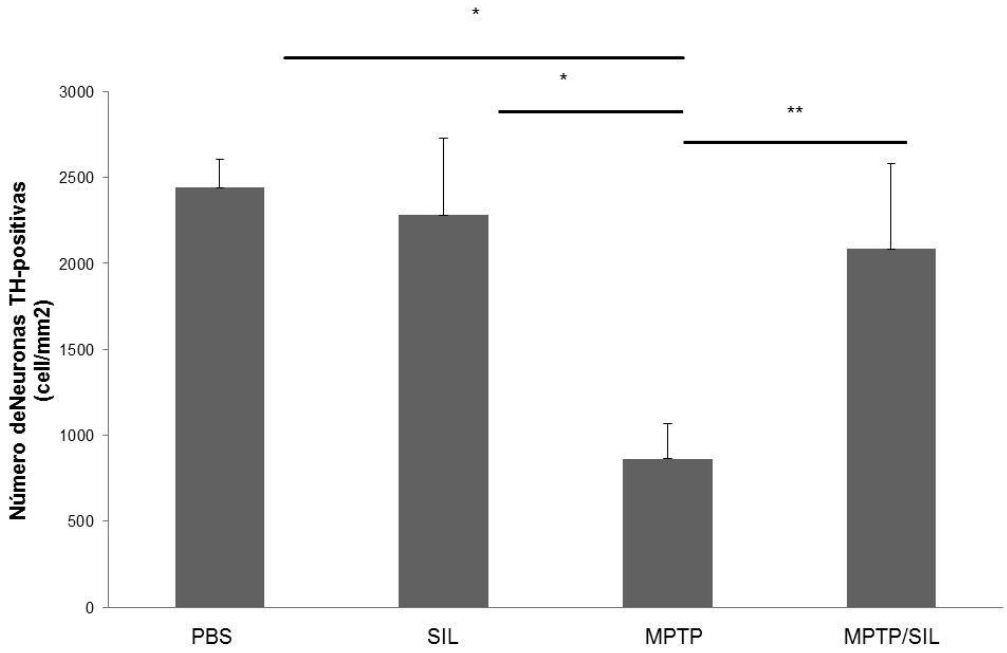

Figura 8. Efecto de la silimarina en el número de neuronas TH+ en la sustancia nigra de ratones intoxicados con MPTP. Los valores son expresados como el promedio +/- desviación estándar. *p≤ *0.001,* **p≤*0.002.*

7.4. *Efecto de la silimarina sobre la peroxidación de lípidos en el estriado de ratones adultos intoxicados con MPTP*

El tratamiento de los ratones con MPTP elevó los niveles de malondialdehído (MDA) el cual es la principal especie molecular que reacciona con el acido thiobarbiturico y fue empleado como indicador de lipoperoxidación (Figura 8). Del mismo modo, el tratamiento con la dosis de silimarina de 100 mg/kg aumenta significativamente los niveles del MDA con respecto al control, de manera semejante a la intoxicación con MPTP ($p \leq 0.002$) (Figura 9). Sin embargo, la administración de silimarina en animales previamente intoxicados con MPTP

reduce significativamente los niveles de MDA (p ≤ 0.015) (Figura 9) comparando contra las lecturas obtenidas de los animlaes intoxicados con MPTP.

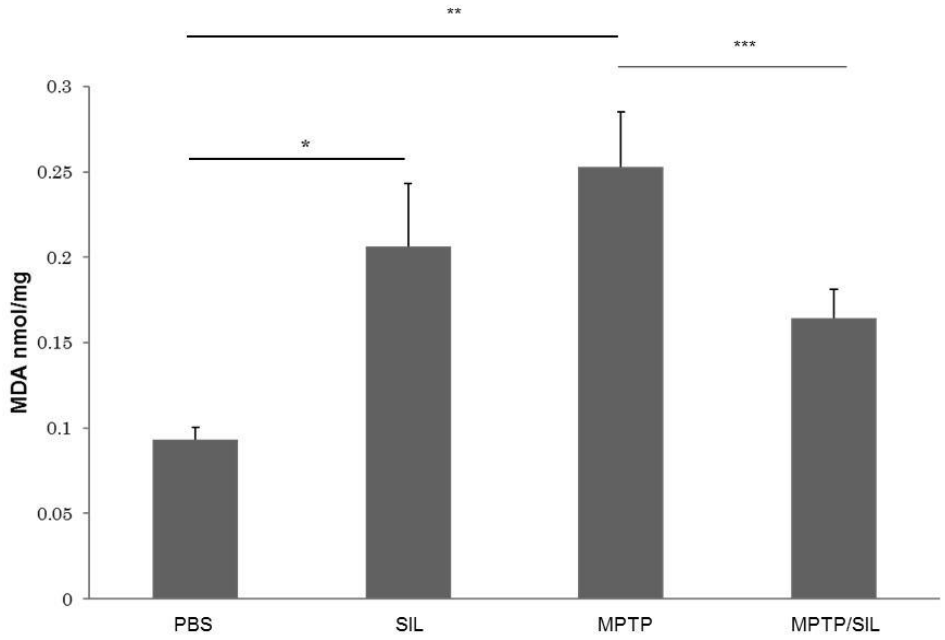

Figura 9. Efecto de la silimarina sobre la peroxidación de lípidos en el estriado de ratones adultos intoxicados con MPTP. Los valores están expresados como las medias +/- SEM. *p≤0.002, **p≤0.0001 y ***p≤0.015.

7.5. Efecto de la silimarina sobre la actividad de la superóxido dismutasa en el mescencéfalo de ratones adultos intoxicados con MPTP

Nuestros resultados muestran que la actividad de la SOD no esta afectada con el tratamiento de la silimarina en la dosis de 100 mg/kg/día; sin embargo, la intoxicación con MPTP reduce significativamente la actividad de la SOD si se compara contra el grupo control (p≤ 0.033) (Figura 10). Los animales intoxicados

con MPTP y tratados con silimarina no mostraron cambios significativos en la actividad de la SOD en comparación con el resto de los grupos (Figura 10).

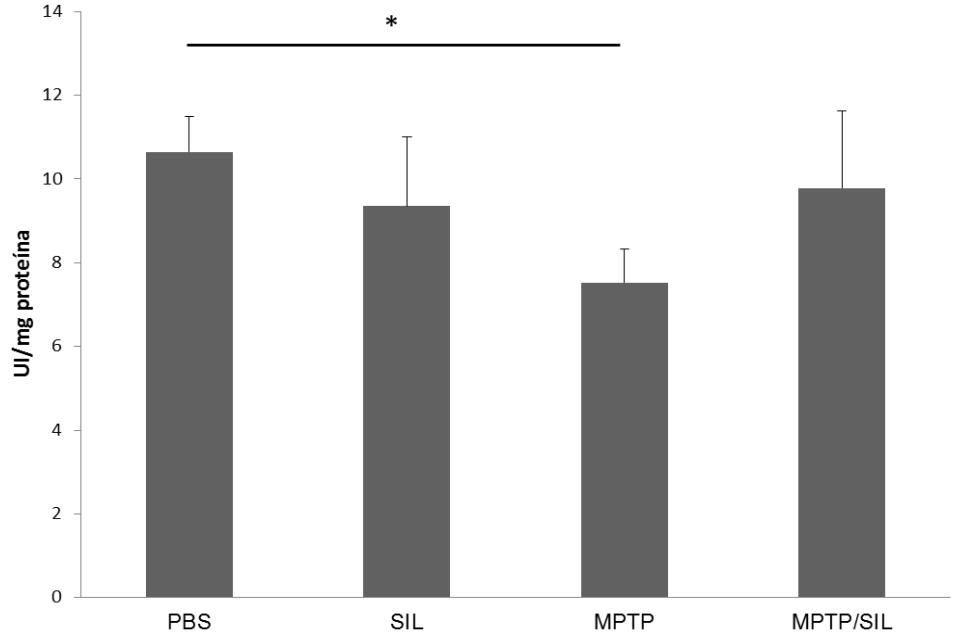

Figura 10. Efecto de la silimarina sobre la actividad de la superóxido dismutasa en el mesencéfalo de ratones adultos intoxicados con MPTP. Los valores están expresados como el promedio de las Unidades Internacionales de la enzima/mg de proteína+/- desviación estándar. *p≤0.033.

8. DISCUSIÓN

Nuestros datos demuestran que la silimarina presenta un efecto neuroprotector al disminuir la cantidad de células apoptóticas presentes en la sustancia nigra después de la intoxicación con MPTP, lo que se asocia con la preservación de

neuronas dopaminérgicas en esta misma región y tiene como efecto la conservación de los niveles de dopamina en el estriado. Nuestros allasgos indican que tal fenómeno puede estar mediado por la actividad anti-oxidante de la silimarina, ya que previno la lipoperoxidacíon asociada al modelo empleado en este estudio. Es importante destacar que el efecto de la silimarina en la conservación de la dopamina presenta una curva dosis-respuesta no-monotónica, siendo las dosis de 50 y 100 mg/kg las que evitan la depleción de este neurotransmisor en nuestro modelo.

El mecanismo por el que la silimarina podría haber ejercido un efecto neuroprotector en el modelo de Parkinson empleado probablemente esta relacionado con la capacidad que tiene para unirse al receptor de estrógenos β (REβ) (Baluchnejadmojarad et al, 2010).

Estudios *in vitro* e *in vivo* han demostrado el efecto protector de los estrógenos contra la toxicidad inducida por el péptido Beta-Amiloide (Aβ) (Green et al., 1996) y toxinas mitocondriales tales como MPTP (De Girolamo et al., 2001). Los estrógenos responden de una manera no-monótona debido a la saturación de sus receptores, fenómeno que se ha observado en líneas celulares de cáncer de mama MCF-7, (Welshons et al., 2003) y en un modelo de MPTP (Ramírez et al., 2003). Es posible que la respuesta no-monotónica observada pueda estar relacionada con la saturación del REβ, imitando una respuesta estrogénica.

En cuanto al efecto anti-apoptótico observado podemos citar un ensayo en el que se empleó un modelo de toxicidad inducido con paracuat y maneb se ha observó que la silimarina favorece la expresión de proteínas anti-apoptóticas como Bcl-2, disminuye la expresión de moléculas pro-apoptóticas como caspasa-3, Bax y la

caspasa-9 en la región nigroestriatal (Pook et al. 2005; Singhal et al., 2011). Es probable que la silimarina al inducir estas proteínas anti-apoptóticas disminuya la apoptosis de las neuronas dopaminérgicas y por lo tanto conserve los niveles de dopamina.

Por otra parte, se ha demostrado que la silimarina y su componente más abundante, la silibinina, presentan propiedades antioxidantes (Köksal et al., 2009; Sangeetha et al., 2009). Muchas reacciones celulares utilizan oxígeno molecular para su catálisis y la producción de energía, lo cual puede conducir a la formación de ROS como son los aniones: súperoxido, peróxido, radicales hidróxilo y peroxirradicales, además en presencia de óxido nítrico (NO por sus siglas en ingles), se producen especies reactivas de nitrógeno como pueden ser peroxinitrito y radicales nitrotirosilo. Aunque estas especies reactivas son importantes para la ejecución de funciones fisiológicas, su excesiva producción es perjudicial para las membranas lipídicas y pueden causar la muerte celular (Loh et al., 2006). La células cerebrales son particularmente susceptibles al daño oxidativo debido a los altos niveles de ácidos grasos poli-insaturados que contienen y a la relativamente poca actividad de enzimas antioxidantes (Mariani et al., 2005). El estrés oxidante se traduce en daño a proteínas celulares, lípidos y DNA, lo cual lleva a la activación de las vías apoptóticas (Mattson et al., 2006).

Estudios post mortem han demostrado el incremento en la oxidación de proteínas, lípidos y DNA en enfermedades neurodegenerativas como Alzheimer, Parkinson, parálisis supra nuclear progresiva, Huntington y esclerosis lateral amiotrófica, entre otras (Mariani et al., 2005). Las neuronas dopaminérgicas constantemente oxidan dopamina a través de la MAO lo que favorece la producción de superóxido

y peróxido de hidrógeno (Cardoso et al., 2005), consecuentemente estas neuronas están perpetuamente en estado de estrés oxidante, lo que lleva a la reducción de los niveles de antioxidantes endógenos haciéndolas susceptibles a la neurodegeneración (Gandhi y Wood, 2005). Este evento también se ha observado en el modelo de intoxicación con MPTP, por lo que el control del estado de estrés oxidante mediante el uso de silimarina constituye un punto clave en la terapéutica de la EP y de otras enfermedades neurodegenerativas.

Evidencias previas demuestran el efecto benéfico de este fitofármaco en cuanto al estrés oxidante en distintos modelos de neurodegeneración (Wang et al., 2002; Nencini et al., 2007; Galhardi et al., 2009; Lu et al., 2009). Sin embargo, nuestras observaciones se contraponen en parte a lo reportado por estos autores, ya que ellos no documentan efecto alguno de la silimarina por sí sola en cuanto al estrés oxidante. Nosotros observamos que la administración de silimarina a una dosis de 100 mg/kg/día, de manera intraperitoneal, eleva las concentraciones de MDA y sólo ejerce su efecto antioxidante en los animales intoxicados con la MPTP. Estudios previos indican que los flavonoides, que poseen varios grupos hidróxilo, pueden tener propiedades pro-oxidantes (Samra et al., 2011). Este efecto pro-oxidante de la silimarina en esta dosis pudiera deberse a que con excepción de la isosilibina, los otros 3 componentes poseen cuatro o más grupos hidróxilo (ver Figura 3). Recientemente se observó que este efecto ocurre de manera dependiente de la dosis, de tal forma que las dosis de 25 y 50 mg/kg/día, administradas de manera oral, previenen el estrés oxidante en el cerebro, pero una dosis de 100 mg/kg/día eleva los niveles de MDA y mediadores pro-inflamatorios como óxido nítrico e IL-1β en el hipocampo (Malekinejad et al.,

2012). Además, se ha reportado que la silimarina tiene un efecto diferente en distintas regiones cerebrales, ya que a dosis de 200 y 400 mg/kg/día se incrementa la cantidad de ROS en el hipocampo y las disminuye en la corteza cerebral; mostrando también una correlación con la edad, ya que en ratas jóvenes presenta un efecto pro-oxidante en estas regiones cerebrales pero no así en ratas viejas donde disminuyó los niveles de ROS (Galardhi et al., 2009).

La SOD protege a las células del radical superóxido (O_2^-) transformándolo en peróxido de hidrogeno (Fridovich, 1982) y presenta aumento en su actividad en distintas regiones cerebrales de pacientes con EP, tales como la corteza temporal, el tálamo, el núcleo rojo y particularmente la sustancia nigra (Marttila et al., 1988). Se ha observado que esta enzima tiene variaciones en cuanto a su actividad y expresión después de la intoxicación con MPTP, se sabe que en el estriado aumenta su acción y disminuye en la sustancia nigra (Thiffault et al., 1995). También se ha documentado que la inhibición *in vivo* de la SOD mediante la administración sistémica de dietilditiocarbonato aumenta la pérdida de neuronas dopaminérgicas y disminuye las concentraciones de dopamina contenidas en el estriado, fenómenos asociados a la intoxicación con MPTP (Corsini et al., 1985). Sin embargo, otros autores han reportado que la actividad de la SOD aumenta en la sustancia nigra conforme transcurren el tiempo con respecto al periodo de intoxicación de tal manera que alrededor del séptimo día post-intoxicación, la SOD no sólo recupera su actividad sino que la acrecienta significativamente (Muralikrishnan y Mohanakumar, 1998).

En experimentos más recientes se observó que la Cu/Zn-SOD residente en células gliales de la sustancia nigra tiene un aumento en su expresión después del

tratamiento con MPTP; sin embargo, la expresión de la Mn-SOD en neuronas de la vía nigro-estriatal es reducida significativamente a los siete días post-tratamiento (Kurosaki et al., 2004). Esto nos permite inferir que la diferencia en resultados puede deberse a la ventana de tiempo en la cual se analizaron las muestras, de tal forma que los exámenes realizados a pocos días después de la intoxicación con MPTP, donde se observa una disminución de la actividad de la SOD total, los resultados se asocian más a la muerte neuronal. En contra-parte con los hallazgos obtenidos después de una semana de la intoxicación, donde se observa un aumento de la actividad de la SOD total, lo que se puede asociar a un evento de gliosis (Kurosaki et al., 2004), nuestros resultados muestran una disminución en la actividad de la SOD en el grupo de animales intoxicados con MPTP: tal allazfo probablemente sea el reflejo de la muerte neuronal, ya que los análisis se realizaron al cuarto día después de la última intoxicación. Las observaciones hechas en los animales pertenecientes al grupo intoxicado con MPTP y tratados con silimarina 100 mg/kg presentan únicamente una tendencia en la conservación de la actividad de la SOD, es probable que dicha tendencia se deba más al efecto neuroprotector que al anti-oxidante de la silimarina.

Asimismo, se ha propuesto que la silimarina tiene un efecto inhibidor de NF-κβ, el cual es mediado vía el receptor de TNFα (Manna et al., 1999), y que el efecto anti-inflamatorio de la silimarina y la silibinina es mediante la inhibición del NF-κβ (Wang et al., 2002; Tyagi et al., 2009; Hou et al., 2010). Es posible que el efecto neuroprotector de la silimarina en el modelo de intoxicación con MPTP pueda estar relacionado con la inhibición de NF-κβ en la microglia, lo que llevaría a una disminución de la concentración de citocinas pro-inflamatorias como TNFα y de la

producción de ROS (Wang et al., 2002). También se ha demostrado que la silimarina inhibe la activación de la microglia tanto *in vitro* como en *in vivo* y por lo tanto disminuye procesos neuroinflamatorios asociados directamente a la activación de la microglia (Wang et al., 2002; Hou et al., 2010).

Nuestros datos muestran que la silimarina tiene un efecto neuroprotector importante ya que evita la depleción de las concentraciones de dopamina estriatales al disminuir la muerte celular por apoptosis, prevenir la pérdida neuronal y la peroxidación de lípidos en el modelo de intoxicación con MPTP.

9. CONCLUSIONES

La silimarina tiene un efecto neuroprotector en el modelo de intoxicación con MPTP al previenir la depleción de la dopamina estriatal, redicir la muerte celular apoptótica, conservar las neuronas dopaminérgicas en la substancia nigra, y disminuir la la peroxidación de lípidos en el estriado (ver Figura 10).

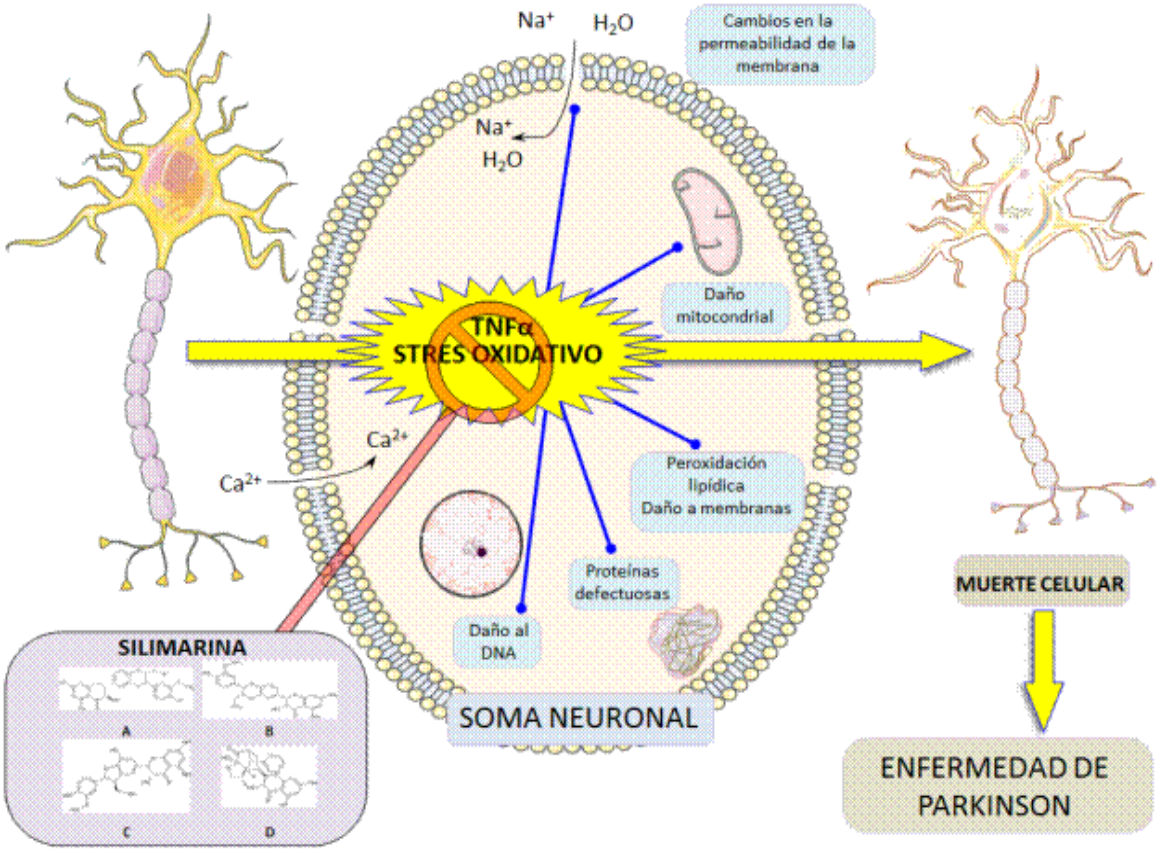

Figura 10. Esquematización de la acción anti-oxidante y neuroprotectora de la silimarinasobre las neuronas dopaminergicas. En la figura se muestra el efecto que tiene la silimarina sobre el estrés oxidante a nivel neuronal y su consecuente acción neuroprotectora. A) Silibina. B) Isosilibina. C) Silicristina. D) Silidianina.

10. PERSPECTIVAS

Derivadas de nuestros resultados, surgen nuevas inquietudes, como es el hecho de analizar de manera más profunda los fenómenos oxidativos en el cerebro asociados al uso de la silimarina en condiciones fisiológicas, describir los fenómenos neuroinmunologicos que puedan estar relacionados con el efecto

neuroprotector de la silimarina en el modelo que empleamos y por supuesto explorar distintos esquemas de tratamiento. Una vez analizadas estas cuestiones y si los resultados son positivos, pudiéramos pensar en explorar el efecto de la silimarina en modelos de la enfermedad de Parkinson en homínidos no humanos y asi explorar la posibilidad de usarla en combinacion con los fármacos empleados en la actualidad para el tratamiento de la enfermedad de Parkinson.

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12. ANEXO

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Short communication

Neuroprotective effect of silymarin in a MPTP mouse model of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease secondary to the loss of dopaminergic neurons in the substantia nigra. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces in mice and primates histopathological changes similar to PD in humans. A common feature of PD and MPTP models is neuronal death and dopamine depletion. Silymarin is a complex of flavonolignans derived from the seeds of the plant *Silybum marianum* and has mainly antioxidant, anti-inflammatory, cytoprotective and neuroprotective effects.

In order to explore whether silymarin has a neuroprotective effects in a mouse model of PD we determined the concentration of striatal dopamine by HPLC, the number of apoptotic cells by *in situ* Tunel assay and the number of tyrosine hydroxylase positive neurons by immunohistochemistry in substantia nigra of vehicle-treated, silymarin-treated, MPTP-intoxicated and MPTP-silymarin treated C57BL/6J male mice. MPTP (30 mg/kg) and silymarin doses (25, 50, 100, 200, 250, 300 or 400 mg/kg) were administered intraperitoneally once daily for five consecutive days. Silymarin treatment showed a non-monotonic dose–response curve and only 50 and 100 mg/kg doses preserved dopamine levels (62% and 69%, respectively) after MPTP intoxication. Additionally, 100 mg/kg silymarin treatment significantly diminished the number of apoptotic cells and preserved dopaminergic neurons in the substantia nigra of MPTP-intoxicated mice. These results show the neuroprotective properties of 100 mg/kg silymarin and may be of interest in the treatment of PD.

1. Introduction

Parkinson's disease (PD) is a frequent neurodegenerative disease characterized by progressive motor function deficits secondary to the specific loss of pars compacta catecholaminergic neurons of the substantia nigra and a dopamine deficit (Dexter and Jenner, 2013).

The primary cause that triggers neurodegeneration remains uncertain, however, several pathophysiological mechanisms have been implicated as aging, high density of microglia in substantia nigra, inflammation, oxidative/nitrosative stress, abnormal protein deposits, decreased neurotrophic factors, among others (Gao et al., 2002; Dexter and Jenner, 2013). These mechanisms lead to microglia activation, which in turn favors an oxidative and inflammatory environment facilitating neuronal death (Gao et al., 2002).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is widely used to induce a Parkinsonian model with biochemical and cellular changes similar to those seen in PD (Smeyne and Jackson-Lewis, 2005). After systemic administration MPTP rapidly crosses the blood brain barrier, is metabolized into 1-methyl-4-phenylpyridine (MPP+) by the enzyme monoamino oxidase B of glial cells, and is selectively transported into nigrostriatal dopaminergic neurons through the dopamine transporter. MPP+ accumulates in the mitochondrial inner membrane inhibiting the complex I of the electron transport chain, and induces apoptotic death of dopaminergic neurons; consequently striatal dopamine

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levels are depleted (Smeyne and Jackson-Lewis, 2005). This event is complemented by oxidative stress and inflammation (Vázquez-Claverie et al., 2009).

Silymarin, a complex of several flavonolignans obtained from the seeds of the plant *Silybum marianum*, has antioxidant, anti-inflammatory, cytoprotective, and anticarcinogenic properties (Nencini et al., 2007). Silymarin acts in CNS as an antioxidant due to its free radical scavenging property, increases superoxide dismutase and reduced glutathione levels (Nencini et al., 2007; Lu et al., 2009; Raza et al., 2011) and inhibits lipid peroxidation (Nencini et al., 2007; Singhal et al., 2011). Additionally, silymarin inhibits microglia activation and production of inflammatory mediators such as TNFα and nitric oxide reducing dopaminergic neurons damage induced by lipopolysaccharide (Wang et al., 2002).

Considering the antioxidant and anti-inflammatory properties that have been reported for silymarin, we evaluated the potential neuroprotective effect of silymarin in a MPTP mouse model of PD, by determining striatal dopamine levels, apoptotic cells and tyrosine hydroxylase positive neurons.

2. Methods

2.1. Animals

All experiments were carried out with male C57BL/6J mice (25–30 g of body weight), maintained under 12:00 h light–dark cycle with food and water *ad libitum*. Animal handling and experimentation followed strictly the Guidelines for Care and Use of Laboratory Animals published by the National Institutes of Health and the Guidelines of the Mexican Law of Animal Protection. All experimental procedures were approved by the research and ethics committees of the Medical School of the National Autonomous University of Mexico. We minimized the number of mice used and their suffering or pain as much as possible.

2.2. Drug administration schemes

In order to induce the Parkinson's model mice (5 animals per group) were treated with MPTP following the subchronic scheme, which consists of MPTP hydrochloride (30 mg/kg, i.p.; Sigma–Aldrich, MO, USA) administered daily for 5 consecutive days. MPTP administration and dosage was selected on the basis of previous studies (Garcia et al., 1992, 2008, 2010), in which dopamine depletion, apoptosis, inflammatory response, and oxidative damage with MPTP were demonstrated in mice under the same experimental conditions. The cumulative dose was 150 mg/kg to avoid necrotic cell death. Control mice received vehicle (phosphate-buffered saline (PBS), pH 7.4). Silymarin (25, 50, 100, 200, 250, 300 or 400 mg/kg, i.p.; Sigma–Aldrich, MO, USA) or vehicle (PBS, pH 7.4) were administered 30 min after MPTP administration during the same 5 consecutive days. Silymarin administration was intraperitoneally in order to avoid silymarin loss due to poor oral absorption and thereby increase its bioavailability. Intraperitoneal administration should assure higher levels of silymarin than oral administration, slightly lower than with intravenous administration due to hepatic metabolism and biliary excretion (Wu et al., 2007; Loguercio and Festi, 2011). To diminish the number of animals employed the dose of 100 mg/kg of silymarin was used for apoptosis and tyrosine hydroxylase determination, because this dose preserved most the dopamine levels and the data showed less dispersal than those derived from the dose of 50 mg/kg. Animals were sacrificed for studying apoptosis 3 days after treatment completion to ensure the presence of apoptotic cells or 7 days for dopamine determination and tyrosine hydroxylase immunohistochemistry when great dopamine depletion and dopaminergic neuron loss are observed (Garcia et al., 1992, 2008; Wang et al., 2012).

2.3. Determination of striatal dopamine content

Dopamine content was analyzed by high resolution liquid chromatography with electrochemical detection in striatal samples obtained from silymarin treated, MPTP-treated or MPTP and silymarin treated mice. Briefly, animals were sacrificed by cervical dislocation; tissue samples were obtained by dissection and immediately sonicated on ice in 300 µl of perchloric acid 0.4 N and 0.1% (w/v) sodium metabisulfite, followed by a 10 min-centrifugation step at 4000 × *g* at 4 °C. The supernatants were filtered and preserved at −70 °C until used for chromatographic analysis with a Perkin Elmer LC-4C liquid chromatograph with a BAS CC-5 electrochemical detector. Peaks were integrated with a Perkin-Elmer Turbochrom Navigator 4.1 data station. Calibration curves were constructed by injecting known concentrations of standard (prepared in perchloric acid metabisulfite solution) into the 20 µl loop of the chromatograph. Dopamine concentrations were obtained by interpolation on its respective standard curve. An Alltech Adsorbosphere Catecholamine (100 × 4.6 mm) column with 3 µ of particle size was used. Mobile phase consisted of an aqueous phosphate buffer solution (0.1 M, pH 3.1) containing 0.9 mM sodium octyl sulfate, 0.1 mM EDTA and 15% (v/v) of methanol. Flow rate was 1.0 ml min^{-1}. Potential was fixed at 0.8 V against an Ag/AgCl reference electrode. All samples were analyzed in duplicate. Twenty microlitres of perchloric acid metabisulfite solution was used as vehicle in samples and standard. The concentration range was 40, 80, 160 and 320 µg/µl and the R2 was 0.9999.

2.4. Tunel cell apoptosis assay

Mice were euthanized with sodium pentobarbital (45 mg/kg), transcardially perfused with isotonic saline solution and afterwards with 4% paraformaldehyde in phosphate-buffered saline solution (PBS), then decapitated and their brains carefully removed to avoid damage. Samples were post-fixed for 2 h with paraformaldehyde 4% in PBS, cryoprotected, frozen by immersion in pre-cooled 2-methylbutane and stored at −80 °C. Serial coronal sections (20 µm) were obtained in a cryostat. Apoptotic cells were detected with an *in situ* apoptosis detection kit following the manufacturer's instructions (ApopTag Red, S7165; Millipore, MA, USA). Labeled cells were detected with a rhodamine-conjugated primary antibody. Slides were mounted with a 4,6-diamidino-2-phenylindole (DAPI) antifade solution (Millipore, MA, USA). Sections were stored at 4 °C and protected from light until analyzed with a fluorescent microscope. Four sections per mouse were analyzed. Apoptotic cells were reported per square millimeter in substantia nigra.

2.5. Immunohistochemistry of tyrosine hydroxylase positive neurons

Serial coronal sections were obtained as described in the previous section, and placed in wells with phosphate-buffered solution (PB) with 0.3% hydrogen peroxide for endogenous peroxidase inactivation. Tissue sections were washed three times with PB-Triton X-100 (PBT) and then incubated with a citrate buffered solution for 60 min at 70 °C. Tissue sections were washed three times with PBT and then incubated with primary antibody mouse anti-tyrosine hydroxylase (1:500, Millipore, MA, USA) diluted in a blocking solution of 3% albumin over night at 4 °C. The next day sections were washed three times with PBT and incubated with a secondary biotinylated antibody goat anti-mouse IgGs (1:500, Millipore, MA, USA) in PB for 90 min at room temperature. The excess of antibody was removed and the sections were washed three times with PBT. The sections were incubated with avidin-peroxidase (AB Elite Kit, Vector Laboratories, CA, USA) for one hour at room temperature and peroxidase activity was revealed with 3,3′-diaminobenzidine and peroxide hydrogen according to the protocol recommended by the supplier (Vector Laboratories, CA, USA). Sections were then mounted on gelatin-covered slides and allowed to dry overnight in dust free environment, mounted in Clarion and observed in a bright field microscope. Images were captured and processed with Image J software for cell quantification. Eight sections per mouse were analyzed. Tyrosine hydroxylase positive neurons (TH+) were reported per square millimeter in substantia nigra.

2.6. Statistical analysis

The results were expressed as mean values ± SEM. Data were processed in Excel 2010 (Microsoft) and SPSS 17.0 for Windows. Statistical differences between experimental groups were determined using a one-way analysis of variance test (ANOVA), followed by *post hoc* Tukey's test. $p \leq 0.05$ was considered significant.

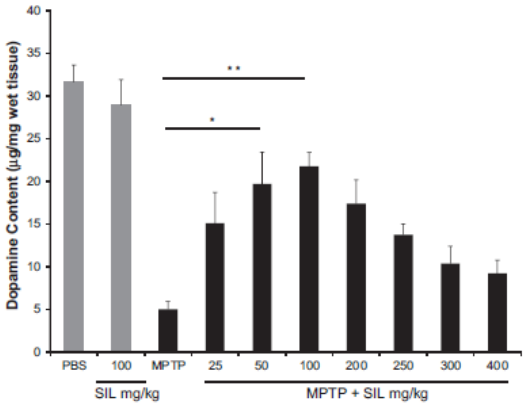

Fig. 1. Effect of silymarin treatment on striatal dopamine levels. Dopamine concentrations were determined 7 days after MPTP and silymarin (SIL) treatment by HPLC. Data are presented as group mean ± SEM. *$p \leq 0.006$, **$p \leq 0.002$.

3. Results

3.1. Silymarin preserves striatal dopamine concentration in MPTP treated mice

Striatal dopamine content was significantly depleted by MPTP treatment (84% below control group), and partially recovered by silymarin (Fig. 1). Silymarin treatment in MPTP-intoxicated mice showed a non-monotonic dose–response curve and only 50 and 100 mg/kg doses preserved significantly dopamine levels (62% and 69%, respectively; Fig. 1). Silymarin alone induced no significant difference in dopamine content (only the data of 100 mg/kg is shown; Fig. 1).

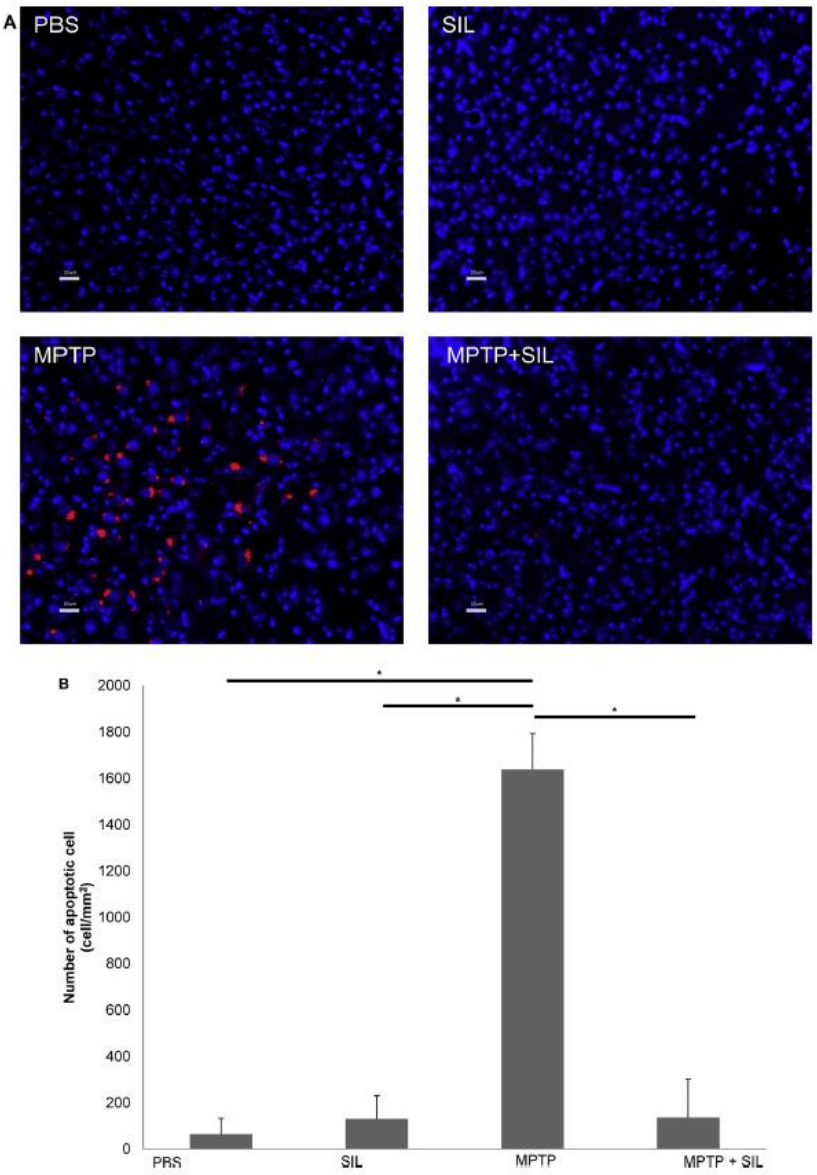

Fig. 2. Silymarin diminishes apoptotic cell counts in the substantia nigra (SN). (A) Low-power photomicrographs of representative sections showing MPTP-induced apoptosis in SN. Apoptosis was determined 3 days after MPTP and silymarin treatment by *in situ* Tunel assay (in red) and nuclei were counterstained with DAPI (in blue). The merged images of representative tissue sections are shown from mice treated with PBS, silymarin (100 mg/kg), MPTP, or MPTP and treated with silymarin (100 mg/kg). (B) Total number of apoptotic cells per square millimeter in SN. Data are expressed as group mean ± SEM. $^*p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

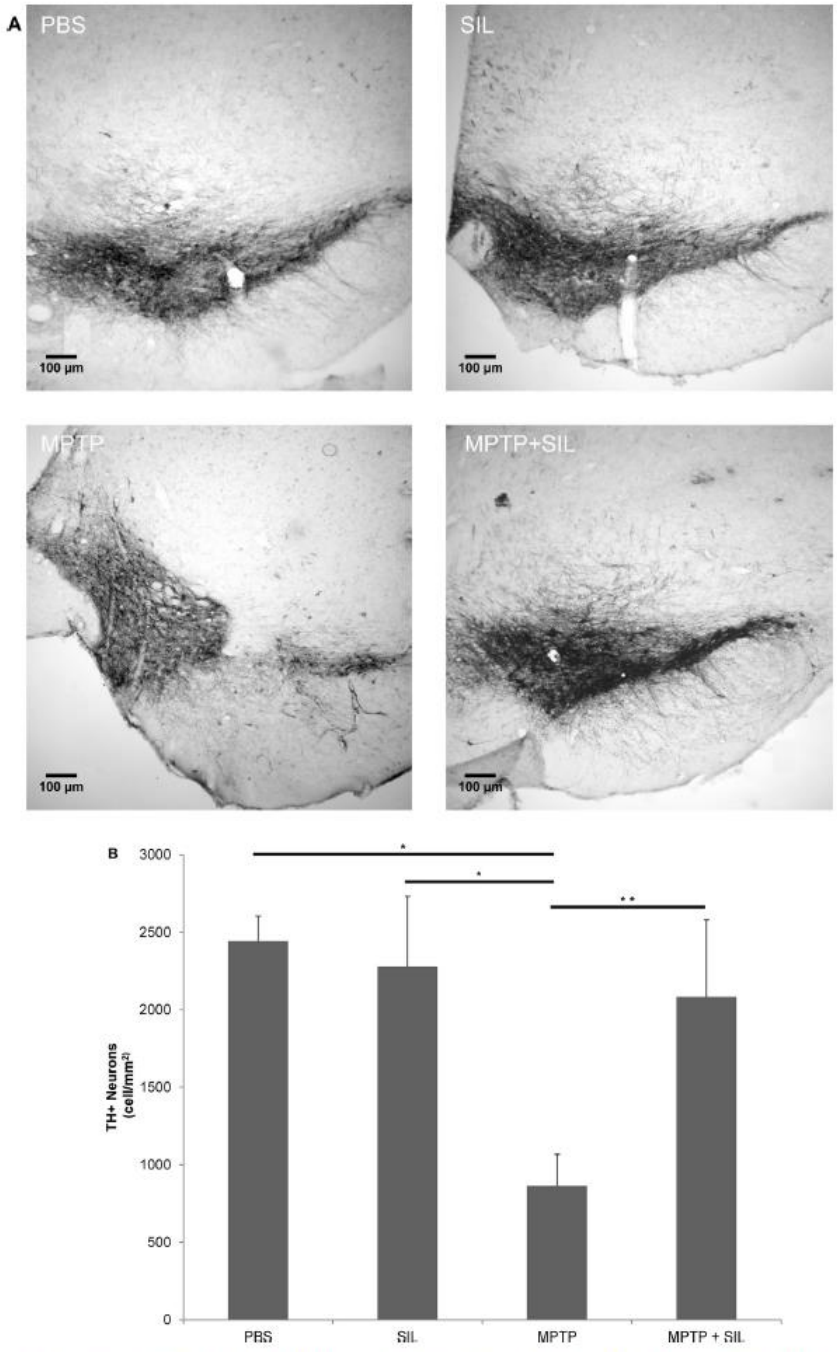

Fig. 3. Silymarin preserves dopaminergic neurons in the substantia nigra (SN). (A) Low-power photomicrographs of SN representative sections showing MPTP-induced loss of tyrosine hydroxylase (TH) neurons. TH expression was determined 7 days after MPTP and silymarin treatment by immunohistochemical staining. Representative tissue sections from mice pretreated with PBS, silymarin (100 mg/kg), MPTP, or MPTP and treated with silymarin (100 mg/kg). (B) Total number of TH+ neurons per square millimeter in the SN. Data are expressed as group mean ± SEM. $^{*}p \leq 0.001$ and $^{**}p \leq 0.002$.

3.2. Silymarin diminishes apoptosis and preserves dopaminergic neurons in the substantia nigra of MPTP treated mice

A reduced number of apoptotic cells in the substantia nigra was observed in the control group and in the group treated with 100 mg/kg of silymarin (Fig. 2) compared to the MPTP-intoxicated mice (Fig. 2). MPTP intoxication depicted a significant increased number of apoptotic cells (Fig. 2), an effect that was significantly diminished in MPTP-intoxicated animals treated with 100 mg/kg of silymarin (Fig. 2). These results are directly related to the number of tyrosine hydroxylase positive neurons (TH+) observed in the sustantia nigra of mice from the different experimental and control groups (Fig. 3). Mice treated with silymarin did not differ in the number of TH+ neurons compared to the control group (Fig. 3). MPTP treatment reduced significantly the TH+ neurons, while silymarin treatment in MPTP-intoxicated mice preserved significantly the TH+ neurons (Fig. 3).

4. Discussion

Silymarin has been widely used as nutritional supplements for liver diseases. However, the variable composition of silymarin preparations has led to controversial results (Gazák et al., 2007). The present study demonstrated the protective properties of a standardized extract of silymarin in a MPTP-induced PD model. Several scientific groups have reported that silymarin and silybin, the most abundant compound of silymarin, are potent antioxidants, have strong free radical-scavenging activity (Nencini et al., 2007; Lu et al., 2009) and anti-inflammatory effects in rodents (Wang et al., 2002). Also, recently Zhu and collaborators showed antioxidant effects of silymarin after 28 days of treatment in healthy humans (Zhu et al., 2013). Moreover, recently it has been shown that silymarin provides neuroprotection. For example, silymarin restores the behavioral and antioxidant profile in focal ischemia/reperfusion (Raza et al., 2011), and diminishes oxidative damage by reestablishing anti-oxidant enzymatic activity (Nencini et al., 2007). In an Alzheimer's disease animal model silybin reduces β-amyloid deposition and improves memory impairment as well the associated oxidative stress (Lu et al., 2009). Furthermore, silybin attenuates dopamine loss in the prefrontal cortex and hippocampus, improving memory deficits associated with the degree of dopamine decrease in methamphetamine-treated mice (Lu et al., 2010). These results are directly related to the neuroprotective effect we observed in MPTP-intoxicated mice treated with 100 mg/kg of silymarin.

We showed that silymarin prevented striatal dopamine depletion in a MPTP model. Silymarin alone had no effect on dopamine levels in control mice displaying a beneficial effect only in the MPTP-intoxicated mice. It is important to highlight that silymarin neuroprotective effect in dopamine conservation presented a non-monotonic dose-response curve, being 50 and 100 mg/kg the only dopamine preserving doses. It is possible that the lack of neuroprotection of the higher silymarin doses in our PD model may be result of a pro-oxidant and pro-inflammatory effect previously reported; silymarin when administered at higher doses favors lipid peroxidation and pro-inflammatory mediators such as nitric oxide and IL-1β (Johnson et al., 2002; Malekinejad et al., 2012). Moreover, *in vitro* and *in vivo* studies have demonstrated estrogen's protective effects against amyloid β peptide (Aβ)-induced toxicity (Green et al., 1996) and mitochondrial toxins such as MPTP (De Girolamo et al., 2001). Silymarin binds to cytosolic estrogen receptor β (ERβ) (Baluchnejadmojarad et al., 2010) and exerts neuroprotective effects through the interaction with ERβ; also, the blocking of this receptor with fulvestrant reverted silymarin's protective effect in a 6-OHDA model (Baluchnejadmojarad et al., 2010). Estrogens respond in a non-monotonic way due to receptor saturation in the MCF-7 breast cancer cell lines (Welshons et al., 2003) and in a MPTP model (Ramirez et al., 2003). It is possible that the non-monotonic response observed with silymarin could be related to the ERβ saturation, mimicking the estrogenic response.

Silymarin protective effect may not only be due to its antioxidant characteristics reported in the CNS but also to an interesting anti-apoptotic property. Recent studies have shown that silymarin and silybin decrease caspase 3 activity in brain damage due to ischemia/reperfusion (Raza et al., 2011). Silymarin also normalizes gene expression of up-regulated Bax, Bcl2, caspase 9, Trp53, Fas, Bak1, NFκB1 and Ripk1, and down-modulates Bax and caspase 9 protein expression in a maneb and paraquat-induced dopaminergic neurodegeneration model (Singhal et al., 2011, 2013). In accordance, our results confirm that silymarin provides a protective effect in MPTP-intoxicated animals by diminishing apoptotic cell death and preserving dopaminergic neurons of substantia nigra, while silymarin given alone has no effect in the control group. The exact mechanism of silymarin protection in this experimental model has not been completely explored and requires further studies for its whole understanding.

Finally, the intraperitoneal route of silymarin administration is limited for the potential clinical use of silymarin to slow down PD progression in humans. Further studies evaluating oral administration are required to set a silymarin dose for future clinical trials.

5. Conclusions

Our findings show the neuroprotective effect of 100 mg/kg silymarin in an MPTP-induced model of PD. Silymarin maintained striatal dopamine levels by diminishing apoptosis in the substantia nigra and preserving dopaminergic neurons. The mechanism of protection provided by this flavonoid in this experimental PD model is possibly through its antioxidant and anti-inflammatory properties, but has yet to be explored.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Invited Review

Central nervous system: A modified immune surveillance circuit?

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ABSTRACT

Immune surveillance in the central nervous system (CNS) was considered impossible because: (i) the brain parenchyma is separated from the blood circulation by the blood–brain barrier (BBB); (ii) the brain lacks lymphatic drainage and (iii) the brain displays low major histocompatibility complex class II (MHCII) expression. In this context, the BBB prevents entry of immune molecules and effector cells to the CNS. The absence of lymphatic vessels avoids CNS antigens from reaching the lymph nodes for lymphocyte presentation and activation. Finally, the low MHCII expression hinders effective antigen presentation and re-activation of T cells for a competent immune response. All these factors limit the effectiveness of the afferent and efferent arms necessary to carry out immune surveillance. Nevertheless, recent evidence supports that CNS is monitored by the immune system through a modified surveillance circuit; this work reviews these findings.

1. Introduction

The immune system protects the organism by constant monitoring by specialized cells. These cells freely circulate between the lymphoid organs and other tissues searching for all kinds of potentially damaging agents of internal or external origin through a process known as immune surveillance (Wekerle, 1993). Immune surveillance occurs in most of the tissues, with few immune privileged exceptions that include the testicles, the anterior chamber of the eye and the central nervous system (CNS; Medawar, 1948; Barker and Billingham, 1977; Wekerle, 1993).

The CNS has structural properties that influence the immune reactivity. Among these features are the presence of the blood–brain barrier (BBB), the absence of lymphatic drainage and the reduced expression of Major Histocompatibility Complex Class II molecules (MHCII). The presence of the BBB interferes with the afferent arm of immune surveillance by preventing immune effector cells and molecules from entering the CNS, which in turn prevents an interaction between T cells and CNS antigens (Wekerle, 1993; Cserr and Knopf, 1990). The absence of lymphatic drainage restricts the efferent arm of the immune surveillance by preventing CNS antigens from reaching nearby lymphatic nodes (LNs), thus restricting the activation of lymphocytes. Finally, the low expression of the MHCII hinders antigen presentation and T cells re-activation. From this perspective, the immune privilege was regarded as a passive non-reactive state associated with the isolation of the CNS from the immune system. Nevertheless, these anatomical and structural elements are much more than passive barriers. For example, the physiological drainage of the cerebrospinal fluid (CSF) into the lymph and the blood circulation provides alternative routes for interstitial liquid antigens draining (Cserr and Knopf, 1990). Previous studies show that the BBB permits the selective access of some T cells (Ben-Nun et al., 1981; Naparstek et al., 1983). Finally, although under normal conditions CNS resident cells have a low or null expression of the MHCII, an inflammatory stimulus is capable of inducing rapidly its expression (Neumann, 2001; Carson et al., 2006).

For all these reasons, the concept of CNS immune privilege should be reassessed and rethought, especially because in the past the entry of immune elements into the CNS has been always associated with damage or disease development (Ransohoff et al., 2003; Bechmann, 2005).

2. CNS antigens draining routes

Adequate immune surveillance requires that both antigens and antigen-presenting cells (APCs) can reach the secondary lymphoid organs; this makes lymphatic drainage essential. In peripheral organs, resident APCs capture local antigens and migrate via afferent lymphatic vessels to the nearby LNs for antigen presentation

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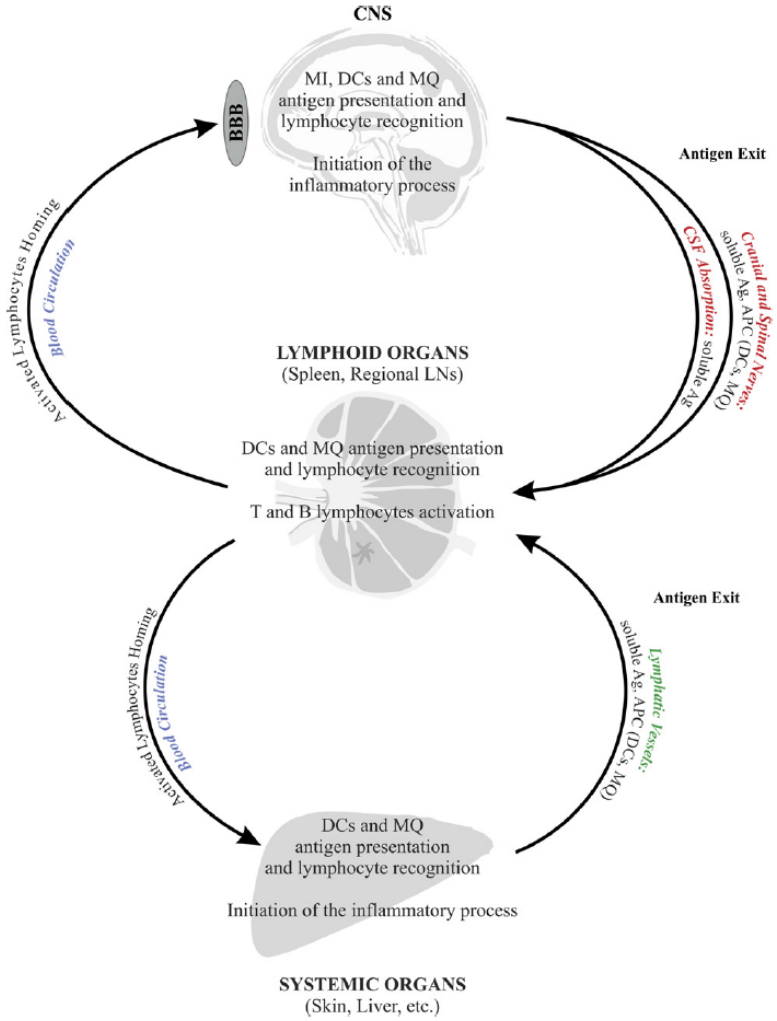

Fig. 1. Differences between systemic and CNS immune surveillance circuits. Antigen draining is normally executed by lymphatic vessels that communicate systemic organs with regional lymph nodes (LNs). Central nervous system (CNS) can drain antigens by alternate routes such as the physiological cerebrospinal fluid (CSF) circulation into the blood and via some cranial and spinal nerves roots into the lymph. Both surveillance circuits share antigen transport by antigen presenting cells (APC) or capture of lymph or CSF solubilized antigen by LNs APCs for further lymphocyte presentation and activation. In order to exert their function lymphocytes need to leave the LNs, home to the different organs and extravasate through a multistep process that involves adhesion molecules in both lymphocyte and endothelial cells. T and B cells must be activated to pass the blood–brain barrier (BBB) in the CNS. Systemic organs and CNS require an additional antigenic presentation to close the immune surveillance circuit. DCs, Dendritic cells; MI, Microglia; MQ, Macrophages.

(Oo et al., 2010; see Fig. 1). The CNS, however, lacks a traditional lymphatic system; consequently CNS antigens draining must occur through alternative routes.

One possible route is the physiological circulation and reabsorption of the CSF through the arachnoid villi towards the venous sinus, allowing CNS soluble antigens to reach the spleen via blood circulation (Harling-Berg et al., 1989; Cserr et al., 1992; Dickstein et al., 1999; also see Fig. 2). Another probable route is the outflow of CSF and interstitial liquid toward the head and neck's lymphatic vessels through the extensions of the subarachnoid space of the olfactory, optic, trigeminal and acoustic nerves (Dickstein et al., 1999). This route favors the arrival of CNS antigens to the deep and superficial cervical LNs (Table 1), thereby potentially promoting a high-level production of antibodies, which can be significantly abolished through surgical obstruction of these cranial nerves (Harling-Berg et al., 1989; Cserr et al., 1992; Gordon et al., 1992).

Local antigens also exit the CNS by APCs such as macrophages or dendritic cells (DCs). These cells uptake and process local antigens and leave the CNS following the same routes as CNS antigens to reach the cervical LNs (Kuhlmann et al., 2001; de Vos et al., 2002; Karman et al., 2004). Although under physiological conditions these cells are absent from the cerebral parenchyma, they are usually present in structures that produce or transport CSF, such as ventricles, meninges and choroid plexuses (Matyszak

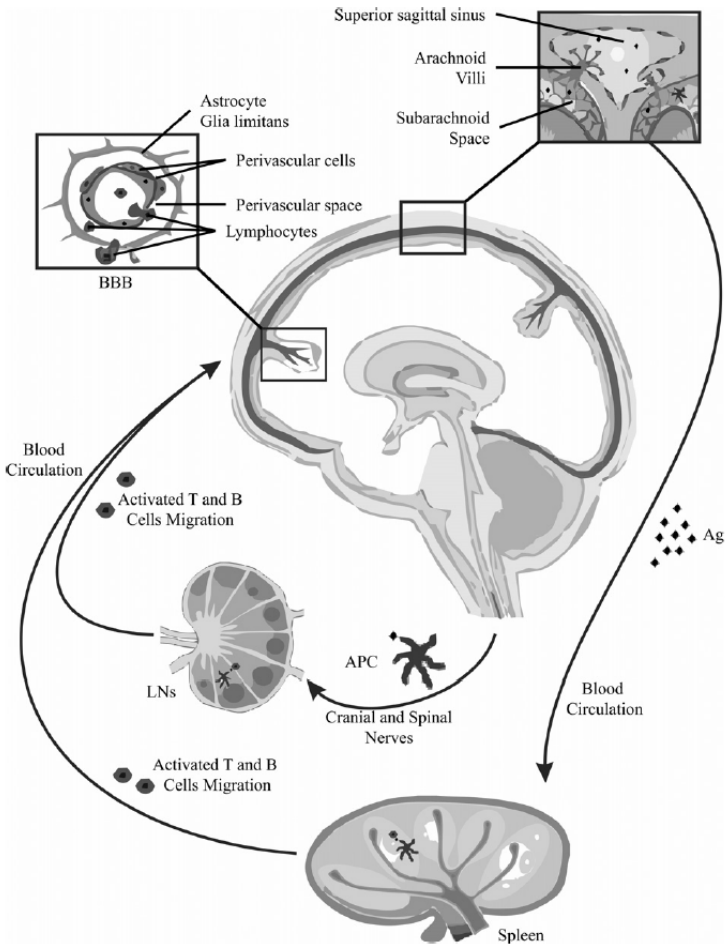

Fig. 2. Modified immune surveillance circuit in the Central Nervous System. Proposed immune surveillance circuit through antigen (Ag) exit, either solubilized in the cerebrospinal fluid or transported by antigen-presenting cells (APC) for its presentation to lymphocytes in the cervical lymph nodes (LNs) and/or the spleen, thus forming the efferent arm of surveillance; and with the subsequent arrival of activated lymphocytes in the central nervous system (CNS) through the blood–brain barrier (BBB), constituting the afferent arm.

and Perry, 1996; McMenamin et al., 2003). In fact during neuroinflammatory conditions, DCs accumulate in the CSF, as well as in perivascular spaces. These findings suggest that the CSF might be a major route for transporting DCs from the CNS to the lymphoid organs (Hatterer et al., 2008). Additionally, it has been reported that DCs injected into the CSF preferentially migrate to B cell follicles within the cervical LNs; suggesting that under neuro-inflammatory conditions, specific mechanisms direct the DCs migration to this location (Hatterer et al., 2006).

Therefore CNS antigens probably access lymphoid tissues rapidly through the CSF or APC transport for subsequent processing and appropriate presentation, in order to stimulate specific antigen responses in immature and memory T cells (Ransohoff et al., 2003).

3. Peripheral stimulation in the lymphatic nodes

Lymphoid organs present a functional specialization for distinct anatomical sites for carrying out immune responses with particular characteristics against local antigens (Wolvers et al., 1999; Kraal et al., 2006). For instance, ileal Peyer's patches provide oral tolerance to many dietary antigens and commensal bacteria. In order to induce tolerance, local DCs capture antigens, migrate to the mucosa-draining LNs and generate antigen-specific suppressive T cells (Fig. 1). The failure to induce tolerance leads to food allergy or celiac disease (Kraal et al., 2006).

In contrast, the CNS does not normally possess any structurally-defined lymphoid tissue. The peripheral immune response to CNS antigens commonly occurs at the cervical LNs and is characterized by a predominant antibody response, a Th2 type response (Harling-Berg et al., 1989; de Vos et al., 2002; Mojtahedi, 2005). One factor that may influence the immature T helper lymphocytes to acquire a Th2 profile in the cervical LNs is the relatively high concentration of antigens in APCs. It could also be induced through the secretion of CNS immune-regulating molecules draining toward the cervical LNs, where these molecules could influence antigen presentation by modulating APC activity (Mojtahedi, 2005). Finally,

Table 1
Reported routes for CNS antigens draining.

Antigen	CNS leaving mechanism	CNS off-site detection	References
MBP	Macrophages Dendritic cells CSF	Cervical lymph nodes Spleen Serum	Massaro et al. (1985), Thompson et al. (1985), Lamers et al. (1998), Liu et al. (2001), de Vos et al. (2002), Ohta et al. (2002) and Fabriek et al. (2005)
PLP	Macrophages Dendritic cells	Cervical lymph nodes	de Vos et al. (2002), Ohta et al. (2002) and Fabriek et al. (2005)
HTLV-I	CSF Lymphocytes CSF cells	Serum Peripheral blood monocytes	Bhagavati et al. (1988), Moritoyo et al. (1999) and Cartier and Ramirez (2005)
HIV	CSF CSF cells	Lymph nodes Spleen Peripheral blood cells Serum	Cashion et al. (1999) and Chiodi et al. (1988, 1992)
VZV	CSF	Serum	Sotelo et al. (2008)
Tænia solium	CSF	Serum	Garcia et al. (2000), Pardini et al. (2001) and Bobes et al. (2006)
Toxoplasma gondhi	CSF	Serum	Requejo et al. (1997), and Chaves-Borges et al. (1999)

MBP, Myelin basic protein; HTLV-I, Human T-lymphotropic virus type I; HIV, Human immunodeficiency virus; VZV, Varicella zoster virus.

DCs preferential migration from CSF to B cell follicles in the cervical LNs could also contribute to an active antibody response (Hatterer et al. 2006). These types of immune responses contribute to less damage than Th1 immune responses, which are usually associated with CNS inflammatory pathologies (Chavarria and Alcocer-Varela, 2004).

4. Lymphocytes migration to CNS

Although it was believed that leukocytes were excluded from the CNS by the BBB making immune surveillance impossible, it is now known that BBB does not prevent CNS leukocyte trafficking. Indeed, T, B and NK lymphocytes as well as cells of the macrophage/monocyte lineage have been detected in the CNS under normal conditions (Hickey, 1999), CNS perivascular macrophages are continually replaced (Hickey and Kimura, 1988) and lymphocytes can gain access to CSF either by traversing BBB to the perivascular space or the choroid plexus (Seabrook et al., 1998; Kivisäkk et al., 2003; Ransohoff et al., 2003).

In order to be capable of passing through the BBB lymphocytes must be activated, independently of their antigenic specificity and MHCII compatibility(Richert et al., 1979; Wekerle et al., 1986; Hickey, 1999). Their retention and participation in CNS inflammation depend on the common patterns of antigen presentation and T cell recognition (Hickey et al., 1991; Knopf et al., 1998; Hickey, 1999). Systemic memory and activated lymphocytes also reach the CNS, thus contributing to the immune surveillance of the CNS (Silva et al., 1999; Kwok et al., 2002; Kivisäkk et al., 2006).

BBB lymphocyte crossing occurs by similar mechanisms described for endothelial transmigration in other tissues; it follows a sequential process of cellular rolling, adhesion and diapedesis; and is mediated and guided by adhesion molecules and chemokines (Drevets and Leenen, 2000). Adhesion molecules are expressed in lymphocytes and endothelial cells (Table 2), and are regulated by immune system and glia cell molecules. Astrocytes and microglia also modulate BBB permeability, increasing or decreasing lymphocyte recruitment (McCarron et al., 1993; Male et al., 1994; Hickey, 1999, 2001). Additionally, adhesion molecules participate in the differential recruitment of several types of lymphocytes and determine lymphocyte final CNS locations, such as parenchyma, meninges or choroid plexus (Baron et al., 1993; Hickey, 1999, 2001).

5. Antigen presentation in the CNS

In physiological conditions, CNS presents low expression of MHCII molecules. Therefore, resident brain cells would be unable to present specific antigens to T lymphocytes. The absence of professional APCs in brain parenchyma could prevent the initiation and propagation of immune responses (Neumann, 2001). Nevertheless, pre-activated T lymphocytes in the immune organs may migrate to the brain parenchyma and release pro-inflammatory cytokines (IFN-γ, TNF-α) inducing MHCII molecules into almost all CNS residing cells (Neumann, 2001; Carson et al., 2006). Also, monocytes/macrophages are recruited and infiltrate the perivascular space as sentinels (Neumann, 2001). Consequently, infiltrated T lymphocytes recognize the antigens presented by these APCs and act as effector cells (Neumann, 2001; Prat et al., 2001).

6. CNS modified immune surveillance: multiple sclerosis as an example

Traditionally immune surveillance involves well-coordinated events between the focus of inflammation and the local lymphoid organs(Fig. 1; Oo et al., 2010).

Despite its structural properties CNS can be monitored by a modified immune surveillance circuit (Fig. 2). Multiple sclerosis clearly shows that immune surveillance is possible in the CNS, though with particular characteristics of its own.

Multiple sclerosis is an autoimmune disease characterized by inflammation, demyelinization and axonal degeneration (Glass et al., 2010). This disease presents characteristic pathological changes such as: an important perivascular infiltration of lymphocytes and plasma cells in the white substance of brain and spinal cord, loss of BBB integrity, astrocyte and microglia activation, and demyelinization (Wekerle, 1993; Glass et al., 2010). Auto-reactive T and B cells are fundamental for disease development. These cells require antigenic presentation of myelin antigens by APCs such as DCs, macrophages and microglia (Fig. 2) in order to activate and differentiate into effector cells (Glass et al., 2010). Several studies have demonstrated that DCs and macrophages can leave the CNS transporting myelin antigens and reach the cervical LNs for antigen presentation to auto-reactive T and B lymphocytes (Fig. 2; Kuhlmann et al., 2001; de Vos et al., 2002; Karman et al., 2004; Fabriek et al., 2005). Also it is probable that CSF and serum soluble myelin antigens could reach cervical LNs and the spleen to be captured there by local APCs and presented to the respective specific lymphocytes (Massaro et al., 1985; Thompson et al., 1985; Lamers et al., 1998). Once activated, the auto-reactive lymphocytes would be able

Table 2
Molecules involved in lymphocyte transmigration to the CNS.

Molecule	Other names	Cell expression	Ligand	Expression	Function	References
Selectin family						
P-selectin	CD62P	CNS endothelial cells	PSGL1	Constitutive Inducible	Rolling Adhesion	Kerfoot and Kubes (2002), Piccio et al. (2002), Kivisäkk et al. (2003), Coisne et al. (2006) and Döring et al. (2007)
E-selectin	CD62E	Activated CNS endothelial cells	PSGL1	Inducible	Rolling Adhesion	Wong et al. (1999), Piccio et al. (2002), Omari and Dorovini-Zis (2003) and Coisne et al. (2006)
PSGL1	CD162	Circulating activated, effector and memory lymphocytes	E-selectin P-selectin	Constitutive Inducible	Rolling	Piccio et al. (2002, 2005) and Kivisäkk et al. (2003)
Integrin family						
LFA-1	CD11a/CD18 α1β2 integrin	Circulating activated, effector and memory lymphocytes	ICAM-1 JAM	Constitutive Inducible	Adhesion Transmigration	Archelos et al. (1993), Piccio et al. (2002) and Yang et al. (2008)
VLA-4	CD49d/CD29 α4β1 integrin	Circulating activated, effector and memory lymphocytes	VCAM-1 Fibronectin	Constitutive Inducible	Adhesion Transmigration	Yednock et al. (1992), Baron et al. (1993), Piccio et al. (2002) and Roffê et al. (2003)
Inmmunoglobulin superfamily						
PECAM-1	CD31	CNS endothelial cells Lymphocyte	PECAM-1	Constitutive	Adhesion Transmigration	Wong et al. (1999), Qing et al. (2001), Graesser et al. (2002), Greenwood et al. (2002) and Coisne et al. (2006)
ICAM-1	CD54	Activated CNS endothelial cells	LFA-1	Constitutive Inducible	Adhesion Transmigration	Archelos et al. (1993), Wong et al. (1999), Piccio et al. (2002, 2005), Greenwood et al. (2002), Kivisäkk et al. (2003), Omari and Dorovini-Zis (2003) and Yang et al. (2008)
VCAM-1	CD106	Activated CNS endothelial cells	VLA-4	Constitutive Inducible	Adhesion Transmigration	Yednock et al. (1992), Baron et al. (1993), Wong et al. (1999), Greenwood et al. (2002), Piccio et al. (2002), Omari and Dorovini-Zis (2003) and Roffê et al. (2003)
JAM-A		Activated CNS endothelial cells	LFA-1	Constitutive Inducible	Adhesion	Del Maschio et al. (1999), Padden et al. (2007), Yeung et al. (2008), Alvarez et al. (2011)
TNF superfamily						
CD40L	CD154	Activated T CD4 lymphocyte	CD40	Constitutive Inducible	Adhesion	Omari and Dorovini-Zis (2003) and Vowinkel et al. (2006)
TNF-R superfamily						
CD40		Endothelial cells	CD40L	Constitutive Inducible	Adhesion	Omari and Dorovini-Zis (2003), Vowinkel et al. (2006) and Ramirez et al. (2010)

ICAM-1, Intercellular adhesion molecule 1; JAM-A, Junctional adhesion molecule A; LFA- 1, Lymphocyte function associated molecule 1; PECAM-1, Platelet-endothelial cell adhesion molecule 1; PSGL-1, P-selectin glycoprotein ligand 1; VCAM-1, Vascular cell adhesion molecule 1; VLA-4, Very late antigen 4.

to home to the CNS and enter the perivascular space of the BBB (Fig. 2; Seabrook et al., 1998; Silva et al., 1999). Still these lymphocytes require an additional specific antigen signal to enable them to migrate from the perivascular space to the cerebral parenchyma in order to begin an inflammatory process in the CNS (Archambault et al., 2005); therefore completing the CNS immune surveillance circuit (Fig. 2).

7. Conclusions

Active immune surveillance in the CNS is integrated by a modified immune circuit resulting in protection from possible brain damage (Baron et al., 1993). This implies a dynamic communication between the CNS and the secondary lymphoid organs. Based on the CNS physiology, three characteristics emerge that are immunologically relevant to the circulation of CNS antigens and their drainage. First, the movement of interstitial fluid and CSF through the subarachnoid space allows antigen access to immune cell sentinels and to lymphatic vessels via cranial and spinal nerves. Second, the drainage of interstitial fluids and CSF makes it possible for antigens to reach at a relatively high concentration a large number of cervical LN APCs. Subsequently, activated lymphocytes migrate to the CNS through the BBB. Third, CNS antigens quickly reach the subarachnoid space to be drained; however, some residual antigens are retained at various sites within the cerebral tissue. This retention presents an opportunity for small but immunologically significant quantities of antigens to interact with recirculating antigen-specific lymphocytes that have been previously activated in the cervical LNs (Harling-Berg et al., 1989).

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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Phytodrugs: a potential alternative against neurodegenerative diseases

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Abstract

The World Health Organization estimates that about 65% of the population uses phytotherapy as the first resource in the treatment of various diseases, and its use has always been deep-rooted in tradition. A large majority of the medicinal plant compounds such as polyphenols, alkaloids, terpenes, among others, have therapeutic properties. Polyphenols are the most common active compounds in herbs and vegetables consumed by man. Neurodegenerative diseases (ND) primarily affect the neurons in the human brain due to oxidative stress and neuroinflammation. The major ND include Alzheimer's and Parkinson's disease. Actually, ND are more common and have a disproportionate impact on countries with longer life expectancies, and represent the fourth highest source of overall disease burden in the high-income countries. Polyphenols biological bioactivity against neurodegeneration is mainly due to its anti-oxidant, anti-inflammatory, as well as anti-amyloidogenic effects. Multiple scientific studies support the use of herbal medicine in the treatment of ND, however, there is still pending to explore other relevant aspects such as phytodrug metabolic analysis, pharmacokinetics, bioavailability in the brain, as well as the metabolic changes they may be subject of on the organism and consequently in the central nervous system. Plant extracts despite of being far its use as a treatment for patients still are important therapeutic potential candidates for ND treatment.

Keywords: Alzheimer's disease; anti-oxidant propreties; neurodegenerative disease; Parkinson's disease; phytotherapy; polyphenols.

Graphical Abstract (for review)
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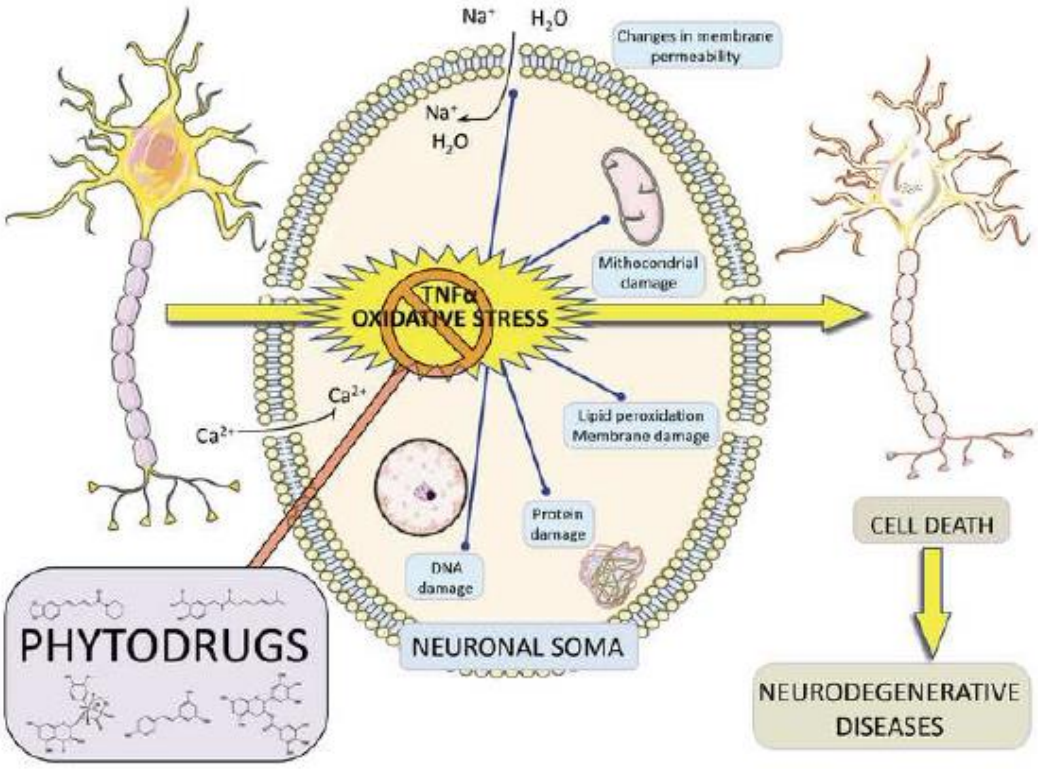

1. Introduction

Neurodegenerative diseases (ND) primarily affect the neurons in the human brain, and are characterized by deterioration of neurons or the myelin sheath, disruption of sensory information transmission, movement control, and more. The main ND include Alzheimer's (AD) and Parkinson's disease (PD), multiple sclerosis (MS), among others. The greatest risk factor for ND is aging, which carries mitochondrial DNA mutations, chronic immune-inflammatory as well as oxidative stress (Talarowska et al., 2014), the major causes of neuronal damage and death. Nowadays, ND represent an enormous disease burden, in terms of human suffering and economic cost. Many ND are chronic and incurable conditions whose disabling effects may continue for years or even decades. These are more common and have a disproportionate impact on countries with longer life expectancies, and represent the fourth highest source of overall disease burden in the high-income countries. According to the World Health Organization (WHO), an estimated 37 million people currently have dementia worldwide, and about 50% of them are being affected by AD and this number is expected to grow up to 115.4 million people by 2050 (Ballard et al., 2011).

Recently, a great number of natural medicinal plants have been tested for their therapeutic properties, showing that the raw extracts or isolated pure compounds from them have more effective properties than the whole plant as an alternative on the treatment of ND. These properties are due mainly to the presence of polyphenols, alkaloids, and terpenes that are micronutrients produced as secondary metabolites by plants (Scalbert, 1993; Joseph et al., 2007; Ramos, 2007). A substantial body of evidence (epidemiological studies, animal studies and human clinical trials) indicates that

polyphenols reduce a range of pathologies associated with cardiovascular disease including thrombosis (Navarro-Núñez et al., 2008), atherosclerosis (Chiva-Blanch et al., 2012) and inflammation (Rieder et al., 2012), as well as displaying anti-cancer (Gali et al., 1991) and neuroprotective (Gatson et al., 2013) properties. The activities of these compounds are achieved via a range of mechanisms including their well-characterized anti-oxidant effects (Rice-Evans et al., 1996; Pignatelli et al., 2006), inhibition of intracellular kinase activity (Wright et al., 2010), binding to cell surface receptors (Jacobson et al., 2002) and disrupting the integrity of cell plasma membranes (Pawlikowska-Pawlega et al., 2007). In addition, recently the polyphenols neuroprotective effects have been described in several models of ND, which involved mainly signaling pathways mediators (Zhong et al., 2009), modulation and related enzymes neurotransmission (Kim, et al., 2009), inhibition of neurotoxicity via ionotropic glutamate receptors (Chuang et al., 2013), anti-amyloidogenic (Ono et al., 2003) and anti-inflammatory effects (Sergent et al., 2010). Currently several scientific studies support the use of herbal medicine in the treatment of ND. However, there is still pending to explore other relevant aspects such as phytodrug metabolic analysis, pharmacokinetics, bioavailability in the brain, as well as any effect they may have on the body and therefore, in the central nervous system (CNS). Therefore, its use for the treatment of ND is still far away, although there are some works for which reports the use of plant extracts are potential candidates for the therapeutic treatment of the ND. This review focusses on the herbal medicines that may hold potential in the treatment of the ND.

2. Etiology of neurodegenerative diseases

ND are incurable and debilitating conditions secondary to neuronal progressive loss, which leads to chronic brain damage and neurodegeneration. The etiology of ND is still unknown, although several animal models of ND have shown mechanisms that are directly involved in the pathogenesis such as damage to the blood brain barrier (BBB), protein aggregation, toxin exposure, mitochondrial dysfunction, which lead to oxidative stress and inflammation, and consequently neuronal death (Cannon and Greenamyre, 2011).

2.1. Vascular contribution to neurodegenerative diseases

The BBB controls the internal environment of the vertebrate CNS and represents the border between the capillary and the extracellular fluid of CNS neurons and glial cells, it also ensures specific brain homeostasis allowing adequate neuronal function (Haseloff et al., 2005). Neurovascular changes normally occur as part of aging, but these are more evident in chronic ND (Zlokovic, 2008). About 20% of blood flow decreases in aged brain, an event associated with reduced protein synthesis (Hossmann, 1994). Interestingly, this blood flow reduction is higher in the presence of any ND, which may lead to changes in intracellular pH, and accumulation of interstitial lactate and glutamate (Drake and Iadecola, 2007; Zlokovic, 2008). These changes can be observed in specific brain regions in diseases such as AD, PD, MS among other CNS disorders (Lo et al. 2003; Drake and Iadecola, 2007; Lok et al., 2007; Hu et al., 2010, Melzer et al., 2011).

2.2. Protein Aggregates in neurodegenerative diseases

Abnormal protein aggregation of specific regions and neuronal populations is a common feature among in ND. For example, in PD the major pathological marker is the presence of cytosolic inclusions called Lewy bodies of dopaminergic neurons in the substantia nigra. The main components of these inclusions are α-sinuclein in Lewy bodies (Spillantini et al., 1997); the beta-peptide amyloid aggregation (Aβ) and the neurofibrils formation composed by Tau protein in AD (Burack et al., 2010; Iqbal and Grundke-Iqbal, 2010); hyperphosphorylated Tau aggregation in demyelization areas in MS (Anderson et al., 2008); and finally, superoxide dismutase 1 (SOD1) aggregations in amyotrophic lateral sclerosis (ALS) (Shaw and Valentine, 2007). The relevance of protein aggregates is that they lead to mitochondrial function loss inducing apoptotic neuronal death.

2.3. Oxidative Stress

The neuroinflammatory diseases are characterized by redox state imbalance and chronic inflammation, a major cause of cell damage and death (Uttara et al., 2009). Reactive oxygen species (ROS) are widely recognized as key mediators of cell survival, proliferation, differentiation, and apoptosis (Dröge, 2002; Bernhardi and Eugenín, 2012). Excessive production of ROS by mitochondria and NADPH oxidase (Nox) in oxidative stress is usually thought to be responsible for tissue damage associated with inflammation and ND (Bernhardi and Eugenín, 2012; Halliwell, 2006; Uttara et al., 2009; Melo et al., 2011). Moreover, many of the well-known inflammatory target proteins, including matrix metalloproteinase-9 (MMP-9), cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and adhesion molecules, are associated with oxidative stress (induced by proinflammatory factors such as cytokines, peptides,

infections, and peroxidants (Lee and Yang, 2012; Bernhardi and Eugenín, 2012; Chiurchiù and MacCarrone, 2011). Several studies have shown that ROS act as a critical signaling molecule to trigger inflammatory responses in CNS through the activation of the redox-sensitive transcription factors, including nuclear factor-κβ (NF-κβ) and activator protein-1 (Bernhardi and Eugenín, 2012; Halliwell, 2006; Uttara et al., 2009; Melo et al., 2011; Chiurchiù and MacCarrone, 2011).

2.4. Mitochondrial Damage

Mitochondrial damage is necessarily an oxidative neuronal damage factor that is involved in ND pathogenesis. This is mediated by ROS and reactive nitrogen species (RNS), which are normal byproducts of mitochondrial respiratory chain activity. ROS concentration is mediated by mitochondrial anti-oxidants such as manganese superoxide dismutase (SOD2) and glutathion peroxidase. Over production of ROS in oxidative stress, mainly induced by aging, as well as environmental toxic agents, is a central feature of all ND. In addition to the generation of ROS, mitochondria are also involved with life-sustaining functions including calcium homeostasis, mitochondrial fission and fusion, lipid concentration of the mitochondrial membranes, and the mitochondrial permeability transition. Mitochondrial disease leading to neurodegeneration is likely, at least on some level, to involve all of these functions (DiMauro and Schon, 2008). There is strong evidence that mitochondrial dysfunction and oxidative stress play a causal role in ND pathogenesis, including in four of the well-known diseases AD, PD, Huntington's, and ALS (Lin and Beal 2006).

Alongside, the proteins aggregation plays an import role in the mitochondrial dysfunction. Mitochondrial Aβ accumulation can be seen both in patients and in transgenic models of AD (Caspersen et al., 2005; Lustbader et al., 2004: Manczak et al., 2006). Inhibition of mitochondrial complex I was observed for decades in PD patients (Parker et al., 1989) and currently two principal models for the study of the disease are induced by rotenone, a isoflavonoid, and *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), both act by inhibiting mitochondrial complex I (Cannon and Greenamyre, 2010). In ALS SOD1 enzyme aggregations found in mitochondria cause loss of mitochondrial function and induce cellular death by apoptosis (Shi et al., 2010). This phenomenon is present in almost all ND and is associated with inflammation, which is one of the points of therapeutic interest and study, which are reviewed below.

2.5. Neuroinflammation

The CNS inflammation is dependent on inflammatory mediators produced by neuronal and glial cells, specifically CNS macrophages and microglia (Streit and Kincaid-Colton, 1995). Microglial activation of is crucial as well in the pathogenesis as the course of several ND such as PD (Kim and Joh, 2006), AD (McGeer and McGeer, 2007), prion disease (Eikelenboom et al., 2002), MS (Sanders and DeKeyser, 2007), and dementia associated with infection by human immunodeficiency virus (Boven, 2000). Uncontrolled microglia activation produces neuronal damage due to overproduction of proinflammatory mediators such as tumor necrosis factor α (TNFα), a cytokine that induces inflammation and apoptotic cell death (Locksley et al., 2001), and nitric oxide (NO), whose excess is cytotoxic either by combining with tyrosine that is essential for

catalytic function of enzyme ribonucleoside diphosphate reductase (Moncada and Higg, 1993) or by forming peroxynitrite (Huie and Padmaja, 1993), which leads to the generation of oxidative stress (Boje and Arora, 1992; Banati et al., 1993, Kim and Joh, 2006).

3. Main therapeutic effects of plant extracts

Plant extracts have become interesting candidates as therapeutic agents due to their anti-oxidant and anti-inflammatory properties. *i) Direct uptake of free radicals*. Primarily polyphenols (Figure 1) and alkaloids (Figures 2) function as scavengers because they possess multiple phenolic hydroxyl and nitrogen groups, respectively, that acts as an electron donor to the aromatic ring, which contributes to the electron-rich property of the aromatic system. These systems are excellent nucleophiles that readily losing electrons and easily being oxidized. Therefore, they can catch free radicals and react with ROS, such as superoxide, peroxyl, hydroxy radicals, nitric oxide, nitrogen dioxide, peroxyl nitrite, and singlet oxygen (Negishi et al., 2004; Skrzydlewska et al., 2002; Yokozawa et al., 2002). *ii) Chelation of the divalent cations in Fenton reactions involved*. Many polyphenol compounds chelates iron cations due to its multiple hidrophyl groups and are efficient scavengers because phenolic groups inhibit iron-mediated oxyradical formation like other iron chelators, such as desferrioxamine, 1,10-phenanthroline and pyridoxal isonicotinoyl hydrazone (Mello-Filho et al., 1991; Schulman et al., 1995; Bhattacharya et al., 1997). *iii) Modulation of enzymes associated with oxidative stress*. Many of the molecular alterations that are associated with ND occur in cell-signaling pathways that regulate cell proliferation and differentiation, such as the intracellular-signaling family of

mitogen-activated protein kinases (MAPK). Abnormal or improper activation or silencing of the MAPK pathway or its downstream transcription factors can result in uncontrolled cell growth, leading to malignant transformation. Some plant compounds 'switch on' or 'turn off' the specific signaling molecule(s), depending on the nature of the signaling cascade they target, preventing abnormal cell proliferation and growth (Kong et al., 2001; Owuor and Kong, 2002).

3.1. CNS anti-oxidant properties

Epidemiological studies indicate that flavonoids, a type of polyphenolic compounds found in fruits, vegetables, red wine and green tea reduced the risk of death from congestive heart failure (Hertog et al., 1993 and 1995), cancer (Steinmetz and Potter, 1991) and also can prevent diabetes mellitus and ND (Scalbert et al., 2005). Specifically in CNS, in 2010, Vuong and colleagues showed that the biotransformation of cranberry juice by the bacteria *Serriata vacinii* increased the polyphenols content with anti-oxidant activity, and in neuronal cultures significantly increased the activity of anti-oxidant enzymes such as catalase and SOD1, protecting neurons against cell death induced by H_2O_2, possibly due to the activation of survival pathways dependent of p38 and blocking death pathway associated with MEK1/2 and ERK1/2 (Vuong et al., 2010). A comparative study of two extracts of *Salvia* species, *S. hydrangea* and *S. mactlenta*, showed that are strong anti-oxidants and at high concentrations ($\geq$ 50 µg/mL) can inhibit DNA damage by free radicals. Moreover, these species not only showed no cytotoxic effect in cultured PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla differentiated with neural growth factor, but also protected them from cell death induced

by peroxide (Asadi et al., 2010). This occurred similarly with selaginellin, a compound extracted from the plant *Saussurea pulvinata*, which showed a neuroprotective effect in a model of neurotoxicity *in vitro* induced by glutamate in PC12 cells (Wang et al., 2010). It is proposed that this effect is mediated by trapping ROS and the regulation of gene expression *klotho*, which has been shown to have an anti-apoptotic role in several cell types (Ikushima et al., 2006; Tang et al., 2009).

3.2. CNS anti-inflammatory activities

Ginger is the root of *Zingiber officinale*, and is an important specie in traditional medicine, which extract has pharmacological properties such as anti-inflammatory, (Mascolo et al., 1989; Grzanna et al., 2005, Lantz et al., 2007), anti-emetic (Sharma et al., 1997), anti-tumor (Katiyar et al., 1996; Surh, 2002), anti-oxidant (Masuda et al. 2004; Eguchi et al., 2005), and analgesic (Mascolo et al. 1989; Aktan et al., 2006, Lantz et al., 2007). The hexane fraction of ginger extract and the methanol extract of *Ficus religiosa* sheet significantly inhibited the production of NO, prostaglandin E2, IL-1β, IL6 and TNFα in cell cultures BV2 line, a mouse microglial cell line, after stimulation with lipopolysaccharide (LPS), through the inhibition of MAPK and NF-κβ (Jung et al., 2008 and 2009a).

Also, methanol extracts of flowers and roots of *Iris pseudopumila* inhibited NO production after stimulation with LPS in RAW 264.7 cell line derived from mouse macrophages. The more effective compounds isolated from the methanol extract of the root were irisolidone (5,7-dihydroxy-6,4'-dimethoxyisoflavone) and 7-methyl-

tectorigenin-4'-O-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside] (Conforti et al., 2009).

Similarly, the ethanol extract of *Knema laurina* exerted an anti-inflammatory and neuroportector effect in microglial cell cultures line MV-2 and in hippocampal neurons HT-22 cells by reducing the microglial production of NO and IL-6 release in a dose-dependt manner. Moreover, in organotypic hippocampal cultures, the extract prevented microglia mediated inflammatory processes by inhibiting ERK-1/2 and Iκβ phosphorylation, and therefore subsequent translocation NF-κβ in microglial cells (Häke et al., 2009).

4. Therapeutic opportunities for plant extracts

In ND such as AD and PD, senescence, among other conditions, oxidative stress and inflammation are the main source for molecules responsible in damaging neural tissue and inducing cell death. Due to their anti-oxidant and anti-inflammatory properties most compounds of medicinal plants are interesting as therapeutic candidates for treating these conditions, either as extracts or purified substances from plants.

4.1. Plant compounds to prevent age-related changes in CNS

It is clear that aging is a critical factor for developing ND and many of these age-related ND are produced by the increased oxidative stress (Metodiewa & Koska, 2000) and the microglial promoted pro-inflammatory environment (Nimmerjahn et al., 2005; Davalos et al., 2005; Fetler and Amigorena, 2005). Therefore, studying potential drugs that prevent or retard age-related changes has become crucial. Natural anti-oxidants such as some

derivatives of cocoa have shown to contain higher flavonoids levels (Adamson et al., 1999). For example, acticoa, a polyphenol extract derived from cocoa, administered daily orally at 24 mg/kg dose in Wistar rats of 15 to 27 months of age, improved cognitive performance, increased life expectancy and preserved free dopamine levels in urine (Bisson et al., 2008). Also, vincamine (Figure 2), an alkaloid purified from *Vinca minor*, which has been used clinically as peripheral vasodilator, has anti-oxidant capacity comparable to vitamin E (Figure 3), also it increased cerebral blood flow, glucose and oxygen utilization in neural tissue and promotes the rise of dopamine, serotonin and noradrenaline levels (Miyamoto et al., 1989; Hadjiev, 2003). In addition, vincamine administration for 14 days at 15 mg/kg per day in rats reduced iron levels in the brains of these animals to about 50%, which suggests a beneficial effect in reducing the oxidative stress associated to the iron deposition in ND (Fayed, 2010) and a possible role as a therapeutic helper in cerebrovascular disease. Moreover, paeonol, compound extracted from the *Paeonta suffruticosa* cortex or *Paeonta lactiflora* root, have been ascribed with anti-pyretic, anti-inflammatory and anti-oxidant (Zhang et al., 1999), and platelet anti-aggregant properties (Lin et al., 1999). Paeonol effects were tested in a model of neurotoxicity induced with D-galactose injected subcutaneously in aged mice. Paeonol prevented memory loss in this model, as it increased acetylcholine and reduced glutathione (GSH) levels, and decreased the activity of acetylcholinesterase (AChE) and SOD1 at hippocampus and cortex, positioning it as a potential drug useful in age-related ND (Zhong et al., 2009). *Magnolia officinalis* compounds, magnolol and honokiol, were tested in a senescence-accelerated prone mice (SAMP8) and prevented learning and

memory deterioration, and acetylcholine deficieny by preserving forebrain cholinergic neurons (Matsui et al., 2009; Chuang et al., 2013).

4.2. Plant compounds used for Alzheimer's disease

AD manifests as cognitive and behavioral disorders and is characterized for an immediate loss of memory and other mental abilities secondary to neuronal loss in the limbic and association cortices. Neuronal death results from oxidative stress, neuroinflammation and abnormal protein deposition. There is no cure for AD, which worsens as it progresses, and eventually leads to death (Berchtold et al., 1998). However, several studies have demonstrated that modulation of oxidative stress and inflammatory response improves AD course (Perry et al., 2002).

The cryptotanshinone is an active component of *Salvia militiorrhiza* with anti-inflammatory, anti-oxidant, anti-apoptotic and platelet anti-aggregant properties (Ng et al, 2000; Kim et al., 2002; Zhou et al., 2005; Park et al., 2007). This compound crossed the BBB, decreased cognitive deficits in mice (Kim et al., 2007) and also provided beneficial effects in ischemia and cerebral infarct (Adams et al., 2006). Additionally, cryptotanshinone reduced Aβ aggregation in brain tissue of APP/PS1 transgenic mouse, improved spatial learning and memory in these animals by promoting amyloid precursor protein metabolism via α-secretase pathway (Mei et al., 2009). Other compounds that can prevent Aβ aggregation by inhibition of the metabolic pathway that generates Aβ are berberine, palmatine, jateorrhizine, epiberberine, coptisine, groenlandicine and magnoflorine alkaloids isolated from *Coptis chinensis* rhizome (Jung et al., 2009b). These compunds also exhibit AChE inhibiting properties (Park et al., 1996 Kim, et al., 2004), anti-depressant (Kong et al., 2001) and enhance cognitive improvements (Hsieh et

al., 2000). Also, jateorrhizine and groenlandicine have significant peroxynitrite scavenging activities, while coptisine and groenlandicine present moderate total ROS inhibitory activities (Jung et al., 2009b).

Similarly, methoxsalen, the main component of the aqueous extract of *Poncirus trifoliate*, inhibited AChE activity in a neurotoxicity model induced *in vivo* by trimetilin (TMT) reducing memory loss and learning problems associated with this toxin (Kim, et al., 2009). The use of *Centella asiatica* in dementia improved memory retention in rodents (Nalini et al., 1992; Veerendra Kumar and Gupta, 2002; Gupta et al., 2003) and decreased amyloid deposition and plaque formation in PSAPP mice, which develop spontaneously Aβ plaque formation (Dhanasekaran et al., 2009).

In a murine transgenic AD model, the grape seed polyphenolic extract from *Vitis vinifera* was tested, which attenuated the cognitive impairment observed in these animals aging and decreased Aβ plaques deposition in their brains (Wang et al., 2008). Another compound with similar properties is nobelitin (Figure 1), a flavonoid purified from the plant *Citrus deppressa*, which prevented memory loss in APP695 transgenic mice and rats treated with Aβ by reducing the Aβ plaques amount in the hippocampus (Onozuka et al., 2008; Nakajima et al., 2007), probably by dimishing the inhibition of PKA and CREB phosphorylation signaling cascade (Nagase et al., 2005). This compaound also stimulated long-term potentiation in hippocampal organotypic cultures (Matsuzaki et al., 2008). L-theanine, a compound present in green tea *Camellia sinensis*, treatment with 2 and 4 mg/kg/day for 5 weeks significantly decreased memory loss associated with intraventricular $A\beta_{1-42}$ AD model, as well as neuronal death in the cortex and hippocampus, also inhibited lipid peroxidation and protein damage, and elevated GSH

levels, suggesting its potential use in the AD prevention and treatment (Kim et al., 2009). Another compound with potential use in AD is *Cassia obtusifolia* ethanol extract, wich reduced memory loss induced by scopolamine administration in mice by inhibiting AChE (Kim et al., 2007). Also, *Dioscorea oppositta* chloroform extract, which has been used to treat memory-related diseases such as AD and others ND (Wu, 2003), prevented neuronal death, and significantly increased spatial learning and memory improvement, probably due to its anti-exitotoxic and anti-oxidant effects (Yang et al., 2009).

In the AD model induced by ethylcholine aziridinium mimicing the cholinergic hypofunction shown in AD (Fisher and Hanin, 1986), the effect of piperine (Figure 2), an alkaloid present in *Piper longum*, was tested. Piperine lowered the cognitive deficits and the hippocampal neurodegeneration associated with this AD model (Chonpathompikunlert et al., 2010), these effects could be probable due to its anti-inflammatory (Gupta et al., 2000) and anti-oxidant activities (Selvendiran et al., 2003).

Sanmjuanhwan (Sjh), a multiherbal formula from oriental traditional medicine, is composed by *Morus alba*, *Linne moraceae*, *Lyctum chinensis*, *Solanum* and *Atractylodes japonica*, and is clinically used as an anti-aging agent for ND (Heo, 1999). Sjh showed neuroprotective effects on primary neuronal cultures, which were stressed with $A\beta_{25-35}$, by increasing the expression of anti-apoptotic proteins such as Bcl-2, and inhibiting the release of cytochrome c and caspase-3 activation (Kim et al., 2010). *Baco pamonnieri*, Brahmi, and its active components bacoside A, bacopaside I and II, and bacosaponine C (Hou et al., 2002; Deepak et al., 2005; Phrompittayarat et al., 2007) have anti-inflammatory, anti-microbial and anti-depressant effects (Sairam et al., 2002; Chaudhuri et al., 2004; Channa et al., 2006). Treatment with Brahmi prevented neuronal death by

the inhibition of AChE activity in primary cortical culture pre-treated with Aβ25-35 (Limpeanchob et al., 2008). Furthermore, animals and volunteers treated with Brahmi presented enhanced memory (Stough et al., 2001; Das et al. 2002; Roodenrys et al. 2002; Kishore and Singh, 2005).

Mono- and di-acetyled cyanidin and peonidin, anthocyanins purple from potatoes (PSPA) extracted from *Ipomoea batatas PotrCv*, can easily attract ROS, which has a very high clinical value as anti-oxidant therapy in AD and other ND (Philpott et al., 2004; Steed and Truong, 2008). For example, pretreatment of PC12 cells with PSPA reduced Aβ toxicity preventing lipid peroxidation, caspase-3 activation and Aβ-induced apoptosis, suggesting a possible use in the treatment of ND (Ye et al., 2010).

Finally, the use of ginseng, *Panax ginseng*, was evaluated in AD patients, those who received a daily dose 9 g of Korean red ginseng for 12 weeks showed a significant improvement in the AD assessment scale and in the clinical dementia rating scale compared to control patients (Heo et al., 2008).

4.3. Plant compounds for Parkinson's disease treatment

PD is the second most frequent ND and is primarily a movement disorder characterized by the loss of dopamine-producing neurons in substantia nigra. Activation of neuronal death pathways involve oxidative stress, neuroinflammation and mitochondrial dysfunction (Fiskum et al., 2003; Mouradian, 2002).

Green tea extract and its isolated (–)-epigallochatechin-3-gallate (EGCG) polyphenol, as well ginseng extract, have demonstrated neuroprotective effects since their use diminished dopaminergic neuron loss in substantia nigra and oxidative damage in a MPTP and its toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) PD animal models

(Levites et al., 2001; Van Kampen et al., 2003). Also, *Chrysanthemum morifolium* (CM), which has anti-oxidant activity (Kim and Lee, 2005; Wang et al., 2001), inhibited MPTP-induced cytotoxicity and maintained cell viability of SH-SY5Y cell line derived from neuroblastoma, preventing ROS formation, decreasing Bax/Bcl2 ratio and caspase-3 activation (Kim et al., 2009). The commercial extract of *Anemopaegma mirandum*, a Brazilian tree, and the crude extract of *Valeriana officinalis* increased cell viability of SH-SY5Y cells after rotenone exposure (Valverde et al., 2008; de Oliveria et al., 2009); while the extract of *Rhus verniciflua* decreased ROS production, preserved the mitochondrial integrity and decreased the number of apoptotic cells (Sapkota et al., 2010). An extract from *Tripterygium regelii*, an anti-oxidant plant (Ahn, 1998), reduced cell death induced by oxidative stress through the inhibition of apoptotic cascades, preserved mitochondrial function, promoted tyrosine hydroxylase (TH) expression and brain-derived neurotrophic factor (BDNF) production in H_2O_2 treated SH-SY5Y cells (Choi et al., 2010). Also, in the MPP^+-intoxicated SH-SY5Y cells, the orchid *Gastrodia elata* increased cell viability, decreased cytotoxicity and ROS production, as well prevented caspase-3 activation by diminishing the Bax/Bcl2 ratio (An et al., 2010). Similarly, in the same model the flavonoid luteolin, a compound present in celery, green pepper, pear leaves and chamomile tea, provided neuroprotection against oxidative stress (Kang et al., 2004). Also, luteolin inhibited LPS induced microglial activation, as well as the production of TNFα, nitric oxide and superoxide in a midbrain mixed primary cultures (Chen et al., 2008). Anther compound, pedicularoside A, a glycosylated phenylethanoid isolated from *Buddleja lindleyn*, has anti-inflammatory properties (Lu et al., 2005) and is a good scavenger of superoxide anions and hydroxyl radicals (Wang et

al., 1996; Shi et al., 2000; Lu et al., 2005), protecting against MPP^+-induced death in mixed midbrain primary culture by increasing TH expression and decreasing caspase-3 cleavage (Li et al., 2008).

Panax notoginseng (PNS) has the property to increase the expression of certain molecules such as Nestin and BDNF (Wang et al., 2007), promoting neural plasticity and recovery after cerebral ischemia (Guo et al., 2003). Also, PNS induces the expression of thioredoxin-1, an oxidoreductase with anti-apoptotic and cell growth promoter effects (Bai et al., 2003), reducing MPTP-induced cell death in PC12 cells (Luo et al., 2010). *Pueraria thomsonii*, a plant containing the isoflavones daidzin, daidzein and genistein (Lin et al., 2009), provided protection in PC12 cells stimulated with 6-hydroxydopamine (6-HODA) through the inhibition of the caspase-3 activation (Lin et al., 2010). Moreover, genistein, a soy phytoestrogen (Figure 3), protected neurons from substantia nigra pars compact and attenuated the rotational behavior in a hemiparkinsonian 6-HODA model (Baluchnejadmojarad et al., 2009). The plant extract from *Uncaria rhynchophylla* (URH) has anti-oxidant (Liu and Mori, 1992; Na et al., 2001), anti-inflammatory (Suk et al., 2002), anti-convulsants (Hsieh et al., 1999), anxiolytic (Jung et al., 2006) and neuroprotective effects (Suk et al., 2002; Liu et al., 2006). This extract decreased cell death, ROS production and increased GSH concentrations in cultured PC12 cells; while 6-HODA-induced caspase-3 activation was attenuated preventing cell death and rotational behavior was significantly reduced in the 6-HODA PD model (Shim et al., 2009). The ethyl extract from *Myracrodruon urundeuva allemão*, has anti-inflammatory (Viana et al., 2003) and neuroprotective properties in mesencephalic cultured cells since it preserved cell viability and attenuated oxidative stress after 6-HODA exposure (Nobre-

Júnior et al., 2009). Pelargonidin, an anthocyanidin with neuroportective effects (Abraham et al., 2007; Vauzour et al., 2008), reduced motor deficit, histological damage and prevented lipid peroxidation in the 6-HODA model (Roghani et al., 2010).

Interestingly, the administration of *Mucuna pruriens* prior to the intoxication of mice whit 6-HODA, was more efficient than levodopa in controlling motor symptoms (Hussain and Manyam, 1997) since it restored dopamine and norepinephrine levels in the nigrostriatal track exhibiting a neuroprotective effect (Manyam et al., 2004). The mechanism of action of *Mucuna pruriens* is not fully understood, however, it has been proposed that increases the mitochondrial complex I activity without affecting the monoamine oxidase B activity, probably due to its high content of NADH and Q-10 coenzyme (Figure 3), and its ability to scavenge DPPH radicals, ABTS radicals and ROS (Manyam et al., 2004). Also, *Mucuna pruriens* significantly inhibited the oxidation of lipids and deoxyribose sugar, and exhibited divalent iron chelating activity and without genotoxic/mutagenic effects on the plasmid DNA (Dhanasekaran et al., 2008).

The root extract of *Withania somnifera* promoted axon and dendrite growth (Kuboyama et al., 2002; Tohda et al., 2005), also increased the levels of SOD1, catalase, and GSH preventing deficit motor in MPTP intoxicated animals (Rajasankar et al., 2009). *Psoralea corylifolia* seeds, specifically $\Delta^{3,2}$-hidroxibacuchiol monoterpene (Figure 3), which has been used for years in Chinese medicine for the treatment of cerebral aging and dementia (Hsieh et al., 2000; Choi et al., 2008), protects SK-N-SH cells form MPP^+ intoxication and prevents the dopaminergic neurons loss in MPTP-intoxicated mice by inhibition of the monoamine transporter (Zhao et al., 2008 and 2009). Echinoside, a compound isolated from *Cistanches salsa*, has anti-inflammatory (Speroni et al., 2002),

anti-oxidant properties, and is also a potent free radical scavenger (Facino et al., 1995). The administration of 20 mg/kg of echinoside prior to MPTP intoxication maintained striatal dopamine levels, reduced cell death, significantly increased the TH enzyme expression and reduced the activation of casapse-3 and -8 expression thus preventing neuronal death (Geng et al., 2007) [198].

A herbal mixture, Toki To (TKT), is prepared of *Angelicae radix*, *Pinelliae tuber*, *Cinnamoni cortex*, *Ginseng radix*, *Magnoliae cortex*, *Paeoniae radix*, *Astragali radix*, *Zanthoxyli fructus*, *Zingiberis siccatum rhizoma* and *Glycyrrhizae radix*, and has excellent results against PD (Sakai et al., 2007). TKT orally administered reduced motor symptoms such as bradykinesia, prevented dopaminergic neurons loss in substantia nigra and increased TH and dopamine transporter expression in MPTP-intoxicated mice. Through microarray was determined that TKT *per se* regulates expression of serum and glucocorticoid kinase gene (*sgk*), which is implicated in the PD pathogenesis (Sakai et al., 2007).

Also worth mentioning is *Rosmarinus officinalis*, a plant used as flavoring in mediterranean cuisine that has anti-oxidant properties (Cheung and Tai, 2007), has a high content of polyphenols and terpenes such as carnosol, carnosic acid and rosmarinic acid, which inhibit NO production (Lo et al., 2002) and protect dopaminergic neurons in different degenerative models (Kim et al., 2006; Lee et al., 2008; Park et al. 2008; Posadas et al., 2009), probably due to its anti-oxidant role and anti-apoptotic effect, also retains TH activity (Park et al., 2010).

4.4. Plant compounds for cerebral ischemia management

In cerebral ischemia severe neuronal damage occurs during the reperfusion period due to excitotoxicity, which consists of an overstimulation of *N*-methyl-D-aspartate receptors leading to glutamate production, which in turn triggers oxidative and inflammatory processes (Lo et al., 2003). The intraperitoneal administration of 200 mg/kg of cactus polysaccharides, the active component of *Opuntia dillent*, prior to the middle cerebral artery occlusion showed neuroprotective effects (Huang et al., 2008 a and b), by significantly reducing infarct volume, decreasing neuronal loss in the cerebral cortex and diminishing importantly the nitric oxide synthase (NOS) synthesis, which is usually induced in the experimental period of reperfusion and ischemia (Huang et al., 2009). Also, oral pretreatment with 30 and 50 mg/kg daily of *Simtlacts chinae* rhizome (SCR) methanol extract reduced the histological changes associated with ischemic injury. It is possible that SCR prevented neuronal death by excitotoxicity by decreasing ROS generation, similarly to the observations made *in vitro* in primary cultures of cortical cells treated with 1 mM *N*-methyl-D-aspartate (Ban et al., 2008). In addition, intravenous pretreatment with 1-10 mg/kg of silymarin, a standardized mixture of polyphenolic (Figure 1) compounds extracted from the fruit and seeds of *Silybum marianum* plant with high anti-oxidant activity (Koksal et al., 2009; Sangeetha et al., 2009), reduced infarcted area size in a 16-40% range, as well as neurological deficits associated with ischemic damage. Similarly, the protein expression associated with inflammation such as iNOS, COX-2, myeloperoxidase, the nuclear transcription factor NF-κβ and proinflammatory cytokines like IL-1β and TNFα were significantly inhibited by silymarin, avoiding neurodegeneration associated with ischemia (Hou et al., 2010).

4.5. Plant compounds used to treat spinal muscular atrophy

Spinal muscular atrophy (SMA) is a congenital condition characterized by muscle weakness, decreased muscle tone, weak cry, and difficulty to swallow down due to injury of the motor neurons of the spinal cord. This disease is caused by a genetic defect in the *smn1* gene that codes SMN1, a protein widely expressed in all eukaryotic cells and that is apparently necessary for survival of motor neurons, and when decreased results in neuronal death and subsequent muscle atrophy (Lefebvre et al., 1995). SMA onset can be prevented by *smn2*, one of the two *smn* gene copies, gene expression of SMN1 protein; therefore, *smn2* is considered a therapeutic target for this disease (Sakla and Lorson, 2008). EGCG stimulated *smn2* gene expression and stimulated SMN1 protein synthesis in cultured fibroblasts derived from patients with SMA (Sakla and Lorson, 2008).

5. Conclusions

Neurodegenerative diseases are chronic and progressive conditions, characterized by neuronal loss secondary to oxidative stress and neuroinflammation. Until now ND have no cure and represent high costs for the health system and patients families. Exploring alternative sources for ND therapy has lead to set eyes on herbal medicine since most herbal compounds have anti-oxidant and anti-inflammatory properties. At present, the use of several plants in the treatment of ND is being supported by numerous scientific investigations (the most relevant herbal plants against ND are listed in Table 1). However, information is still missing on relevant aspects such as metabolism, pharmacokinetics, and bioavailability in the brain as well as any changes that they may

have in the CNS. Nevertheless, plant compounds or extracts remain interesting therapeutic candidates for ND management.

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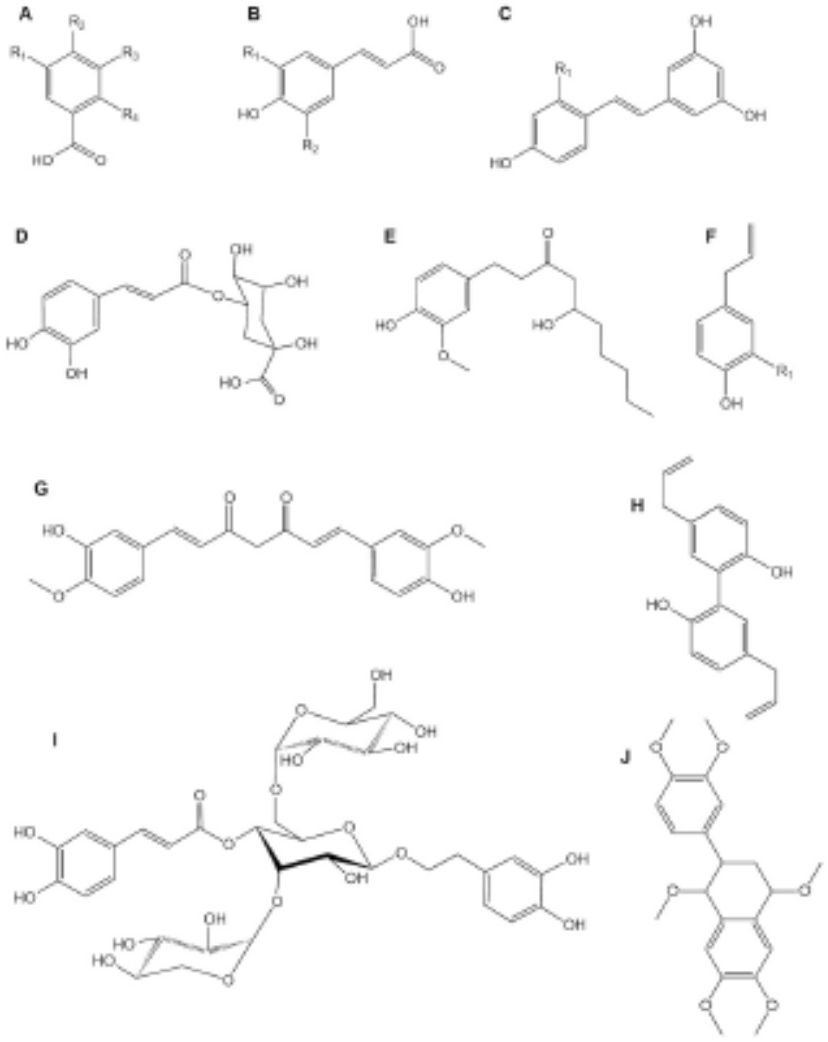
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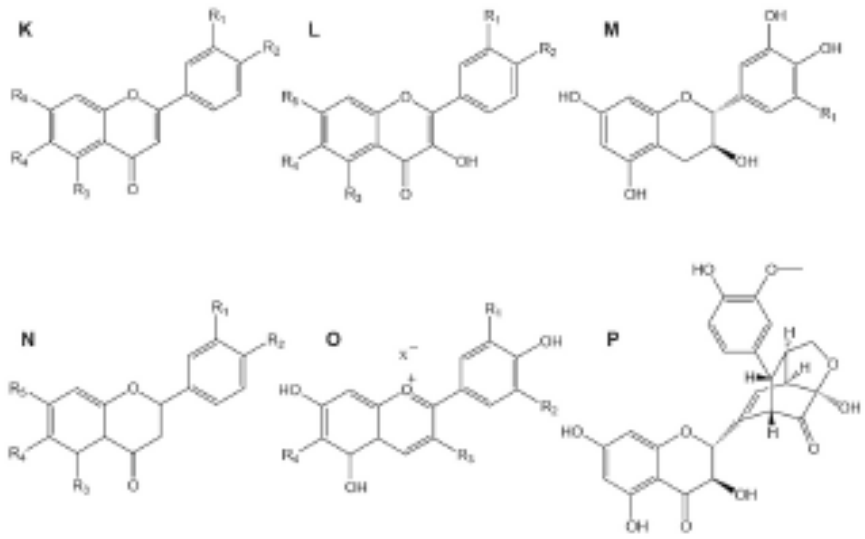

Figure 1. Representative polyphenol compounds. A) Benzoic acids: *p*-hydroxybenzoic acid $R_1 - R_3 - R_4 -$ H, $R_2 -$ OH; protocatechuic acid $R_1 - R_4, -$ H, $R_2 - R_3 -$ OH; gallic acid $R_1 - R_2 - R_3 -$ OH, $R_4 -$ H; salicylic acid $R_1 - R_2 - R_3 -$ H, $R_4 -$ OH. **B**) Hydroxyccinamic acids: coumaric acid $R_1 - R_2 -$ H; caffeic acid $R_1 -$ OH, $R_2 -$ H; ferulic acid $R_1 -$ OMe, $R_2 -$ H; sinapic acid $R_1 - R_2 -$ OMe. **C**) Stilbenes: resveratrol R_1 – H; oxyresveratrol $R_1 -$ OH. **D**) Hydroxyccinamoyl ester: chlorogenic acid. **E**) Hydroxyccinamoyl derivates: gingerol; **F**) Chavicol $R_1 -$ H; eugenol $R_1 -$ OMe; **G**) curcumin; **H**) magnolol; **I**) echinacoside; **J**) nobelitin. **Flavonoid compounds**. **K**) Flavones: apigenin $R_1 - R_4 -$ H, $R_2 - R_3 - R_5 -$ OH; baicalein $R_1 - R_2 -$ H, $R_3 - R_4 - R_5$ – OH; crysin $R_1 - R_2 - R_4 -$ H, $R_3 - R_5 -$ OH; luteolin $R_4 -$ H, $R_1 -$ OMe, $R_2 - R_3 - R_5$ – OH. **L**) Flavonols: kaemferol $R_1 - R_4 -$ H, $R_2 - R_3 - R_5 -$ OH; quercetin $R_4 -$ H, $R_1 - R_2 - R_3 - R_5 -$ OH. **M**) Flavanols (+)-catequin $R_1 -$ H; (+)-gallocatequin $R_1 -$ OH. **N**) Flavanones: hesperetin $R_4 -$ H, $R_1 - R_3 - R_5 -$ OH, $R_2 -$OMe; naringerin $R_1 - R_4 -$ H, R_2 – $R_3 - R_5 -$ OH; pinocembrin $R_1 - R_2 - R_4 -$ H, $R_3 - R_5 -$ OH. **O**) Anthocyanins: aurantinidin $R_1 - R_2 -$ H, $R_3 - R_4 -$ OH; cyanidin $R_2 - R_4 -$ H, $R_1 - R_3 -$ OH; pelargonidin $R_1 - R_3 - R_4 -$ H, $R_2 -$ OH; peonidin $R_2 - R_4 -$ H, $R_1 -$ OMe, $R_3 -$ OH. **P**) Flavanolignans: silydianin.

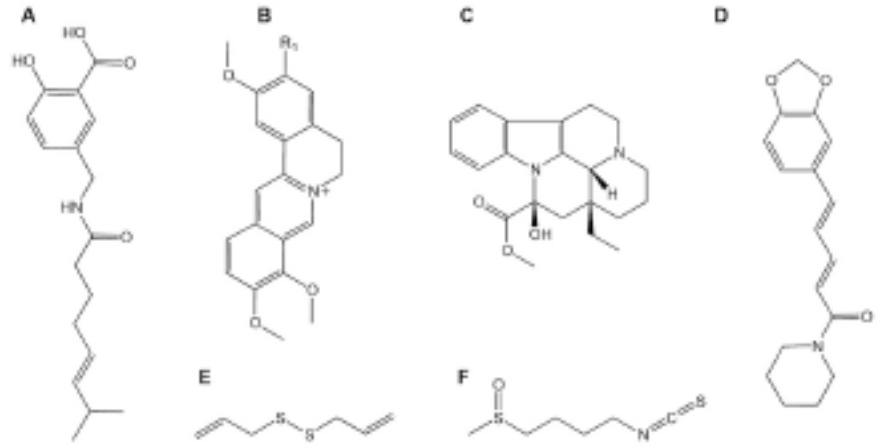

Figure 2. Some alkaloid compounds in plants. A) Capsaicin; **B**) protoberberines: jateorrhizin R_1 – OH, palmitine R_1 – OMe; **C**) vincamin; **D**) piperine; **E**) diallyl sulfide; **F**) sulphoraphane.

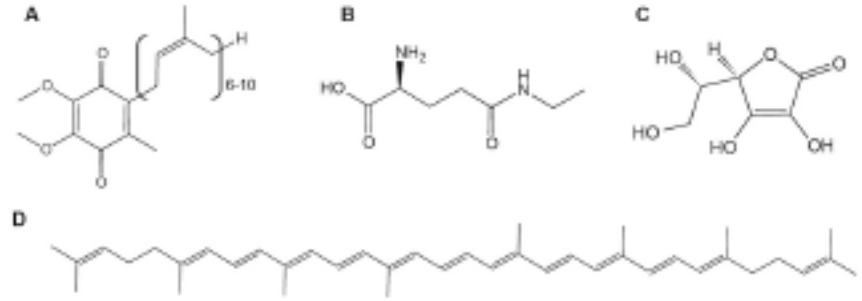

Figure 3. Miscellaneous antioxidant compounds from plants. A) Coenzyme Q_{10}; **B**) l-theanine; **C**) ascorbic acid; **D**) licopene.

Table 1

Table 1. Biological effects of phytodrugs (extracts and/or compounds) in neurodegenerative diseases models, both *in vivo* and *in vitro*.

Compounds	*Main neuroprotective, anti-oxidant anti-inflammatory effects*	*Models*	
Extracts of *Anemopaegma mirandum*.	• ↑ Cell viability • ↓ Cellular morphology changes associated with apoptosis • Preserved mitochondrial membrane	*In vitro*, human neuroblastoma SH-SY5Y cells exposed to rotenone.	Valverde et al., 2009
Δ^3,2-hydroxybakuchiol, isolated from *Psoralea corylifolia*.	• ↓ Dopamine and norepinefrine transporters in striatal and hippocampal synaptosomes • ↑ Cell viability • Preserved mitochondrial activity • ↓ Loss of TH positive neurons.	*In vitro*, transporter transgenic CHO cells and SK-N-SH cells exposed to MPP^+. *In vivo*, male Sprague–Dawley rats and male C57BL/6J mice MPTP intoxicated.	Zhao et al., 2008
Biotransformed blueberry juice by *Serriata vacinii* bacteria.	• ↑ Antioxidant enzyme activities • ↑ p38- and c-Jun *N*-terminal kinase-dependent survival pathways • ↓ MEK1/2- and ERK1/2-mediated cell death.	*In vitro*, neuronal cell culture exposed to H_2O_2.	Vuong et al., 2010
Bacoside A3, bacopaside I and II, bacopasaponin C contained in *Bacopa monnieri* extract (Brahmi).	• ↑ Neuronal survival • ↓ Lipid peroxidation • ↓ Damage by AChE	*In vivo*, primary cortical cultured neurons treated with Aβ.	Limpeanchob et al., 2008
Carnosol, carnosic acid, and rosmarinic acid, phenolic compounds from *Rosmarinus officinalis*.	• ↓NO production • ↓ Cerebral catalase activity • ↓ Lipid peroxidation a • ↓ ROS level • ↓ Bax, Bak, caspase-3 and -9 activation • ↑ Bcl-2 expression	*In vitro*, human dopaminergic cell SH-SY5Y exposed to H_2O_2 or rotenone, dopaminergic neuron SN4741 cells exposed to dieldrin. *In vivo*, aged Wistar rats.	Park et al., 2010
Alcoholic extract of *Cassia obtusifolia*.	• ↓ Memory impairment • ↓ AChE	*In vivo*, male ICR mice exposed to scopolamine or transient cerebral hypoperfusion.	Kim et al., 2007
Extract of *Centella asiatica*.	• ↓ ROS damage of proteins, sugars and nucleic acids • ↓ Aβ plaques formation	*In vivo*, PSAPP mice.	Dhanasekaran et al., 2009

Polyphenolic compounds extracted from Cocoa.	• ↑ Cognitive performance • ↑ Life expectancy • ↑ Dopamine levels	*In vivo*, aged Wistar rats.	Bisson et al., 2008
Alkaloids berberine, palmatine, jateorrhizine, epiberberine, coptisine, groenlandicine and aporphine isolated of *Coptidis Rhizoma*.	• ↓ Aβ-induced neurotoxicity • ↓ AChE, BChE and BACE1 • $ONOO^-$ scavenging effect • ↓ ROS	*In vitro* assays.	Jung et al., 2009b
Chrysanthemum morifolium extract.	• ↓ Cytotoxicity • ↑ Cell viability • ↓ ROS • ↓ Bax/Bcl-2 ratio • ↓ Cleavage of caspase-3 • ↓ PARP proteolysis	*In vitro*, human SH-SY5Y neuroblastoma cells exposed to MPP^+.	Kim et al., 2005
Curcumin, epigallocatechin galate and resveratrol.	• ↑ *smn2* gene expression.	*Ex vivo*, SMA patients type I fibroblasts cells.	Sakla et al., 2008
Cryptotanshinone from *Salvia miltiorrhiza*.	• ↓ APP metabolism and • ↓ Aβ-plaque deposition • ↓ α-secretase protein expression • ↑Activated PI3K pathway.	*In vitro*, cultured cortical neurons overexpressing Swedish mutant human APP695. *In vivo*, APP/PS1 transgenic mice.	Mei et al., 2009
Cyanidin and peonidin, purple sweet potato anthocyanins (PSPA) extracted from *Ipomoea batatas PoirCv*.	• Scavenger effect • ↓ ROS generation • ↓ Aβ-induced lipid peroxidation • ↓ Aβ-induced caspase-3 activtion • ↑ Intracellular calcium level • ↑ Mitochondrial function	*In vitro*, PC12 cells treated with Aβ.	Ye et al., 2010
Daizein and genistein isolated from *Pueraria thomsonii*.	• ↓ caspase-8 and caspase-3 activation	*In vitro*, PC12 cells exposed to 6-OHDA.	Lin et al., 2010
Chloroformic-soluble extract from *Dioscorea opposita*.	• ↓ Neuronal apoptosis • ↑ Spatial learning and memory • ↓ Excitotoxic activity	*In vitro*, primary rat cultured cortical neurons exposed to H_2O_2 or glutamate. *In vivo*, male ICR mice exposed to scopolamine.	Yang et al., 2009

Chloroformic-soluble extract from *Dioscorea opposita*.	• ↓ Neuronal apoptosis • ↑ Spatial learning and memory • ↓ Excitotoxic activity	*In vitro*, primary rat cultured cortical neurons exposed to H_2O_2 or glutamate. *In vivo*, male ICR mice exposed to scopolamine.	Yang et al., 2009
Echinacoside, a phenylethanoid glycoside extracted from *Cistanches salsa*.	• ↓ Caspase-3 and caspase-8 activation • ↑ Motor behavior • ↑ Striatal dopamine	*In vivo*, male C57BL/6 mice MPP^+ intoxicated.	Geng et al., 2007
Methanol extract of *Ficus religiosa* leaf.	• ↓ NO • ↓ TNF-α and IL-6	*In vitro*, LPS-stimulated BV-2 microglia cells.	Jung et al., 2008
Gastrodia elata extract.	• ↓ ROS • ↓ Caspase-3 cleavage • ↓ PARP proteolysis • ↑ Cell viability	*In vitro*, human dopaminergic SH-SY5Y cells exposed to MPP^+.	An et al., 2010
Korean red ginseng.	• ↑ Cognitive and functional performance	Clinical trial in AD patients.	Heo et al., 2008
Hexane extract of ginger	• ↓ NO	*In vitro*, LPS-stimulated BV-2	Jung et al., 2009a
Irisolidone isolated from *Iris pseudopumila*	• ↓ NO	*In vitro*, LPS-stimulated murine monocytic macrophage cell line RAW 264.7.	Conforti et al., 2009
Alcoholic extract of *Knema laurina*.	• ↓ NO • ↓ IL-6	*In vitro*, LPS-stimulated BV-2 microglia cells.	Häke et al., 2009
Luteolin, a polyphenolic compound found in foods of plants.	• ↑ Dopamine uptake • ↑ TH positive neurons • ↓ Activation of microglia • ↓ TNFα • ↓ NO and superoxide	*In vitro*, LPS-treated primary mesencephalic neuron-glia cultures and microglia-enriched cultures.	Chen et al., 2008
Magnolol and honokiol, from *Magnolia officinalis*.	• ↑ Forebrain cholinergic neurons	*In vivo*, SAMP8 mice.	Matsui et al., 2009
Methoxsalen from *Poncirus trifoliate*.	• ↓ AChE	*In vivo*, male ICR mice exposed to trimethyltin.	Kim et al., 2009

7-methyl-tectorigenin-4'-O-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside] isolated from *Iris pseudopumila*.	• Scavenger effect of DPPH radicals, ABTS radicals and ROS • ↓ Oxidation of lipids and deoxyribose sugar • Iron chelating activity	*In vivo*, 6-OHDA nigrostriatal tract lesioned rats. Clinical trial of PD patients.	Conforti et al., 2009
Mucuna pruriens.	• ↑ cAMP/PKA/extracellular signal-regulated kinase/cAMP • ↓ Aβ plaques formation.	*In vivo*, transgenic mice overexpressing human APP695 harboring the double Swedish and London mutations (APP-SL 7-5).	Manyam et al., 2004. Dhanasekaran et al., 2008
Nobiletin, a flavonoid from citrus peels.	• ↓ Neurological deficit score • ↓ Infarct volume • ↓ Neuronal loss in cerebral cortex • ↓iNOS expression • ↑ Cell viability • ↓ ROS • ↓ Bax/Bcl-2 ratio	*In vitro*, PC12 cells exposed to H_2O_2. *In vivo*, male Sprague-Dawley rats with ischemia following middle cerebral artery occlusion.	Onozuka et al., 2008
Cactus polysaccharides from *Opuntia dillenii*.	• ↓ Neuronal death • ↓ ROS • ↑ Intracellular calcium • ↓ Infarct size	*In vitro*, cortical neurons exposed to NMDA. *In vivo*, rats with middle cerebral artery occlusion.	Huang et al., 2009
Oxyresveratrol and resveratrol extracts and isolates from *Smilacis chinae* rhizome.	• ↑ ACh • ↑ GSH and SOD a • ↑ Na^+, K^+-ATPase activities	*In vivo*, d-galactose treated male ICR mice.	Ban et al., 2008
Paeonol from *Paeonia suffruticosa* or *Paeonia lactiflora*.	• ↑ Nestin • ↑ BDNF • ↑ Thioredoxin-1 expression • ↑ Neural plasticity	*In vitro*, PC12 cells exposed to MPP^+. *In vivo*, female Kunming mice exposed to MPP^+.	Zhong et al., 2009
Panaxatriol saponins extracted from *Panax notoginseng*.	• ↓ Caspase-3 gene expression • ↓ Cell death • ↑ TH positive neurons	*In vitro*, rat mesencephalic neuron primary cultures exposed to MPTP.	Luo et al., 2010

Pedicularioside A from *Buddleia lindleyana*.	• ↓ Lipid peroxidation • ↓ Rotational behavior • ↑ Substantia nigra neurons	*In vivo*, male Wistar rats exposed to 6-OHDA.	Li et al., 2008
Pelargonidin, an anti-oxidant anthocyanidin from red fruits and plants.	• ↓ Memory impairment • ↓ Hippocampal neuronal degeneration • ↓ Lipid peroxidation • ↓ AChE	*In vivo*, adult male Wistar rats exposed to ethylcholine aziridinium ion (AF64A).	Roghani et al., 2010
Piperine, an alkaloid from *Piper nigrum*.	• ↑ BDNF and GDNF • ↑ TH positive neurons • ↓ Caspase-9 and -3 cleavage • ↓ Bax expression • ↑ Bcl-2 expression	*In vitro*, human dopaminergic cells, SH-SY5Y exposed to rotenone.	Chonpathompikunlert et al., 2010
Extract of *Rhus verniciflua*.	• ↓ Free radical DNA damage	*In vitro*, PC12 cells exposed to H_2O_2.	Sapkota et al., 2011
Rosmarinus officinalis extract.	• ↓ Aβ plaques formation • ↓ Bax/Bcl-2 ratio • ↓ Caspase-3 activation	*In vitro*, primary cultured rat cortical neurons treated with Aβ.	Park et al., 2010
Methanolic extracts of six species of *Salvia* (*S. hydrangea, S. lachnocalyx, S. macilenta, S. multicaliz, S. sclarea and S. xanthocheila*).	• ↓ ROS • ↑ *klotho* gene expression	*In vitro*, rat PC12 cells exposed to glutamate.	Asadi et al., 2010
Sanjunghwan, multi-herbal extract from *Morus alba Linne, Lycium chinensis,* and *Atractylodes japonica*.	• ↓ Lipid peroxidation • ↓ Protein nitrosylation • ↓ iNOS • ↓ COX-2 • ↓ NF-κβ and STAT-1 • ↓ IL-1β and TNFα	*In vivo*, rats with acute ischemic stroke.	Kim et al., 2010
Selaginellin from *Saussurea pulvinata*.	• ↓ ERK and p38 MAPK • ↓ NF-κβ • ↓ Memory impairment	*In vivo*, male ICR mice treated with Aβ.	Wang et al., 2010

Silymarin extract and its bioactive component silibinin isolated from *Silybum marianum*.	• ↑ Hidroxylase and dopamine transporter expression • ↓ Serum- and glucocorticoid regulated kinase expression • ↓ Bradikinesia • ↑ Dopaminergic neurons	*In vivo*, mice exposed to MPTP.	Hou et al., 2010
L-theanine, from green tea.	• ↑ Tyrosine hydroxylase expression • ↓ Cellular morphology changes associated with apoptosis • ↓ Bax, caspase-3 and -9 activation • ↑ Bcl-2 expression • ↑ BDNF	*In vitro*, human dopaminergic SH-SY5Y cells exposed to H_2O_2	Kim et al., 2009
Toki-to, a Japanese/Chinese herbal remedy from mixed medicinal herbs: *Angelicae Radix, Pinelliae Tuber, Cinnamoni Cortex, Ginseng Radix, Magnoliae Cortex, Paeoniae Radix, Astragali Radix, Zanthoxyli Fructus, Zingiberis Siccatum Rhizoma* and *Glycyrrhizae Radix*.	• ↓ ROS • ↑ GSH • ↓ Caspase-3 activity. Moreover • ↑ Behavioral recovery • ↓ Cell death • ↓ Dopaminergic neuron loss	*In vitro*, PC12 cells exposed to 6-OHDA. *In vivo*, Male Sprague–Dawley rats exposed to 6-OHDA.	Sakai et al., 2007
Methanolic extract of *Tripterygium regelii*.	• ↓ NO • ↓ ROS • ↓ TBARS formation • ↑ TH positive neurons	*In vitro*, rat mesencephalic cells exposed to 6-OHDA.	Choi et al., 2010
Water extract of *Uncaria rhynchophylla*.	• ↑ Cell viability	*In vitro*, human neuroblastoma SH-SY5Y cells exposed to rotenone.	Shim et al., 2009
Urundeuvines A, B and C chalcones isolated from *Myracrodruon urundeuva*.	• ↑ Cerebral blood flow • ↓ Fe concentration reduction of the brain.	*In vivo*, rats.	Nobre-Júnior et al., 2009
Extract *of Valeriana officinalis*.	• ↓ Aβ-amyloid plaques formation • ↓ Cognitive deterioration	*In vivo*, Tg2576 transgenic mice.	de Oliveria et al., 2009

Extract *of Valeriana officinalis*.	• ↓ Aβ-amyloid plaques formation • ↓ Cognitive deterioration	*In vivo*, Tg2576 transgenic mice.	de Oliveria et al., 2009
Vincamine from *Vinca minor*.	• ↓ Superoxide dismutase, catalase and malondialdehyde	*In vivo*, male Swiss albino mice.	Fayed et al., 2010
Polyphenolic extract from *Vitis vinifera*.	• ↑ Cell viability • ↓ Cellular morphology changes associated with apoptosis • Preserved mitochondrial membrane	*In vitro*, human neuroblastoma SH-SY5Y cells exposed to rotenone.	Wang et al., 2008
Aqueous extract of *Withania somnifera*.	• ↓ Dopamine and norepinefrine transporters in striatal and hippocampal synaptosomes • ↑ Cell viability • Preserved mitochondrial activity • ↓ Loss of TH positive neurons.	*In vitro*, transporter transgenic CHO cells and SK-N-SH cells exposed to MPP^+. *In vivo*, male Sprague–Dawley rats and male C57BL/6J mice MPTP	Rajasankar et al., 2009