**UNIVERSIDADE ESTADUAL DE FEIRA DE SANTANA**

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**ROXANA BRAGA DE ANDRADE TELES**

# EFEITO ANTIDEPRESSIVO DO COMPLEXO DE INCLUSÃO CONTENDO O FLAVONOIDE NARINGENINA EM β-CICLODEXTRINA: EVIDÊNCIAS PARA O ENVOLVIMENTO DO SISTEMA MONOAMINÉRGICO

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia, da Universidade Estadual de Feira de Santana como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Orientador: Prof. Dr. Jackson Roberto Guedes da Silva Almeida

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus não sou o que era antes”

Marthin Luther King

## RESUMO

A naringenina é um flavonoide presente em frutas cítricas e possui várias atividades farmacológicas comprovadas como anti-inflamatória, antidiabética, hepatoprotetora, cardioprotetora, neuroprotetora, entre outras. Entretanto, a naringenina possui baixa solubilidade que pode ser minimizada com carreadores como as ciclodextrinas. O objetivo do trabalho foi avaliar a atividade antidepressiva do flavonoide naringenina e do complexo de inclusão naringenina-β-ciclodextrina (NR-βCD) nas doses de 20 e 40 mg/kg v.o. em camundongos. Os animais receberam tratamento agudo e foram avaliados nos testes de nado forçado (NF), suspensão de cauda (SC) e campo aberto (CA). O pré-tratamento com haloperidol (0,2 mg/kg, i.p., antagonista do receptor $D_2$), ioimbina (1 mg/kg, i.p., antagonista do receptor adrenérgico $\alpha_2$) e ondansetron (1 mg/kg, i.p., antagonista do receptor 5-$HT_3$) foram utilizados na investigação do envolvimento do sistema monoaminérgico. Foi observado que a NR e NR-βCD (20 e 40 mg/kg, v.o), produziram uma redução significativa na duração do tempo de imobilidade nos testes de NF e SC. O pré-tratamento com haloperidol e ioimbina no NF demonstrou que a redução no tempo de imobilidade observada no tratamento com NR (20 mg/kg, v.o.) foi revertida, mas não pela ondasentrona. Os resultados demonstraram que o tratamento agudo com flavonoide e seu complexo possuem efeito antidepressivo em ambos os testes sem interferência da atividade locomotora dos animais no teste de CA, com possível mediação dos sistemas dopaminérgico (receptor $D_{2)}$ e noradrenérgico (receptor adrenérgico $\alpha_{2).}$ Adicionalmente, sugere-se que o complexo NR-βCD é um agente promissor e tem valor biotecnológico no tratamento da depressão.



**Palavras-chave:** Sistema Nervoso Central. Depressão. Ciclodextrina. Naringenina.

## ABSTRACT

Naringenin is a flavonoid present in citrus fruits and has several pharmacological activities proven as anti-inflammatory, antidiabetic, hepatoprotective, cardioprotective, neuroprotective, among others. However, naringenin has low solubility that can be minimized with carriers such as cyclodextrins. The objective of this study was to evaluate the anti-depressant activity of flavonoid naringenin and naringenin-β-cyclodextrin (NR-βCD) inclusion complex at doses of 20 and 40 mg/kg p.o. in mice. The animals received acute treatment and were evaluated in forced swimming test (FST), tail suspension test (TST) and open field (OFT) tests. Pretreatment with haloperidol (0.2 mg/kg, i.p., $D_2$ receptor antagonist), yohimbine (1 mg/kg, i.p., $\alpha_2$ adrenergic receptor antagonist) and ondansetron (1 mg/kg, i.p., 5-$HT_3$) were used to investigate the involvement of the monoaminergic system. It was observed that NR and NR-βCD (20 and 40 mg/kg, p.o), produced a significant reduction in the duration of immobility time in FST and TST. Pretreatment with haloperidol and yohimbine in NF demonstrated that the reduction in immobility time observed in NR treatment (20 mg/kg, p.o.) was reversed, but not by the ondansetron. The results demonstrated that the acute treatment with flavonoid and its complex have antidepressant effect in both tests without interference of the locomotor activity of the animals in the OFT, with possible mediation of the dopaminergic ($D_2$ receptor) and noradrenergic ($\alpha_2$ adrenergic receptor) systems. Additionally, it is suggested that the NR-βCD inclusion complex is a promising agent and has biotechnological value in the treatment of depression.



**Keywords:** Central Nervous System. Depression. Cyclodextrin. Naringenin.

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## INTRODUÇÃO GERAL

O crescimento da morbi-mortalidade por doenças não transmissíveis (DNTs) fomenta a transição epidemiológica que ocorre mundialmente. Os transtornos mentais e de comportamento estão em ascensão. Estes representam um desafio complexo, tendo em vista a sua alta prevalência e carga de incapacidade laboral e para as atividades da vida diária, além de serem estimados como preponderantes entre as DNTs (ATUN, 2015; GBD, 2015).

Como um grupo importante nessa esfera, as doenças neurodegenerativas Alzheimer e Parkinson se apresentam como condições neurológicas de etiologia multifatorial que implicam num curso clínico progressivo (KWOK, 2010; CÍCERO et al., 2017). Mutações genéticas são conhecidas nessas patologias, porém a etiologia pode combinar-se entre fatores genéticos, ambientais (metais derivados das poluições industriais e químicas) e estilo de vida. Adicionalmente, a geração de radicais livres, o estresse oxidativo e a disfunção mitocondrial produzem prejuízos celulares que são frequentemente mencionados como fatores importantes na etiologia das doenças neurodegenerativas (AHMED et al., 2013).

Doenças psiquiátricas como depressão, ansiedade, esquizofrenia e transtorno bipolar impõem uma morbimortalidade relevante (WEAVER; PETRONIS, 2014). Dentre os transtornos mentais mais comuns e prevalentes destacam-se a depressão e ansiedade (WHO, 2017). Estas patologias implicam em grande impacto na saúde pública, com estigmas generalizados, práticas desatualizadas e fragmentação organizacional que resultam em respostas lamentavelmente inadequadas dos sistemas de saúde às doenças mentais (WALKER; MCGEE; DRUSS, 2015).

A depressão é um transtorno heterogêneo com sintomas emocionais, neurovegetativos e cognitivos que podem se apresentar de diferentes formas e combinação de sintomas como extrema tristeza, baixa auto-estima, sentimentos de culpa, anedonia que podem se associar o sono ou apetite alterados, fadiga e baixa concentração (NABAVI et al., 2017; WHO, 2017). Estima-se que 4,4% da população mundial, equivalente a 300 milhões de pessoas, sofram de depressão. Outro dado alarmante é o número de suicídios que nesses pacientes chegam a 800 mil pessoas/ano (WHO, 2017).

Os transtornos depressivos podem subdividir-se em transtorno depressivo maior/episódio depressivo ou distimia. O primeiro é determinado com perda de interesse e prazer (anedonia), astenia e a depender dos sintomas pode ser enquadrado como leve, moderado ou grave; o segundo tem sintomas semelhantes, porém menos intensos e com maior duração, é como uma forma mais persistente da depressão leve. Ainda se enquadram nessa

categoria o transtorno bipolar (alternância de humor) e episódios maníacos de humor elevado, resultando em hiperatividade (WHO, 2017).

A compreensão da fisiopatologia da depressão ainda não está totalmente elucidada, apesar da progressão nas pesquisas, nenhum modelo ou mecanismo pode explicar sumariamente todos os aspectos da doença, culminando em uma etiologia multifatoral. Algumas hipóteses incluem a neuroplasticidade e neurogênese, diminuição dos níveis de monoaminas no Sistema Nervoso Central (SNC), alterações cerebrais estruturais e funcionais, interações gene-ambiente e mudanças no eixo hipotalâmico-hipofisário-adrenal (MALHI; MANN, 2018).

A hipótese monaminérgica da depressão é predominante e propõe que o deficit de monoaminas como serotonina (5-HT), dopamina (DA) e norepinefrina (NE) esteja envolvido. A diminuição da função gabaérgica também é sugerida nesta patologia e está associada à redução da neurotransmissão de ácido gama aminobutírico (GABA) em circuitos corticais (PEHRSON; SANCHEZ, 2015).

A teoria colinérgica está associada à hiperativação do sistema colinérgico e, como consequência, diminui a atividade no sistema noradrenérgico. Na hipofunção do sistema dopaminérgico, o estresse crônico reduz a liberação basal de DA que leva a um quadro depressivo (DREVETS, ZARATE JÚNIOR, FUREY, 2013).

Atualmente a hipótese das neurotrofinas tem sido estudada, o fator neurotrófico derivado do cérebro (BNDF), em conjunto com outras neurotrofinas tem efeitos tróficos e sobre a plasticidade neuronal. Algumas linhas de evidências implicaram distúrbios na neurotransmissão glutamatérgica na depressão. Estudos pré-clínicos demonstraram que a exposição a eventos estressantes aumenta drasticamente a liberação de glutamato dentro do SNC, enquanto diminui o número de astrócitos que estão criticamente envolvidos na remoção e reciclagem deste neurotransmissor. O aumento do estímulo glutamatérgico combinado com uma capacidade reduzida para eliminar o neurotransmissor é significativo, uma vez que o glutamato pode diminuir os níveis de BDNF e é conhecido por ser neurotóxico em níveis elevados (JOHN et al., 2012).

Ainda pode ser atribuída à plasticidade prejudicada de circuitos neurais e conexões com envolvimento da neurogênese hipocampal reduzida, de-ramificação dendrítica e perda de espinhas dendríticas e sinapses (SERAFINI, 2012), e à desregulação do eixo pituitário hipotalâmico (SICKMANN et al., 2014), as vias pró-inflamatória são ativadas nessa patologia contribuindo para a fisiopatologia da depressão (RAEDLER, 2011).

A anedonia, um sintoma central da depressão, é atribuída a um circuito de recompensas interrompido, no qual o sistema opióide cerebral desempenha um papel regulador fundamental. O estresse induz a liberação de dinorfina, que ativa os receptores opióides *kappa* e regula o nível da DA (KNOLL, CARLEZON JÚNIOR, 2010). Desta forma, diferentes circuitos podem estar alterados no transtorno depresssivo.

O tratamento busca a remissão dos sintomas e inclui psicoterapia, terapias farmacológicas ou associações dessas modalidades terapêuticas (GARTLEHNER et al., 2017). Na abordagem farmacológica, a maioria dos tratamentos se concentra em drogas que utilizam o sistema monaminérgico. Essas drogas que aumentam a biodisponibilidade de 5-HT, DA e NE são eficientes para reduzir sintomas depressivos. Atuam nos efeitos iniciais dentro da sinapse, que então impactam vias de sinalização intracelular e segundos mensageiros, que culminam em mudanças na expressão gênica, neurogênese e plasticidade sináptica cujas mudanças adaptativas levam ao benefício terapêutico (SHARP, 2012).

Os antidepressivos tricíclicos (ADTs), os inibidores de monoaminooxidade (IMAOs), inibidores de recaptação de noradrenalina (ISRN), os inibidores seletivos da recaptação da serotonina (ISRSs) e inibidores de recaptação de norepinefrina e serotonina (IRSNs) são amplamente utilizados no tratamento da depressão por alterar a sinalização monoaminérgica bloqueando a recaptação de 5-HT, a recaptação de NE ou ambos (VIETA; COLOM, 2011).

Apesar de um enorme progresso ter sido feito para entender a patogênese da depressão, a maioria dos antidepressivos usados na prática clínica não só tem baixa eficácia, mas também podem causar efeitos colaterais como efeitos anticolinérgicos (boca seca, visão, obstipação e retenção urinária), tonturas, sedação, convulsões, disfunção sexual e ganho de peso (BRUNTON et al., 2012). Principalmente, os antidepressivos baseados na hipótese de deficiência monoaminérgica têm um papel fundamental na prática clínica. Além disso, estão associados por retratar resistência ao tratamento em um terço dos pacientes. Portanto, o desenvolvimento de medicamentos mais seguros e eficazes é uma busca constante na comunidade científica (SINGH; SINGH, 2015).

A biodiversidade dos vegetais constitui a principal fonte de biomoléculas para a produção de uma gama de produtos de importância econômica, incluindo modelos para a síntese de um grande número de fármacos. As plantas produzem metabólitos primários e secundários, os metabólitos secundários incluem diversas classes de compostos como alcaloides, cumarinas, ácidos fenólicos, flavonoides, óleos voláteis, quinonas, saponinas, taninos e terpenos, cujos teores e composição podem ser alterados em resposta a variações de diferentes fatores internos e ambientais (CARDOSO et al., 2010). Eles são alvos de pesquisa

de interesse para a busca de moléculas novas e melhor toleradas a partir de fontes vegetais naturais devido a gama de efeitos adversos nos tratamentos farmacológicos das desordens neuropsiquiátricas (ZHANG, 2004).

Mais de 6000 flavonoides possuem efeitos farmacológicos conhecidos (WANG et al., 2017) que estão ligados à sua estrutura (HARBORNE; WILLIAMS, 2000). A extração dos flavonoides canfenol, quercetina, apigenina e crisina das folhas de *Ginkgo biloba*, demonstrou na análise *in vitro* uma inibição pronunciada da monoamino oxidase-A (MAO-A) relacionada ao efeito antidepressivo (SLOLEY et al., 2000). Estudo realizado *in vivo* com os flavonoides extraídos do *Hypericum perforatum*, popularmente conhecida como Erva de São João (isoquercetina, hiperosídeo e quercetina-3-*0*-miquelianina e rutina) demonstraram efeito semelhante à imipramina no teste de nado forçado, demonstrando uma potente ação antidepressiva (NÖLDNER; SCHÖTZ, 2002). Da classe dos antidepressivos tricíclicos, a atividade antidepressiva da imipramina resulta de aumentos nos níveis sinápticos de 5-HT e NE através da inibição da recaptação dessas monoaminas (RIBEIRO; PUPO, 2005).

Bahramsoltani et al.(2015) apontaram em sua revisão que os constituintes fitoquímicos, naringenina, derivados da quercetina, eugenol, piperina, alcaloides diterpênicos, berberina, hiperforina, derivados de riparina, ginsenosídeos, bem como, alcaloides β-carbolínicos, estão entre os mais relevantes como futuros antidepressivos. A naringenina representou seu efeito antidepressivo pela elevação da 5-HT, NE, BDNF e receptores de glicocorticoides.

A naringenina (NR) é um composto amargo e incolor abundante em frutas cítricas, como limão, laranja (AL-DOSARI, 2017). Possui estrutura típica de uma flavanona que contém grupos hidroxila (nas posições 5, 7 e 4'). Substituições podem ocorrer nesses grupos hidroxila por diferentes grupos funcionais resultando em análogos estruturais (KRAUZE-BARANOWSKA et al., 2013). A naringina administrada por via oral é metabolizada em naringenina que atravessa a barreira hematoencefálica (ZBARSKY et al., 2005).

Muitos estudos sobre a naringenina a destacam com grande potencial para diversas atividades biológicas. Exerce atividade antidiabética (ANNADURAI; THOMAS; GERALDINE, 2013), atividade citotóxica (TUNDIS et al., 2011), efeito anti-inflamatório (DOU et al.,2013) e atividade antibacteriana (AGUS; ACHMADI; MUBARIK, 2017).

Além dessas atividades tem-se observado os efeitos neuroprotetores desse flavonoide que incluem a inibição da monoaminooxidase (MAO) (OLSEN et al., 2008), combate a neurotoxicidade induzida por inseticida (carbaryl) (MUTHAIAH et al.,2013), prevenção de déficits cognitivos (BALUCHNEJADMOJARAD; ROGHANI, 2006), antioxidante contra o

estresse oxidativo (SACHDEVA; KUHAD; CHOPRA, 2014) e confere proteção contra doenças neurodegenerativas (ZAIDUN; THENT; LATIFF, 2018). Apesar disso, o uso da naringenina como droga possui limitações devido à sua baixa solubilidade em água e consequente baixa biodisponibilidade (KRISHNAKUMAR et al., 2011), necessitando de alternativas para melhora das propriedades físico-químicas como a utilização das ciclodextrinas (CDs).

Devido à sua versatilidade e vantajosas propriedades, as CDs se tornaram um cobiçado alvo de pesquisas em diferentes áreas de aplicação, com especial interesse no âmbito farmacêutico. A microencapsulação é um processo tecnológico para envolver materiais sólidos, líquidos ou gasosos em cápsulas de milímetros de tamanho que liberam seu conteúdo sob condições controladas (TYAGI et al., 2011). Na classe de polifénois, essa técnica é uma metodologia alternativa para melhorar o armazenamento e estabilidade de bioativos, além de, mascarar o sabor do gosto amargo e adstringência (TYAGI et al., 2011).

Forças físico-químicas, como ligações de hidrogênio, forças de Van der Waals ou interações hidrofóbicas estão envolvidas no processo de encapsulação à nível molecular, consistindo no aprisionamento do composto hóspede (ativo) por um hospedeiro (polímero), ocorrendo a reação em meio aquoso (MARQUES, 2010).

As moléculas carreadoras mais comuns são as CDs compostas por polissacarídeos cone-truncados principalmente composto os de seis a oito *D*-glucosemonômeros unidos por ligações α-1,4-glicosídicas que originam estruturas cíclicas troncocônicas. As mais comuns são as α, β e γ-ciclodextrinas, com seis, sete e oito unidades de glicopiranose, respectivamente. Eles têm uma cavidade central hidrofóbica e uma superfície externa hidrofílica e pode encapsular várias moléculas inorgânicas ou orgânicas para formar complexos de inclusão ou espécies supramoleculares. Essa característica geralmente aumenta a solubilidade do fármaco em soluções aquosas e afeta as características químicas do medicamento encapsulado na indústria farmacêutica atuando como um carreador de drogas (YANG et al., 2017).

Entre as CDs mais comuns a β-CD é a mais utilizada, pois sua cavidade pode hospedar moléculas de massa molecular entre 100 e 400 g/mol, faixa de massa molecular da maioria das moléculas de interesse. Na área farmacêutica, este excipiente tem sido explorado principalmente no incremento da solubilidade, estabilidade e biodisponibilidade de medicamentos. Além disso, pode-se destacar sua utilização para mascarar odores e sabores desagradáveis de certos fármacos, para reduzir ou eliminar irritações oculares ou

gastrintestinais e na prevenção de interações e incompatibilidades (PALEM et al., 2012; AGUIAR et al.,2014).

Na literatura, muitas moléculas com baixa solubilidade têm sido encapsuladas visando à melhora das propriedades. A crisina (CHR) possui muitas atividades farmacológicas entre elas anticancerígenas, entretanto possui baixa solubilidade aquosa e biodisponibilidade. Kulkarni e Belgamwar (2017) obtiveram o complexo CHR-βCD que apresentou um incremento nas propriedades farmacocinéticas como solubilidade e pH. No estudo realizado por Chakraborty e colaboradores (2010) o complexo da CHR-βCD mostrou a interação de forças de Van der Waals e ligações de hidrogênio e aumentou a capacidade antioxidante do composto com o carreador.

Como intuito de mapear o desenvolvimento tecnológico e identificar tecnologias relevantes, inovações e produtos, foi realizada uma prospecção tecnológica sobre o uso da naringenina no tratamento de distúrbios do SNC. A busca utilizou os termos *naringenin, central nervous system, flavonoid*, sozinhos ou combinados, sendo considerados válidos os documentos que apresentassem esses termos no título e/ou resumo nas bases de pedidos de patentes depositados no *European Patent Office* (EPO), na *World Intellectual Property Organization* (WIPO), no *United States Patent and Trademark Office* (USPTO), Banco de Patentes Latinoamericanas (LATIPAT) e no banco de dados do Instituto Nacional de Propriedade Industrial do Brasil (INPI) no período de janeiro de 2005 a janeiro de 2015. Os registros na China e República da Corea estão em maior número com 43 e 42 patentes, respectivamente. Os primeiros registros ocorreram em 2005 (16), atingindo o pico máximo de patentes em 2014 (21), o que representa 26,58% do total de registro dos últimos anos. A classificação internacional preponderante na prospecção tecnológica foi A61K, que trata de preparações para finalidades médicas, odontológicas ou higiênicas. Quanto à associação desse flavonoide com o SNC não foram encontradas patentes. A pesquisa demonstrou que a naringenina possui potencial tecnológico ainda a ser explorado, principalmente em relação à sua atividade sobre o SNC. Além disso, não foram encontrados estudos que retratam o complexo de inclusão com ciclodextrinas e o flavonoide naringenina com relação aos efeitos sobre a atividade neuroprotetota.

Desta forma, o presente estudo promoveu a obtenção do complexo de inclusão do flavonoide naringenina em β-ciclodextrina e avaliou o potencial antidepressivo por meio de modelos animais para depressão com os testes de nado forçado e suspensão de cauda, e atividade locomotora com o teste de campo aberto, amplamente utilizados para entender a

base molecular da fisiopatologia, bem como, na identificação de alvos terapêuticos (WILLNER, 1984).

O nado forçado é um dos modelos mais usados para testar a eficácia antidepressiva e avaliar o comportamento depressivo em ratos e camundongos. É baseado no estresse ambiental (PORSOLT et al., 1977). O tempo de imobilidade durante o teste é medido como comportamento depressivo e é considerado como o tempo gasto pelo rato flutuando na água sem lutar e fazendo apenas os movimentos necessários para manter a cabeça acima da água. Este comportamento pode ser revertido pelo tratamento com antidepressivos (BHATTACHARYA; SATYAN; RAMANATHAN, 1999).

O teste de suspensão de cauda também é utilizado na avaliação pré-clínica de drogas com propriedades antidepressivas. Nesse teste, o animal é colocado em uma situação estressante (suspenso pela cauda) e após tentativas de escape mantém uma postura imóvel. O parâmetro é o tempo de imobilidade que quanto menor, melhor o potencial antidepressivo da substância testada. O teste reflete a resposta do indivíduo a uma situação sem solução aparente (STERU et al., 1985).

O esclarecimento das respostas locomotoras induzidas por drogas é essencial, pois os efeitos sobre a atividade geral são um potencial fator de confusão em muitos testes comportamentais (BURKE et al., 2019). O teste do campo aberto fornece evidências da ação exploratória e efeitos locomotores do animal envolvendo o animal em situação de confronto com o novo ambiente aberto (CHOLERIS et al., 2001). O número de cruzamentos de linha e a frequência de *rearing* (levantar sobre patas traseiras) e *grooming* (auto-limpeza) são geralmente usados como medidas da atividade locomotora, mas também são medidas de exploração e ansiedade. A alta frequência de tais comportamentos indica aumento da locomoção e exploração, e refere-se a um menor nível de ansiedade. O aumento da locomoção pode ser considerado um efeito estimulante do SNC (PRUT; BELZUNG, 2003).

Diante do que foi exposto, o capítulo 1 refere-se ao artigo de revisão que aborda o uso de flavonoides como agentes terapêuticos  nas doenças neurodegenerativas Alzheimer e Parkinson. O capítulo 2 apresenta o efeito antidepressivo do flavonoide naringenina complexado em β-ciclodextrina e o possível envolvimento do sistema monoaminérgico.

# OBJETIVOS

## OBJETIVO GERAL

Avaliar o efeito antidepressivo do flavonoide naringenina e de seu complexo de inclusão com β-ciclodextrina em modelos animais.

## OBJETIVOS ESPECÍFICOS

- ✓ Desenvolver, obter e caracterizar físico-químicamente o complexo de inclusão contendo o flavonoide naringenina em β-ciclodextrina;
- ✓ Investigar o perfil de segurança do flavonoide naringenina por meio do protocolo de toxicidade aguda;
- ✓ Avaliar atividade antidepressiva da naringenina e naringenina-βCD nos testes de suspensão de cauda e nado forçado;
- ✓ Avaliar atividade locomotora da naringenina e naringenina-βCD no teste de campo aberto;
- ✓ Avaliar os possíveis mecanismos de ação envolvidos na atividade antidepressiva.

# CAPÍTULO 1

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# Flavonoids as therapeutic agents in Alzheimer's and Parkinson's disease: a systematic review of preclinical evidences

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**Abstract**

Alzheimer’s and Parkinson’s diseases are considered as the most common neurodegenerative disorders, representing a major focus of neuroscience research to understand the cellular alterations and pathophysiological mechanisms involved. Several natural products including flavonoids considered as able to cross the blood-brain barrier are known for their central nervous system-related activity. Therefore, studies are being conducted with these chemical constituents to analyze their activity in slowing down the progression of neurodegenerative diseases. The present systematic review summarizes the pharmacological effects of flavonoids in animal models for Alzheimer and Parkinson’s diseases. A PRISMA model for systematic review was employed in this search. The research was conducted in the following databases: PubMed, Web of Science, Bireme and Science Direct**.** Based on the inclusion criteria, 31 articles were selected and discussed in this review. The studies listed revealed that the main targets of action for Alzheimer's disease therapy were the reduction of reactive oxygen species and production of amyloid beta protein. Regarding in Parkinson's disease, it was the reduction of the cellular oxidative potential and the activation of mechanisms of neuronal death. Results showed that a variety of flavonoids have been studied and can be promising for the development of new drugs to treat neurodegenerative diseases. Thus, this review is promising in that it suggests more experimental studies should be conducted to consolidate the studies in the clinical area of such promising compounds.

## 1. Introduction

Neurodegenerative diseases are multifactorial conditions in which many biological processes become unregulated [1], mediated by endogenous, genetic and environmental factors [2] and intimately associated with progressive brain damage [3]. For many years, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases have been a major focus of neuroscience research to understand the cellular alterations and pathophysiological mechanisms [4]. The generation of free radicals, oxidative stress and mitochondrial dysfunction producing cellular impairments are often mentioned as important factors in the etiology of neurodegenerative diseases [5, 6].

Alzheimer's disease (AD) is a chronic progression characterized by loss of memory and cognitive deficits such as agnosia, aphasia, apraxia, among others, facts that cause interference in the daily life and in the individual's work. The mechanism of the disease is not fully understood yet. However, in 1991, it was believed that extracellular deposits of beta amyloid (Aβ) were the underlying cause of Alzheimer's disease [7]. Currently, there is a change in the tau protein, which is hyperphosphorylated to associate with other tau chains, forming medley neurofibrillary inside the neurons [8]. The prevalence rate is about 7% for individuals aged 65 or more, with the risk doubling every 5 years [9].

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's. PD is a chronic neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra, which leads to decreased levels of dopamine in the striatum and disrupted motor control [10, 11]. This neuronal loss / death may be caused by the presence of protein inclusions called Lewy body and neurites that are predominantly composed of misfolded and aggregated forms of the presynaptic protein α-Synuclein (α-Syn). Furthermore, morphologically, α-syn aggregates can be grouped into oligomers of different shapes or sizes [12]. Its incidence is usually comprised between 10 and 50/100,000 person-years, and its prevalence between 100 and 300/100,000 population increases progressively after 60 years of age [13].

Recently, studies showed considerable efforts in the search for antioxidant plant-derived polyphenol compounds with neuroprotective potential for the treatment of neurodegenerative diseases [14]. Dietary flavonoids have been suggested to prevent and treat neurodegenerative diseases [15].

Flavonoids are found in numerous plants, fruits and vegetables [16] and are known as the most common phytochemicals that possess a multiple range of pharmacological effects [17]. These secondary metabolites have been described as potent antioxidants, free radical scavengers and metal chelators [18], also presenting anticholinesterase [19], anti-aging [20], neuroprotective [1,20] and anti-inflammatory properties [5,21,22], neurotrophic roles [23], capacity of ameliorating learning and memory [23], potent antidepressant, anti-amyloidogenic effects [17], suppressors of the activation of microglia and, last but not least, mediators of inflammatory processes in the central nervous system (CNS) [24]. Moreover, flavonoids are able to cross the blood-brain barrier with chronic or acute administration suggesting that these compounds can feasibly have a direct effect on the brain, so this chemical compound could be used as a prophylactic in order to slow down the progression of diseases such as AD and PD [10].

Neurodegenerative diseases are considered as a public health problem because they are limiting and debilitating diseases. Due to the lack of clarification on the etiology and neurodegenerative mechanisms of the diseases, the pharmacological treatment is complex and limited. The use of flavonoids can be seen as a factor in the prevention and treatment of neurodegenerative diseases, since they act especially on oxidative stress and mitochondrial dysfunction. Therefore, the objective of this systematic review was to relate the use of flavonoids in conditions of neurodegenerative diseases, especially Parkinson's disease and Alzheimer's disease.

## 2. Materials and Methods

### *2.1 Search Strategy*

The present systematic review was conducted according to the guidelines for Transparent Reporting of Systematic Reviews and Meta-Analyses (PRISMA statement) [25]. In this review, the specialized databases Pubmed, Web of Science, "Biblioteca Regional de Medicina" (BIREME) and Science Direct were used for literature search in September and October 2017, using the terms "flavonoids, bioflavonoids, flavonols, flavonoid, anthocyanin, flavone, flavones, 2-phenyl-benzopyrans, 2-phenyl-chromenes, isoflavonoids or flavonones", combined with "neurodegenerative diseases, Alzheimer´s disease or Parkinson´s disease". We did not contact investigators, nor did we attempt to identify unpublished data.

*2.2 Study Selection*

In this step, two independent researchers (T.C.C.P. and T.C.D.) in charge of the selection followed electronic search titles and selected the abstracts. Full-text articles were independently reviewed by a third researcher who made the analysis of the full text. Disagreements on study inclusion/exclusion were resolved with a consensus meeting with more reviewers. The following inclusion criteria were applied: studies with flavonoids with action on CNS in animal models. Studies were excluded according to the following exclusion criteria: review articles, meta-analyses, abstracts, conference proceedings, editorials/letters, case reports, studies in humans and articles published over 5 years ago. Additional papers were included in our study after analyses of all references from the selected articles.

*2.3 Data Extraction*

Data were extracted by one reviewer using standardized forms and were checked by a second reviewer. Extracted information included the substance, animal models, doses and concentrations and parameters evaluated.

*2.4 Methodological quality assessment*

The risk of bias and quality of preclinical evidences were assessed using a checklist adapted from Hooijmans et al. [26] and Siqueira-Lima et al. [27]. This analysis allowed the evaluation of the methodological quality of the manuscripts selected regarding the outcome measurements, randomization of treatment allocation, blinded drug administration, blinded outcome assessment, appropriate description of the doses, and routes of administration used and appropriate reporting of the data statistical analysis. Studies that reported randomization of animals, blinding, outcome measurements and statistical analysis were considered as a higher methodological quality.

# 3 Results and Discussion

*3.1 Study selection*

The primary search identified 2,456 articles, including 347 from PubMed, 1,338 from BIREME, 595 from Web of Science and 176 from Science Direct. Among these, 1,157 documents had been published more than five years before and were therefore excluded from the review. In addition, 305 manuscripts were indexed in two or more databases and were considered only once, resulting in 994 eligible articles. After an initial screening of titles and abstracts, followed by a full-text analysis, 31 articles were included in this systematic review, while the remainings (n = 728) was excluded since they did not meet the inclusion criteria. A flowchart illustrating the progressive study selection and numbers at each stage is shown in Figure 1.

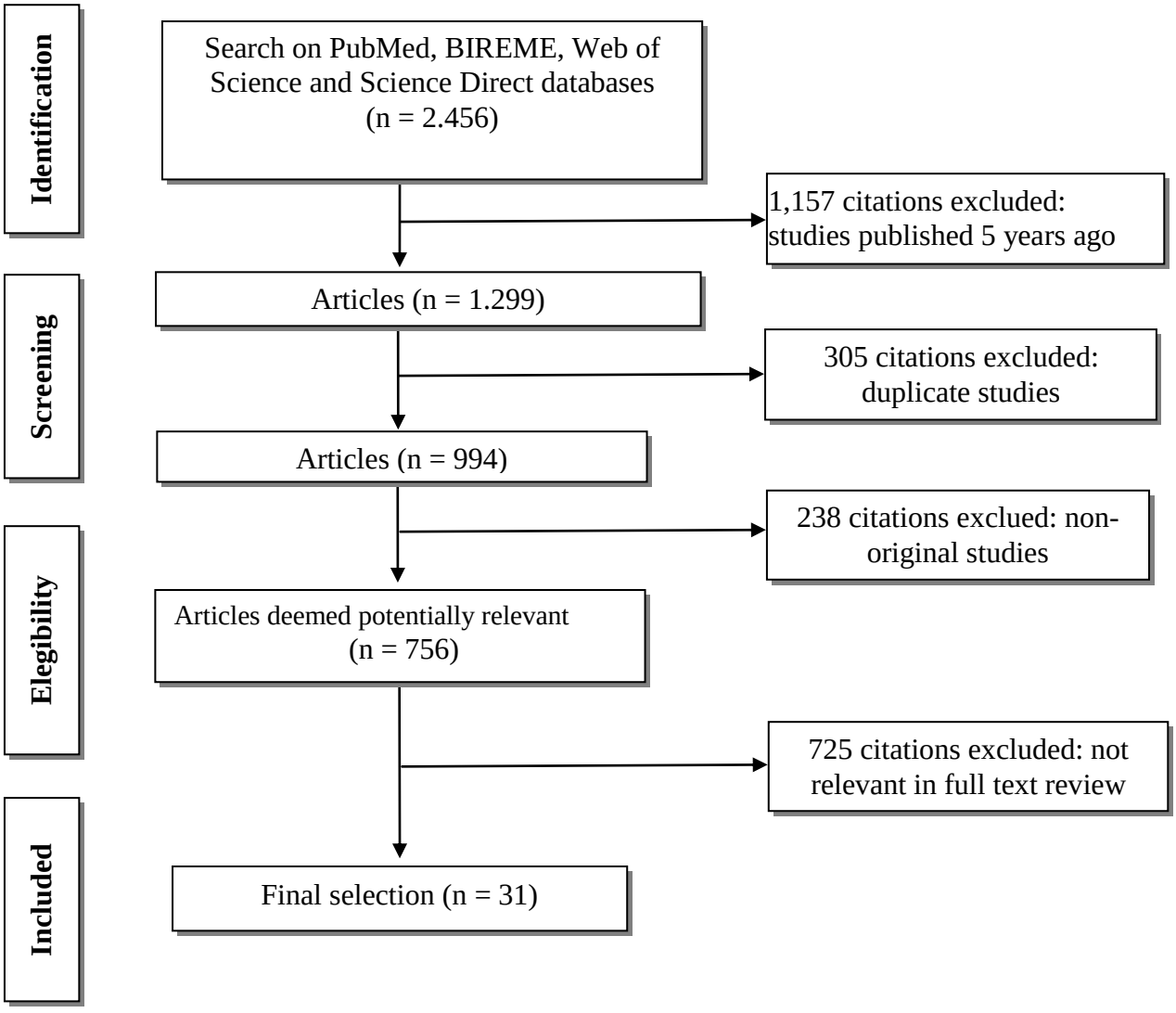


Figure 1: Flowchart for literature searching and screening.

*3.2 Characteristics of included studies*

In general, the studies were conducted by research groups from several countries, mainly China (10 reports, 32.3%), Republic of Korea (07 reports, 22.6%) and India (05 reports, 16.1%). These findings reflect the contribution of Oriental medicine in the search for new drugs from natural products. In fact, Ayurveda and Traditional Chinese Medicine (TCM) are major traditional treatment systems used not only in India and China, but also in several countries [28,29]. These systems have provided important information for the development of new pharmaceutical products based on plant extracts or even small molecules of original chemical structure and with innovative mechanisms of action. Their byproducts have been applied in a number of diseases, including central nervous system disorders such as AD and PD [30,31].

Chinese herbal medicines have been clinically used to treat AD for a long time with significant effectiveness [32]. Recently, Jiang et al. [33] have described the progress of TCM in the treatment of AD, highlighting traditional Chinese medicinal herbs as potential AChE inhibitors for anti-AD approach. Among bioactive molecules, numerous flavonoids have been cited, including quercetin, apigenin, epigallocatechin-3-gallate, catechin, epigallocatechin, epicatechin-3-*O*-gallate, icariin, procyanidin and silibinin. Similarly, TCM has also been reported for the treatment of PD. Several Chinese plant derivatives have been shown to be promising neuroprotective agents in PD, including resveratrol, curcumin and ginsenoside [34, 35]. In this context, such information justifies the significant number of Chinese authorship studies included in this review.

Although most of the manuscripts was focused on AD (20 reports), a relevant number of studies involving experimental protocols in PD was also found (13 reports). Many studies have evaluated important behavioral parameters related to memory, motor functions and cognitive activities of animals. However, biochemical and molecular targets were also verified by colorimetric and enzymatic assays; histological, biochemical and hematological analyzes; western blot, immunohistochemical and immunofluorescence techniques. The parameters evaluated and main outcomes are summarized in Table 1.

TABLE 1. Characteristics of studies inserted in the review.

| Authors, year, country | Substance (s) | Disease | Animals, (strain/sex) n (per group) | Doses, route, administration, period | Preclinical models | Parameters evaluated | |
|---|---|---|---|---|---|---|---|
| | | | | | | Behavior | Biochemical/molecular |
| Wei et al., 2013 [36], China | (2S)-5, 2', 5'-trihydroxy-7-methoxyflavanone – TMF | Alzheimer | Mice (Kunming/ NR) (n=8) | 4 or 8 mg/kg, i.p., 1 week | D-galactose | - Spatial learning and memory (Morris water maze test) | - Colorimetric assay: GSH and GSSG<br>- ELISA: AP-1 and BDNF<br>- Western blot: CREB and p-CREB |
| Mani et al., 2013 [37], India | Naringin | Alzhemier | Rats (W/M), n=6 | 100 mg/kg, p.o., 21 days | Deltamethrin | - NR | - Agarose gel electrophoresis: DNA fragmentation<br>- Biochemical: LDH, CK, AChE, SOD, CAT, GPx, GR, GSH, vitamin C, vitamin E and lipid peroxidation level<br>- Histological analysis<br>- Native gel electrophoresis: SOD and CAT |
| Nakajima et al., 2013 [38], Japan | Nobiletin | Alzheimer | Rats (SAMP8/M) n = 47 | 10 or 50 mg/kg, i.p., unclear | Senescence-accelerated prone mouse (SAMP8) model | -Non-spatial memory (Novel-object test)<br>- Contextual and auditory fear memory (Fear conditioning test)<br>- Emotional reactivity, Anxiety (Elevated-plus maze test) | - Biochemical: MDA, protein carbonyl level, SOD and GPx<br>- HPLC: GSH and GSSG<br>- Real-time RT-PCR: GPx1 and GPx4<br>- Western blot: $A\beta_{1-42}$, tau, p-tau, GPx1, and GPx4 |
| Moghbelinejad et al., 2014 [39], Iran | Rutin | Alzheimer | Rats (W/M) n=30 | 100 mg/kg, i.p., 3 weeks | Amyloid beta ($A\beta_{1-42}$) | - Memory retrieval (Passive avoidance apparatus test) | - Biochemical: MDA and total SH groups<br>- Real-time RT-PCR: BDNF, ERK1, ERK2 and CREB1 |

*(Continued)*

TABLE 1*(Continued)*

| Authors, year, country | Substance (s) | Disease | Animals, (strain/sex) n (per group) | Doses, route, administration, period | Preclinical models | Parameters evaluated | |
|---|---|---|---|---|---|---|---|
| | | | | | | Behavior | Biochemical/molecular |
| Li et al., 2014 [40], Germany | Hesperidin | Alzheimer | Mice (APP/PS1–21/ M), n=12 | 100 mg/kg, p.o., NR | Transgenic APP/PS1–21 mouse model | - Nesting behavior (Nest-building assay)<br>- Social behavior, degree of interaction (Social interaction assay) | - Double-immunostaining<br>- Immunohistochemistry: Aβ, GFAP, TGF-β1, Alzheimer precursor protein A4 |
| Javed et al., 2014 [41], India | Hesperidin | Alzheimer | Mice (S/M), n=10 or n=12 | 100 and 200 mg/kg , i.p, 15 days | Streptozotocin | - Spatial learning and memory (Morris water maze test) | - Biochemical: TBARS, GSH, AChE, ganglioside and phospholipids<br>- Immunohistochemistry: GFAP, NF-κB, iNOS and COX-2 |
| Walker et al., 2015 [42], USA | Epigallocatechin gallate | Alzheimer | Mice (TgCRND8 (Tg) and wild type (nTg)/M and F), n=10, n=11 or n=12 | 50 mg/kg, p.o., 4 months | TgCRND8 amyloid precursor protein transgenic mice | - Acquisition experience (Nest building)<br>- Locomotor activity and exploratory (Open field test)<br>- Novelty-seeking and anxiety-like behaviors (Light-dark box)<br>- Learning (Barnes maze) | - ELISA: $A\beta_{1-42}$ |

*(Continued)*

TABLE 1*(Continued)*

| **Authors, year, country** | **Substance (s)** | **Disease** | **Animals, (strain/sex) n (per group)** | **Doses, route, administration, period** | **Preclinical models** | **Parameters evaluated** | |
|---|---|---|---|---|---|---|---|
| | | | | | | **Behavior** | **Biochemical/molecular** |
| Kou et al., 2016 [43], China | Dihydromyricetin | Alzheimer | Rats (SD/M), n=10 | 100 and 200 mg/kg, p.o., 6 weeks | D-galactose | - Spatial learning and memory (Morris water maze test) | - Histological analysis<br>- Real-time PCR: miR-34a<br>- SA-β-gal staining<br>- Western blot: caspase-3, Bcl-2 SIRT1, p53, p21, Atg7, LC3-II/LC3-I, GFAP and mTOR |
| Alim et al., 2016 [44], Republic of Korea | Anthocyanins and anthocyanin-loaded PEG-AuNPs | Alzheimer | Mice (C57BL/M), n=15 | 12 μg/g/day, p.o., 14 days | Amyloid beta ($A\beta_{1-42}$) | - Spatial learning and memory (Morris water maze and Y-maze tests) | - Immunohistochemical Nissl and FJB Staining<br>- Immunofluorescence<br>- Western blot: Aβ, BACE-1, SNAP23, synaptophysin, p-AMPARs, p-PI3K, p-Akt, p-GSK3β, p-tau, PSD95, caspase-3, Cyt c, Bax, Bcl2, poly (ADP-ribose) polymerase-(PARP-1) |
| Ramalingaya et al.,2016 [45], India | Naringin and Rutin | Alzheimer | Rats (W/F), n= 12 | 50 and 100 mg/kg, p.o., 15 days | Donzepil and scopolamine | - Locomotor activity and time spent in the center zone (Open field test)<br>- Non-spatial memory (Object recognition task) | - Hematological |

*(Continued)*

TABLE 1*(Continued)*

| Authors, year, country | Substance (s) | Disease | Animals, (strain/sex) n (per group) | Doses, route, administration, period | Preclinical models | Parameters evaluated | |
|---|---|---|---|---|---|---|---|
| | | | | | | Behavior | Biochemical/molecular |
| Chen et al., 2016 [46], China | Quercetin | Alzheimer | Mice (C57BL/6J/ M), NR | 30 mg/kg, p.o., NR | Cognitive disorders per high-fat diet (HFD) | - Spatial learning and memory (Morris water maze test) | - Immunohistochemistry: p-PERK, p-IRE1α, NLRP3 and p-tau<br>- Light microscopy: CA1<br>- Western blot: AMPK, p-AMPK, IRE1α, p-IRE1α, eIF-2α, p-IF-2α, TXNIP, NLRP3, GSK-3β, p-GSK-3βser9, tau, p-tau |
| Song et al., 2017 [47], China | Silibinin | Alzheimer | Rats (SD/M), NR | 25, 50 and 100 mg/kg, p.o., 28 days | Amyloid beta ($A\beta_{25-35}$) | - Anxiety and locomotor activity (Elevated plus maze test)<br>- Spatial learning and memory ability (Morris water maze test)<br>- Learning and memory (Novel object-recognition test)<br>- Memory (Memory flexibility test) | - Biochemical: MDA and GSH<br>- ELISA: IL-1β, IL-4<br>- Flow cytometric analysis<br>- Transmission electron microscopy<br>- Western blot: NF-κB, COX-2, iNOS, p53 and p-p53 |
| Moreno et al., 2017 [48], Spain | Quercetin and quercetin-loaded nanoparticles | Alzheimer | Rats (SAMP8/M), n=8 | Quercetin (25 mg/kg, p.o., 2 months); Quercetin-loaded nanoparticles (25 mg/kg every 48 hours, p.o., 2 months) | Senescence-accelerated prone mouse (SAMP8) model | - Locomotor activity (open field test)<br>- Motor coordination and balance (Rotarod test)<br>- Exploratory motivation (marble burying test)<br>- Spatial memory and both the working and reference memory functions (Morris water maze test) | - Western blot: GFAP and CD11b |

*(Continued)*

TABLE 1*(Continued)*

| Authors, year, country | Substance (s) | Disease | Animals, (strain/sex) n (per group) | Doses, route, administration, period | Preclinical models | Parameters evaluated | |
|---|---|---|---|---|---|---|---|
| | | | | | | Behavior | Biochemical/molecular |
| Godoy et al., 2016 [49], Chile | Quercetin | Alzheimer | Rats (B6.129S7-Sod2tm1Leb/ J/ NR), NR | 50 mg/kg, two times a week, p.o., 4 weeks | Amyloid beta ($A\beta_{25-35}$) | - NR | - Eletrophysiology |
| Palle; Neerati, 2016 [50], India | Quercetin and Quercetin nanoparticles | Alzheimer | Rats (W/M), n=6 | 30 mg/kg, i.p., 8 days | Scopolamine | - Conditioning, avoidance responses (Conditioned avoidance test)<br>- Learning, memory (Rectangular maze test) | - Biochemical: MDA, GPx, AChE and CAT<br>- Histological analysis |
| Ahmad et al., 2016 [51], Republic of Korea | Fisetin | Alzheimer | Mice (C57BL/6N/ M), n =12 | 20 mg/kg, i.p., 2 weeks | Amyloid beta $A\beta_{1-42}$ | - Spatial memory and both the working and reference memory functions (Morris water maze test) | - FJB staining<br>- Immunofluorescence: Aβ (B4), synaptophysin, PSD95, p-tau, GFAP and Iba-1<br>- Immunohistochemistry: caspase-3<br>- Western blot: caspase-9, SYN, p-AMPAR1, p-CREB, p-CAMKII, p-PI3K, p-Akt |
| Rehman et al., 2017 [52], Republic of Korea | Anthocyanins | Alzheimer | Rats (SD/M), n =13 | 100 mg/kg, i.p., 7 weeks | D-galactose | - Spatial learning and memory (Morris water maze and Y-maze tests) | - Biochemical: ROS, MDA<br>- Immunofluorescence: Aβ, 8-OxoG, p-JNK, GFAP, Iba-1<br>- Western blot: Aβ, BACE-1, RAGE, 8-OxoG, TNF-α, iNOS, p-JNK, Bax, Bcl2, PARP-1, Syntaxin, Synaptophysin, SNAP-23, p-CREB, GFAP, Iba-1 |

*(Continued)*

TABLE 1*(Continued)*

| Authors, year, country | Substance (s) | Disease | Animals, (strain/sex) n (per group) | Doses, route, administration, period | Preclinical models | Parameters evaluated | |
|---|---|---|---|---|---|---|---|
| | | | | | | Behavior | Biochemical/molecular |
| Kim et al., 2017 [53], Republic of Korea | Anthocyanins alone and anthocyanin-loaded poly (ethylene glycol)-gold nanoparticles (PEGAuNPs) | Alzheimer | Mice (C57BL/6N/M), n=8 | 10 mg/kg, i.v., 14 days | Amyloid β ($A\beta_{1-42}$) | - NR | - ICP-AES<br>- Immunofluorescence: GFAP, Iba-1, RAGE<br>- Nissl staining<br>- TEM<br>- Western blot: Aβ, BACE-1, GSK3β, CDK5, tau, NF-kB, iNOS, p-JNK, Bcl2, Bax, Cyt.c, FJB, COX-2, NOS3, IL-1β, and TNF-α |
| Sharma et al., 2016 [54], India | Quercetin | Alzheimer and Parkinson's | Rats (W/M), n=6 | 10 mg/kg, p.o., 12 weeks | Aluminum | - NR | - Biochemical: ROS, MnSOD<br>- DNA isolation for DNA fragmentation,<br>- Electron microscopy analysis<br>- Histological analysis<br>- Immunohistochemistry: MnSOD, cyt-c and caspase-3<br>- RT-PCR: MnSOD;<br>- Western blot: MnSOD, cyt-c, Bax, Bcl-2, p53 and caspase-3 |
| Jeong et al., 2015 [55], Republic of Korea | Naringin | Epilepsy, Parkinson and Alzheimer | Mice (C57BL/6/M) NR | 80 mg/kg , i.p., 7 days | Kainic acid | - NR | - Immunohistochemical: NeuN and Iba-1<br>- Light microscopy: CA1<br>- Western blot: LC3B and TNF*α* |

*(Continued)*

TABLE 1*(Continued)*

| Authors, year, country | Substance (s) | Disease | Animals, (strain/sex) n (per group) | Doses, route, administration, period | Preclinical models | Parameters evaluated | |
|---|---|---|---|---|---|---|---|
| | | | | | | Behavior | Biochemical/molecular |
| Lee et al., 2014 [56], Republic of Korea | Baicalein | Parkinson | Mice (C57B/6/M), n= 8–14 | 1 and 10 mg/kg (i.p.), 7 days | MPTP | - Motor coordination and balance (Rotarod test) | -DAB Immunostaining: TH<br>- Double-Label Immunostaining: TH, GFAP, Iba-1<br>- Histological analysis<br>- Western Blot: GFAP |
| Antunes et al., 2014 [57], Brazil | Hesperidin | Parkinson | Mice (C57 BL/6/F ), n=10 | 50 mg/kg, p.o., 28 days | 6-OHDA | - Depression (Tail-suspension test)<br>- Spatial learning and memory (Morris water maze)<br>- Locomotor activity and time spent in the center zone (Open field test) | - Biochemical: GSH, ROS, TRAP, SOD, CAT, GR, GPx, GST, DA, DOPAC and HVA |
| Lou et al., 2014 [58], China | Naringenin | Parkinson | Mice (C57BL/6/ F), n =10 | 70 mg/kg, p.o., 4 days | 6-OHDA | - Rotational behavior - numbers of rotations (Apomorphine-induced circling behavior) | - HPLC-MS: DA, DOPAC and HVA<br>- Immunohistochemistry: TH<br>- Western blot: Nrf2, HO-1, GCLM, GCLC, Lamin A, cleaved caspase-3, p-JNK, JNK, p-p38 and p38 |
| Wang et al., 2015 [59],China | Tanshinone I | Parkinson | Mice (C57BL/6/M) , NR | 5, 10 mg/kg, p.o., 7 days | MPTP | - Motor coordination and balance (Rotarod test) | - Biochemical: ALT, AST and ALP<br>- ELISA: TNF-α and IL-10<br>- HPLC: DA, DOPAC, HVA and MPP+<br>- Immunohistochemistry: TH and Iba-1 |

*(Continued)*

TABLE 1*(Continued)*

| Authors, year, country | Substance (s) | Disease | Animals, (strain/sex) n (per group) | Doses, route, administration, period | Preclinical models | Parameters evaluated | |
|---|---|---|---|---|---|---|---|
| | | | | | | Behavior | Biochemical/molecular |
| Chen et al., 2015 [60], China | Silibilin | Parkinson | Rats (M and F), NR | 25 and 50 mg/kg, p.o. in second day | Increased neonatal iron intake | - Locomotor activity and time spent in the center zone (Open field test)<br>- Motor coordination and balance (Rotarod test) | - Biochemical: MDA and GSH<br>- HPLC-ECD: DA and 5-HT |
| Mu et al., 2016 [61], China | Quercetin | Parkinson | Rats (SD/ M), NR | 100, 200 and 400 mg/kg, i.g., NR | 6-OHDA | - Rotational behavior - numbers of rotations (Apomorphine-induced circling behavior) | - HPLC-ECD: DA, DOPAC, HVA, 5-HT and 5-HIAA |
| Lee et al., 2015 [62], Korea | Silibinin | Parkinson | Mice ( C57B/6/M), n=10 or 12 | 1 or 10 mg/kg, i.p., 5 days | MPTP | - Motor coordination and balance (rotarod test) | - DAB Immunostaining: TH<br>- Double-Label Immunostaining: GFAP and Iba-1 |
| Hu et al., 2016 [63], China | Baicalein | Parkinson | Mice (C57BL/6/ M), NR | 100 mg/kg, i.p., 7week to 12week | Rotenone | - Motor coordination and balance (Rotarod test)<br>- Motor dysfunctions (grid test) | - HPLC: DA, DOPAC and HVA<br>- Immunofluorescence: α-syn, TH and ChAT<br>- Real-time PCR: α-syn<br>- TEM<br>- Western blot: α-synuclein and GAPDH |

*(Continued)*

TABLE 1*(Continued)*

| Authors, year, country | Substance (s) | Disease | Animals, (strain/sex) n (per group) | Doses, route, administration, period | Preclinical models | Parameters evaluated | |
|---|---|---|---|---|---|---|---|
| | | | | | | Behavior | Biochemical/molecular |
| Zhang et al., 2017 [64], China | Baicalein | Parkinson | Rats (SD/ M), n=15 or 10 | 100, 200 and 400 mg/kg, p.o., 28 days | Rotenone | - Locomotor activity (Open field test)<br>- Motor coordination and balance (Rotarod test)<br>- The inclined plane assessment | - Immunohistochemistry: TH<br>- TEM<br>- TUNEL staining<br>- Western blot: caspase 3, PGC-1α, NRF-1 and TFAM |
| Goes et al., 2017 [65], Brazil | Chrysin | Parkinson | Mice (C57B/6J/M), n=6 | 10 mg/kg, p.o., 28 days | 6-OHDA | - Motor coordination and balance (Rotarod test)<br>- Rotational behavior - numbers of rotations (Apomorphine-induced circling behavior) | - ELISA: IFN-γ, TNF-α, IL-6, IL-10, NF-kB, S100B, BDNF, GDNF and NGF<br>- HPLC: DA, DOPAC and HVA<br>- TRAP and TAR<br>- Immunohistochemistry: $TH^+$ neurons |
| Ay et al., 2017 [66], USA | Quercetin and quercetin-containing formulation (QB3C) | Parkinson | Mice (MitoPark and C57BL/6/M/F), n=8 or n=9 | Quercetin (25 mg/kg, p.o., 6 weeks) QB3C comprising quercetin (175 mg/kg, p.o., 8 weeks) | MitoPark Transgenic Mouse Models | - Locomotor activity (Open field test)<br>- Motor coordination and balance (Rotarod test) | - HPLC: DA, DOPAC and HVA<br>- DAB Immunostaining: TH |

*(Continued)*

Animals: SD: Sprague–Dawley; W: Wistar; S: Swiss; SAMP8: Senescence Accelerated Mouse-Prone 8; NR: Not reported

Parameters assessed: DPPH: 2,2-diphenyl-1-picrylhydrazyl radical; MDA: malonaldehyde; TBARS: thiobarbituric acid-reactive substances; AAPH: 2,2_-azobis(2-methylpropionamidine) dihydrochloride; FeSO4: ferrous sulphate; 6-OHDA: 6-hydroxydopamine; MPTP: 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine; FJB: Fluoro-Jade B; GSH: reduced glutathione; GSSG: oxidized glutathione; AP-1: activator protein-1;

BDNF: brain derived neurophic factor; CREB: cAMP-response, element-binding protein; p-CREB phosphorylated; SOD: superoxide dismutase; CAT: catalase, GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; LDH: lactate dehydrogenase; CK: creatinine kinase; AChE: Acetylcholinesterase; MDA: malondialdehyde; p-tau: phosphorylated tau; TGF-β1: transforming growth factor beta 1; SYN: Synaphtophysin; p-AMPAR1: phospho-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; p-CRE: phosphorylated-cAMP response element-binding protein; p-CAMKII: phosphorylated calcium/calmodulin-dependent protein kinase II; p-PI3K: phosphorylated phosphatidylinositol-4,5-bisphosphate 3-kinase; p-Akt: phosphorylated protein kinase B; GFAP: Antiglial fibrillary acidic protein; Iba-1: anti-ionized calcium-binding adapter molecule 1; 8-OxoG: 8-oxoguanine; p-JNK: C-jun N-terminal kinase; ICP-AES: Inductively coupled plasma-atomic emission spectrometer; TEM: transmission electron microscopy; Aβ: Brain expression levels of amyloid-beta; BACE-1: beta-site APP cleaving enzyme-1; GSK3β : glycogen synthase kinase-3β; CDK5: cyclin-dependent kinase5; GFAP: glial fibrillary acidic protein; NF-Kb: nuclear factor kappa B; iNOS: inducible nitric oxide synthase; COX-2; NOS3; IL-1β; TNF-α; p-JNK: phospho-JNK; Bcl2; Bax; Cyt.c: cytochrome c; FJB. RAGE receptor for advanced glycation end products; MnSOD: Mitochondrial superoxide dismutase; NeuN: Neuronal nuclei; LC3B: Microtubule-associated protein light chain 3 isoform B; MS: Mass Spectrometry; DA: Dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; HO-1: heme oxygenase; Nrf2: nuclear factor E2-related factor 2; GCLC: glutathione cysteine ligase regulatory subunit; GCLM : glutathione cysteine ligase modulatory subunit; JNK: c-Jun N-terminal kinase; MPP+: 1-Methyl-4-phenylpyridinium; ALT: alanine aminotransferase ; AST: aspartate aminotransferase; ALP: alkaline phosphatase ; HPLC: High-performance liquid chromatography; ECD: equipped with electro chemical detector; TRAP: total reactive antioxidant potential; TAR: total antioxidant reactivity; S100B: Calcium-binding protein B; GDNF: glial cell line-derived neurotrophic factor; NGF: nerve growth factor; DAB: Diaminobenzidine; *SA*-β-*Gal*: senescence-associated β-*galactosidase*.

*3.3 Methodological quality*

Concerning the methodological quality, all reports were carefully assessed through a standard checklist. As shown in Figure 2, only one of the included articles reported sample size calculations. Only 48.4% of the studies reported randomization of animals for outcome assessment. Allocation of the animals in different treated groups was adequately reported by 64.5% of the studies. Blinding of investigators during animal treatment and outcome evaluation was found only in 16.1 and 12.9% of the studies, respectively. The remaining studies did not report any blindness strategy.

Although these parameters are most often applied in clinical trials, the need of randomization, blinding, allocation concealment and attempting to minimize variation have been discussed and strongly recommended for preclinical studies, especially when the experimental protocols involve the evaluation of behavioral parameters. Since human studies are often justified based on results from animal studies, it is extremely important to plan preclinical protocols that follow the same rigor of clinical trials. In addition to reducing the risk of bias, this research strategy avoids obtaining distinct results between the two study designs (preclinical and clinical) [67-69]. In this context, the methodological quality of the studies included in our review was considered low to moderate, which limits the interpretation of the results.

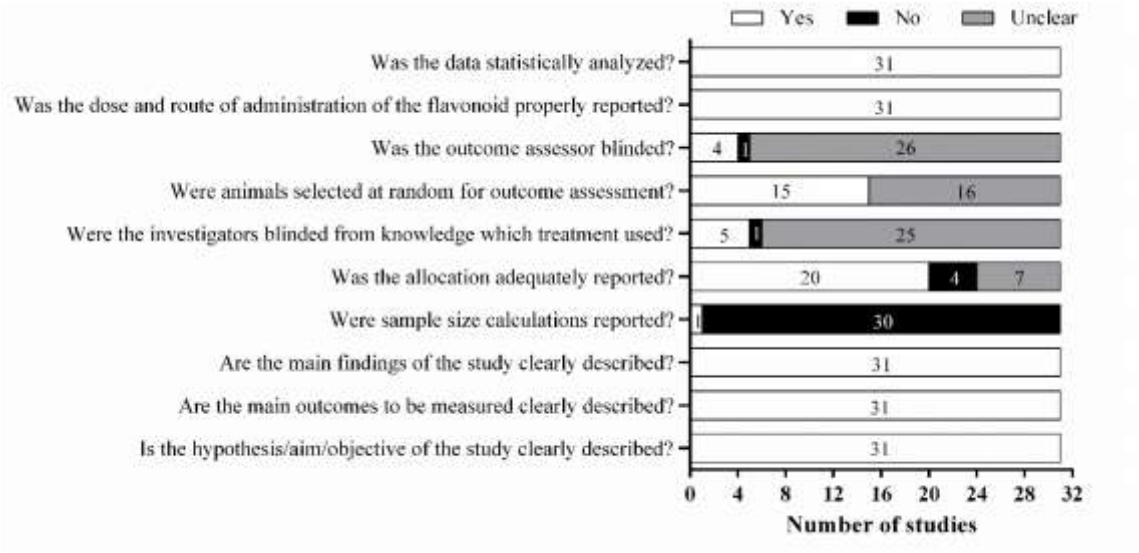


FIGURE 2: Methodological quality of studies included. *White bars* indicate the proportion of articles that met each criterion; *Black bars* indicate the proportion of studies that did not and *Gray bars* indicate the proportion of studies with unclear answers.

*3.4 Animal models in Alzheimer´s disease*

AD is a gradual and highly prevalent neurodegenerative disease that has as pathological characteristics neuroinflammation, neuronal loss and impairment of cognitive function [47, 70]. AD is classified into two subtypes: early-onset familial outcome, related to specific mutations in genes that code for presenilin 1 (PS1), presenilin 2 (PS2) and amyloid precursor protein (APP); and late-onset sporadic disease, associated with mutations in genes that code for apolipoprotein E (ApoE), which include several environmental and genetic risk factors, yet unknown [71].

Among the pathological features, there is an amyloid dense core of β-amyloid peptide (Aβ) and intracellular neurofibrillary tangles (NFTs) composed of an abnormally phosphorylated form of the tau protein [41]. The deposition of amyloid β peptide culminates in pathological processes of synaptic and cognitive dysfunction and neuronal death [47, 70].

Animal models recapitulate characteristic pathological features of AD in addition to several phenotypic traits simulating the disease, acting as therapeutic targets and in their preclinical validation. According to Laurijssens [72], animal models used in AD can be divided into three categories: natural (dog, mouse lemur, Octodon, Rhesus monkey), genetic (APP mice, Tg2576 mice, PSAPP mice) and interventional models (rat, intrahippocampal amyloid infusion model). In the studies selected, interventional models were the most used

[39, 44, 47, 49, 51, 53]. In fact, interventional models would generally be better at identifying symptomatic or corrective treatment. These models can provide important insights such as the Aβ pharmaco-chemical substance-induced model and the understanding of inflammation, neurotoxicity, neurodegeneration and synaptic function, specific brain lesions and neuronal mechanisms underlying memory dysfunction [72, 73].

*3.5 Animal models in Parkinson disease*

PD is a progressive neurodegenerative disease defined by the selective loss of dopaminergic neurons in the nigrostriatal pathway [62]. A disruption of synaptic activity may represent the primary event in the PD pathogenesis. Recently, studies on the neurodegeneration mechanisms in PD have revealed pathological features and important genetic influences. Common pathogenic pathways are present in PD, such as proteostatic deficits, mitochondrial dysfunctions, oxidative stress and inflammation process [74, 75].

As well as the knowledge of new pathophysiological mechanisms involved, the development of experimental models in PD has also advanced. As in other neurodegenerative disorders, the PD experimental protocols have become increasingly sensitive and accurate, allowing not only the evaluation of new drugs, but also the understanding of the molecular mechanisms that play an important role in the disease [74]. However, it is important to note that animal models still cannot express the complexity of human pathological hallmarks and clinical features. Each one has specific limitations and the choice must be conditioned to what best responds to the research objective [74, 76].

The papers selected in this review reported animal models to evaluate pathogenic mechanisms, motor and non-motor manifestations of PD by administering different neurotoxic agents such as 6-hydroxydopamine (6-OHDA) [57, 58, 61, 65], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [56,59,62] or rotenone [63, 64]. The most cited animal model in the selected studies was 6-OHDA. It exerts toxic effects on catecholaminergic neurons and is characterized as a neurotoxin structural analogue of catecholamines, dopamine and noradrenaline. It is toxic at the peripheral and central level although its toxicity in the CNS is only possible through direct stereotaxic surgery. Its neurotoxicity involves the accumulation of toxin in the catecholaminergic neurons triggering the alteration of the cellular homeostasis and neuronal damages. 6-OHDA oxidation by MAO-A generates $H_2O_2$, which, besides being highly cytotoxic, triggers the production of oxygen radicals [77-79]. Other models included the alteration of dopaminergic neurotransmission by

drugs, such as reserpine [80], or by genetic manipulation to study the progression of dopaminergic cell degeneration and motor signs [81].

The models that use toxins are widely employed and act in the replication of most pathological and phenotypic features of the disease. However, as a limitation of this tool, the loss of approximately 70 to 80% of the dopaminergic neurons in the acutely induced neurodegeneration is difficult to achieve, making it difficult to explore dysfunction and progression. In light of the early synaptic dysfunction and neurodegeneration that are inherent to the pathology, new models are necessary to help the early stages of the disease, as well as new therapeutic strategies for the treatment of PD [76].

### *3.6 Flavonoids in the AD and PD treatment*

The plants use adaptation systems in extreme conditions, such as high temperatures, lighting and water scarcity, which culminates in oxidative damages leading to the excessive production of free radicals. Among the main natural antioxidant agents, we highlight carotenoids, flavonoids, anthocyanins and phenolic derivatives that act in several mechanisms against oxidative stress [82].

Flavonoids are a class of secondary metabolites that always attract the attention of the pharmaceutical industry due to their versatility in therapeutic properties. Additionally, they are part of the human dietary due to their abundance in vegetables, fruits, seeds and beverages such as coffee, tea and red wine, as well as in medicinal herbs. More than 9,000 different flavonoids have been identified, which are divided into six subclasses based on their molecular structure. These subclasses include flavonols (rutin, quercetin), flavanols (catechin, epicatechin, epigallocatechin), isoflavones (genistein, daidzein, glycetin, formanantine), anthocyanidins (cyanidin, malvidine, delphinidine), flavanones (hesperetin, naringenin) and flavones (apigenin, luteolin) [83].

Studies indicate that phenolic compounds play an important role in the prevention and treatment of age-associated neurodegenerative diseases. Among the neuroprotective actions of dietary flavonoids, we highlight their potential to modulate cell signaling pathways, protect neurons against oxidative stress, inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and subsequently decrease the production of reactive oxygen species, downregulate pro-inflammatory transcription factors, as well as their ability to suppress neuroinflammation through the reduction of the release of cytokines [84].

In our systematic review, quercetin, rutin, silibinin, naringin, baicalein, hesperidin and anthocyanins (Figure 3) were the most studied flavonoids for PD and AD treatment. In this sense, we describe bellow the pharmacological effects of these compounds to better understand the role of flavonoids in the treatment of AD and PD (Figures 4 and 5, respectively). In general, they act to improve the progression of the disease, but in the reports included, there is no consensus of dose, treatment duration and route of administration of these flavonoids. However, the researchers argue that they have good bioavailability.

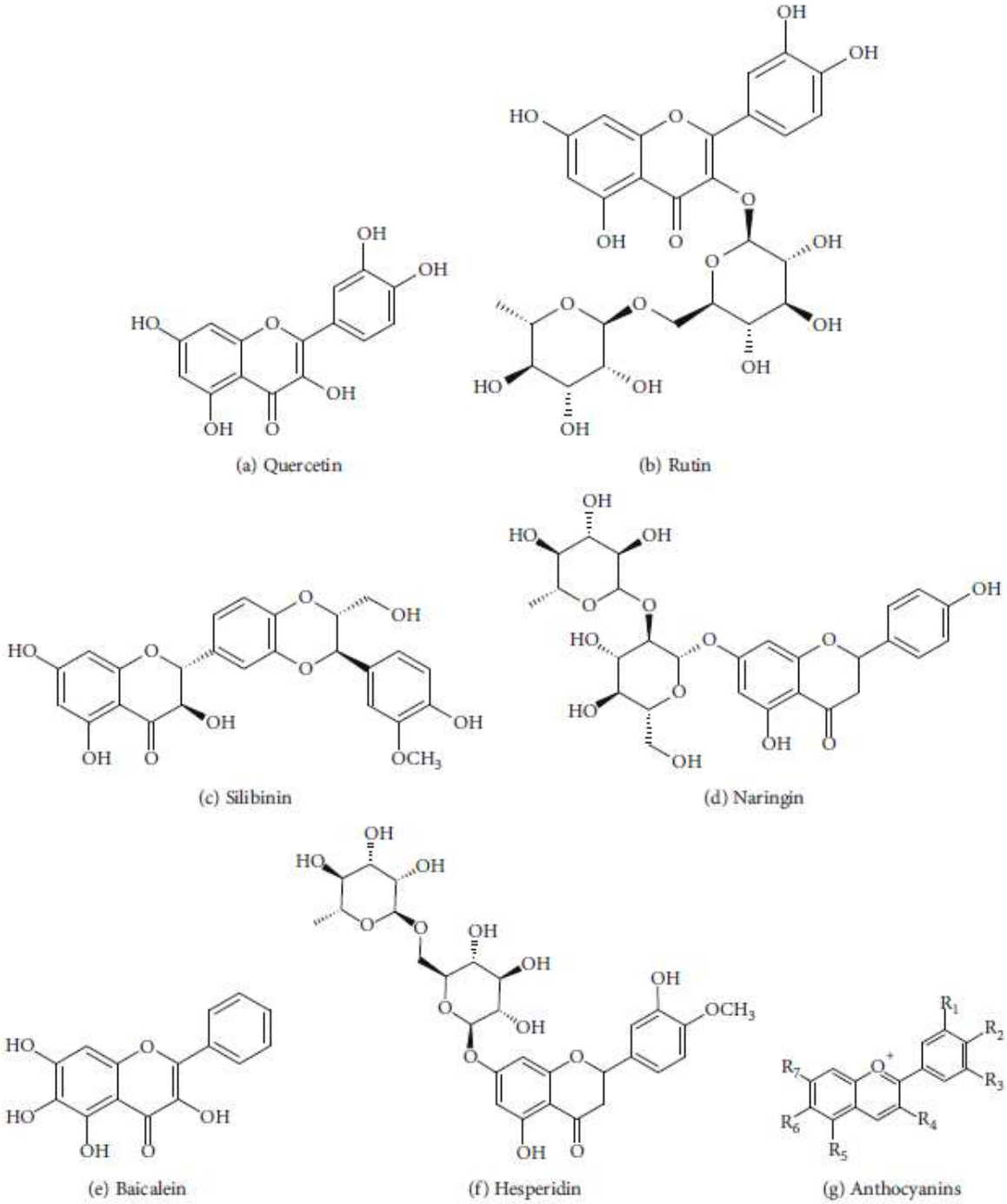


FIGURE 3: Chemical structures of the flavonoids most cited in this review.

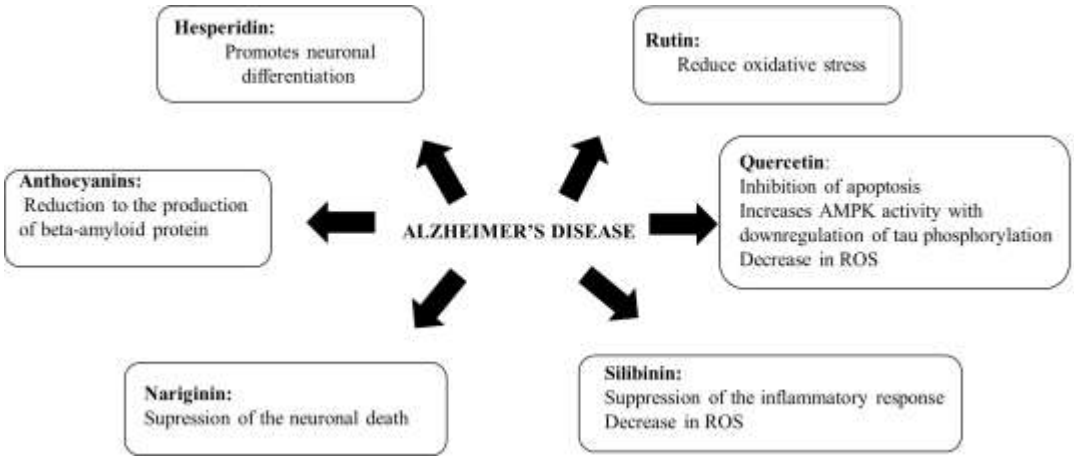


FIGURE 4: Possible mechanisms of action of flavonoids against Alzheimer's disease.

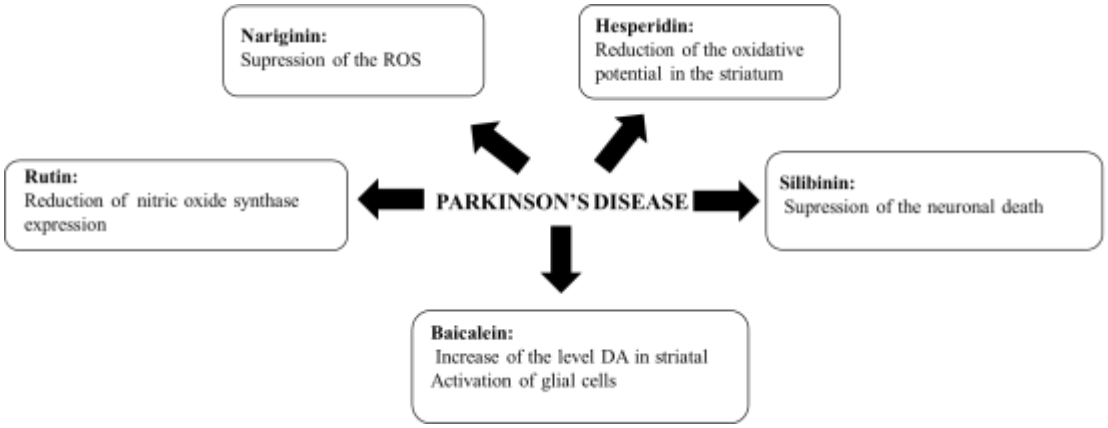


FIGURE 5: Possible mechanisms of action of flavonoids against Parkinson's disease.

### *3.6.1 Quercetin*

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonoid found naturally in plants and among other foods such as onions, apples, broccoli and red wine. It presents pharmacological, antioxidant, cardioprotective and antiapoptotic properties [85]. Some studies relate their neuroprotective action in the prevention of neurodegenerative diseases like AD and PD [86]. Sharma et al. [54] evaluated the effect of the manipulation of quercetin in young rats treated with aluminum on isolated mitochondrial hippocampus. A decrease in the levels of reactive oxygen species (ROS) was observed; however, there were no significant changes in mitochondrial superoxide dismutase activity (MnSOD) [54].

Expression of the apoptotic markers in the mitochondrial fraction revealed levels of reduced Bax and increased Bcl. When the cytosolic fractions were evaluated, the expression of Bcl-2 and cyt-c was decreased, reducing the activation of caspase-3 suggesting that the

administration of quercetin inhibits apoptosis. Quercetin prevents the release of cyt-c and the subsequent activation of caspase-3, and decreases p53 expression. The histopathological analysis revealed no significant degeneration [54]. Thus, the study performed by Sharma et al. [54] has showed that quercetin attenuates aluminum-induced mitochondrial turgor, loss of ridge and chromatin condensation.

Environmental factors such as hyperlipidemic diet consumption may increase the inflammatory response in the brain by increasing the release of cytokines such as TNF-α and IL-6 [87]. Studies have showed that the action of these cytokines promotes neuronal death by activating the apoptotic cascade [88]. Quercetin is also involved in the activity of AMPK, an energy sensor, which has the ability to prevent the phosphorylation of tau protein [89]. Tau protein facilities the polymerization of tubulin in the cell, resulting in the formation of microtubules. In the neurofibrillary tangles, the aggregation of Tau is due to irreversible phosphorylation suffered by this protein. This prevents its normal function and at the same time facilitates its aggregation in fibrils, preventing the normal functioning of the neuron [90].

Chen et al. [46] evaluated whether the activation of AMPK through the action of quercetin is able to block the phosphorylation of tau protein in animals submitted to a hyperlipidic diet. It was seen that quercetin was able to promote downregulation in blood levels of TNF-α and IL-6. Also, it particularly prevented tau phosphorylation by restoring AMPK activation. Additionally, quercetin inhibits GSK3β enzyme activity by dephosphorylation and attenuates the expression of P-PERK and P-IRE1α (membrane sensors present in the endoplasmic reticulum whose activation indicates oxidative stress) and normalizes the inflammation of NLRP3 (inflammation promoter). Improvement in learning and spatial memory was also seen [46].

In a study that evaluated the manipulation of quercetin nanoparticle facilitating oral absorption, it was seen that there was no change in coordination or locomotor activity [48]. The nanoparticle treatment of quercetin is capable of promoting reversal of abnormal exploratory behavior by improving learning and memory assessed by Morris water maze test. In addition, the expression of an inflammatory marker GFAP (glial fibrillary acidic protein) in the hippocampus was significantly reduced, which was not observed with free quercetin. On the other hand, the expression of CD11b, a member of the complement cascade, whose function is adhesion and leukocyte migration in response to inflammation, did not present significant results. Therefore, manipulation of quercetin-loaded nanoparticles has been shown to increase the concentration of this flavonoid in the brain of animals [48].

In the study performed by Palle and Neerati [50], the protective effect of quercetin nanoparticles compared to free quercetin against the induction of spatial memory deficiency promoted by scopolamine in animals was assessed. This work highlighted a relevant role of quercetin nanoparticles on oxidative stress, revealing that these nanoparticles are able to significantly reduce malonaldehyde levels, as well as increased levels of glutathione peroxidase and catalase in the brain. In observing the effect of the treatment with quercetin nanoparticles on the pharmacological action of scopolamine, it has been seen that they are able to reduce the induction of elevation of scopolamine-promoted anticholinesterase activity. In addition, treatment with quercetin nanoparticles significantly reduces morphological abnormalities revealing the cellular protective effect of this flavonoid [50].

It is believed that changes in mitochondrial activity are the main cause of the occurrence of neurodegenerative disorders because they are the main producers of ROS [76]. Godoy et al. [49] observed that quercetin exhibits antioxidant activity protecting against neuronal toxicity induced by hydrogen peroxide, although this protection has been partial in the rat hippocampal neurons. Animals treated with quercetin had a reduction in ROS levels, recovered normal mitochondrial morphology and prevented mitochondrial dysfunction in neurons that were manipulated with hydrogen peroxide [49].

Of all the works selected in this review, only one demonstrated the role of quercetin on Parkinson's disease. Mu et al. [61] investigated the anti-tremor effect of quercetin in the experimental model of PD induced by the application of 6-hydroxydopamine (6-OHDA). The study demonstrated that injection of (6-OHDA) into the striatum induces a marked decrease in the serotonin levels and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA). Therefore, it was seen that these animals, when treated with quercetin, had attenuated serotonin levels - which may be related to an improvement in the tremor level of these animals [61].

Quercetin seems to act by inhibiting the cell oxidative potential, in addition to exhibiting anti-inflammatory action. Both events may minimize the progression of PD and AD.

### *3.6.2 Silibinin*

Silibinin (2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydro-4*H*-chromen-4-one) is a flavonoid derived from milk thistle of the species *Silybum marianum* [92]. Recent research

investigated the effects of silibinin on the inflammatory process, oxidative stress and autophagy [93, 94]. Following this subject, Song et al. [47] investigated the effect of the treatment with silibinin on the locomotor activity, learning and spatial memory. In addition to the concentration of the proinflammatory cytokines IL-1β, IL-4, and the levels of the antioxidant enzyme (GSH) and malondialdehyde (MDA), the lipid peroxidation marker, which occurs in response to excess free radicals the NF-κB (nuclear factor kappa B), was performed. Cyclooxygenase-2 (COX-2) and i-NOS (nitric oxide synthase) are products of glial cells contributing to an inflammatory response in the brain. The expressions of Cox-2 and i-NOS were significantly suppressed by the treatment with silibinin, what suggests the protective effect of this flavonoid. P-53, a tumor suppressor agent, is a critical component of the acute stress cell response and p-p53 is its phosphorylated component. It has been seen that silibinin decreases anxiety-like behavior, reverses memory damage and spatial learning caused by the treatment of Aβ25-35 and improves the ability to recognize new objects and memory flexibility. Silibinin is able to suppress the inflammatory response and improve oxidative stress levels in the hippocampus, in addition to suppressing the expression of p-p53 and p-53 [47].

Chen et al. [60] analyzed the levels of glutathione and malonylaldehyde in animals that underwent neonatal manipulation with carbonyl-iron dose, and its consequence in young adulthood and old animals as well as the treatment with silibinin. It was first seen that iron consumption resulted in abnormal behavior of coordination and locomotor activity only in the old animals. Also in these animals, iron in the neonatal period also increased levels of malonylaldehyde and reduced those of glutathione enzyme. Treatment with silibinin in aging animals decreasing dopamine depletion in the striatum, improving motor behavior, was also able to significantly reduce the content of MDA and increase the content of GSH in the nervous system. It is concluded that silibinin acts as a neuroprotective factor preventing oxidative stress, one of the consequences of neurodegenerative diseases, among them Parkinson's disease [60].

Using a model of dopaminergic neuronal death caused by the pharmacological agent MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin capable of inducing Parkinsonism, Lee et al. [62] investigated the neuroprotective mechanism of sibilinin. Treatment with silibinin prevented motor dysfunction in the pharmacological parkinsonism (MPTP) model, as well as neuronal loss (TH positive neurons) in the striatum and substantia nigra. Silibinin effectively protects dopaminergic neurons in the striatum and black matter of neuronal death caused by MPTP, but does not stop the inflammatory response, since it does

not act effectively on glial cell activation or modulation of oxidative stress. The latter result differs from the previous work probably due to the dose difference used in the present study, from 1 to 10 mg/kg body weight. This demonstrates that the neuroprotective action of silibinin is dose dependent [62].

It can be concluded that silibinin is a flavonoid with a neuroprotective characteristic especially acting upon the inflammatory response, on components of oxidative stress and neuronal death, thus having a potential therapeutic agent in neurodegenerative diseases.

In summary, silibinin is a flavonoid with neuroprotective potential, especially acting upon the inflammatory response, on components of oxidative stress and neuronal death.

### *3.6.3 Anthocyanins*

Anthocyanins are polyphenolic flavonoids widely found in fruits, flowers, grains and vegetables [95]. This flavonoid has anti-oxidant, anti-inflammatory and anti-apoptosis properties, in addition to improving memory and cognition [96], characterized by the basic flavilium core (2-phenylbenzopyryl cation) consisting of two aromatic rings joined by a three-carbon unit and condensed by an oxygen. The anthocyanin molecule consists of two or three portions, an aglycone (anthocyanidin), a group of sugars and often a group of organic acids [97].

There are several evidences that relate the role of this flavonoid in cognition and memory, being characterized with a neuroprotective factor in the dementias, among them AD. Ali et al. [44] observed in genetically modified mice ($A\beta_{1-42}$ mouse model of AD) the ability of anthocyanin particles and (ethyleneglycol)-gold nanoparticles (PEGAuNPs) (polyphenolic flavonoid anthocyanins for conjugation to PEG-AuNPs) to improve memory loss and synaptic deficit and neurodegeneration. The behavioral assessment showed that the latency times required to reach the hidden platform were shorter and also increased the number of platform crossings and time spent in the target quadrant during the probe test in mice treated with anthocyanins and anthocyanin-loaded PEG-AuNPs. Furthermore, anthocyanins and anthocyanin-loaded PEG-AuNPs increased the spontaneous alteration behavior. Anthocyanin-loaded PEG-AuNPs reduced the levels of Aβ (β-amyloid protein), BACE-1 (a beta secretase, a key enzyme in the formation of the β-amyloid protein) and APP (protein precursor anti-amyloid). This demonstrates a potential action of this flavonoid on the production of beta-amyloid protein. The administration of free anthocyanins and anthocyanin-loaded PEG-

AuNPs mitigated the effect of $A\beta_{1–42}$ and increased the expression levels of synaptophysin, PSD95 and SNAP23, molecules that are related to the synapse process between the neurons. Anthocyanins and anthocyanin-loaded PEG-AuNPs increased the phosphorylation of GluR1 at Ser 845 and increased the expression level of p-CREB (Ser133), which can improve the memory process. The administration of free anthocyanins and anthocyanin-loaded PEG-AuNPs increased phosphorylation and elevated the levels of p-PI3K and p-Aktat Ser 473, increased the level of p-GSK3β at Ser9 and reduced the level of p-tau at Ser 413 and Ser 404, which consequently may reduce the level of formation of fibrillar components. The results showed a reduction of the ratio of Bax/Bcl2 and Cyt c, but anthocyanin-loaded PEG-AuNPs were more effective than free anthocyanin. A reduction in the caspase-9, cleaved caspase-3 and PARP-1 levels in the hippocampus was also observed and these results demonstrate the neuroprotective action of this flavonoid. Finally, an increase was seen in the number of surviving neurons, as well as a reduction in the $A\beta_{1–42}$-induced degenerated neuronal cells in the hippocampus and cortex [44].

In the work of Kim et al. [53], the therapeutic efficacy of anthocyanins alone and anthocyanin-loaded PEG-AuNPs in $A\beta_{1-42}$-induced AD mouse model were also investigated. It was observed that the anthocyanin-loaded-PEG-AuNPs can cross the blood brain barrier and accumulate in the Aβ-injected mice. Furthermore, the anthocyanin-loaded-PEG-AuNPs reduced-β-amyloid and BACE-1 expressions and also prevented tau hyperphosphorylation GSK-3β/CDK5 pathway. Anthocyanin-loaded-PEG-AuNPs also reduced $A\beta_{1-42}$-induced microglia and activation of astrocyte cells [53].

In another study, anthocyanins inhibited activated astrocytes and various inflammatory markers including p-NF-κB, inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) in the hippocampus and cortex regions of D-gal-treated rats [52].

Anthocyanins are able to inhibit the cascade of amyloid beta protein production, and to decrease synaptogenesis and neuronal death. They also induce microglial activation in areas important to the process of memory, such as the hippocampus and the cortex.

### *3.6.4 Naringin*

Naringin (4′,5,7-trihydroxyflavanone-7-rhamnoglucoside) belongs to a family of $C_6$-$C_3$-$C_6$ polyphenol compounds and is present in grapefruit and other citrus fruits. This flavonoid has been shown to possess numerous biological benefits, such as antioxidant and

anti-inflammatory [98]. Preclinical models of atherosclerosis, cardiovascular disorders, diabetes mellitus, neurodegenerative disorders, osteoporosis and rheumatological disorders were established in a few studies of naringin *in vitro* and *in vivo* [99]. Recently studies showed a neuroprotective effect of naringin by the modulation of endogenous biomarkers and the downregulation of the free radical and cytokines, including tumor necrosis factor-α (TNF-α) in streptozotocin-induced painful diabetic neuropathy [100]. Mani et al. [37] investigated the effect of naringin against deltamethrin-induced neurotoxicity in male Wistar rats. The result showed that the treatment leads to a significant revival of the oxidative status, which confirms the protective effect of naringin.

The behavioral analysis of the effect of naringin on memory deficit in a pharmacological model (donezepil and scopolamine) in animals has demonstrated a significant difference in the locomotor activity, confirming that naringin has no confounding influence on locomotion in the improving potential for episodic memory; in the familiarization trial, no preference or discrimination towards any of the objects used was seen. The flavonoid reversed the increase in time-induced episodic memory deficits in novel object exploration time compared with familiar objects and improvement in recognition and discriminative indices. Therefore, naringin reversed the scopolamine-induced short-term episodic memory deficits and improved discrimination and recognition [45].

In a model of animal excitotoxicity by treatment with Kainic acid (KA), a potent agonist of excitatory amino acids, especially glutamate, the prevention effect of autophagy and neuroinflammation of naringin was investigated [55]. Excess KA becomes a neurotoxin leading to neuronal death due to excitotoxicity. The Naringin treatment significantly decreased the frequency of chronic spontaneous seizures in KA-treated mice compared with KA alone suggesting that naringin might have beneficial properties as an antiepileptic agent. Additionally, treatment with naringin attenuated the loss of hippocampal neurons in the KA-treated CA1 region, suggesting that naringin might have the property of reducing autophagic stress, which could be involved in neuronal cell death. In addition, the naringin treatment attenuated an increase in TNF$\alpha$ within Iba1-positive microglia in the KA-treated hippocampus characteristic of diseases such as Parkinson's and Alzheimer's [55].

Naringenin (5,7-Dihydroxy-2-(4-hydroxyphenyl) chroman-4-one) is a flavonoid of citrus fruits, predominantly found in grapefruit. It has already been demonstrated the antinociceptive, anti-inflammatory and antioxidant effects of naringenin [101]. Lou et al. [58] investigated the effect of naringenin on PD through the pharmacological parkinsonism model (6-OHDA). Treatment with naringenin resulted in an increase in nuclear factor E2-related

factor 2 (Nrf2) protein (regulatory factor of the expression of antioxidant protein genes) levels and subsequent activation of antioxidant response element (ARE) pathway genes. Additionally, the pretreatment with naringenin protected mice against 6-OHDA-associated ROS damage in striatum. The 6-OHDA-induced loss of TH-positive neurons in the striatum and SNC were remarkably attenuated by the treatment with naringenin. Therefore, there was a profound reduction in striatal DA and its metabolites after 6-OHDA lesioning that was attenuated by the treatment with naringenin, which produced a significant elevation in striatal DA and the metabolites DOPAC (dihydroxyphenylacetic acid) and HVA (homovanillic acid). Furthermore, apomorphine-induced asymmetrical rotations contralateral to the 6-OHDA injection site were significantly reduced by the treatment with naringenin as compared to mice lesioned with 6-OHDA [58].

Narigin seems to act especially through the inhibition of oxidative cellular stress, which results in reduction of neuronal loss (autophagia stress). It is known that oxidative stress is a strong mechanism of neuroinflammation with an important consequence for neurodegenerative diseases.

### *3.6.5 Baicalein*

Baicalein (5,6,7-trihydroxy-2-phenyl-chromen-4-one) is a flavonoid originally isolated from the roots of *Scutellaria baicalensis* and *Scutellaria lateriflora* [102]. It has neuroprotective properties against PD, such as antioxidant and anti-inflammatory [103]. Hu et al. [63] noted that the treatment with baicalein attenuated the motor deficits and also increased in striatal neurotransmitters: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid and HVA, homovanillic acid. Analysis of fluorescence intensity by microscopy and the amount of α-syn in enteric nervous system was also lower and there was an increase in the number of TH-positive neurons. There was a decrease in α-synoligomers, not monomers in ileum, thoracic spinal cord and midbrain; baicalein had no effect upon α-syn mRNA expression. Therefore, it can be inferred that this flavonoid can prevent the progression of α-syn accumulation in PD, by inhibiting the formation of α-syn oligomers [63].

Upon the investigation of the therapeutic effects of baicalein on rotenone-induced PD rats as to whether the neuroprotective potential practice by baicalein was through intervening in mitochondrial function and mitobiogenesis, it was found that baicalein partially ameliorated the motor dysfunction and increased the number of TH+ cells in the substance nigra (SN) in

rotenone-induced PD rats [64]. This flavonoid also protected neurons in the SN against rotenone-induced apoptosis. The baicalein ameliorated the dysfunction of mitochondrial complex I in the ventral midbrain that was damaged by rotenone [64]. In addition, the administration of baicalein increased the protein levels of PGC-1α (a regulator of mitochondrial mitobiogenesis), NRF-1 (transcription factor that regulates the expression of antioxidant proteins) and TFAM (mitochondrial transcription factor) in the ventral midbrain, which may improve the brain's response to oxidative stress and consequent neuronal loss observed in Parkinson's disease [64].

Lee et al. [56] through a model of pharmacological parkinsonism in animals (MPTP administration) observed that the in low doses, baicalein improved motor ability and prevented the loss of dopaminergic neurons caused by MPTP. In addition, microglial and astrocyte activation was reduced in the animals with pre-treated baicalein PD. This study revealed the importance of astrocyte activation for the occurrence of the neurodegenerative process. It has also been reported that baicalein reduces MPP (a toxic molecule that interferes with oxidative phosphorylation in mitochondria), which is capable of inducing the activation of NF-κB, ERK (protein kinase intracellular signaling), JNK (c-jun N-terminal kinase) in the astrocyte leading to a mechanism of neuroinflammation considered as a potent inducer of PD [56].

These studies taken together reveal the neuroprotective action of baicalein especially on mitochondrial activity and the activation of glial cells. Both processes are recognized with potential mechanisms capable of increasing the risk of neurodegenerative diseases. This flavonoid was proven to be effective as a therapeutic strategy especially against PD disease.

### *3.6.6 Hesperidin*

Hesperidin (2*S*)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromen-7-yl 6-*O*-(6-deoxy-α-*L*-mannopyranosyl)-β-*D*-glucopyranoside isthe major flavanone glycoside present in citrus fruits. This compound has an important neuroprotective property and radical scavenging properties related to diverse neuronal insults, such as ischemia [104], stroke [105] and oxidative-induced damage [106], as well as pathology related to AD [107] and Huntington’s disease [108]

Nones et al. [109] showed that hesperidin promotes neuronal differentiation and survival and also enhances the neuroprotective capacity of astrocytes, by inducing them to secrete soluble factors involved in neuronal survival *in vitro*.

Li et al. [40] investigated the potential therapeutic effect of hesperidin on behavioral dysfunction, Aβ deposition and neuro-inflammation in the transgenic APP/PS1–21 mouse model. In this research, the treatment with hesperidin caused a decrease in the nesting ability and social interaction. Furthermore, the improvement of amyloid beta accumulation and APP expression, with a reduction of microglial activation, suggests that hesperidin might be a potential candidate for the treatment of AD or even other neurodegenerative diseases.

An investigation of the anti-inflammatory potential, antioxidants and protective effects of hesperidin was performed by Javed et al. [41] using the mouse model of sporadic dementia of Alzheimer's type (SDAT). In this model, researchers showed that hesperidin can be used for the treatment of cognitive disorders because of its neuronal cell death modulation by inhibiting the over expression of inflammatory markers such as nuclear factor κB, coupled with the inducible nitric oxide synthase, cyclooxygenase-2 and glial fibrillary acidic protein positive astrocytes.

Antunes et al. [57] evaluate the role of the flavonoid hesperidin in an animal model of PD induced by 6-OHDA and demonstrated that treatment with hesperidin (50 mg/kg) was effective in preventing memory impairment and depression-like behavior with a reduction in glutathione peroxidase and catalase activity, total reactive antioxidant potential and the dopamine and its metabolite levels in the striatum of aged mice.

Matias et al., 2017 [110] described hesperidin as a new drug able to improve memory in healthy adult mice through two main mechanisms: by inducing synapse formation and function between hippocampal and cortical neurons. In addition, other mechanisms enhance the synaptogenic ability of cortical astrocytes due to increased secretion of transforming growth factor beta-1 (TGF-β1) by these cells.

Hesperidin seems to present neuroprotective role through its action on glial cells, microglia and astrocytes, promoting reduction of neuroinflammation and oxidative stress. In addition, it may induce synapse formation in brain regions involved with memory and decision-making.

### *3.6.7 Rutin*

Rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl 6-*O*-(6-deoxy-α-*L*-mannopyranosyl)-β-*D*-glucopyranoside**)** is a glycone of quercetin with a flavonol structure. This substance modify the cognitive and various behavioral symptoms of neurodegenerative diseases due the ability of rutin and/or its metabolites to cross the blood brain barrier this way causes effects on the various cellular functions under pathological conditions [111, 112].

Some reports have showed that rutin scavenges superoxide radicals, increases antioxidant enzymatic activity *in vitro*, reduces lipid peroxidation and cytokine production and prevents cognitive disorders (including CNS injuries) in rat models [113, 114].

In recent studies, Rodrigues et al. [115] investigated the effect of the treatment with rutin after induction of focal cortical ischemia and the results showed rutin as a putative candidate to treat stroke. Few studies have evaluated the treatment with rutin in models of global and focal brain ischemia, showing positive effects [116].

According Hhan et al. [117], similar to other flavonoids, the main expected mechanisms of action of rutin are its anti-inflammatory and antioxidative potential. Thus, anti-inflammatory action of rutin was demonstrated with a reduction of inducible nitric oxide synthase expression in a model of PD.

Moghnelinejad et al. [39] investigated the possible effects of rutin on MAPK and BDNF gene expression and memory retrieval in β-amyloid-injected rats. Their results demonstrated improved memory impairment caused by injection of Aβ in rats through activation of MAPK and BDNF, and also reduced oxidative stress in the hippocampus of rats by reducing MDA levels and increasing thiol content in the hippocampus. Further studies are necessary to clarify the effects and molecular mechanisms of this flavonoid.

Rutin has antioxidant and anti-inflammatory roles, its mechanism of action is not yet completely elucidated but may be related to the MAPK pathway and reduction of nitric oxide synthase activation.

## 4 Conclusion and Perspectives

This systematic review suggests that the flavonoids herein reported have a potential for the treatment of neurodegenerative diseases such as PD and AD and stand out as drug

candidates in future clinical research. The studies listed in this review revealed that the main targets of action for Alzheimer's disease therapy were the reduction of reactive oxygen species and production of amyloid beta protein. As for Parkinson's disease, the reduction of the cellular oxidative potential and the mechanisms of neuronal death are often involved in the neuroprotective potential of flavonoids.

It was observed that flavonoids had been studied using various *in vivo* animal models, including the evaluation of their mechanism of action and effects on the molecular level. However, it is essential to improve the rigor of study design and data in light of the fact that most of the studies included presented low to moderate methodological quality, what limits the interpretation of the results and the continuity of the studies.

**Competing Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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# CAPÍTULO 2

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# Antidepressant-like activity of naringenin/β-cyclodextrin inclusion complex in mice: evidence for the involvement of monoaminergic system

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**ABSTRACT**

To improve the pharmacological properties of naringenin (NR) an inclusion complex with β-cyclodextrin was used. The complex was characterized by several methods. The present study was designed to investigate the antidepressant-like effect of NR and the inclusion complex obtained with its incorporation into β-cyclodextrin (NR-βCD) in mice using forced swimming test (FST), tail suspension test (TST) and open-field test (OFT). The involvement of the monoaminergic system in the antidepressant-like activity of NR was also studied. Our results showed that NR and NR-βCD, when administered acutely to mice (20 and 40 mg/kg, p.o.), produced a significant reduction in the duration of immobility time, with a profile comparable to that of imipramine (30 mg/kg, p.o.). The reduction in immobility time observed with NR treatment (20 mg/kg, p.o.) was prevented by pretreatment with haloperidol (0.2 mg/kg, i.p., $D_2$ receptor antagonist) and yohimbine (1 mg/kg, i.p., an $\alpha_2$-adrenoceptor antagonist), but not with ondansetron (1 mg/kg, i.p., a 5-$HT_3$ receptor antagonist). Our results suggest that NR and NR-βCD produced an antidepressant-like effect which was unrelated to its locomotor activity. Furthermore, this anti-immobility effect seems most likely to be mediated through an interaction with the monoaminergic system (dopaminergic and noradrenergic systems).

Keywords: Naringenin; Cyclodextrin; Antidepressant-like; Pharmacological mechanisms; Central Nervous System.

## 1 Introduction

Depressive and anxiety are among the disorders of the Central Nervous System (CNS). According to the World Health Organization these mental disorders are the most prevalent in the population, and it is estimated that 300 million people worldwide suffer of depression and that this number increases in low-income countries. Anxiety disorders affect 264 million of people [1]. The depression is a multifactorial disease asymptomatically, psychologically and biologically heterogeneous. It's considered chronic and disabling mental illness which is a leading cause of morbidity and mortality worldwide [2,3].

The pathological mechanisms of depression are still unclear, despite many studies of the negative impact on public health. One of the hypotheses is that environmental stress is a risk factor for mood disorders [4]. In line with this hypothesis, exist metabolic dysfunction of monoamine neurotransmitters, the levels of serotonin (5-HT), noradrenaline (NA), and dopamine (DA) in the Central Nervous System (CNS) are decreased. In addition, includes hypothalamic-pituitary-adrenal (HPA) axis dysfunction, neuroinflammatory, neurotrophic, neuroimmune, and oxidative stress [5].

The pharmacological category antidepressants are described to treat this pathology. However, some patients do not respond to treatment with market availability, 30% of patients do not respond to treatment, even after exhaustive attempts at therapeutic regimens. Thus, it is essential to discover new drugs as alternatives for the treatment [6,7].

In this regard, a study carried out with Brazilian plants with actions in the CNS, from the phytochemical point of view, the chemical constituents flavonoids, alkaloids, phenolic acids, tannins and essential oils were identified as most recurrent in the plants. Flavonoids are among the natural products that arouse interest in the pharmaceutical industry. They constitute a family of natural polyphenolic compounds and are normal constituents of the human diet

due to their abundance in vegetables, fruits, seeds and beverages such as coffee, tea and red wine, as well as in medicinal herbs [8].

Phenolic compounds have important health benefits; however, they need to improve the pharmacokinetic profile before oral administration. Distributed in abundance in plants, flavonoids have low absorption in the intestine in its natural form. During their process of formation they form different metabolites that if they are absorbed they undergo to the hepatic enzymatic system being able to form new metabolites favoring the bioactivity. Additionally, the low solubility of flavonoids restricts its pharmaceutical use [9,10].

Among flavonoids, naringenin; NR ($C_{15}H_{12}O_5$) 5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one); is a widely distributed flavanone in citrus fruits and grape fruits and presents a larger potential therapeutic for the management of a variety of diseases [11]. Naringenin has a recognized antioxidant activity which is attributed to its structure-activity relationship [8]. Naringin is hydrolyzed by the intestinal microflora to produce naringenin. This is readily absorbed and also has good penetration through the blood-brain barrier. It has been investigated for its pharmacological properties, including antitumor, hepatoprotective, anti-inflammatory, antihyperglycemic, antihyperlipidemic and antimicrobial [12]. Furthermore, naringin confers neuroprotection on neurodegenerative diseases, affective disorders, and neuroinflammation because the antioxidants and inflammatory mechanisms [13]. However, the use of naringenin as a drug is quite limited, due to its low solubility in water and consequent low bioavailability [14].

Thus, microencapsulation is an alternative methodology to improve bioactive storage and stability. One of the pharmaceutical alternatives used to improve the bioavailability of low solubility substances is the use of such as cyclodextrins (CDs) [15]. CDs constitute a class of pharmaceutical excipients composed of *D*-glucopyranose units which, together, give rise to cyclic frustoconical structures. The most common are α, β and γ-cyclodextrins,

composed of 6, 7 or 8 glucopyranose units with primary and secondary hydroxyl groups facing the outer face, giving some hydrophilicity to the external surface, while the cavity of the molecule is hydrophobic forming inclusion complexes. CDs can be incorporated a variety of inorganic and organic molecules, which may increase physicochemical, pharmacodynamic and pharmacokinetic properties of the drug for the pharmaceutical industry [16-18].

To the best of our knowledge, there is no data about naringenin complexed with CDs assessed in experimental behavior protocols. Thus, the aim of our study was to perform a neuropharmacological investigation for evaluation of the antidepressant-like effects of NR and the inclusion complex obtained with its incorporation into β-cyclodextrin (NR-βCD) in animal models. The participation of the dopaminergic, noradrenergic and serotonergic systems was also investigated.

## 2 Material and methods

### *2.1 Chemicals*

Naringenin (NR) (purity 98% by HPLC), β-cyclodextrin, haloperidol, ondansetron, yoimbine, and diazepam were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and imipramine (EMS, Brazil).

### *2.2 Preparation of the inclusion complex*

The complex was obtained by the method of physical mixture (PM) and co-evaporation according to the methodology described by Pinto et al. [19] which consisted of the following steps: a) PM: Naringenin 97% (272 g/mol) and βCD 98% (1135 g/mol); 1) were mixed (1:1 mol ratio) mechanically at ambient conditions each and 2) NR and βCD were mixed (1:1 mol ratio) in 20 mL of water under constant stirring (36 h) in 400 rpm (Quimis Q

261A21, Brazil) until equilibrium. Afterwards, the samples were dried in a glass desiccator, and after that were removed and stored in glass. The samples were characterized by Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and Nuclear Magnetic Resonance (NMR) spectroscopies. The complex that presented the best results in the characterization was used in the pharmacological tests.

### *2.3 Characterization of the inclusion complex*

#### *2.3.1 Scanning electron microscopy (SEM)*

The surface morphology of samples β-CD, naringenin, naringenin:β-CD physical mixture and Naringenin:β-CD inclusion complex were examined by a scanning electronic microscopy (SEM) in a Tescan-VEGA3 model. For this, powdered samples were mounting into carbon tape attached to aluminum stub and metallized with gold powder for 250 s and examined using SEM at 5 kV.

#### *2.3.2 FTIR analysis*

The FTIR spectra of β-CD, naringenin, naringenin:β-CD physical mixture and naringenin:β-CD inclusion complex were performed in a Perkin Elmer Spectrum, Version 10.4.00 using KBr pellets, scan between 4000 and 650 $cm^{-1}$ and KBr as spectroscopic blank.

#### *2.3.3 NMR investigations*

$^{1}H$ NMR and $^{1}H$-$^{1}H$-ROESY spectra were recorded for samples β-CD, naringenin and naringenin:β-CD inclusion complex, dissolved in $D_2O$ at 289 K. All experiments were developed in a Bruker AvanceIII 400 MHz Spectrometer and the $D_2O$ resonance at 4.80 ppm was used as internal reference to report chemical shifts values, according to equation:

$$\Delta\delta = \delta_{(complex)} - \delta_{(free)}$$

A rotating-frame Overhauser spectroscopy (ROESY) for detection of intermolecular nuclear Overhauser effects (NOEs) between β-CD and naringenin was conducted for inclusion complex. The 2D ROESY spectrum was collected with mixing time of 200 ms under spin lock condition [20].

### *2.3.4 Docking study*

Molecular docking procedures [21] were performed to verify the possible molecular interactions profile between naringenin and β-CD carrier. The crystallographic 3D structure of the β-CD was taken from the RCSB-PDB database (www.rcsb.org) [22] (PDB ID: 5MK9) [23]. The complex was edited by UCSF Chimera program [24], removing the co-crystallized protein and adding the hydrogens to the β-CD. The ligand was builder using ACD/Chem Sketch 12.01 software [25], followed by a initial geometry optimization at semi-empirical PM3 level through Gauss View 6.0 and Gaussian 09 packages [26,27], at CENAPAD cluster environment (http://www.cenapad.ufc.br/).

Autodock v4.2 and Autodock tools (ADT) v1.5.4 (Autodock, Autogrid, Autotors, Copyright-1991–2000) packages [28] were used to perform the docking algorithms. Initially, Gasteiger charges and polar hydrogens were assigned to β-CD and naringenin ligand. Nonpolar hydrogens were merged. The ligand was considered flexible on analysis and the rotatable bonds were chosen automatically by ADT. The affinity maps was calculated into a grid box containing 30 Å, 30 Å, 30 Å, which involved all the structure of β-CD, with an internal spacing of 0.375 Å between the grid points. This box was centered on the Cartesian center of β-CD. Lamarckian Genetic Algorithm (LGA) was used to investigate the most stable conformations of the ligand [29]. Initial population was chosen as 150, with 27,000 as the maximum number of generations. The best value for the maximum number of energy evaluations was 25,000,000 (long). For mutation and crossover rates, the 0.02 and 0.8 default values were chosen, respectively, and the elitism rate was 1.0. In the final docking analysis,

was observed the best cluster containing conformational similarity, similar binding energy and little relative values of RMSD (Root Mean Square Deviation) between the geometries. Thus, the lowest energies conformations of the most populated cluster were analyzed and considered as the most trustable solutions. The final goals of the docking results were discussed at the light of other experimental data.

### *2.4 Pharmacological evaluation*

#### *2.4.1 Animals*

All experiments were conducted using 6-8-week-old male Swiss mice (*Mus musculus*) (30-35g). The animals were randomly separated in six groups of six (n=6) animals in polypropylene cages at a temperature of 22 ± 1 °C and relative humidity of 60-80% with a light/dark cycle of 12:12 h (start 7:00 AM and end 7:00 PM) and free access to food (Purina Labina®) and water. Animals were allowed to have a period of acclimation (24 h) before pharmacological test and were deprived of food but given free access to water for 4 h before the experiment. All efforts were made to minimize animal suffering and to reduce the number of animals in the experiments. This study was performed in accordance with the Conselho Nacional para o Controle de Experimentação Animal (CONCEA, Brazil). Experimental protocols (number 0004/221015) were approved by the Animal Care and Use Committee at the Federal University of Vale do São Francisco (CEUA-UNIVASF, Brazil).

#### *2.4.2 Acute toxicity*

The oral acute toxicity test was performed according to the methodology described in Organisation for Economic Co-operation and Development (OECD) Guide 423 (OECD, 2001). For this, female Swiss albino (*Mus musculus*) mice were divided into two groups containing three animals each (n=3) kept in their cages for 5 days before the test to allow acclimatization to laboratory conditions and fasted (water *ad libitum* only) for 3-4 hours.

After the fasting period the animals were weighed and treated. The negative control group received only the vehicle (saline solution) orally (gavage) and the other groups received the flavonoid and their complex. The highest dose of 2000 mg/kg was tested, based on the protocol proposed by the OECD [30].

Mice were also evaluated for potential neurotoxic effects through open field test (exploratory and locomotor activity) and then in the elevated plus-maze for observation of the potential anxiolytic effect. Also, behavioral, motor and sensory functions were evaluated. The respective tests were performed 60 minutes after administration.

After administration of the treatments, individual observations of the animals were performed in the 30 minutes, 1, 2, 3 and 4 hours periods, and daily during the following 14 days [31].

The body mass of the animals was evaluated weekly (days 0, 7 and 14) and water and feed intake was recorded daily throughout the experiment. At the end, the animals were anesthetized with a mixture of ketamine 15 mg/kg and xylazine 150 mg/kg and euthanized by cervical dislocation. After euthanasia, the organs were removed, evaluated macroscopically and weighed.

*2.5 Central nervous system effects*

Sixty minutes after the treatment, behavioral assessments were carried out for neurobehavioral phenotypes representing spontaneous motor activity (openfield test) and antidepressant-like behavior (forced swim test and tail suspension test) were assessed. All behavioral tests were conducted between 10:00 AM and 16:00 PM. The mice were habituated to the 24h room before starting the test.

### *2.5.1 Open field test (OFT)*

The OFT was used to evaluate the exploratory activity of the animals, considering that once placed in an unknown environment. The apparatus consisted of a circular arena with 50 cm in height x 60 cm in diameter, with the base divided into 12 circumcentric quadrants (Insight, Brazil). The mice were randomly distributed into six groups of 6 animals. Sixty minutes after the treatment with saline, NR (20, 40 mg/kg, p.o.) and NR-βCD at the same doses and thirty minutes after the treatment with diazepam (1.0 mg/kg, i.p.), each mouse was placed in the central square of the open field and observed for 5 min to record locomotor (number of segment crossed with four paws), immobility time and explore activities (expressed by number of rearing on the grooming) [32].

### *2.5.2 Forced swimming test (FST)*

For the forced swim test, a glass cylinder containing 11 cm of water at a temperature of 23 to 26 °C (height, 20 cm; internal diameter, 15 cm) was used [33]. An initial swimming training was conducted in a 15-minute session. After 24 hours, the animals were submitted a 6-minutes test session in which the duration of immobility duration parameters were observed during the final four minutes of the test. For this test, the immobility period was observed as the time spent by the mice floating in the water without struggling and making only those movements necessary to keep its head above the water. The test sessions were scored by an observer blind to treatment.

### *2.5.3 Tail suspension test (TST)*

For this test was used a manufactured tail suspension box with the dimensions (55 height X 60 width X 11.5 cm depth). In order to prevent animals from observing or interacting each other, each mouse is suspended within its own three-walled rectangular compartment (55 height X 15 width X 11.5 cm depth). The mice were suspended by tail with

a clamp (in the middle of this compartment and the width and depth are sufficiently sized so that the mouse cannot make contact with the walls). The approximate distance between the mouse's nose and the apparatus floor is 20-25 cm. Mice were considered immobile only when they hung passively and completely motionless. Mice were suspended for 6 minutes, and the parameter was recorded during the final 4 min interval the test [34].

*2.6 Mechanism involved in the antidepressant-like effect of NR*

To investigate the possible involvement by serotoninergic system of 5-$HT_3$ receptors in the antidepressant-like effect of NR, animals were pretreated with ondansetron (1 mg/kg i.p., 5-$HT_3$ receptor antagonist). After 30 min, they received NR or vehicle and were tested with the FST 30 min later.

To test the antidepressant-like effect of NR mediated of noradrenergic system, animals were pretreated with yohimbine (1 mg/kg, i.p., a α2-adrenoceptor antagonist). After 30 min, they received NR or vehicle and were tested in FST.

Furthermore, the involvement of the dopaminergic system in antidepressant-like effect of NR was performed with animals pretreated with haloperidol ($D_2$ receptor antagonist). The doses used in this study are in agreement with previous studies [35-37].

*2.7 Statistical analysis*

The results are presented as the mean ± standard error of the mean (SEM), and statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant if $p < 0.05$. All analyses were performed using GraphPad Prism® 6.0 (Graph Pad Prism Software, Inc., San Diego, CA, USA).

# 3 Results

## *3.1 Characterization of the inclusion complex*

### *3.1.1 Scanning electron microscopy (SEM)*

The Figure 1 (a and b) depicts the morphology of individuals materials naringenin and β-CD. As can be seen, naringenin exhibits as needle-like crystal form, whereas β-CD shows a structure of large irregular blocks. The physical mixture (c) revealed some similarities with the isolated compounds where the characteristic naringenin crystals and irregular blocks lower than β-CD blocks can be observed. The naringenin:β-CD inclusion complex (d), showed smaller and distinct structures of isolated components or physical mixture. The comparison among images, literature data [38] and observations allow us to infer that a new material was obtained.

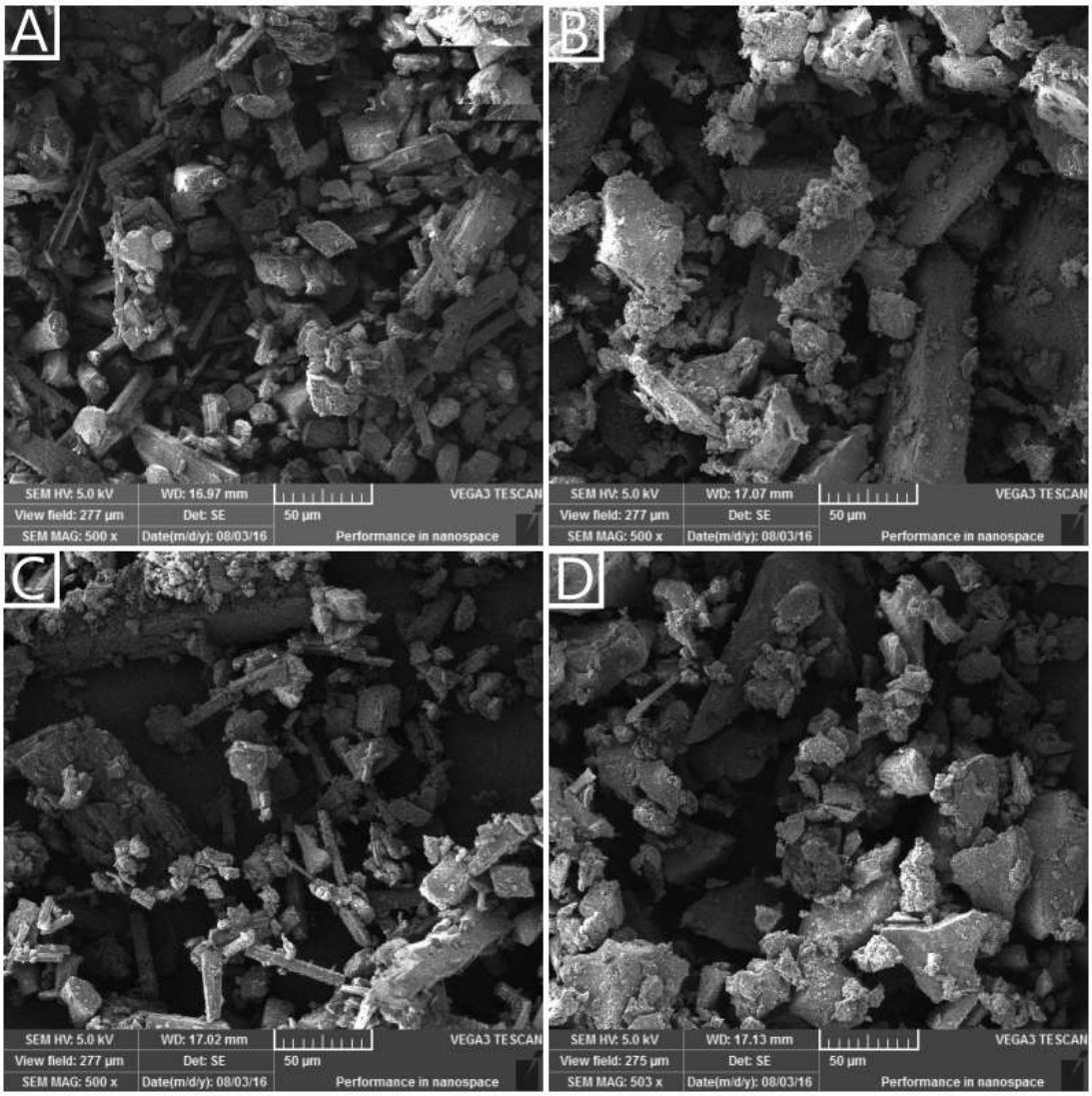



**Figure 1.** SEM micrographs of naringenin (**a**) β-CD (**b**) naringenin:β-CD physical mixture (**c**) and naringenin:β-CD inclusion complex (**d**).

*3.1.2 FTIR analysis*

Several alterations were observed in FTIR spectra of naringenin:β-CD inclusion complex compared to spectra of isolated compounds (Figure 2). Among these, it is possible to list: the peaks disappearance at 3271 $cm^{-1}$ (O-H axial deformation), 2925 $cm^{-1}$ and 2835 $cm^{-1}$(C-H $sp^3$, axial deformation) of guest, the band intensity increase at intensity at 3303 $cm^{-1}$ (O-H axial deformation of intermolecular bonds) and the maintenance of the peak at 1024 $cm^{-1}$ (axial deformation of free O-H) of host [39].

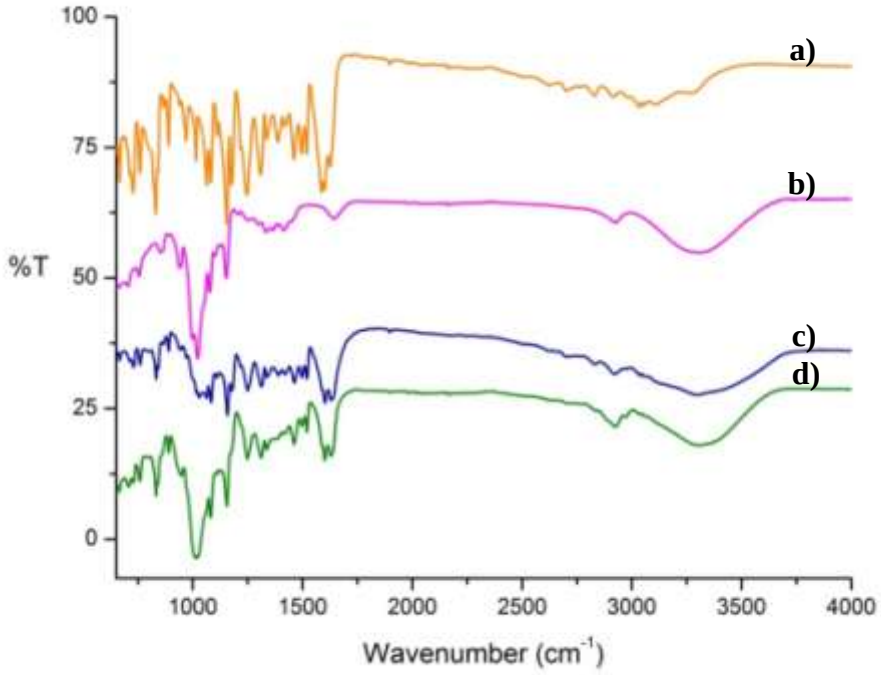



**Figure 2.** FTIR spectra a) naringenin; b) β-cyclodextrin; c) naringenin:β-CD physical mixture; d) naringenin:β-CD inclusion complex.

*3.1.3 NMR analysis*

Alterations at chemical shifts of β-CD internal protons (H-3 and H-5) are often used to confirm inclusion complexes formation [20, 38, 41-43]. In our study, changes in chemical shifts of β-CD were observed after naringenin complexation (Table 1). With the exception of H-5, all protons suffered a slight, but not significant changes (-0.0015 - 0.0433 ppm) in chemical shifts. The greater displacements was observed for protons H-5 (-0.1233 ppm), showing a larger interaction among the internal proton of β-CD and hydrogens of naringenin thus confirming the inclusion of flavonoid in the host. In order to confirm the suggestions of the mode of interaction between the guest and host molecules, from the $^{1}$H-$^{1}$H ROESY experiment was developed.

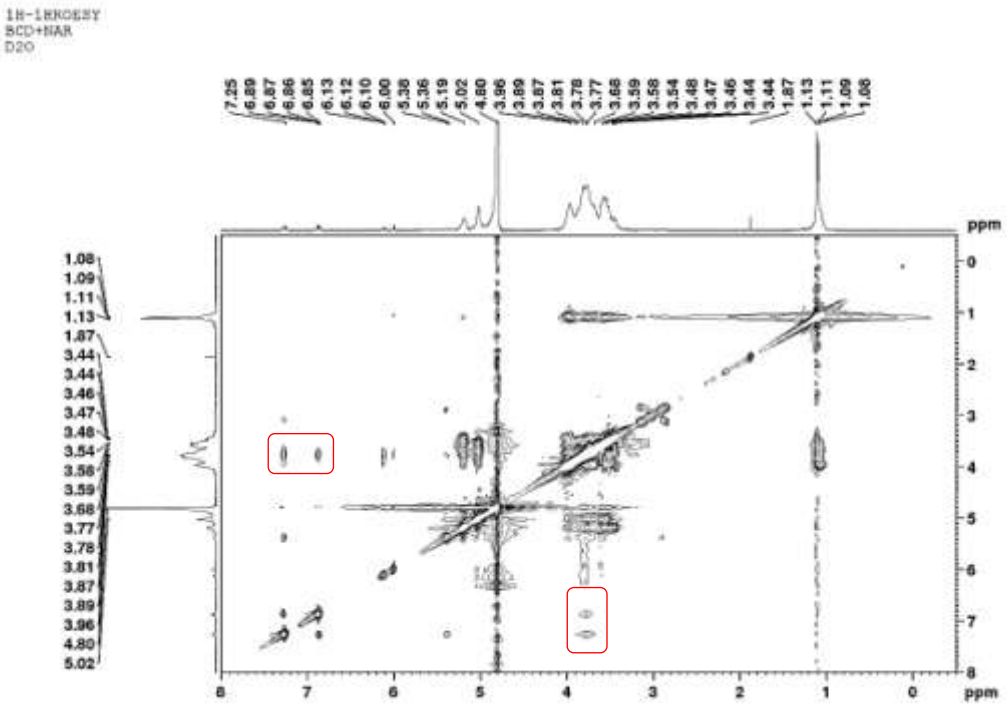
**Table 1**. Chemical shifts for the protons of CD and CD-NR inclusion complex

| **Proton** | ***δ*β-CD** | **Naringenin:β-CD inclusion complex** |
|---|---|---|
| H-1 | 4.9805 | 4.9790 |
| H-2 | 3.5580 | 3.5344 |
| H-3 | 3.8816 | 3.9249 |
| H-4 | 3.4977 | 3.4168 |
| H-5 | 3.7703 | 3.6470 |
| H-6 | 3.7912 | 3.7371 |

Samples solubilized in deuterium oxide and values express in ppm; Data obtained from Bruker Avance III 400 MHz. β-CD=beta-cyclodextrin; Naringenin:β-CD= inclusion complex (1:1) naringenin-β-cyclodextrin.

As can be seen in Figure 3a, the $^{1}$H-$^{1}$H ROESY spectra shows strong correlations among the ring B aromatic protons (H-2'/H-6' and H-3'/H-5' at 6.85 ppm and 7.25 ppm, respectively) of flavonoid structure and the protons H-5 at 3.77 ppm of β-cyclodextrin. These results confirm one more time the formation of inclusion complex and the interaction mode of two molecules (Figure 3b).

a)




b)

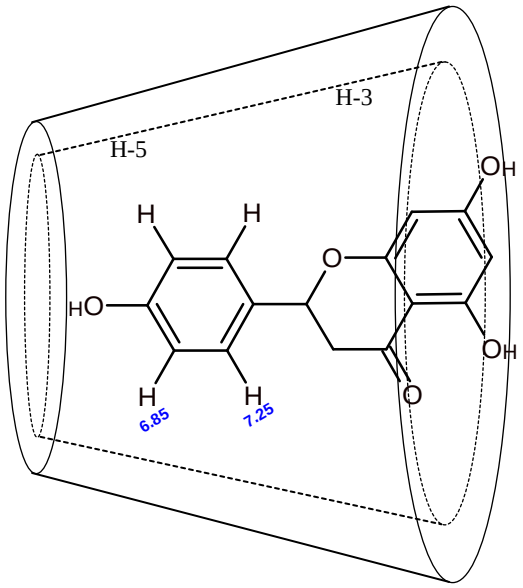



**Figure 3.** Rotation Overhauser effect (ROESY) of Naringenin:β-CD inclusion complex in $D_2O$ at 289K (**a**). Interaction mode among naringenin and β-cyclodextrin (**b**).

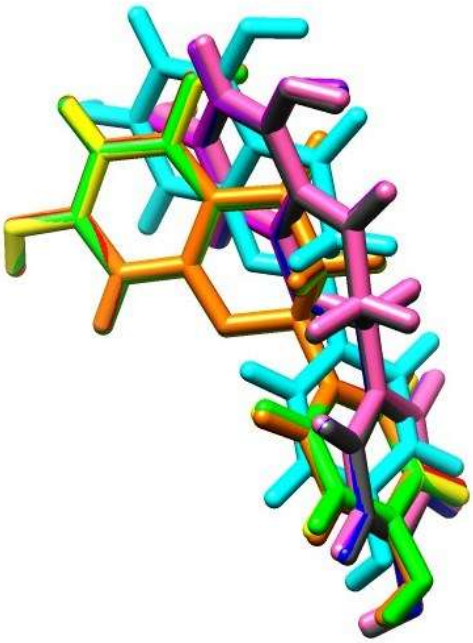
*3.1.4 Molecular docking results*

Figure 4a shows the the ten best conformations after docking results, demonstrating that the naringenin has a medium geometric orientation and a preferential occupancy volume

within the βCD carrier, forming relatively stable complexes. This result demonstrates a similar conformational profile in the same region, which gives confidence to the reasonableness of the docking result, considering that the geometries with lower interaction energy should not be very different.

Figure 4b presents the particular molecular interactions for the lowest energy conformation of naringenin into the cavity of βCD (binding energy = -6.0 Kcal/mol). It can be noted that the ligand is preferably inserted through the phenolic ring, which have a more lipophilic profile than the condensed rings. This is noteworthy, since that the interior of the βCD have a nonpolar nature, favoring lipophilic interactions. Additionally, the value of the binding energy can be justified considering the more weak nature of the predominant dispersion interactions. However, this featureit shows interesting given that the carrier must to transport the ligand but should also release it without difficulties.

a)



b)

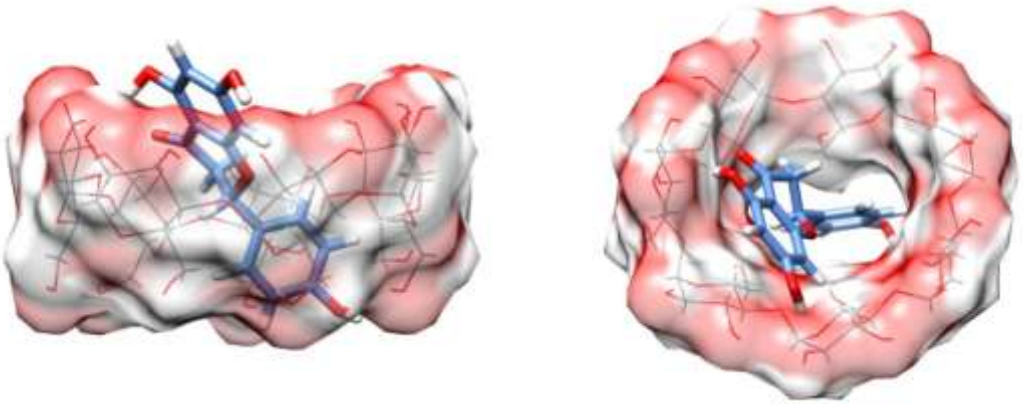


**Figure 4.** The ten best conformations of naringenin after molecular docking (**a**). Best docking conformation for naringenin-βCD in two visualization modes (the surfaces denotes the occupation volume of βCD based on Van der Waals radii) (**b**).

*3.2 Acute toxicity*

No significant changes were found in the acute toxicity test with regard to the behavior parameters. The relative weight of the organs did not have significant difference between the groups, as well as there were not any macroscopic alterations in the organs of the females. The body weight of the mice was not significantly changed due to the single dose of the treatment even after 14 days. There was no case of death for any of the treated animals. Thus, the results indicate that was no neurobehavioral alterations in the mice (data not shown).

*3.3 Effects of NR and NR/β-CD in the open field test*

In the open-field test (Figure 5), NR (20 mg/kg) and diazepam reduced the immobility time when compared to control group. NR (40 mg/kg) and diazepam significantly reduced the number of crossings when compared with the control group. In addition, NR-βCD (40 mg/kg)

treated group increased the frequency of rearing while diazepam caused a significative reduction of this parameter.

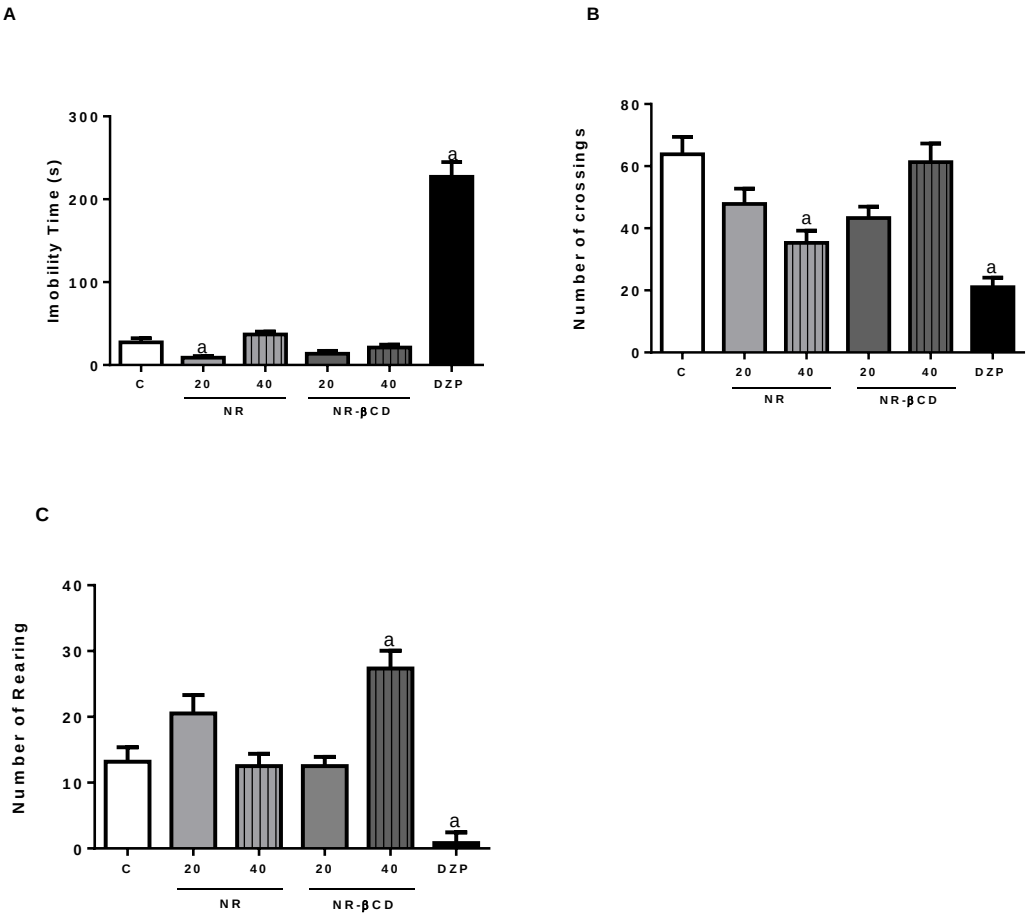



**Figure 5**. Effects of (20 and 40 mg/kg, p.o.), NR-βCD (20 and 40 mg/kg, p.o.) and diazepan (1 mg/kg, i.p.) treatment on (A) immobility time, (B) the number of total squares crossed, (C) number of rearing in the OFT. Results are shown as mean ± SEM (n = 6) by vertical bars, where $^{a}p < 0.05$ (compared with normal control group) by ANOVA, followed by post-hoc Tukey.

### *3.4 Influences of NR and NR/β-CD on depressive-like behavior in mice*

#### *3.4.1 Effects of NR and NR-βCD in the tail suspension test after acute treatment.*

According to Figure 6, NR (40 mg/kg), NR-βCD (20 and 40 mg/kg) and imipramine significantly decreased the immobility time [F(5, 30)=17.49, p<0,0001] from 118.2 ± 6.55 s

(control) to 63.83 ± 4.81 s (p=0.0001), 68.0 ± 6.84 s (p=0.0003), 65.5 ± 6.82 s (p=0.0001), respectively in the tail suspension test.

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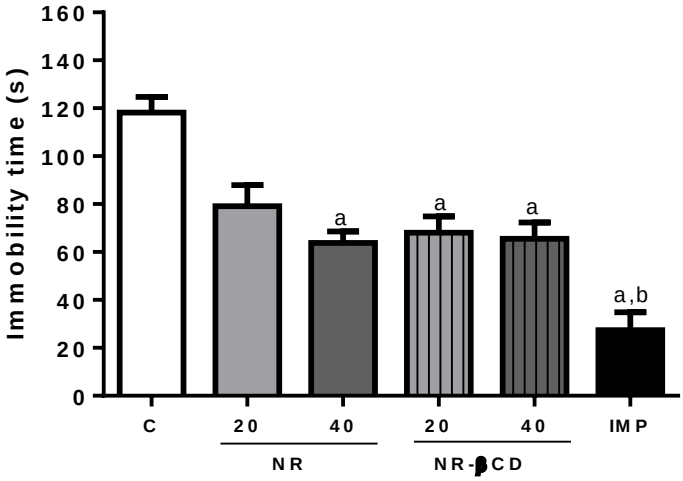


**Figure 6**. Effects of NR (20 and 40 mg/kg, p.o.), NR-βCD (20 and 40 mg/kg, p.o.) and imipramine (30 mg/kg, i.p.) in the tail suspension test. Values are expressed as mean ± standard error of the mean (n = 6). [a]p <0.05, compared to the negative control group; [b]p <0.05, compared to the NR 20 mg/kg by ANOVA followed by Tukey.

*3.4.2 Effects of NR and NR-βCD in the forced swimming test after acute treatment.*

As shown in Figure 7 the results showed that all treated groups has shown a significantly decrease [F(5, 30)=63.96, p=0.001] the immobility time compared to control group. The group treated with the dose of 20 mg/kg of NR has a significantly lesser (p=0.0001) immobility time (3.33 ± 2.17 s) than 40 mg/kg of NR (24.33 ± 4.58 s). Similarly, the group treated with the dose of 20 mg/kg of NR-βCD has a significantly lesser (p=0.0007) immobility time (10.33 ± 1.52 s) than 40 mg/kg of NR-βCD (24.0 ± 5.0).

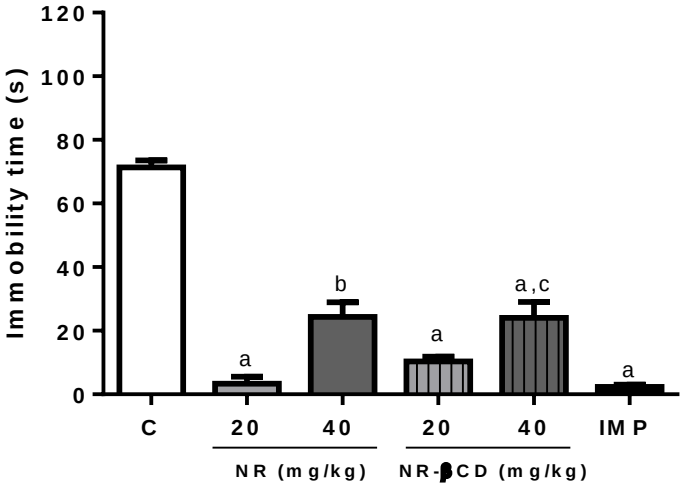


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**Figure 7**. Effects of NR (20 and 40 mg/kg, p.o.), NR-βCD (20 and 40 mg/kg, p.o.) and imipramine (30 mg/kg, i.p.) in the forced swimming test. Values are expressed as mean ± standard error of the mean (n=6). [a]p<0.05 compared to the negative control group; [b]p <0.05 NR 20 mg/kg vs. NR 40 mg/kg; [c]p <0.05 NR-βCD 20 mg/kg vs. NR-βCD 40 mg/kg by ANOVA followed by Tukey.

### *3.5 Mechanisms involved in the antidepressant-like effect of the NR*

#### *3.5.1 Involvement of the serotoninergic system in the anti-depressant like effect of NR*

The statistical analysis revealed a significantly reduction [F(3, 20)=9.27, p=0.0005] in the immobility time induced by NR 20 mg/kg (11 ± 2.71 s), NR+OND (20.0 ± 7.52 s) and OND (5.67 ± 2.49 s), as present in Figure 8. However, the pretreatment with ondansetron (antagonist of 5-$HT_3$ receptor) had no effect on the immobility time produced by of 20 mg/kg of NR in FST. It's suggested that there is no participation of 5-$HT_3$ serotonergic receptors in the antidepressant like effect of NR.

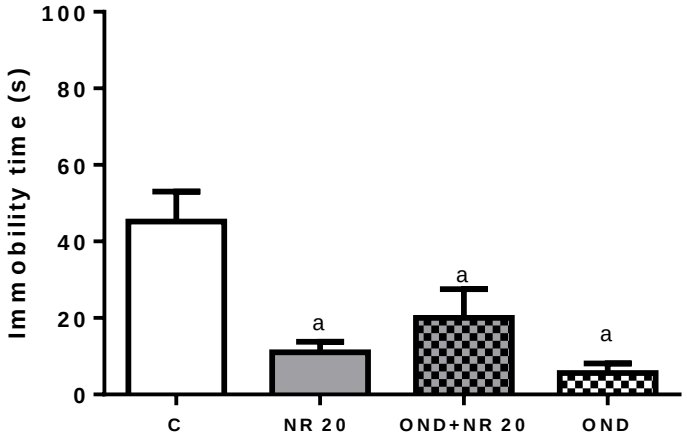


**Figure 8**. Effect of pretreatment of mice with ondansetron (1 mg/kg, i.p. a 5-$HT_3$ receptor antagonist) on the anti-immobility effect of NR (20 mg/kg) in the FST. Values are expressed as mean ± standard error of the mean (n = 6). $^{a}p < 0.05$, compared to the negative control group by ANOVA followed by Tukey.

*3.5.2 Involvement of the noradrenergic system in the anti-depressant like effect of NR*

As shown in Figure 9, the immobility time in the FST was significantly reduced [F(3, 20)=11.36, p=0.0001] in the NR treated group (11.00 ± 2.78 s) when compared to the control group (45.17 ± 7.85 s). Interestingly, the effect of NR 20 mg/kg was abolished (46.17 ± 7.36 s) than control group (p=0.007), when the animals were pre-treated with yohimbine, suggesting that there is participation of noradrenergic receptors, especially α-2, in its antidepressant activity.

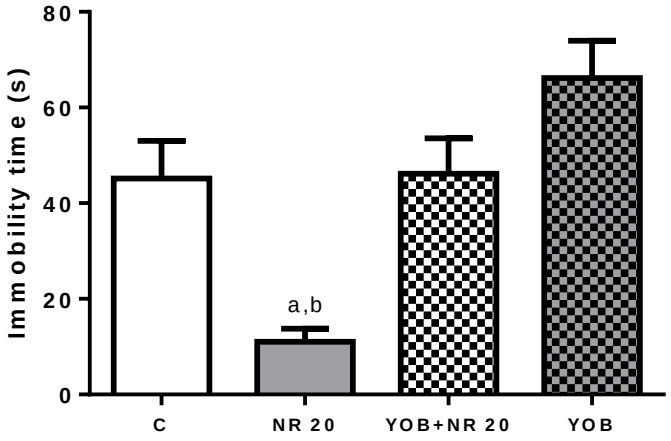


**Figure 9**. Effect of pretreatment of mice with yohimbine (Yob) (1 mg/kg, i.p.) on the anti-immobility effect of NR (20 mg/kg) in the FST. Values are expressed as mean ± standard error of the mean (n = 6). [a]p <0.05, compared to the negative control group; [b]p <0.05, compared to the Yob group 1 mg/kg + NR 20 mg/kg per ANOVA followed by Tukey.

*3.5.3 Involvement of the dopaminergic system in the anti-depressant like effect of NR*

As shown in Figure 10, the immobility time in the FST was significantly reduced [F(3, 20)=32.90, p=0.0012] in the NR treated group (11.00 ± 2.90 s) when compared to the control group (86.33 ± 14.59 s). Interestingly, the effect of NR 20 mg/kg was not only abolished, but also was significantly greater (161.5 ± 12.53 s) than control group (p=0.0012), when the animals were pre-treated with haloperidol. Additionally, haloperidol itself has a significantly effect (p=0.0119) in the immobility time in the FST (144.5 ± 13.55 s) when compared to the control group (86.33 ± 14.59 s).

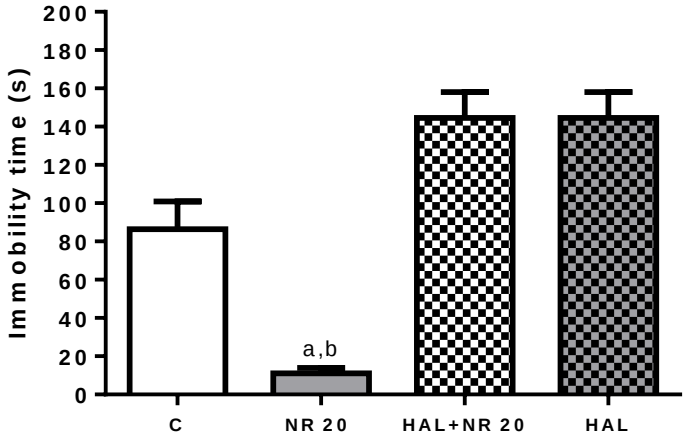


**Figure 10.** Effect of pretreatment of mice with haloperidol (0,2 mg/kg, i.p. an dopaminergic $D_2$-receptor antagonist) on the anti-immobility effect of NR (20 mg/kg, p.o.) in the FST. Values are expressed as mean ± standard error of the mean (n = 6). $^{a}p < 0.05$, compared to the negative control group; $^{b}p < 0.05$, compared to HAL 0.2 mg/kg + NR 20 mg/kg.

## 4 Discussion

The present study evaluated the effects of acute treatment of flavonoid naringenin and its beta-cyclodextrin complex after exposure to inescapable stressors or behavioral despair such as tail suspension test and forced swim. These tests have been widely used to study the neurobiology of depression [44].

Naringenin, a citrus flavonoid that possesses various biological activities, has emerged as a potential therapeutic agent for the management of a variety of disease [45]. However, the use of naringenin as a natural herbal medicine is greatly limited due to its low water solubility and bioavailability [41].

Inclusion complexes of β-CD have successfully used to improve solubility, chemical stability and bioavailability of a number of poorly soluble compounds [38]. Thus, several methods can be used to obtain inclusion complexes, a range of analytical techniques can be used to as certain the formation of β-CD complexes, such as scanning electron microscopy (SEM), infrared spectroscopy (FTIR), differential scanning calorimetry (DSC),

thermogravimetry (TG), ultraviolet spectroscopy (UV-vis), X-ray diffractometry (XRD) and nuclear magnetic resonance spectroscopy (NMR) [20,38,40-43]. The combination of the information generated by all these techniques or at least three of these is able to prove whether or not β-CD complexes have formed. In our study, the inclusion complex between naringenin and β-CD was monitored using SEM, FTIR, $^{1}$H NMR and $^{1}$H-$^{1}$H ROESY NMR.

SEM is a qualitative method often used to study the structural aspects of raw materials as β-CD, phytocompounds as naringenin and derivatives of this as the inclusion complexes [20,38,40-43,46]. The accented changes in morphology, size of naringenin:β-CD inclusion complex and similarities with inclusion complex obtained by Yang et al. [41], allow us to affirm that an new material was obtained.

In FTIR spectra, alterations in shift, shape, intensity and extinction of peaks are used for verification of inclusion complexes formation [20,38,40-43]. In naringenin:β-CD inclusion complex spectra, the band intensity increase at 3303 $cm^{-1}$, suggest a strong interaction between host and guest. The maintenance of the peak at 1024 $cm^{-1}$ reinforces the hypothesis that no chemical interaction is taking place on the external face of the host [39]. All observed alterations confirm that naringenin inclusion in β-CD cavity and, the disappearance of bands at 2925 $cm^{-1}$ and 2835 $cm^{-1}$ (C-H sp3, axial deformation) of guest suggest that the flavonoid enters the β-CD by means of the condensed rings A and C portions, portion with lower polarity of the molecule.

In NMR spectra of β-CD, the alterations in proton H-5, located in cavity of host, confirms once more that the guest is in their cavity. The protons H-5 are located inside the cavity, which are near the narrow side of the cavity and the greater displacement suffered by this proton allow to affirm that naringenin penetrated deeply into cavity of CD by wider side. In $^{1}$H-$^{1}$H ROESY experiments, the NOE interactions among spins separated by shorter distances that 4 Å, enables the formation of cross-peaks which given important information

about the structure of inclusion complex. The transfer of nuclear spin polarization from one spin to another spin via cross-relaxation allows verifying how the guest molecule enters the cavity of the host [20].

As obtained by Yang et al. [41], ours $^{1}$H-$^{1}$H ROESY results showing the strong correlation of the H-2'/H-6' and H-3'/H-5' protons of naringenin with H-3 and H-5 of β-CD. These results indicate that the ring C of naringenin was included in the β-CD as suggested by FTIR analysis.

Molecular docking is a computational procedure used to study and understand the interaction profiles between small ligands and macromolecules, providing an accessible 3D molecular visualization commonly inaccessible by the usual analytical techniques. Interestingly, the docking results presented here are in agreement with analytical evidences, corroborating the possibility of complex formation and allowing to a better understanding of this process.

Our docking results ratify the analysis made for FTIR spectra (extinction of peaks from axial deformation attributed at C-H, $sp^3$), 1D$^{1}$H NMR that suggest the interaction of internal hydrogens from host (H-3 and H-5) with guest molecule and still, while $^{1}$H-$^{1}$H ROESY NMR suggest that interaction among the ring B aromatic protons of flavonoid structure and the protons H-5 of β-cyclodextrin. Evidence demonstrates satisfactory complexation. The study by Yang et al. [41] showed that the water solubility and thermal stability of naringenin were increased in the inclusion complex with cyclodextrins. In view of the limitations of NR application, this complex is useful for the development of drugs derived from medicinal plants.

In order to determinate the toxicity, NR was evaluated using OECD protocol (OECD guideline 423, 2001). According to this protocol, NR falls within Category 5 (substance with $LD_{50}$ greater than 2000 mg/kg and less than 5000 mg/kg), being considered of low toxicity.

These results are in agreement with the findings reported by Oritz-Andrade et al. [47] which used male mice.

After the characterization of NR-βCD complex and toxicity evaluation, the antidepressant effect was assessed for two experimental models, the tail suspension test and forced swimming test. Our results demonstrate that NR an NR-βCD complex administered by oral route produces significant antidepressant-like action. The open field test was also performed because the locomotor activity can influence in the time of immobility due to central nervous system stimulants effects.

The OFT has been described as a well-established paradigm to measure locomotion, depression-like states, and anxiety-like behavior in animals by when confronted with a stressful or threatening situation and commonly used for behavioral phenotyping studies [48]. The test has been shown to be sensitive to the anxiolytic-like effects of classical benzodiazepines and 5-$HT_{1A}$ receptor agonists and is therefore a validated procedure to assess anxiety-like behavior in rodents [48, 49].

FST and TST have the same observation parameter, immobility time, measuring the coping behavior of animals and are widely used in CNS surveys. The variability in response to certain antidepressants indicates potentially different substrates and neurochemical pathways mediating performance [50, 51]. In this study, imipramine was used as standard drug. This antidepressant induces significant reduction of immobility at lower doses in the tail suspension test than in the forced swimming test [51, 52].

In order to investigate antidepressant-like effects, mice were treated orally with NR and its complex and performed to TST and FST. In FST, there was observed a significant reduction in immobility time caused by NR (20 and 40 mg/kg) and NR-βCD (20 and 40 mg/kg) relative to the control group. NR 20 mg/kg had a satisfactory result reduction in immobility time, followed by NR-βCD 20 mg/kg, NR-βCD 40 mg/kg and NR 40 mg/kg. In

the OFT the NR 20 mg/kg not alter the number of crossing and rearing and promoted a decrease in the immobility time, that reinforces their anti-depressant effect.

In the TST, NR (40 mg/kg) significant reduced the immobility time, followed by NR-βCD 40 mg/kg and NR-βCD 20 mg/kg. The parameters found in this study for OFT are similar to those of anxiolytic drugs such as diazepam (benzodiazepine receptor agonist) [48]. Another study suggested that repeated treatment with naringin at small dose (2.5 and 5 mg/kg, i.p. once daily for 7 days) range increased locomotor activity and may be relevant in the treatment of psychiatric conditions characterized of behavioral inhibition and decreased exploratory activity [53].

Many studies demonstrated that naringenin possessed different biological activities as antioxidant, anti-inflammatory, anticancer, antidiabetic, cardiovascular and central nervous system [45]. The antioxidant and anti-inflammatory potential of naringenin found in previous research [54]. Among other properties, Peng and colleagues [55] found in their study the possible degree of neuroprotection in the CNS due to its permeability between 250 and 350 nm/s and its transport in the cerebral cortex and striatum, with a reduced level in the later. The neuroprotective properties in NR in animal models have been reported in some studies. Its ability to traverse the blood-brain barrier (BBB) and exert a diverse array of neuronal effects through its ability to interact with protein kinase C (PKC) signaling pathways is well known and makes it the subject of research in the CNS area [56].

Depression is a complex disorder with symptoms at behavioral level, but also at the physiological. Some hypotheses include the pathophysiology of depression as the monoaminergic system is one of the most important targets in the pathophysiology and treatment of depression [57]. Still unknown pathophysiology, the monoaminergic hypothesis that relates the monoamine deficiency, the reduction of the gabaergic function, the hypofunction of the dopaminergic system and the neurotrophins with the neurotrophic factor

derived from the brain (BDNF) of trophic effects and on the plasticity neuronal [58]. Thus, it is believed that several neuronal systems are involved in the neurochemistry and neurobiology of depression [59].

The serotoninergic system associated with activity in the hippocampus and the prefrontal cortex (PFC) mechanisms suggested a role in depression development as neural regulation of mood, emotion, appetite and cognition and normalities in 5-HT neurotransmission includes low neuronal production of serotonin or of postsynaptic receptors, reduced excitatory inputs or excessive self-inhibition, reduced 5-HT synthesis [60]. Attention, memory, stress, decision making and arousal process involving several behaviors a land cognitive functions are modulated by the noradrenergic system associated with activity in the PFC, amygdala, and hypothalamus [61,62]. The dopaminergic system modulates the function of point-to-point neurotransmission influences, the limbic system and striatum and affects complex processes like mood, attention and emotion [62,63].

Thus, we investigated the involvement of serotonergic, noradrenergic and dopaminergic systems in the antidepressant-like effect caused by NR on the FST.

The data of this study suggest that there isn't involvement of the serotoninergic system of 5-$HT_3$ receptors in the antidepressant-like effect induced by NR. It was observed that ondansetron, a selective antagonist of 5-$HT_3$ receptors, was ineffective in reversing the decrease in immobility time caused by NR in the FST. Although it is less investigated than 5-$HT_{1a}$ and 5-$HT_{2a/2c}$, the performance of 5-$HT_3$ receptors may contribute to the action of antidepressants [64]. In the brain, 5-$HT_3$ receptors control dopamine, acetylcholine release and GABAergic system and their excitation also excites cortical GABAergic neurons [65]. Besides that, 5-$HT_3$ antagonists are used for postoperative nausea and vomiting treatment though have also been suggested to be a possible adjunctive treatment option for obsessive-compulsive disorder [65,66]. Our results contrast with the obtained by Yi et al. [67] that

demonstrate that NR possess potent antidepressant-like property via central serotonergic and noradrenergic system in TST.

Interesting finding in the present study is the fact that antidepressant-like effect of NR on the FST was blocked by pretreatment with yoimbine, a α2-adrenoceptor antagonist, suggesting the involvement of the noradrenergic system in the antidepressant-like effect of this flavone. Pharmacological evidences of treatment with antidepressants generally acts on serotonergic and noradrenergic neurotransmission, the latter being an important therapeutic alternative in the treatment of depression [68,69].

In the investigation of dopaminergic systems, haloperidol ($D_2$ receptor antagonist) reversed the reduction in immobility time observed with NR in FST suggesting the involvement of this system. In Alzheimer disease NR (25–100 μM) considerably decreased Aβ-induced free radical-mediated neurotoxicity in PC12 cells [70]. Parkinson's disease demonstrated strong anti-oxidant properties of NR for dopaminergic neurons against oxidative stress caused by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium hydrochloride (MPP +). According to previous reports 6-OHDA is a neurotoxin of which toxicity is relatively selective for catecholaminergic neurons. It induces efficient and long lasting noradrenaline and dopamine depletion [71]. In this study, we demonstrated the hypothesis that naringenin would modulate the noradrenergic and dopaminergic system of mice in FST.

These findings are in agreement with several pharmacological reports demonstrating that the antidepressant potential of flavonoids. The naringenin and NR-βCD reduced immobility time, appears to be mediated by dopaminergic and noradrenergic system. In addition, the pharmacological effects are improved compared to CD complexation when compared to the free substance. The complexation presented has biotechnological value in the treatment of depression; however, it needs more studies regarding clinical feasibility.

## 5 Conclusion

This is the first study which reports the antidepressant-like activity of naringenin/β-cyclodextrin complex in animal behavior despair models. The acute treatment with this bioflavonoid produced anti-immobility effect in FST and TST, which are widely used tests in the screening of antidepressant activity. In addition, this study provides evidence that the antidepressant-like effect of NR in FST is dependent on the interaction with the dopaminergic ($D_2$ receptors) and noradrenergic (α-2 adrenoceptors) systems.

### Authors' contributions

RBAT prepared the manuscript. RBAT, MGS, EML, RGOJ, LAAR, TCD and JRGSA participated in the statistical analysis, correction and with their expertise in the discussion and finalization of the manuscript. RBAT, MGS, EML, RGOJ, TCD and FPRAR made behavior models and investigated the mechanisms of action. LJQJ, LMMM and APO contributed to the inclusion complexes and their characterizations. EBAF performed the docking docking study.

### Conflicts of interests

The authors declare no conflict of interest.

### Acknowledgments

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## CONCLUSÃO GERAL

A otenção do complexo naringenina-βCD (NR- βCD) por co-evaporação foi satisfatória com comprovada efetividade da complexação pelos métodos de caracterização.

Com relação ao perfil de segurança, foi observado que a naringenina não foi capaz de provocar mortes nos animais submetidos à avaliação da toxicidade aguda. Com isso, foi caracterizada de baixa toxicidade o que certifica a realização de estudos farmacológicos.

Com relação aos efeitos farmacológicos, o tratamento agudo com a naringenina livre e complexada apresentou atividade antidepressiva nos modelos animais de comportamento (suspensão de cauda e nado forçado). Além disso, os efeitos farmacológicos da complexação em βCD foram melhorados quando comparados com a substância livre.

Na investigação do mecanismo envolvido, este parece estar relacionado à sua ação em receptores noradrenérgicos e dopaminérgicos, entretanto, outros estudos são necessários para completa elucidação.

Os resultados obtidos sugerem que o complexo naringenina-βCD é um agente promissor e tem valor biotecnológico no tratamento da depressão, porém mais estudos com administração subcrônica e crônica são necessários para confirmação do efeito observado e estabelecimento do mecanismo de ação para viabilidade clínica.

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