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Illana Kemmerich Martins

EFEITO NEUROPROTETOR DE *Anacardium microcapum*-Duke EM DANO INDUZIDO POR 6-OHDA EM CÓRTEX CEREBRAL DE PINTAINHOS

Dissertação de Mestrado

São Gabriel, 2017.

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Dissertação apresentada ao programa de Pós-Graduação Stricto sensu em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Thaís Posser

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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". (Martin Luther King)*

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas
Universidade Federal do Pampa – *Campus* São Gabriel

EFEITO NEUROPROTETOR DE *Anacardium microcapum*-Duke EM DANO INDUZIDO POR 6-OHDA EM CÓRTEX CEREBRAL DE PINTAINHOS

Autora: Illana Kemmerich Martins

Orientadora: Prof.ª Dr.ª Thaís Posser

Data e local da defesa: São Gabriel, 02 de março de 2017.

A Doença de Parkinson (DP) é uma doença degenerativa, crônica e progressiva, acomete o sistema nervoso central e é responsável pela degeneração dos neurônios dopaminérgicos. Sabe-se que alterações genéticas e ambientais como exposição a agroquímicos, estresse oxidativo e disfunção mitocondrial estão associados à progressão da doença. A neurotoxina 6-hidroxidopamina (6-OHDA), é um análogo estrutural da catecolamina dopamina, servindo como um modelo de neurotoxicidade por mecanismos semelhantes ao observado na DP. O mecanismo de citotoxicidade atribuído a 6-OHDA está diretamente ligado com a produção de espécies reativas de oxigênio (EROs) oriundas da inibição da respiração mitocondrial e sua auto-oxidação. A busca por terapias alternativas como os antioxidantes tem crescido ao longo dos anos, buscando atenuar a progressão da DP através de compostos bioativos de plantas. Neste estudo buscou-se avaliar o efeito neuroprotetor de *Anacardium microcarpum* frente ao dano induzido pela neurotoxina 6-OHDA em fatias corticais de pintainhos. Fatias foram incubada por 2h na presença da neurotoxina e diferentes concentrações do extrato hidroalcoólico (AMHE) e frações acetato de etila (AMEAF) e metanólica (AMMF) de *A. microcarpum.* AMHE, AMMF e AMEAF (1-1000 µg/mL) não apresentaram citotoxicidade *per se* nas fatias corticais. AMMF e AMEAF restauraram a queda da viabilidade induzida por 6-OHDA (500 µM) a partir da concentração de 100 µg/mL, sendo a AMMF nesta mesma concentração, capaz de reverter a peroxidação lipídica causada pelo composto. 6-OHDA

aumentou a atividade de GST e TrxR e diminuiu a atividade da GPx, além de diminuir os níveis de GSH total e aumentar a razão GSH/GSSG. Tais efeitos não foram observados na presença de AMME. Ainda, 6-OHDA inibiu o complexo I da cadeia respiratória mitocondrial, sendo que a fração não reverteu este efeito. Além disso, a auto-oxidação de 6-OHDA não foi revertida pela planta. A fosforilação de p38, JNK1/2, ERK1/2 e AKT bem como clivagem de PARP foi avaliada frente ao tratamento com neurotoxina e fração metanólica. A fração não levou a aumentos significativos na fosforilação das MAPKs bem como na expressão destas, também não levou à clivagem da proteína PARP. Entretanto, na presença de fração e 6-OHDA houve aumento significativo na fosforilação de ERK1/2 sem alterar sua expressão. Averiguou-se o envolvimento de ERK1/2 e AKT, proteínas envolvidas nos mecanismos de sobrevivência, na neurotoxicidade induzida por 6-OHDA através do uso de inibidores. Observou-se que na presença de inibidor, o extrato não foi capaz de proteger contra o dano promovido por 6-OHDA. Nossos resultados sugerem que o extrato tem ação antioxidante contra o estresse oxidativo decorrente da inibição da respiração mitocondrial e auto-oxidação da neurotoxina, sem no entanto interferir nestes processos. Além disso, sugere-se que as proteínas anti-apoptóticas ERK1/2 e AKT estejam envolvidas no efeito neuroprotetor da fração por mecanismos ainda não conhecidos. Nossos dados mostram pela primeira vez a ação neuroprotetora de *A. microcarpum* frente ao dano neuronal induzido pela 6-OHDA em fatias cerebrais e ressaltam o potencial desta planta como fonte de compostos bioativos com potencial terapêutico.

Palavras-chave: *Anacardium microcarpum*, 6-hidroxidopamina, espécies reativas de oxigênio, disfunção mitocondrial, MAPK, AKT.

ABSTRACT

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas
Universidade Federal do Pampa – *Campus* São Gabriel

NEUROPROTECTIVE EFFECT OF *Anacardium microcapum*-Duke ON 6-OHDA INDUCED DAMAGE IN CEREBRAL CORTEX OF CHICKS BRAIN

Autora: Illana Kemmerich Martins

Orientadora: Prof.ª Dr.ª Thaís Posser

Data e local da defesa: São Gabriel, 02 de março de 2017.

Parkinson's disease (PD) is a degenerative, chronic and progressive disease, which affects the central nervous system and it is responsible for degeneration of dopaminergic neurons. It is known that genetic and environmental factors such as exposure to agrochemical, oxidative stress and mitochondrial dysfunction are associated with progression of the disease. 6-hydroxydopamine (6-OHDA) is a structural analogue of catecholamine dopamine, used as a model of neurotoxicity by similar mechanism in PD. The mechanism of cytotoxicity attributed to 6-OHDA is linked to the production of reactive oxygen species (ROS) from inhibition of mitochondrial respiration and its autoxidation. The search for alternative therapies such as antioxidants has grown over the years, seeking to mitigate the progress of PD through bioactive plant compounds. This study aimed to evaluate the neuroprotective effect of *Anacardium microcarpum* on the damage induced by neurotoxin 6-OHDA in cortical slices of chicks. Slices were incubated for 2 h in the presence of neurotoxin and different concentrations of hydroalcoholic extract (AMHE) and ethyl acetate (AMEAF) and methanol (AMMF) fractions of *A. microcarpum*. AMHE, AMMF and AMEAF (1-1000 μg/mL) did not show cytotoxicity *per se* in the cortical slices. AMMF and AMEAF restored the drop in viability caused by 6-OHDA (500 μM) from the concentration of 100 μg/mL. 6-OHDA increased GST and TrxR activity while GPx activity and total GSH levels was decreased with an augmented ratio GSH / GSSG. These effects were not observed in the presence of AMMF and 6-OHDA. Furthermore,

6-OHDA inhibited the complex I of the mitochondrial respiratory chain but this effect was not reversed by fraction as well as the self-oxidation of 6-OHDA was not avoided by the plant. Phosphorylation of p38, JNK1/2, ERK1/2 and AKT as well as PARP cleavage was evaluated against treatment with neurotoxin and methanolic fraction. The methanolic fraction and 6-OHDA did not alter phosphorylation of MAPKs, as well as expression of these proteins, not did it result in the cleavage of the PARP protein in the time studies. However, in the presence of fraction and 6-OHDA there was a significant increase in ERK1/2 phosphorylation without altering its expression. The involvement of ERK1/2 and AKT proteins in protective mechanism of fraction was analyzed through the use of inhibitors. It was observed that in presence of inhibitor, the extract was not able to protect against the damage promoted by 6-OHDA. Our results suggest that the extract presented antioxidant action against oxidative stress resulted from the inhibition of mitochondrial respiration and neurotoxin autoxidation. In addition, it is suggested that antiapoptotic proteins ERK1/2 and AKT are involved in neuroprotective effect of the fraction. Our data show for the first time a neuroprotective action of *A. microcarpum* against neuronal damage induced by 6-OHDA in cerebral slices and highlights the potential of this plant as a source of bioactive compounds with therapeutic potential.

Key words: *Anacardium microcarpum*, 6-hydroxydopamine, reactive oxygen species, mitochondrial dysfunction, MAPK, AKT.

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LISTA DE ABREVIATURAS

6-OHDA	6-hidroxidopamina
AKT	proteína quinase B / homóloga ao oncogene v-AKT
AMEAF	fração acetato de etila de *Anacardium microcarpum*
AMHE	extrato hidroalcoólico de *Anacardium microcarpum*
AMMF	fração metanólica de *Anacardium microcarpum*
CAT	Catalase
DAT	transportador de dopamina
DP	doença de parkinson
ERK1/2	proteína quinase regulada por sinal extracelular ½
EROs	espécies reativas de oxigênio
GF	fator de crescimento
GPCR	proteína G acoplada a receptor metabotrópico
GPx	glutationa peroxidase
GSH	glutationa reduzida
GSSG	glutationa oxidada
GST	glutationa S-transferase
H_2O_2	peróxido de hidrogênio
HO-1	heme oxygenase 1
JNK1/2	c-Jun N-terminal quinases
MAO	monoamina oxigenasse
MAPK	proteína quinase ativada por mitógeno
NAT	transportador de noradrenalina
Nrf-2	nuclear factor erythroid 2-related factor
$O^{\circ-}$	radical superóxido
$OH^{\circ-}$	radical hidroxila
OMS	Organização Mundial de Saúde
ONU	Organização das Nações Unidas
PH	plecstrina homóloga
Pi3K	fosfatidil inositol 3 quinase
PIP3	fosfatidil inositol 3,4,5 trifosfato
RTK	receptor de tirosina quinase

SAPK	Proteína quinase ativada pelo estresse
SNC	sistema nervoso central
SOD	superóxido dismutase
TrxR	tiorredoxina redutase

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma breve revisão da literatura sobre os temas trabalhados nesta dissertação. A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados sob a forma de manuscrito, que se encontra no item **MANUSCRITO**. No mesmo constam as seções: Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas. O item **CONCLUSÕES**, encontrado no final desta dissertação, apresenta interpretações e comentários gerais sobre os resultados dos manuscritos presentes neste trabalho. As **REFERÊNCIAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO.**

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1. INTRODUÇÃO

1.1 Espécies Reativas de Oxigênio (EROs) e Defesas Antioxidantes

Radicais livres são átomos ou moléculas que contêm a última camada eletrônica desemparelhada, formados através de reações de óxido-redução. Devido à lacuna na órbita externa do átomo, tornam-se espécies altamente reativas, agindo como eletrófilos (FERREIRA & MATSUBARA, 1997). Radical superóxido (O°-), radical hidroxila (OH°-) e o não radical, peróxido de hidrogênio (H_2O_2), são espécies reativas a oxigênio (EROs) e são formadas e degradadas pelo metabolismo natural da célula mantendo os níveis fisiológicos necessários para a sobrevivência da mesma, atuando em sistemas de sinalização celular e nos sistemas redox. Porém uma geração excessiva de EROs está implicada no aumento de radicais livres, e quando associada a uma diminuída capacidade antioxidante celular leva ao estresse oxidativo (LI et al., 2013; SALIM, 2016).

O estresse oxidativo acontece quando existe um desequilíbrio entre os fatores pró-oxidantes e os antioxidantes. O desequilíbrio entre moléculas oxidantes e antioxidantes resulta na indução de danos celulares podendo atacar lipídios, proteínas, carboidratos, ácidos nucléicos e outras substâncias oxidáveis (LI et al., 2013; SALIM, 2016). Inúmeras condições patológicas estão relacionadas ao estresse oxidativo, tais como artrite, câncer, inflamações, doenças cardíacas e degenerativas, além do envelhecimento (BRYK et al, 2017; FORCADOS et al., 2017; LI et al., 2013).

No decorrer da evolução, os seres vivos desenvolveram mecanismos adaptativos capazes de lhes permitirem co-existir com a exposição a EROs. Entre os mecanismos de defesa antioxidante celular, sistemas não enzimáticos como o tripeptídeo glutationa (GSH) e enzimas antioxidantes como a superóxido dismutase (SOD), glutationa peroxidade (GPx), glutationa S-transferase (GST), catalase (CAT) e Tiorredoxina redutase (TrxR) podem ser destacados (BIRBEN et al., 2012). Quando há limitação nestes sistemas antioxidantes, podem ocorrer diversas lesões cumulativas, levando a disfunção e morte celular através de cascatas de sinalização (HOTAMISLIGIL & DAVIS, 2016).

1.2 Sinalização Celular

1.2.1 Vias de Sinalização celular de proteínas quinases ativadas por mitógenos (MAPKs)

As proteínas quinases ativadas por mitógenos (MAPKs) são compostas por proteínas serina-treonina quinases, que medeiam a sinalização intracelular e estão associadas na diferenciação celular, proliferação, sobrevivência, transformação e morte celular (MCCUBREY et al., 2006). A resposta celular a estímulos depende de redes integradas de sinalização celular, que funcionam de uma maneira coordenada. O controle dessa rede de sinalização se dá através de proteínas quinases e fosfatases (CHANG & KARIN, 2001).

As MAPKs são reguladas por uma cascata de fosforilação, estabelecendo uma via sequencial de ativação composta de três proteínas ou mais. A transmissão de sinais através desta cascata é normalmente iniciada pela ativação de uma pequena proteína G, como a Ras, ou pela interação e ativação de componentes superiores da cascata com proteínas adaptadoras. Então, os sinais são passados ao longo da via, por proteínas quinases citosólicas, organizadas em três a cinco níveis. As quinases de cada nível fosforilam e ativam quinases localizadas abaixo, permitindo uma comunicação controlada e rápida dos sinais para vários alvos nas cascatas (Figura 1). Uma vez acionadas, as MAPKs fosforilam resíduos de serina e treonina em seus alvos específicos, nos casos em que estes aminoácidos forem seguidos por uma prolina (HOTAMISLIGIL & DAVIS, 2016; KIM & CHOI, 2015; SEGER & KREBS, 1995).

A família das MAPKs inclui proteínas quinases regulada por sinal extracelular (ERK1/2) e proteínas quinases ativadas pelo estresse (SAPKs), JNK1/2 e $p38^{MAPK}$ (CHANG & KARIN, 2001; CHEN et al., 2001). A ativação de MAPKs tem sido observada em resposta a estresse osmótico, estresse oxidativo, exposição às citocinas e injúria tóxica (COWAN & STOREY, 2003).

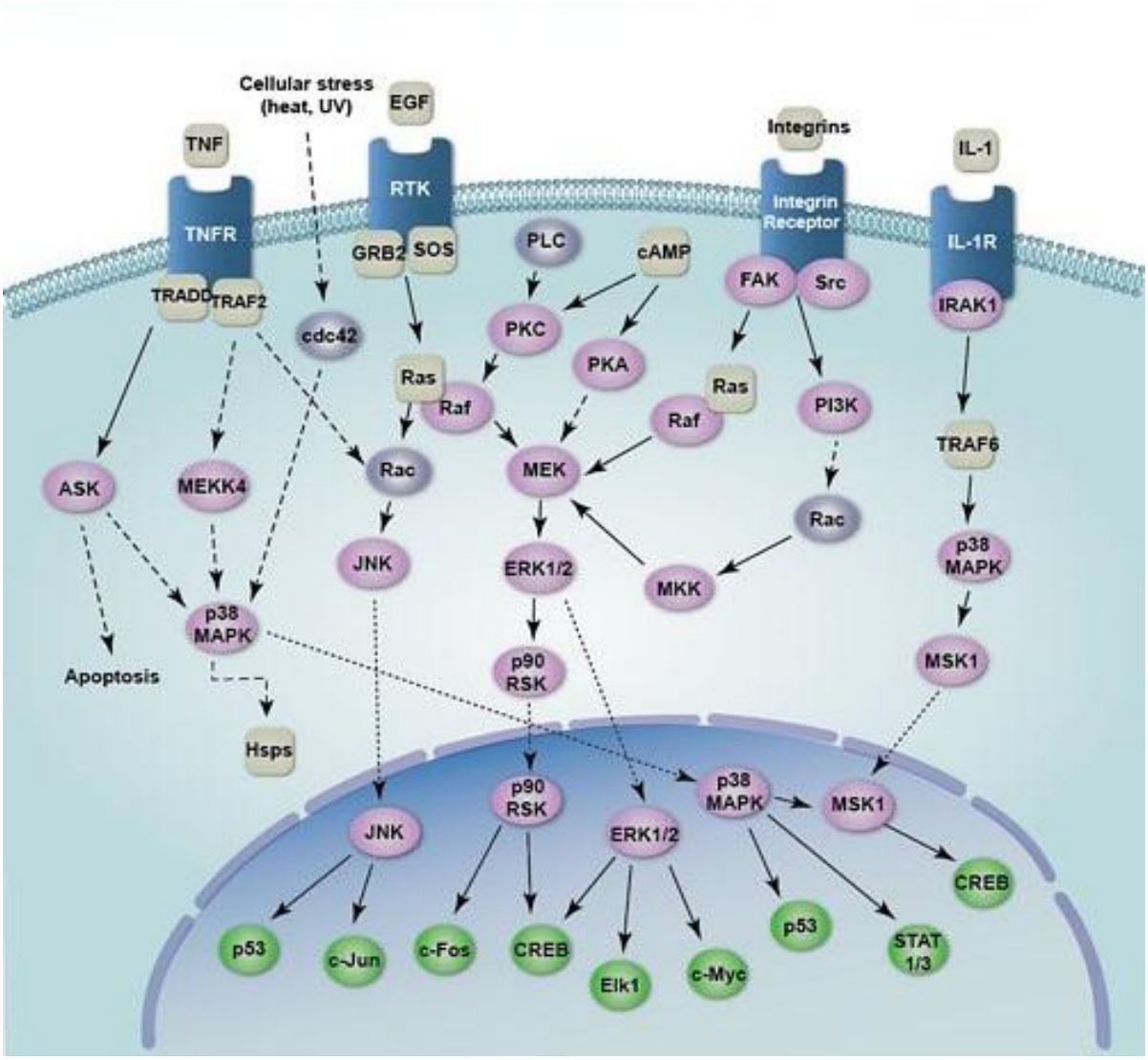

Figura 1: Via de sinalização das proteínas da família MAPK. Fonte: https://www.tocris.com

ERK1 e ERK2 são proteínas quinases serina/treonina que participam na cascata de transdução de sinal Ras-Raf-MEK-ERK (Figura 2). Esta cascata participa na regulação de uma grande variedade de processos incluindo adesão celular, progressão do ciclo celular, migração celular, sobrevivência celular, diferenciação, metabolismo, proliferação e transcrição. MEK1/2 catalisam a fosforilação de ERK1/2 humana em Tyr204/187 e Thr202/185 respectivamente. A fosforilação tanto da tirosina como da treonina é necessária para a ativação enzimática. Uma vez fosforilada ERK1/2 catalisa a fosforilação de centenas de substratos citoplasmáticos e nucleares, incluindo moléculas reguladoras e fatores de transcrição (CHU et al., 2004; ROUX & BLENIS, 2004).

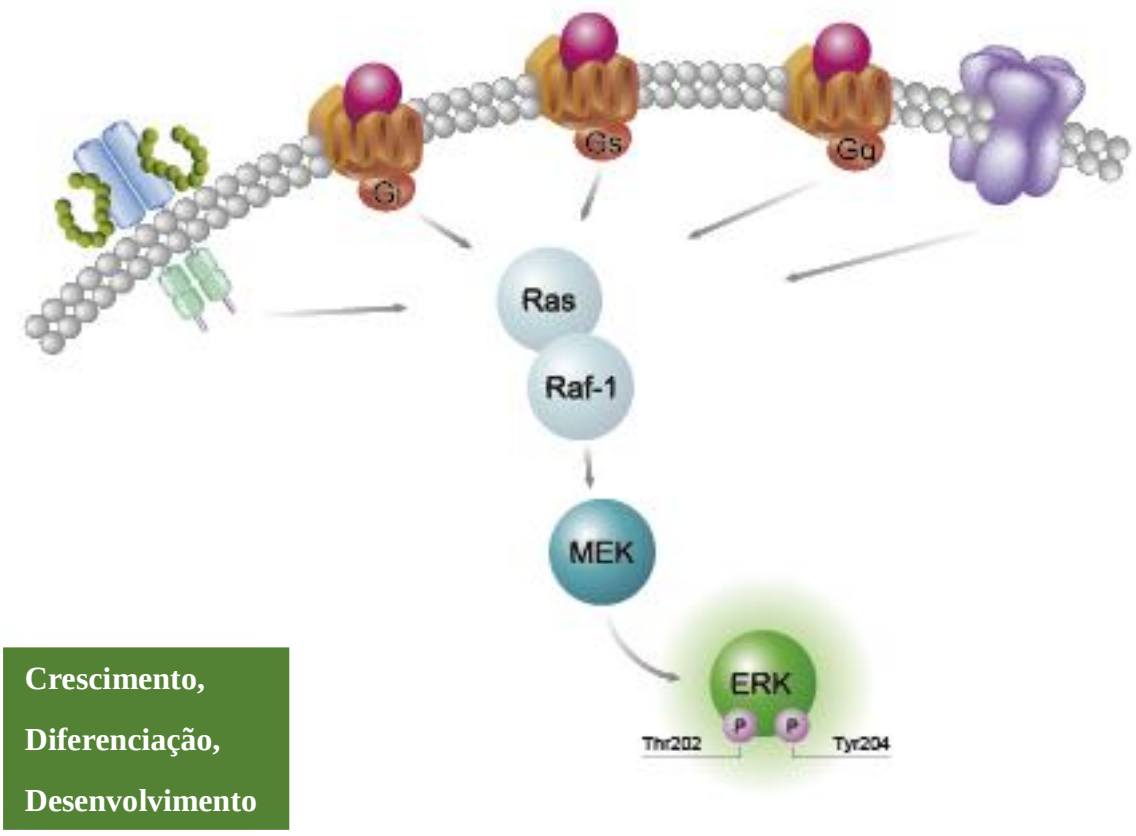

Figura 2: Ativação da quinase ERK1/2 via ativação de receptores associadas à proteínas G. Fonte: http://www.cisbio.com

A de ERK1/2 é especialmente importante no dano oxidativo em neurônios, sendo envolvida em diversas condições neuropatológicas, como isquemia, trauma cerebral e doenças neurodegenerativas (CHU et al., 2004), foi demonstrado que a indução de genes dependentes de Nrf-2 é dependente da proteína ERK1/2 embora sem participar diretamente da fosforilação deste (RICHTER et al., 2014).

O grupo de proteínas quinases p38 desempenham um papel vital em numerosos processos biológicos entre estes inflamação, ciclo celular, morte celular, desenvolvimento, diferenciação celular, senescência e tumorogênese em tipos celulares específicos (CUENDA & ROUSSEAU, 2007; ZARUBIN & HAN, 2005). A via de sinalização $p38^{MAPK}$ é mediada por proteínas quinases, respondendo a diferentes estímulos, como fatores de crescimento, citocinas inflamatórias e espécies reativas de oxigênio e podem desencadear a fosforilação em cascata de moléculas efetoras e/ou ativadoras da sinalização $p38^{MAPK}$ (NEBREDA & PORRAS, 2000). O estímulo externo é transmitido para o meio intracelular ativando membros da subfamília MKK3 ou MKK6, que são altamente específicos para $p38^{MAPKs}$. MKK6 fosforila todas as isoformas de

p38 (p38α, p38ß p38δ e p38γ), enquanto que MKK3 é seletiva para as isoformas p38α, p38δ e p38γ. MKK3/6 transmitem esse sinal à p38 que acaba sedo translocada do núcleo para o citoplasma, ativando fatores de transcrição que controlam a expressão de enzimas envolvidas em rotas antioxidantes, na proliferação, diferenciação, inflamação e apoptose (Figura 3).

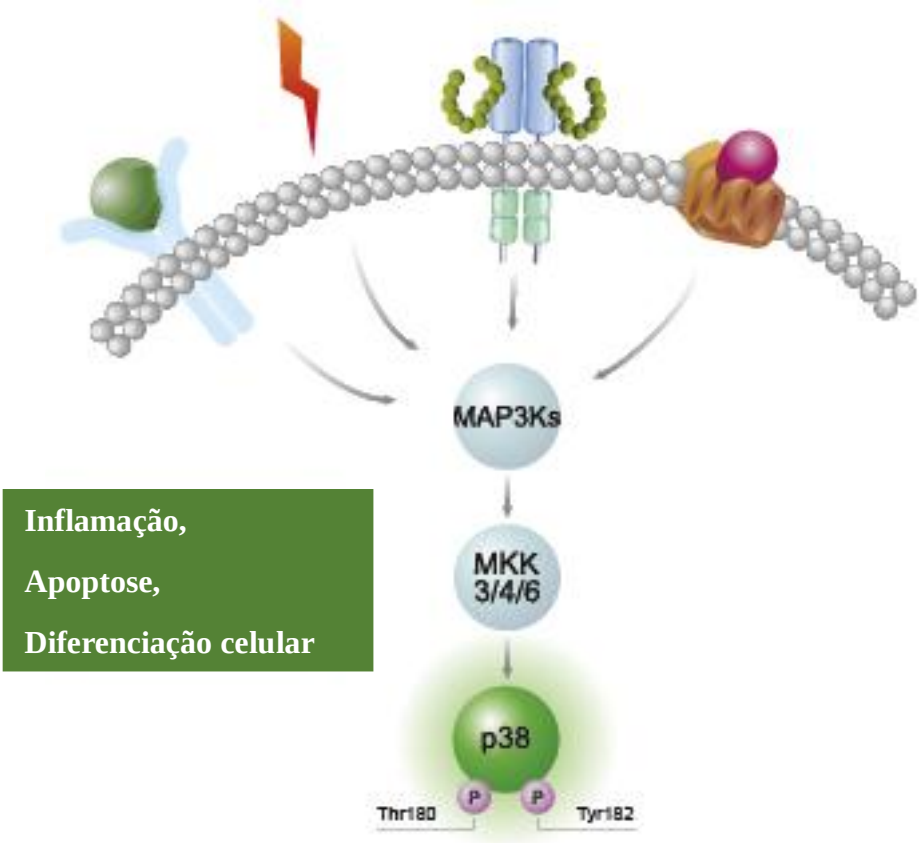

Figura 3: Via de sinalização p38^{MAPK}. Fonte: http://www.cisbio.com

As proteínas c-Jun Quinases N-terminais (JNKs), também chamadas proteínas quinase ativada por estresse (SAPKs) são membros da família MAPK. Proteínas quinase JNK são ativados por uma variedade de estresses ambientais, citocinas inflamatórias e, em alguns casos, por fatores de crescimento e agonistas de GPCR. As proteínas JNK são codificadas por três genes, JNK1, JNK2 e JNK3, que sofrem um *splicing* alternativo para gerar várias isoformas para cada gene. Estímulos específicos desencadeiam a ativação de MAPKKKs, que então fosforilam as MAPKKs MKK4 e MKK7, que por sua vez ativam SAPK/JNK por fosforilação de Thr183 e Tyr185 (COFFEY, 2014; DHANASEKARAN & REDDY, 2008). O JNK ativado transloca-se para o núcleo onde pode fosforilar múltiplos fatores de transcrição, tais como c-

Jun, ATF-2 e p53 (Figura 4). A via JNK regula numerosas respostas celulares incluindo proliferação, sobrevivência e apoptose, desenvolvimento neural, inflamação, metabolismo e reparação do DNA (LIU & LIN, 2005).

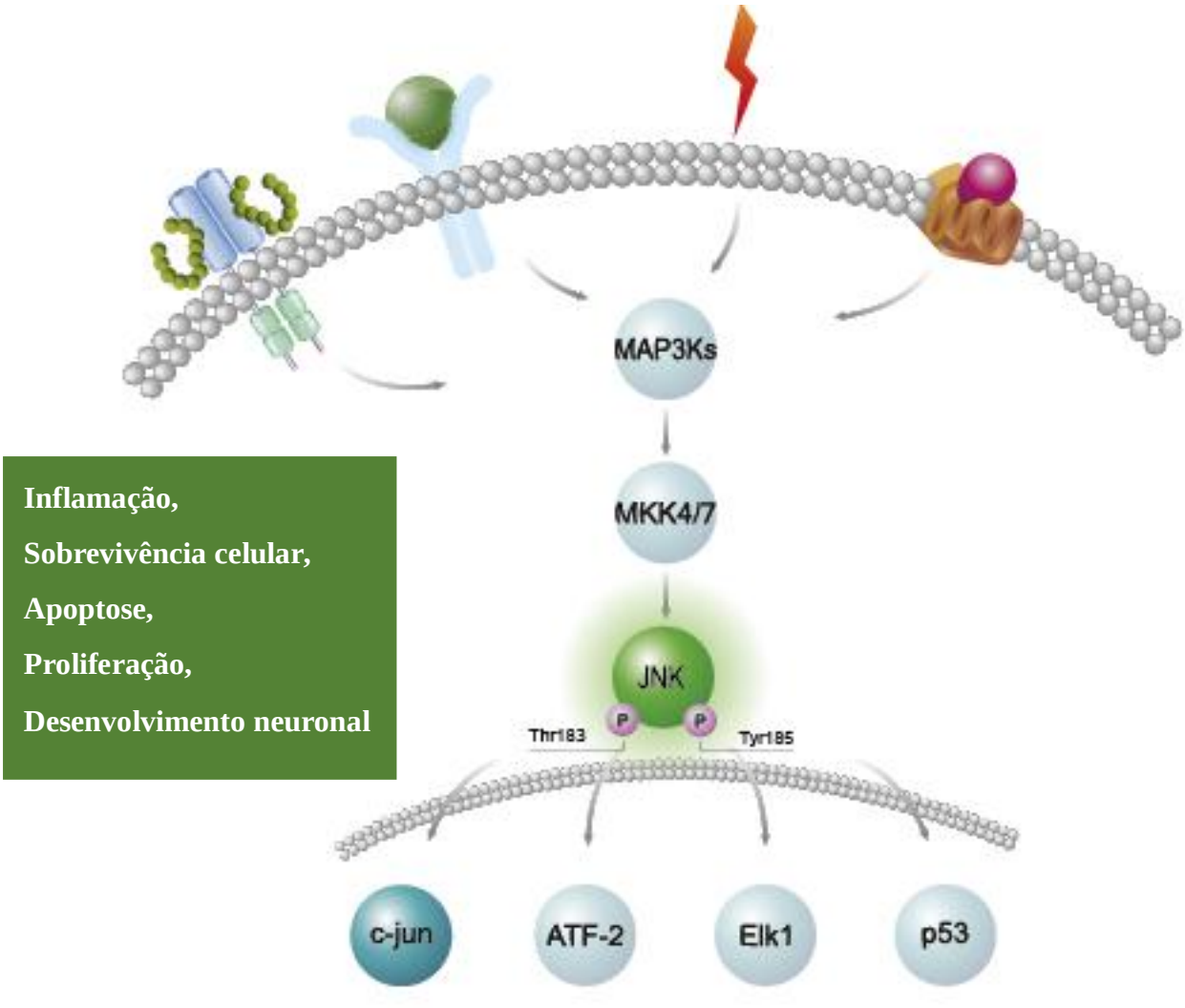

Figura 4: Via de sinalização JNK1/2. Fonte: http://www.cisbio.com

1.2.2 Akt / Proteína Quinase B (PKB)

AKT é uma proteína serina/treonina quinase atuando como regulador da sobrevivência celular e proliferação. A família Akt/PKB compreende três membros altamente homólogos conhecidos como PKBα/Akt1, PKBβ/Akt2 e PKBγ/Akt3 em células de mamíferos (BACURAU, 2013). Muito similar com outras proteínas quinases, Akt/PKB contém um

domínio estrutural conservado incluindo o domínio específico PH (domímio de homologia plecstrina), um domínio regulatório carboxil-terminal, mediando a interação entre moléculas de sinalização (CHEUNG & TESTA, 2013). Akt/PKB desempenha importante papel na via de sinalização em resposta a fatores de crescimento e outros estímulos extracelulares que regularão várias funções celulares, incluindo metabolismo de nutrientes, crescimento celular, apoptose e sobrevivência (SONG, OUYANG, BAO, 2005).

É regulada por múltiplos processos biológicos incluindo sobrevivência celular, proliferação, crescimento e metabolismo do glicogênio. Vários fatores de crescimento, hormônios e citocinas ativam Akt através da ligação no seu receptor tirosina quinase (RTK), receptor de citocina ou proteína G acoplada a receptor metabotrópico (GPCR) desencadeando ativação do lipídio quinase Pi3K. A ativação de Pi3K gera PIP3 na membrana plasmática, que consequentemente se ligará a Akt no domímio de homologia plecstrina (PH) resultando na translocação da Akt para a membrana (Figura 5) (SONG, OUYANG, BAO, 2005). Sua ativação via Pi3K inibe sinais apoptóticos, como por exemplo promovendo a ativação de Nrf2 e genes regulados por ARE ("*Antioxidant responsive element*"), como HO-1 (Heme oxigenasse-1), Trx (Tioredoxina) e Prx-1 (Peroxiredoxina) que promovem respostas celulares antioxidantes (NAKASO et al., 2003).

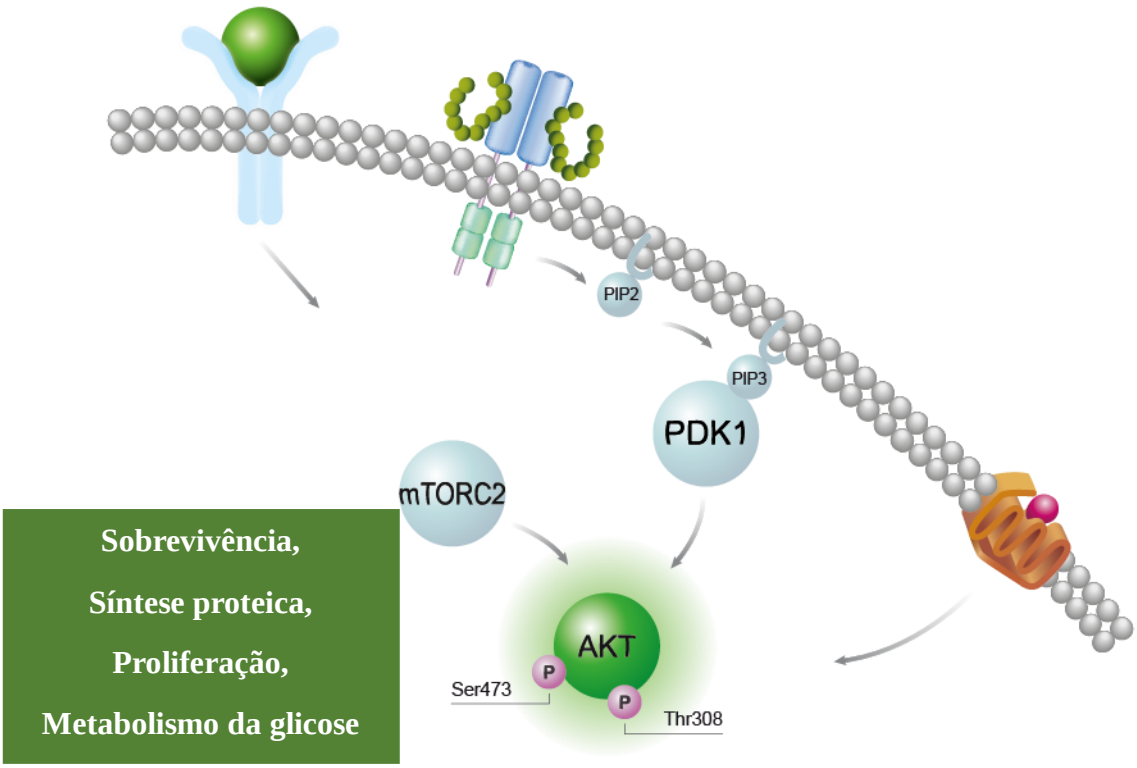

Figura 5: Via de sinalização Pi3K/Akt. Fonte: http://www.cisbio.com

1.3 Doenças Neurodegenerativas

Segundo relatório da ONU, no ano de 2010, 35,6 milhões de pessoas em todo o mundo eram portadoras de doenças neurodegenerativas, incluindo Doença de Alzheimer e outras demências, mal de Parkinson e mal de Huntington. Segundo a ONU este número tende a triplicar até o ano de 2050. Neste relatório o Brasil é apresentado como 9º país com maior número de pessoas acometidas por tais doenças, com 1 milhão de pessoas, em primeiro lugar apareceu a China, com 5,4 milhões de pessoas. Doenças neurodegenerativas estão entre as principais causas de invalidez na vida adulta, levando à dependência de cuidados de terceiros e incapacidade entre os idosos, isto tanto em países de alta renda, como em média e baixa renda. Estima-se um gasto mundial de 604 bilhões de dólares associado aos cuidados com pacientes acometidos por tais doenças (WORLD HEALTH ORGANIZATION, 2012).

Patologias do sistema nervoso central (SNC) como as doenças de Alzheimer e Parkinson, podem ser desencadeadas através do processo de envelhecimento, exposição a metais pesados, agroquímicos (BELLOU et al., 2016; BOSE & BEAL, 2016; UTTARA et al., 2009), levando ao aumento da produção de EROs, dando início ao processo oxidativo e por consequência, causando danos celulares irreversíveis ao SNC, no qual denominamos neurodegeneração (FAHN & SULZER, 2004).

Tais doenças são caracterizadas por alterações fisiológicas e bioquímicas do cérebro, além do acúmulo de componentes proteicos anormais e perda neuronal. Por exemplo: acúmulo da proteína β-amilóide nas placas senis e de proteína tau hiperfosforilada nos emaranhados neurofibrilares é observado na Doença de Alzheimer (KOCAHAN & DOĞAN, 2017); acúmulo da α-sinucleína nos corpos de Lewy é observado na Doença de Parkinson (KLINGELHOEFER & REICHMANN, 2017); agregados da proteína Huntingtina é observado na Doença de Huntington (NOPOULOS, 2016).

1.3.1 Doença de Parkinson

A Doença de Parkinson (DP) foi descrita pela primeira vez em 1817 pelo médico inglês James Parkinson, com o nome de “paralisia agitante” (FRITSCH et al., 2012). É uma doença degenerativa, crônica e progressiva, acomete o sistema nervoso central envolvendo os gânglios

da base, causando deficiência do neurotransmissor dopamina na via nigroestriatal e cortical, intervindo principalmente no sistema motor (FAHN & SULZER, 2004), consequência da degeneração dos neurônios dopaminérgicos, responsável pela produção da dopamina. Na maioria dos casos é esporádica e idiopática resultante de alguma combinação de herança poligênica, exposições ambientais, provocando disfunção neural combinada com o envelhecimento (LANDGRAVE-GÓMEZ, MERCADO-GÓMEZ, GUEVARA-GUZMÓN, 2015).

O crescimento da população idosa traz consigo um volume crescente de doenças crônicas e degenerativas, traçando um novo perfil epidemiológico. A Doença de Parkinson (DP) ou parkinsonismo primário tem uma distribuição universal, não tendo distinção de raça e classe social. Segundo a Organização Mundial de Saúde (OMS), calcula-se que até 2030, a enfermidade terá acometido cerca de 9 milhões de indivíduos no mundo (WIRDEFELDT et al., 2011). A DP acomete ambos os sexos, mas a incidência da doença é prevalente em homens, com uma proporção de 3:1 com relação a mulheres, manifestando-se principalmente em pessoas idosas acima dos 60 anos. Tal preferência é atribuída aos efeitos protetores do estrogênio e doenças recessivas relacionadas ao cromossomo X (DEXTER & JENNER, 2013; SHULMAN, DE JAGER, FEANY, 2011).

Clinicamente, a DP caracteriza-se por tremor de repouso, rigidez, bradicinesia e comprometimento da marcha, conhecidos como os principais sintomas da patologia. Tendo ainda manifestações não dopaminérgicas da DP como congelamento da marcha, instabilidade postural, dificuldade na fala, disfunção do sono, transtornos do humor, comprometimento cognitivo e demência (JANKOVIC, 2008). Ao aparecerem os primeiros sintomas, a substância negra já perdeu cerca de 60% dos neurônios dopaminérgicos e o conteúdo de dopamina no estriado está aproximadamente 80% abaixo do normal (FAHN & SULZER, 2004) (Figura 6). Os sintomas motores podem ser atenuados com medicamentos que agem principalmente no sistema dopaminérgico. Porém, a eficácia diminui à medida que a degeneração progride.

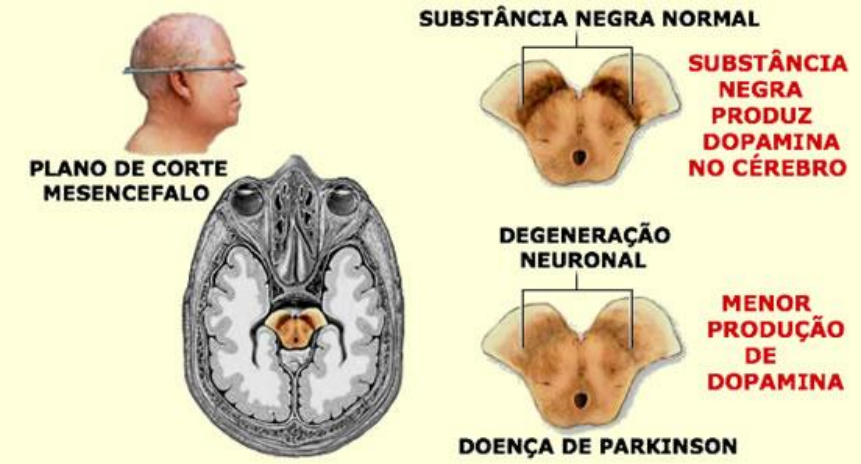

Figura 6: Corpo estriado de paciente normal (acima) e portador da DP (abaixo) mostrando a depleção da dopamina na doença. Fonte: https://museudinamicointerdisciplinar.wordpress.com/tag/dopamina/

Outra característica da doença é a presença de corpos de Lewy. A morte neuronal dopaminérgica está associada diretamente aos corpos de Lewy, resultando na depleção da produção de dopamina no corpo estriado. Dentro dos corpos de Lewy encontra-se a proteína α-sinucleína e ubiquitina, que juntamente com outros fatores contribuem para a degeneração dopaminérgica (WIRDEFELDT et al., 2011), como mostra a Figura 7. O acúmulo de α-sinucleína gera uma reação em cascata, resultando no bloqueio do sistema de degradação celular ubiquitina-proteossoma (ROGERS et al., 2010).

A sua etiologia ainda é desconhecida, mas sabe-se que alterações genéticas como nos genes PARK1, PARK2, LRRK2, fatores ambientais como exposição a herbicidas e pesticidas, estresse oxidativo e disfunção mitocondrial estão associados ao aparecimento da doença (Figura 6) (BOSE & BEAL, 2016; UTTARA et al., 2009; WIRDEFELDT et al., 2011).

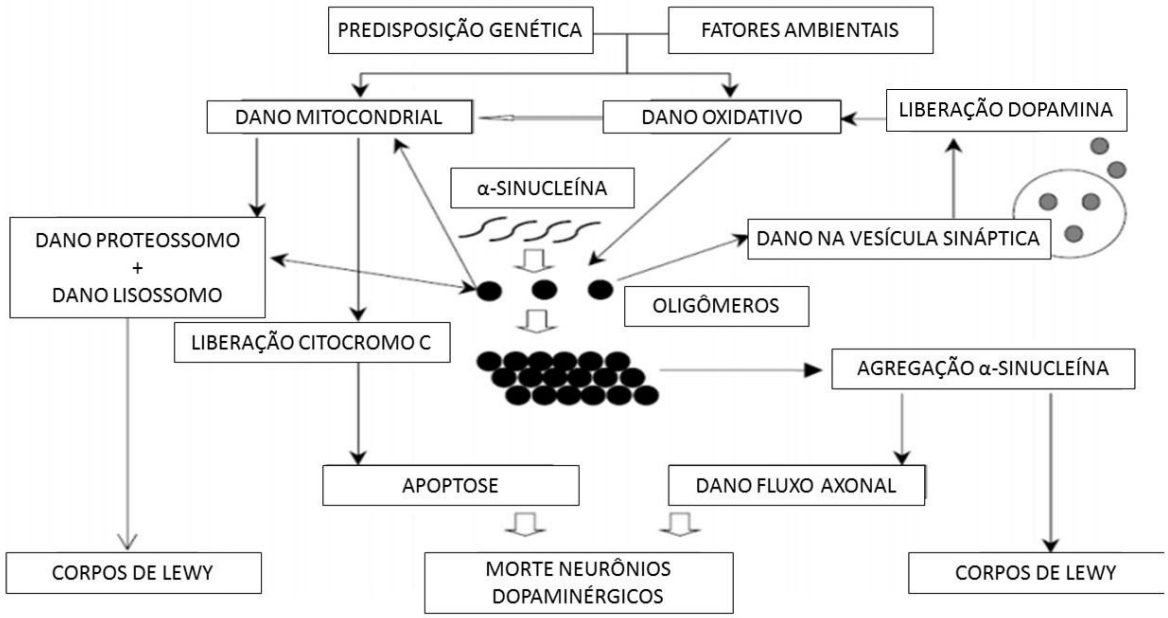

Figura 7: Apresentação esquemática da morte neuronal na Doença de Parkinson. Adaptado de Minuzo et al., 2008.

1.3.2 Modelos de Doença de Parkinson

O conhecimento sobre a patologia da DP contribui para o desenvolvimento de modelos animais para um melhor entendimento sobre os mecanismos de morte neuronal, assim podendo identificar possíveis alvos para terapia. Modelos da DP foram desenvolvidos a partir da utilização de neurotoxinas dopaminérgicas capazes de destruir seletivamente neurônios dopaminérgico e modelos de linhagens genéticas com deleção genética para mimetizar a doença (BOVÉ & PERIER, 2012).

Entre as toxinas usadas para indução da neurodegeneração dopaminérgica, estão o MPTP (1-metil-4-fenil-1,2,3,6-tetraidropiridina), paraquat, rotenona e 6-OHDA, sendo que todas tem como característica comum a capacidade de gerar estresse oxidativo, tendo como consequência a morte dos neurônios dopaminérgicos (BLESA & PRZEDBORSKI, 2014; BOVÉ & PERIER, 2012).

1.3.3 6-Hidroxidopamina (6-OHDA)

Descrita pela primeira vez em 1959, a 6-hidroxidopamina (6-OHDA), também conhecida como 2,4,5-trihidroxifeniletilamina, é a neurotoxina mais utilizada para mimetizar os sinais da DP tanto *in vivo* como *in vitro*. É um análogo estrutural das catecolaminas dopamina e noradrenalina, exercendo efeitos tóxicos aos neurônios catecolaminérgicos (BLANDINI et al., 2008) (Figura 8).

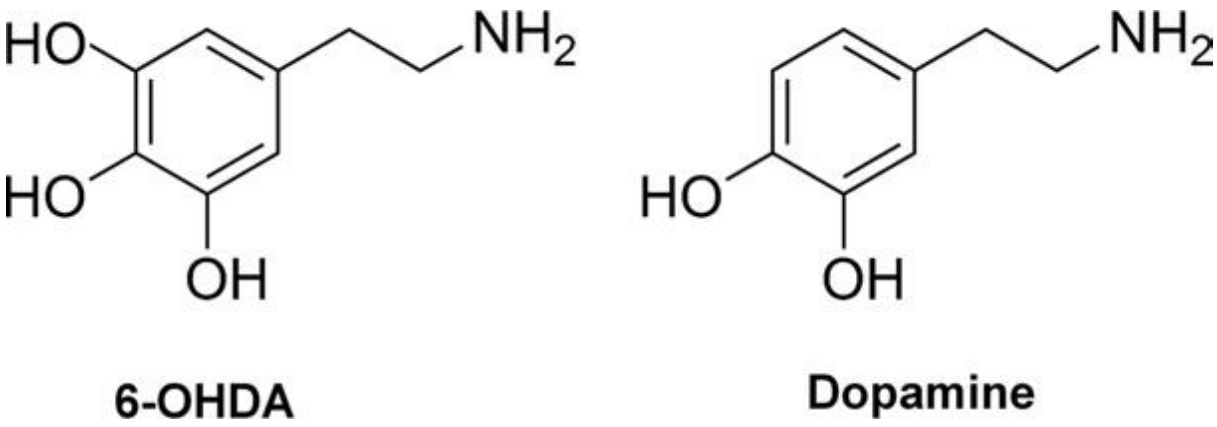

Figura 8: Comparação da estrutura química da neurotoxina 6-OHDA com o neurotransmissor Dopamina. Adaptado de Bové; Perier, 2012.

Os efeitos neurotóxicos da neurotoxina estão relacionados à acumulação da 6-OHDA em neurônios catecolaminérgicos seguido da alteração da homeostase celular e danos neuronais irreversíveis. O armazenamento ocorre por meio de transportadores de membrana de dopamina (DAT) ou noradrenalina (NAT), devido a sua semelhança estrutural com as catecolaminas endógenas (Figura 9).

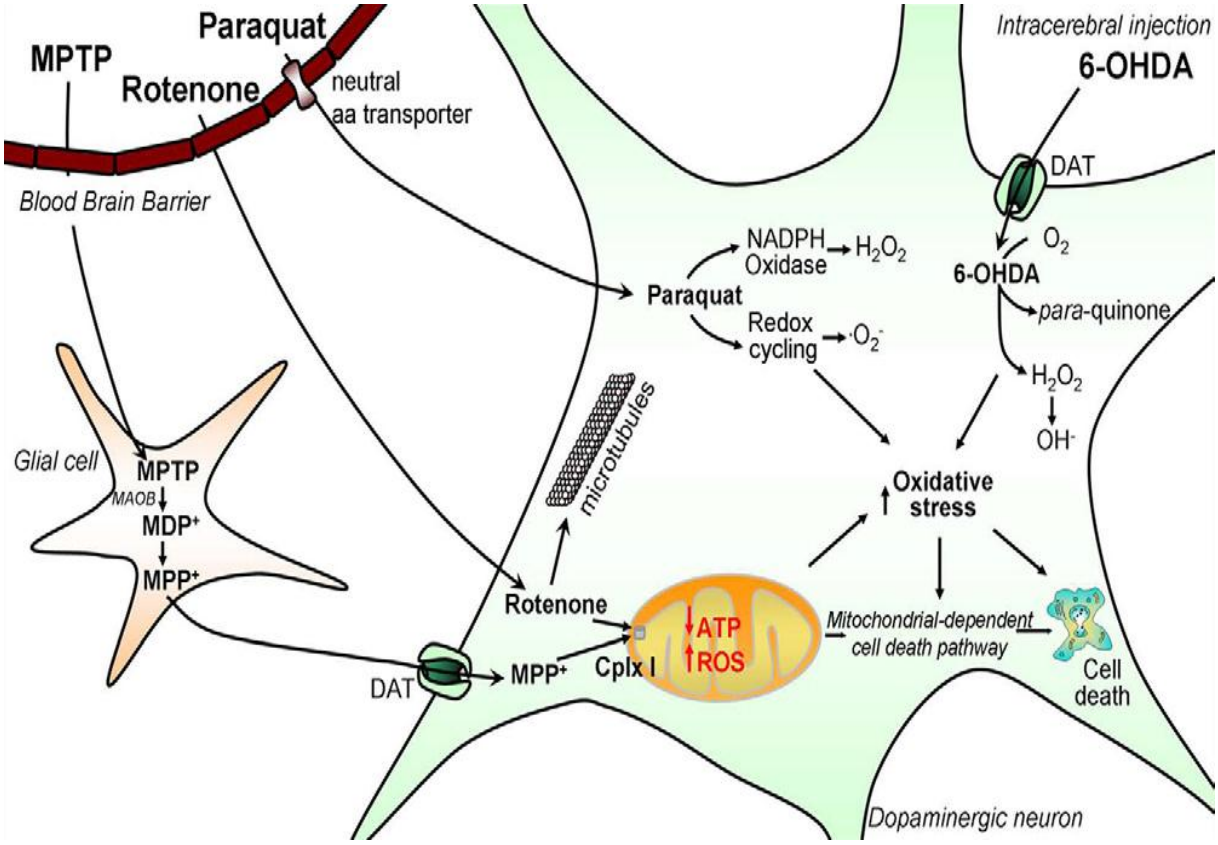

Figura 9: Mecanismo de ação da 6-OHDA e outras neurotoxinas em neurônio dopaminérgico. 6-OHDA é transportado para dentro do neurônio via transportador da dopamina (DAT). No meio citoplasmático, sofre oxidação dando origem a seus metabólitos secundários: peróxido de hidrogênio (H_2O_2) e para-quinona. O estresse oxidativo consequente da oxidação da neurotoxina leva a danos a nível mitocondrial e morte celular por vias apoptóticas. Adaptado de Bové; Perier, 2012.

Em condições fisiológicas, 6-OHDA sofre reações enzimáticas e não-enzimáticas. A oxidação da neurotoxina pela monoamina oxigenase (MAO) gera peróxido de hidrogênio (H_2O_2), induzindo a produção de outros radicais. Quando se auto-oxida (Figura 9), gera espécies tóxicas como quinonas e peróxido de hidrogênio (H_2O_2) (SIMOLA et al., 2009) (Figura 10). O aumento das espécies reativas de oxigênio leva à depleção de enzimas endocelulares, ampliando a neurotoxicidade, resultando em dano neuronal (BLUM et al., 2001). A neurotoxicidade da 6-OHDA pode induzir disfunções mitocondriais através da inibição dos complexos I e IV da cadeia respiratória (GLINKA, GASSEN, YOUDIM, 1997; GLINKA & YOUDIM, 1995). A sinergia da 6-OHDA com o Fe na neurotransmissão dos neurônios dopaminérgicos, compromete o sistema de transporte de elétrons (TOBÓN-VELASCO et al., 2013) e a homeostase de Ca^{2+} mitocondrial (BERRETTA et al., 2005), tendo como consequência a peroxidação lipídica e consequentemente a morte celular (SHICHIRI, 2014).

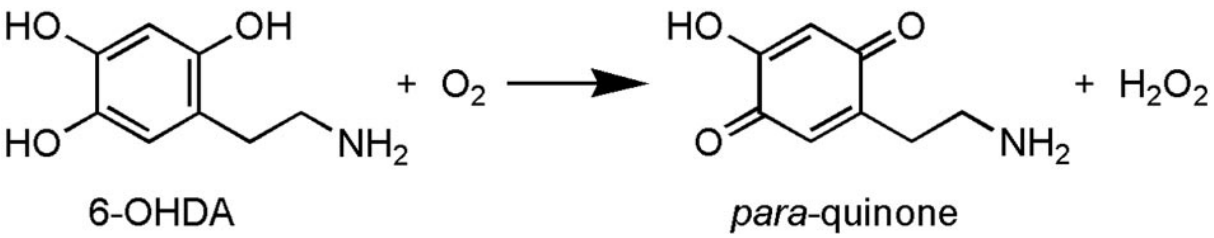

Figura 10: Oxidação da neurotoxina 6-OHDA. Através da oxidação pelo O_2, perde dois átomos de hidrogênio, gerando H_2O_2 e para-quinona. Adapatado de Przedborski e Ischiropoulos, 2005.

1.4 Compostos Naturais

O Brasil possui cerca de 20% do número total de espécies da biodiversidade mundial, sendo esta uma fonte rica em produtos naturais oriundos de plantas superiores, toxinas e microrganismos (CALIXTO, 2003). Vários estudos têm sido conduzidos objetivando a busca por novos compostos naturais para aplicação biotecnológica e na saúde (ANTUNES et al., 2014; ROSADO, 2016; SANTOS, 2014). Parte destas pesquisas buscam descobrir suas atividades antioxidantes, com potencial para reverter o estresse oxidativo e assim atuar na cura e prevenção de uma série de doenças, bem como encontrar compostos com atividades citotóxicas.

1.4.1 *Anacardium microcarpum*-Duke

O Cajuizeiro (Figura 11) é uma espécie da família Anacardiaceae nativa do Brasil, dispersa na Amazônia, no Nordeste, Goiás, Mato Grosso e Guianas e que habita na mata alta de terra firme ou de várzea, sendo raramente cultivada. No trabalho de MITCHEL & MORI (1987), esta espécie foi agrupada, pela taxonomia numérica, como variabilidade da espécie cultivada, *Anacardium occidentale* L. A casca do caule (Figura 12) de *A. microcarpum* é utilizada na medicina popular brasileira como um tônico para o tratamento de inflamação, reumatismo, doenças tumorais e infecciosas, em que os radicais livres e as espécies reativas de

oxigênio têm sido implicadas em sua etiologia (BARBOSA FILHO *et al*., 2014). Apesar do uso popular, há poucos estudos descrevendo seu potencial biológico.

Figura 11: *Anacardium microcarpum.* Adaptado de Valter Menezes Barbosa Filho, 2014.

Figura 12: Casca do caule de *Anacardium microcarpum.* Adaptado de Valter Menezes Barbosa Filho, 2014.

Os frutos do gênero *Anacardium* (Figura 13) são ricos em vitamina C e o suco de cajuí apresenta teores consideráveis de açúcares, compostos fenólicos e minerais, destacando-se entre eles cálcio, ferro e fósforo (RUFINO *et al.*, 2007).

Figura 13: Frutos de *Anacardium microcarpum*. Fonte: http://www.onordeste.com/onordeste

Dentre os compostos descritos para esta espécie, nos últimos anos foi descoberta uma série de atividades biológicas para o principal componente desta família, os ácidos anacárdicos; além de atividade antitumoral, destaca-se a habilidade em inibir as enzimas tirosinase (KUBO et al., 1994), prostaglandina sintase e lipo-oxigenase (PARAMASHIVAPPA et al., 2001). Estes ácidos também são conhecidos por suas atividades antiacne (KUBO et al., 1994), antibacteriana (KUBO et al., 1993) e antifúngica (PRITHIVIRAJ et al., 1997). Recentemente outros compostos presentes nas cascas de *A. microcarpum* foram descritos por nosso grupo como ácido gálico, ácido caféico, ácido clorogênico, ácido elágico, catequina, epicatequina, quercetina, isoquercitrina, quecitrina, rutina, dentre outros (BARBOSA FILHO et al., 2014).

Estudo realizado em nosso grupo de pesquisa, avaliando o efeito do extrato etanólico e frações acetato e metanólica obtidos das cascas do caule de *A. microcarpum* em modelo de Parkinsonismo induzido por Paraquat em *Drosophila melanogaster*, demonstrou baixa toxicidade e seu potencial neuroprotetor atribuído ao potencial antioxidante da planta (dados não publicados). Também foi observado efeito protetor do extrato bruto e fração metanólica frente ao dano oxidativo *in vitro* promovido pelo H_2O_2 em fatias (dados não publicados). BARBOSA FILHO et al., 2014 e colaboradores demonstrou potencial antioxidante *in vitro* para o extrato bruto e frações frente ao radical DPPH e proteção *in vitro* na lipoperoxidação induzida por Fe/EDTA em fígado e homogenato de cérebro de rato.

Tendo em vista estudos prévios do nosso grupo de pesquisa e a presença de compostos antioxidantes na planta, pode-se considerar *A. microcarpum* como uma espécie promissora na busca por propriedades farmacológicas e protetoras frente a danos neurotóxicos.

1.5 Modelo experimental

O desenvolvimento da técnica de fatias cerebrais, é um dos procedimentos mais importantes a nível mundial no que diz respeito a neurociência. Foram desenvolvidas na década de 1950 por McIlwain e colaboradores com o intuito e estudar a bioquímica do SNC, mostrando que neurônios poderiam ser mantidos em estado saudável para serem estudados. A técnica de fatias oferece uma grande vantagem de se poder controlar a viabilidade do meio, o pH, aplicação de drogas, além de manter a estabilidade física e metabólica dos mesmos. O modelo de fatias cerebrais tem sido muito utilizado para estudos imunocitoquímicos, imunohistoquímicos, hibridização *in situ* e de radicais livres. Diferentes estruturas cerebrais animais têm sido estudados neste modelo, destacando-se fatias de hipocampo e de córtex cerebral por mimetizar o que acontece em uma sinapse pela presença dos neurônios e estruturas cerebrais de interesse (MELLO, MACHADO, FUNCHAL, 2014).

O SNC de pintainhos, modelo utilizado neste estudo, possui projeções dopaminérgicas distribuídas no diencéfalo, telencéfalo, mesencéfalo e rombencéfalo, estando em maior quantidade nas regiões do diencéfalo e mesencéfalo (MOONS et al., 1995; SARTSOONGNOEN et al., 2008), sendo portanto um modelo bastante útil no estudo de mecanismos bioquímicos mediadores de doenças neurodegenerativas que envolvam o neurotransmissor dopamina (MOONS et al., 1994).

2. OBJETIVOS

2.1 Objetivo Geral

Investigar o possível potencial neuroprotetor do extrato vegetal de *Anacardium microcarpum*, e suas frações metanólica e acetato de etila, frente a exposição *in vitro* da neurotoxina 6-OHDA em modelo de fatias corticais de *Gallus gallus*.

2.2 Objetivos Específicos

Através da exposição das fatias corticais ao extrato hidroalcoólico, fração metanólica e fração acetato de etila de *A. microcarpum*, avaliar:

- a toxicidade pela análise da viabilidade celular;
- a neuroproteção frente à neurotoxicidade induzida por 6-OHDA;
- o potencial em inibir a auto-oxidação da neurotoxina 6-OHDA *in vitro.*

Através da exposição das fatias corticais à fração metanólica e fração acetato de etila de *A. microcarpum* na presença e / ou ausência da neurotoxina 6-OHDA, avaliar:

- os níveis da peroxidação lipídica.

Através da exposição das fatias corticais a fração metanólica de *A. microcarpum* na presença e / ou ausência da neurotoxina 6-OHDA, avaliar:

- a atividade de enzimas antioxidantes (Glutationa S-transferase, Glutationa Peroxidase e Tiorredoxina Redutase);
- os níveis de glutationa;
- a atividade mitocondrial;
- a modulação das vias de sinalização MAPK, Pi3K/Akt;
- o envolvimento de ERK1/2 e AKT na neuroproteção pelo uso de inibidores.

3. RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscrito, o qual foi submetido à revista *Journal of Ethnopharmacology* ISSN: 0378-8741 no dia 23/02/2017. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se no manuscrito, **o qual está disposto na forma em que foram submetidos para publicação.**

Methanolic fraction of *A. microcarpum* – Duke protects from oxidative damage induced by 6-OHDA in chicken brain slices

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Abstract

Parkinson's disease (PD) is a degenerative and progressive disease characterized by degeneration of dopaminergic neurons. This neuronal loss is related with oxidative stress and mitochondrial disruption. Neurotoxin 6-OHDA is a useful model for screening of therapeutic compounds, since it reproduces biochemical events observed in Parkinson. *Anacardium microcarpum* is a Brazilian plant used in traditional folk medicine for the treatment of infectious diseases, inflammation, and tumor. This study aimed to evaluate the neuroprotective effect of *Anacardium microcarpum* extract and methanolic and acetate fraction on the damage induced by the neurotoxin 6-OHDA in brain cortical slices of chicken. 6-OHDA decreased cellular viability and induced lipid peroxidation. The neurotoxin also induced an increasing in GST and TrxR activity and inhibited GPx activity. Total GSH was decreased and the ration GSH/GSSG was increased by 6-OHDA. All these events were avoided by methanolic fraction of *A. microcarpum*. A mild protective effect was attributed to acetate fraction and no effect was demonstrated by hydroalcoholic extract. 6-OHDA autoxidation and 6-OHDA mitochondrial dysfunction was not blocked by methanolic fraction. Additionally, ERK1/2 and AKT pathways were involved in the neuroprotection mediated by fraction, since it was ineffective in the presence of inhibitors. Our data show by the first time a protective effect of a specific fraction

of *A. microcarpum* against 6-OHDA damage and this effect seems to be related to its antioxidant action without avoiding mitochondrial damage or autoxidation in an AKT and ERK dependent manner.

Keywords: *Anacardium microcarpum;* 6-OHDA; slices; Parkinson; MAPK; neuroprotection.

1. Introduction

Parkinson's disease (PD), described by James Parkinson in 1817, is the second most common neurodegenerative disease being associated with progressive nigrostriatal neurodegeneration. It has a wide distribution and it reaches all ethnic groups and socioeconomic classes. Data show approximately 1% of the world population over the age of 60 present this disease (Fritsch et al., 2012). Clinically, PD is characterized by resting tremor, stiffness, bradykinesia and gait impairment (Balestrino and Martinez-Martin, 2017). When clinical symptoms appear, the pathology is already well advanced, and almost half of the dopaminergic cells of the substantia nigra is lost with a depletion of approximately 80% of striatum dopamine (Michel et al., 2016). The etiology of Parkinson's Disease remains undefined, but it is known that genetic, environmental and aging factors contributes for disease (Fahn and Sulzer, 2004). According with literature, factors as oxidative stress, mitochondrial dysfunction, inflammation, and apoptotic cell death in the brain play important roles in the pathogenesis of PD (Bhattacharjee and Borah, 2016; Kempuraj et al., 2016).

Considering the complexity of events implied in neurodegenerative disease etiology, animal models as cerebral slices can be considered an appropriate model to study biochemical events *in vitro* in the brain, since they maintain the natural extracellular matrix, neuronal connectivity and neuronal–glial interactions (Rodnight et al., 1991) that has made possible a better understanding of the mechanisms of neuronal death. Furthermore, the use of substances capable of selectively destroying dopaminergic neurons, mainly by the induction of oxidative stress, has been very useful tools for study of PD. The major toxins used to induce dopaminergic neurodegeneration are MPTP, paraquat, rotenone and 6-hydroxydopamine (Bové and Perier, 2012).

6-hydroxydopamine (6-OHDA) is a toxic oxidative dopamine metabolite which is rapidly and non-enzymatically oxidized by molecular oxygen to form *p*-quinone and hydrogen peroxide (Soto-Otero et al., 2000). It has been propose as a putative neurotoxic factor in PD pathogenesis. The main mechanism of cytotoxicity attributed to 6-OHDA has been linked to the production of reactive oxygen species (ROS). A high content of unsaturated fatty acids, iron and an elevated consumption of oxygen, which leads to damage and dysfunction of mitochondria (Shichiri, 2014) make the brain tissue very susceptible to oxidative stress associated with as exacerbated production of ROS.

The MAP kinase (MAPK) pathway is a highly conserved route implied in a diversity of cellular functions, including cell proliferation, cell differentiation and apoptosis (Chang and Karin, 2001). PI3K/Akt plays an important role in neuronal apoptosis associated with environmental stress and neurotoxin (Cui et al., 2016). Oxidative stress injury caused by 6-OHDA has been attributed to modulation of MAPK p38, JNK and ERK and AKT pathway (Cui et al., 2016; H. C. Kuo et al., 2014).

L-DOPA is a drug more frequently used by Parkinson's disease patients, however, studies have shown that it may be toxic to dopaminergic neurons and give rise to 6-OHDA neurotoxin by non-enzymatic reactions (Blum et al., 2001). In spite of this, the search for alternative therapies such as antioxidants has grown greatly over the years to prevent and mitigate the symptoms of the disease. Besides, there is evidence that plant extracts have a beneficial potential, attenuating the progression of PD, through antioxidant compounds present in the extracts (Abushouk et al., 2016; Gao et al., 2015; Komolafe et al., 2014).

The brazilian plant *Anacardium microcarpum*, popularly known as "cajui", belongs to the Anacardiaceae family. It is found in the Northeast Region of Brazil and is used in traditional folk medicine for the treatment of infectious diseases, inflammation, rheumatism, and tumor (Barbosa Filho et al., 2014). A study carried out by BARBOSA FILHO et al., 2014, characterized the phytochemical constitution of *A. microcarpum* stem bark crude extract showing its composition of phenols and flavonoids, as gallic acid, caffeic acid, quercetin among other components. Recently, BARBOSA-FILHO et al., 2014 showed the potential antioxidant *in vitro* of extract and fractions of this plant against Fe2+-induced lipid peroxidation. Moreover, antibactentrial potential was attributed to ethyl acetate and methanolic fractions of *A. microcarpum* (Barbosa-Filho et al., 2015). In spite of scarce studies, *A. microcarpum* extract and fractions demonstrated to represent a good source of natural antioxidants and protective potential, which might be helpful in preventing or delaying the progress of PD. Therefore, the aim of this study was to evaluate the neuroprotective potential of hydroalcoholic extract (AMHE), methanolic (AMMF) and ethyl acetate fractions (AMEAF) against the neurotoxicity induced by 6-hydroxydopamine (6-OHDA) and the involvement of oxidative stress and MAPK and AKT pathways on its protective effect using cortical slices of *G. gallus* as a model of study.

2. Materials and methods

2.1 Chemicals

Dimetil sulfoxide, Folin-Ciocalteu, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, sodium acetate, reduced glutathione (GSH, G4251); oxidized glutathione, tetramethylethylenediamine (TEMED, T9281); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, M2128); D- Manitol (M9647); K_2KO_4P (1110216); KH_2PO_4 (P0662); Hepes Minimum 99,5% (Titration, H3375); Albumin from bovine serum (BSA, A6003); Triton X-100 (T8532); β-mercaptoethanol (M6250); anti-rabbit immunoglobulin (HRP peroxidase-linked antibody) were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). Anti-phospho-p38 (Thr180/Tyr182), anti phospho-AKT, anti PTEN, anti-phospho JNK1/2 (Thr183/Tyr185), anti-phospho ERK1/2 (Thr202/Tyr204) and anti-total-ERK1/2 and β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA). SDS, acrylamide, bis-acrylamide, hybond nitrocellulose were obtained from GE Healthcare Life Division (Uppsala, Sweden). Poly (ADP)-ribose polymerase (PARP) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Kit Caspase-Glo 3/7 (G7790) was obtained from Promega. All other reagents were commercial products of the highest purity grade available.

2.2 Animals

For this study were used little chicks of *Gallus gallus* species with age between 5-15 days (both genders). The animals were maintained in the biotery of University Federal do Pampa with temperature between 22°C – 25° C with food and water *ad libitum*. All the procedures were performed in accordance with CEUA/UNIPAMPA approval under protocol n° 011/2012.

2.3 Plant collection and extractions

The stem barks of *A. microcarpum* were collected from Barrero Grande, Crato-Ceará (7◦22_S; 39◦28_W; 892 m sea level), Brazil, in November 2011. The plant material was identified by Dr. Maria Arlene Pessoa da Silva of the herbarium Caririense Dárdano de Andrade – Lima (HCDAL) of the Regional University of Cariri (URCA) and a voucher specimen was deposited (number 6702). The fresh barks of *A. microcarpum* were macerated with 99.9% of ethanol and water (1:1, v/v) for 3 days. The suspension was filtered, solvent evaporated under reduced pressure and lyophilized to obtain 490 g of ethanolic extract. One hundred and fifty

grams (150 g) of this was partitioned with ethyl acetate and methanol to obtain 12.5 g of ethyl acetate fraction and 105.23 g of methanolic fraction. All the fractions were stored in the freezer and resuspended in water prior to experiments. The phytochemical characterization of the extract was previously performed and is described in (Barbosa Filho et al., 2014).

2.4 Tissue slice preparation and treatment

The animals were euthanized by decapitation under anestesia with halothane. The brain was dissected and placed in cutting solution oxygenated at 4°C (110 mM sucrose, 60 mM de NaCl, 3 mM KCl, 0.5 mM $CaCl_2$, 7 mM $MgSO_4$, 5 mM glicose, 25 mM HEPES, pH 7.4). The cortical region of the brain were separated and 400 µm thickness slices were prepared in a McIlwain tissue slicer (Posser et al., 2009). The diameter of slices was standardized using punch 3 mm. Briefly slices were transferred to 96-well plates containing HEPES-saline buffer (124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 12 mM glucose, 1 mM $CaCl_2$ and 25 mM HEPES pH 7.4) previously oxygenated during 30 minutes (200 µL/slice). After 30 min of pre-incubation, the buffer was removed and fresh buffer was added. Tissue slices were subsequently incubated for 120 minutes at 37°C in presence/absence of 6-OHDA 500 µM and/or hydroalcoholic extract (AMHE), methanolic (AMME), and ethyl acetate *Anacardium microcarpum* fractions (AMEA) (0.1 – 1mg/mL concentrations). All dissolved in the HEPES-saline buffer.

2.5 Cell viability

Cell viability was determined by reduction of 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) (0.05% HEPES-saline). For this assay, three slices per group (n=3) were incubated during 120 minutes with treatment solutions. After, slices were incubated for 30 minutes at 37°C in the presence of MTT according POSSER et al., 2009. Subsequently, MTT was removed and samples were incubated in DMSO for 30 min (37ºC). The absorbance resulted from formazan dilution by addition of DMSO was read in an EnsPire® (PerkinElmer, USA) multimode plate reader at 540 nm.

2.6 Spectrophotometric studies of 6-OHDA autoxidation

The autoxidation of 6-OHDA was followed spectrophotometrically by monitoring the formation of *p*-quinone at 490 nm (Soto-Otero et al., 2000). A Cary 60-UV-Visible

Spectrophotometer by Agilent Technologies was used for the assay. The cuvette holder was thermostatically maintained at 37°C. For each assay, 1 mL of phosphate buffer (pH 7.4) was incubated in a quartz cuvette for 10 min to reach the set temperature. Then, the autoxidation was initiated with addition of 5 µL of stock solution of 6-OHDA (100 mM) at a final concentration of 0.5 mM. The monitoring of the corresponding kinetics was immediately initiated and maintained for the subsequent 3 min. To verify if AMHE, AMMF and AMEAF could prevent autoxidation of the compound, it was added different concentrations of plant extract or fractions (1 µg/mL, 10 µg/mL and 100 µg/mL) in presence or absence of 6-OHDA. 10 mM GSH was used as positive control.

2.7 Lipid peroxidation

The final product of lipid peroxidation was determined according OHKAWA, 1979, with some modifications, having thiobarbituric acid as reactive substance (TBARS). Tissue slice were incubated for 120 minutes at 37°C in different concentrations of the fractions (0.1 – 1 mg/mL) in presence/absence of 6-OHDA (500 µM). For the next step, five slices per treatment group were homogenized in 150 µL of 20 mM HEPES buffer. Further, all content was incubated during 60 minutes at 95°C into 0.45 M acetic acid/HCl buffer, 0.8% thiobarbituric acid (TBA), 8.1% SDS to promote the coloring and after, the absorbance was measured in a wavelength of 532 nm.

2.8 Enzyme assays

All spectrophotometric assays were performed in an Agilent Cary 60 UV/VIS spectrophotometer with a 18 cell holder accessory coupled to a Peltier Water System temperature controller (Santa Clara, CA). Six slices were homogeneized in 20 mM HEPES pH 7.4 and centrifuged at 20.000 g for 30 minutes at 4°C. The supernatant was isolated in aliquots for determination antioxidant enzymes activity. Glutathione S-transferase activity (GST) was assayed following the procedure of HABIG; JAKOBY (1981) using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate. The assay is based on the formation of the conjugated complex of CDNB and GSH at 340 nm. The reaction was conducted in a mix consisting of 0.1 M phosphate buffer pH 7.0, 1 mM EDTA, 1 mM GSH, and 2.5 mM CDNB. Glutathione peroxidase activity (GPx) was measured as described by WENDEL (1981) defined as the rate

of NADPH oxidation at 340 nm by the coupled reaction with gluthatione reductase. One unit of GPx will consume 1.0 μmoL of $NADP^+$ from NADPH per minute (ε= 6.22 M^{-1} cm^{-1}). TrxR activity was measured according to HOLMGREN; BJÖRNSTEDT (1995) which consists in measuring the rate of reduction of DTNB by NADPH. One unit of enzyme activity was considered the amount of enzyme that catalyzes the formation of 1.0 μmoL of TNB per minute at 25 °C, pH 7.0 (ε= 13.60 M^{-1} cm^{-1}).

2.9 Determination of reduced (GSH) and oxidized glutathione (GSSG)

For the measurement of GSH and GSSG levels, five slices were homogenated and treated with 0.5 mL of 13% trichloroacetic acid and centrifuged at 100.000 g for 30 min at 4ºC. Aliquots (10 μL) of the supernatant were mixed with 180 μL of 100 mM NaH_2PO_4 buffer, pH 8.0, containing 5 mM EDTA. Ten microliters of *O*-phthalaldehyde (OPT) (1 mg/mL) was added and fluorescence was measured 15 min later using the 350/420 nm excitation/emission wavelength pair in Perkin Elmer inspire (Hissin and Hilf, 1976). For measurement of GSSG levels, a 25 μL of the brain supernatant was incubated at room temperature with 10 μL of N-ethylmaleimide (NEM) (0.04 M) for 30 min at room temperature, and after that, 14 μL of the mixture, were added to 176 μL of NaOH (0.1 N) buffer, following of added 10 μL OPT and incubated for 15 min in the dark, using the procedure outlined above for GSH assay. Results were presented as the GSH/GSSG ratio.

2.10 High-resolution respirometry (HRR) in vitro

For the respirometry determination, the little chicken brain homogenate (400 mg) was weighed and transferred to 1 mL of cold homogenization buffer containing 5 mM Tris-HCl, 250 mM sucrose and 2 mM EGTA (pH 7.4), and brain homogenate (0.1 mg/mL protein) was used to the HRR. Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) was employed for all measurements of respiration. The experiments were performed in 2 mL of MiR05 buffer (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES and 0.1% BSA essentially fatty acid free pH 7.1) (Gnaiger, 2009). All experiments were performed at 37ºC using DatLab 4.0 software (Oroboros Inc., Austria), with continuous stirring at 750 rpm and all experiments started by registering the

endogenous substrate supported respiration, following protocols established in the literature (Kuznetsov et al., 2004).

All the experiments of mitochondrial bioenergetics analysis in brain homogenate were performed following PICHAUD et al., 2013 with minor modifications at the O2k-chamber. All concentrations of compounds (control group without treatment, 100 µg/mL of methanolic fraction, 6-OHDA 500 µM in the absence or presence of methanolic fraction) were added at the O2k-chamber after signal stabilization of the basal respiration supported by endogenous substrates. Four individuals preparation of the brain homogenate were performed per group.

2.11 Mitochondrial respiration assays

Titration protocols of multiple substrates and inhibitors were used to assess mitochondrial function in terms of different respiration states. The routine of electron transport system activities in brain homogenate was carried out in according to literature (Pesta and Gnaiger, 2012). Malate, glutamate and succinate were used as oxidizable substrates in all experiments. Complex I (CI)-mediated Leak (LEAK) respiration was determined using 2 mM malate and 10 mM glutamate. CI-mediated OXPHOS (OXPHOS) was determined using ADP (2.5 mM). Respiratory control ratios (RCR = CI_{OXPHOS}/CI_{LEAK}) was used as quality control of isolated mitochondria. The convergent electron flow during the maximal OXPHOS respiration ($CI+CII_{OXPHOS}$) was determined with substrates of CI and CII (10 mM Succinate). CI+CII mediated ETS (electron transfer system) ($CI+CII_{ETS}$) was determined using Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) (optimum concentration reached between 0.5 and 1.5 µM). CII mediated ETS respiration (CII_{ETS}) was determined with 0.5 µM rotenone. Addition of 2.5 µM antimycin A inhibited complex III, resulting in non-mitochondrial respiration (ROX) with small contributions from electron leak in the uncoupled state.

2.12 Western Blotting Analysis

Analysis of protein phosphorylation in cortical slices was performed using western blotting according to POSSER et al., 2011 with slight modification. After the homogenate preparation and protein quantification, was added to samples 4% SDS stop solution (4% SDS, 50 mM Tris, 100 mM EDTA, pH 6.8), 25% Glycerol sample and 8% β-mercaptoethanol. The proteins were separated by SDS–PAGE using 10% gels and then electrotransferred to nitrocellulose membranes. The membranes were washed in Tris-buffered saline with Tween

containing 100 mM Tris–HCl, 0.9% NaCl and 0.1% Tween-20, pH 7.5, and incubated overnight (4ºC) with primary antibodies anti-rabbit anti total and phospho p38, anti-total and phospho ERK1/2, anti-total and phospho JNK1/2, anti-phospho and total AKT, anti PTEN. Subsequently, membranes were washed in Tris-buffered saline with Tween and incubated for 1 hour at 25 ºC with horseradish peroxidase-linked anti-IgG secondary specific antibodies. The immunoblotting's were visualized on the IS4000MM Pro Bruker imaging system using ECL-detection reagent and the band density was quantified using the Scion Image® software. The density of the bands was measured and expressed as a % of increase in relation to control (slices treated only with media).

2.13 PI3K/AKT and ERK 1/2 Inhibitors

To determine the implication of signaling pathway involved in cell survival in the neuroprotective effect of *A. microcarpum* methanolic fraction, PI3K/AKT inhibitor LY294002 at final concentration of 20 µM and ERK1/2 inhibitor PD98059 at final concentration of 50 µM were added to the medium 30 min prior the treatment, after it was added the 6-OHDA plus AMMF for 2 hours. After treatment, was performed MTT assay according with protocol already described to verify cell viability. Inhibitors were diluted in DMSO, the final concentration of DMSO in the wells was 0.5%.

2.14 Protein quantification

Protein concentration was performed by the method of BRADFORD, 1976 using albumin from bovine serum as the standard to almost all tests, except to Western Blotting, in which it was used PETERSON, 1977 for the measurement quantification.

2.15 Statistical Analysis

All the procedures were made in triplicates. Statistical analysis was performed using a one-way ANOVA followed by Newman-Keuls test. Differences were considered to be significant at the $p < 0.05$ level.

3. Results

3.1 Evaluation of toxicity of A. microcarpum hydroalcoholic extract and fractions in vitro and neuroprotection against 6-OHDA

In order to investigate a possible neurotoxic effect of *Anacardium microcarpum*, cortical slices were incubated for 2 hours with different concentrations of hydroalcoholic extract and fractions: control group, 1 µg/mL, 10 µg/mL, 100 µg/mL and 1000 µg/mL. After the end of treatment, we performed cell viability assay (MTT) to verify any change on mitochondrial viability that could be caused by *A. microcarpum*. Our data showed that *A. microcarpum* per se was unable to affect viability of slices (Figure 1).

To investigate the neuroprotective potential of *A. microcarpum*, slices were incubated with the neurotoxin 6-OHDA 500 µM for 2 h in presence or absence of different concentrations of extract or fractions (1 – 100 µg/mL). The concentration of neurotoxin was based in a concentration curve, and the concentration able to cause approximately 30% decrease in cell viability was used in further studies. AMMF and AMEAF avoided the drop in cell viability promoted by 6-OHDA only at the concentration of 100 µg/mL ($p < 0.0001$) (Figure 2B and Figure 2C, respectively). Hydroalcoholic extract was unable to protect against damage caused by 6-OHDA (Figure 2A). The fractions were used for analysis of antioxidant effect against 6-OHDA.

3.2 Analysis of 6-hydroxydopamine auto-oxidation in presence of methanolic fraction of A. microcarpum

According to the Figure 3, it is possible to visualize an augmented absorbance of 6-OHDA, representing its auto-oxidation with formation of its respective *p*-quinone read at 490 nm. *A. microcarpum* (1-100 µg/mL) was unable to avoid 6-OHDA (500 µM) auto-oxidation, which was completed blocked by presence 10 mM of GSH.

3.3 Lipid peroxidation in response to the treatment with methanolic fraction of A. microcarpum and 6-OHDA

Considering the involvement of oxidative stress in dopaminergic cell death induced by 6-OHDA (Shichiri, 2014), it was analyzed possible induction of lipid peroxidation. The

neurotoxin induced in 25% the lipid peroxidation on cortical slices ($p< 0.0001$) when compared to control group (Figure 4). AMMF but not AMEAF was able to avoid the lipid peroxidation inducted by toxin (Figure 4A).

3.4 Activity of antioxidant enzymes and redox state of cell

Considering the production of ROS a mechanism implicated in 6-OHDA neurotoxicity, general antioxidant cell status was investigated. Activity of antioxidant enzymes GST and GPx were evaluated. Additionally to the GSH and GSSG content in slices submitted to the treatment. As shown at Table 1, 6-OHDA caused a 1.68 fold increase in GST activity and this effect was not observed in the presence of fraction and 6-OHDA. TrxR was presented a 1.9 fold increase in the activity when in presence of 6-OHDA, such effect was avoided by the presence of fraction. On the other hand, GPx was 1.71 fold inhibited by 6-OHDA when comparing to control, and this effect was not observed in the presence of fraction (Table 1). The total glutathione content and oxidized glutathione was decreased in 34% and 39%, respectively by 6-OHDA treatment. However, the ratio GSH/GSSG was increased by the 6-OHDA. When in presence of fraction, this effect was reverted. The plant per se induced the levels of GSH without affecting other parameters (Table 2).

3.5 Evaluation of Mitochondrial respiration in response to 6-OHDA and A. microcarpum methanolic fraction.

It was demonstrated 6-OHDA disrupts mitochondrial respiration by complex IV inhibition, thus leading to augmented ROS generation (Glinka and Youdim, 1995). In this study, basal respiration was unchanged in brain slices by treatments. After glutamate and malate substrate (CI_{Leak}) addition, a significant decrease ($p< 0.05$) on CI activity was caused by 6-OHDA. This drop in CI activity persisted when fraction was present ($p< 0.05$). AMMF per se did not change activity of CI. In order to see CI_{OXPHOS}, it was added succinate and ADP (CI_{OXPHOS}), this parameter was also inhibited by 6-OHDA and the fraction was unable to avoid it. The convergent electron flow during the maximal oxidative phosphorylation ($CI+CII_{OXPHOS}$) was also significantly decreased by 6-OHDA and the fraction did not avoid this effect. Maximal mitochondrial respiration ($CI+CII_{ETS}$) was determined with the addition of the uncoupler FCCP. A significant decreasing ($p< 0.05$) in this parameter has occurred after administration of 6-OHDA, which was not prevented by AMMF addiction ($p< 0.001$). CII_{ETS} activity was analyzed

after inhibition of CI by rotenone. As visualized in Figure 5, no significant changes occurred by treatments. The presence of AMMF per se, induced CI_{LEAK}, CI_{OXPHOS}, $CI+CII_{OXPHOS}$, $CI+II_{ETS}$ and CII_{ETS}.

3.6 Analysis of MAPKs and AKT phosphorylation in response to the treatment with 6-OHDA and methanolic fraction of A. microcarpum.

MAPK and AKT signaling pathways are involved in the regulation of cell proliferation, differentiation, survival and apoptosis (Liu and He, 2017). In this study, the effect of 6-OHDA on phosphorylation of proteins MAPK was analyzed by Western blotting technique. The neurotoxin 6-OHDA stimulated the JNK1 phosphorylation in 20% ($p< 0.0001$), this effect was reverted by 100 µg/mL of methanolic fraction (Figure 6B). While $p38^{MAPK}$ phosphorylation was inhibited in 25% when both extract and 6-OHDA was present in the medium (Figure 6C) ERK phosphorylation was increased in 50% by extract and neurotoxin (Figure 6A). There was no alteration in AKT phosphorylation (Figure 6D). AMMF per se caused no changes in the phosphorylation of ERK1/2, JNK1/2, $p38^{MAPK}$ and AKT proteins. The total content of these proteins was not altered by treatments.

3.7 Involvement of ERK1/2 and Akt signaling pathways on neuroprotection of methanolic fraction of A. microcarpum against 6-OHDA

Considering involvement of ERK and AKT in survival of cells, it was investigated the participation of these pathways in protective potential of methanolic fraction. Slices were incubated with synthetic inhibitors of ERK1/2 phosphorylation (PD98059) and LY294002 a synthetic PI3k/Akt inhibitor in the presence of extract. As shown in Figure 7, inhibitors did not change the cortical slices viability, however, they blocked the protective potential of fraction.

4. Discussion

Oxidative stress, mitochondrial dysfunction, genetic and environmental factors are mechanisms associated with neuronal damage observed in PD (BELLOU et al., 2016; UTTARA et al., 2009; LIN; BEAL, 2006). A number of studies have proposed antioxidant therapies to attenuate PD symptoms (Abushouk et al., 2016; Solayman et al., 2016; Zhao et al.,

2016). In this study, methanolic and acetate fraction but not hydroalcoholic extract protected against neurotoxicity induced by 6-OHDA in brain slices and methanolic fraction was more effective in revert the lipid peroxidation induced by 6-OHDA. Our group has already demonstrated a list of flavonoids and phenolic compound that are expressed in both extract and fractions in different amount. The major components of hydroalcoolic extract were quercetin, caffeic acid and ellagic acid, for methanolic fraction was quercetin, isoquercetrin and caffeic acid and for Acetate fraction was caffeic acid, quercetin and ellagic acid. The synergistic behavior of components of extract or fraction may be accounting for the differential potential observed; however, the presence of other classes of more lipophilic compounds in fractions could not be discarded.

The cytotoxicity attributed to 6-OHDA could to be in part by forming reactive oxygen species from enzymatic reaction or by autoxidation of 6-OHDA. generating superoxide radical, *para*-quinone and hydrogen peroxide (GLINKA et al., 1997). It this study, plant extract or fractions did not avoid the autoxidation of 6-OHDA, although glutathione blocked this reaction. Based in this finding, it can be inferred the mechanism implied in the protection by the fraction could be the neutralization of the reactive species secondary to autoxidation reaction. Other important mechanism implied in 6-OHDA toxicity, is induction of mitochondrial dysfunction by inhibition of I and IV complexes, compromising the mitochondrial function and producing superoxide, which, in turn, may form hydroxyl radicals which reacts with nitric oxide forming peroxynitrite (Brown and Borutaite, 2004; Chaturvedi and Beal, 2013; Glinka and Youdim, 1995; Golpich et al., 2016; Tobón-Velasco et al., 2013). In this study, 6-OHDA inhibited mitochondrial complex I in brain homogenate, which was not avoided by AMMF. Taking into account this data, it can be supposed the AMMF is neutralizing reactive species resulted from mitochondrial dysfunction thus protecting against damage caused by those species, without impeding mitochondrial inhibition caused by 6-OHDA.

Reactive oxygen species production and detoxification, and signaling pathways have been considered interesting targets for intervention in neurodegenerative diseases (Kim and Choi, 2015; Uttara et al., 2009). Endogenous enzymatic and non-enzymatic antioxidants, such as GSH, glutathione S-transferase, glutathione peroxidase and thioredoxin reductase, delay or prevent oxidative damage to proteins, lipids, and DNA (Gutteridge and Halliwell, 2010; Robaczewska et al., 2016). Glutathione peroxidase is an intracellular antioxidant that reduces hydrogen peroxide to water at expenses of GSH and limits its harmful effect. In this study, 6-OHDA caused a substantial inhibition in GPx activity that was not observed in presence of methanolic fraction. Similar data was demonstrated in neuroblastome cells treated with 6-

OHDA (H.-C. Kuo et al., 2014). In PD patients, the degree of symptom severity correlates with intracellular GSH loss in substantia nigra (Riederer et al., 1989). In our study, GSH content was not altered and GSSG was decreased by OHDA, this effect may be related to GPx inhibition and consequently a lower oxidation of GSH by this system. On the other hand, the TrxR activity was stimulated by 6-OHDA, suggesting the participation of peroxiredoxins catalyzing peroxides reduction as a compensatory mechanism to replace the inhibited activity of GPx. Herein, 6-OHDA increased the activity of GST, which is implied in neuronal detoxification quinones resulted from catecholamine oxidation and free radicals (Mazzetti et al., 2015) and this effect was not observed when AMMF was present. All these effects returned to control level in the presence of methanolic extract of *A. microcarpum*, showing a protection against oxidative stress induced by 6-OHDA.

MAPK including extracellular signal–regulated kinases (ERKs) have been implicated in the cellular response to reactive oxygen species (GUYTON et al., 1996; BONNI et al., 1999; DUDEK et al., 1997; HARADA et al., 2004). Growth factors and other extracellular stimuli activate the kinase MEK1/2 by Ras/Raf pathway; MEK1/2 then phosphorylates and activates ERK1/2 (Seger and Krebs, 1995). ERK1/2 activates transcription factors such as cAMP response element–binding protein (CREB) and Elk, thereby increasing transcription of neurotrophic factors and prosurvival genes such as Bcl-2 (Lin et al., 2008). In this study, the use of MEK1/2 inhibitor weakened the protective potential of methanolic extract against the 6-OHDA, suggesting that the antioxidant potential of fraction per se is not sufficient to protect the brains slices, but the activation of pro-survival factors plays an important role in its effect. In this study, 6-OHDA did not increase ERK1/2 phosphorylation in brain slices after two hours of incubation with 6-OHDA, however, Lin et al demonstrated a ERK1/2 phosphorylation peak after 10-15 min of exposure of dopaminergic cells to 6-OHDA and the phosphorylation of pro survival protein CREB followed this temporal profile. In that study, the inhibition of early phosphorylation by ERK1/2 inhibitor abolished CREB activation and increased 6-OHDA toxicity. Thus, it is not discarded an early activation of ERK1/2 in slices and the contribution of this activation for a self-protective response of cells (Lin et al., 2008).

Recent studies have suggested that several signal transduction pathways, including phosphatidylinositol 3 kinase (PI3K) pathways and MAPKs, are involved in releasing transcription factor Nrf2 from the complex Keap1-Nrf2 promoting Nfr2 translocation to the nucleus (KIM et al., 2010). Nrf2 promotes transcriptional activation of a variety of antioxidant genes (Qi et al., 2017). AMMF mediated cytoprotection against 6-OHDA was abolished by ERK and AKT pathways inhibitors, these data supports a possible involvement of Nrf2

activation leading to expression of downstream antioxidant genes through modulation of Akt and Erk pathways by the fraction. According with previous study, inhibition of AKT and ERK has also abolished the neuroprotective effect of a triterpenoid isolated from plant (Qi et al., 2017).

In conclusion the present work shows by the first time the potential of *A. microcarpum* to protect against 6-OHDA induced damage in brain slices preparation, and propose that several mechanism might be implied in its protective action, including scavenging of reactive species resulted from mitochondrial damage and dopamine autoxidation and involvement of ERK and AKT in this effect.

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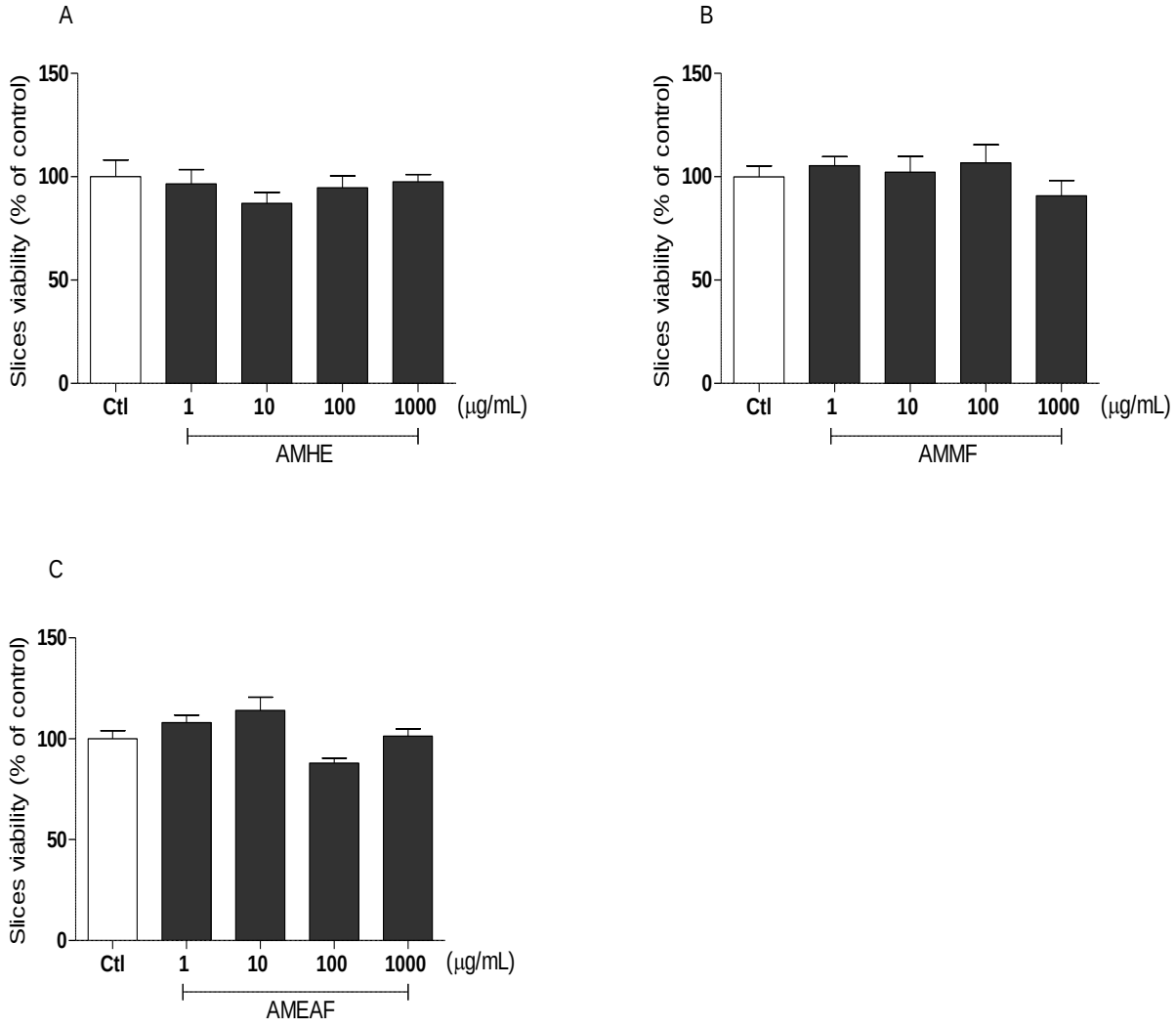


Figure 1. Effects of *A. microcarpum* on the viability of cortical slices. Cortical slices were incubated for 2 h in different concentrations (1 – 1000 µg/mL) of (A) AMHE, (B) AMMF and (C) AMEAF. Cell viability was measured by MTT test. Data are expressed as percentage of the untreated control ± SEM (n = 3).

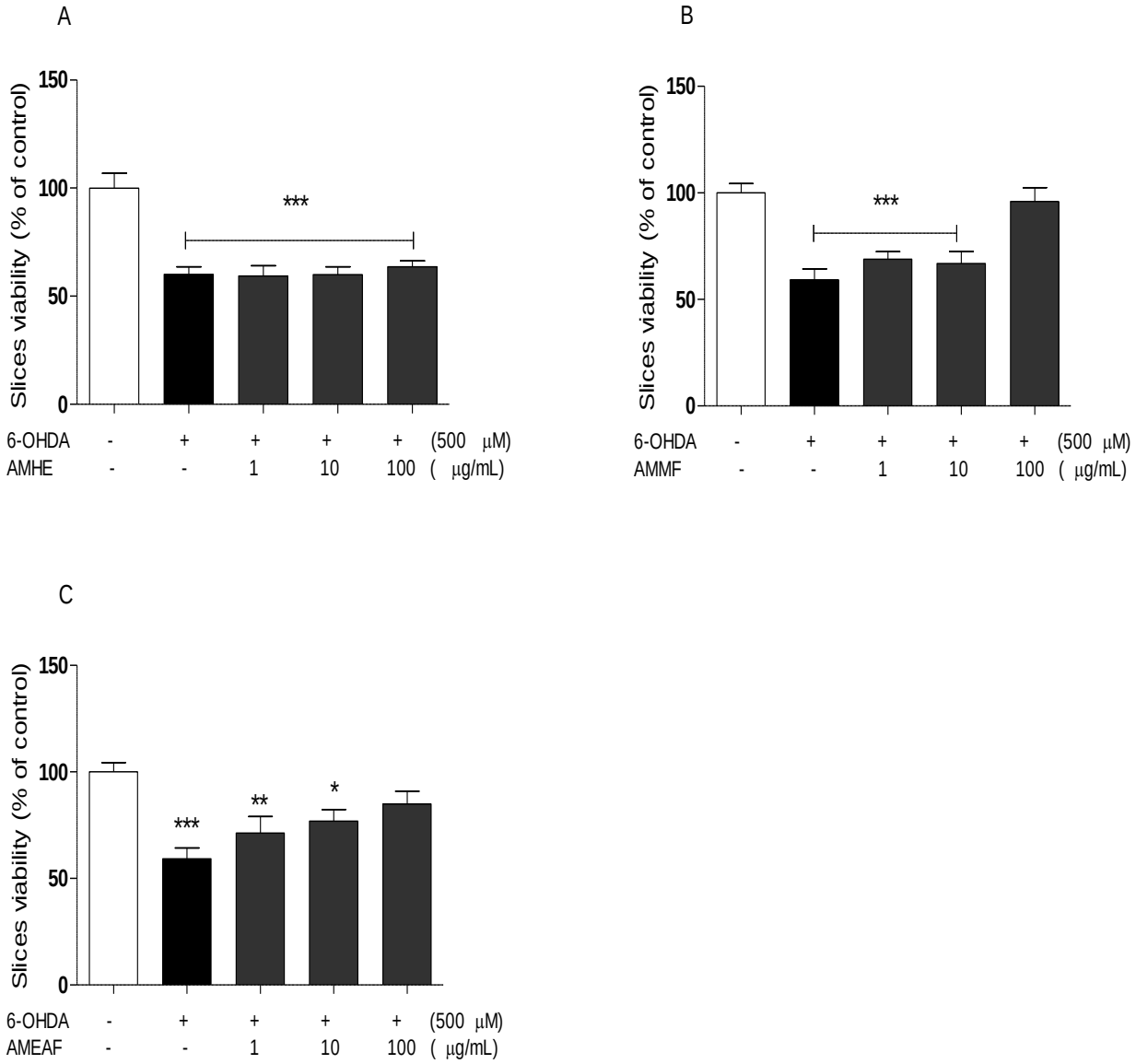


Figure 2. Effects of *A. microcarpum* and 6-OHDA on the viability of cortical slices. Cortical slices were incubated with (A) AMHE, (B) AMMF and (C) AMEAF in the presence or absence of 6-OHDA (500 µM) during 2 h. Data are expressed as percentage of the untreated control ± SEM (n = 3). *p < 0.05; **p < 0.001 and *** p < 0.0001 different from control group.

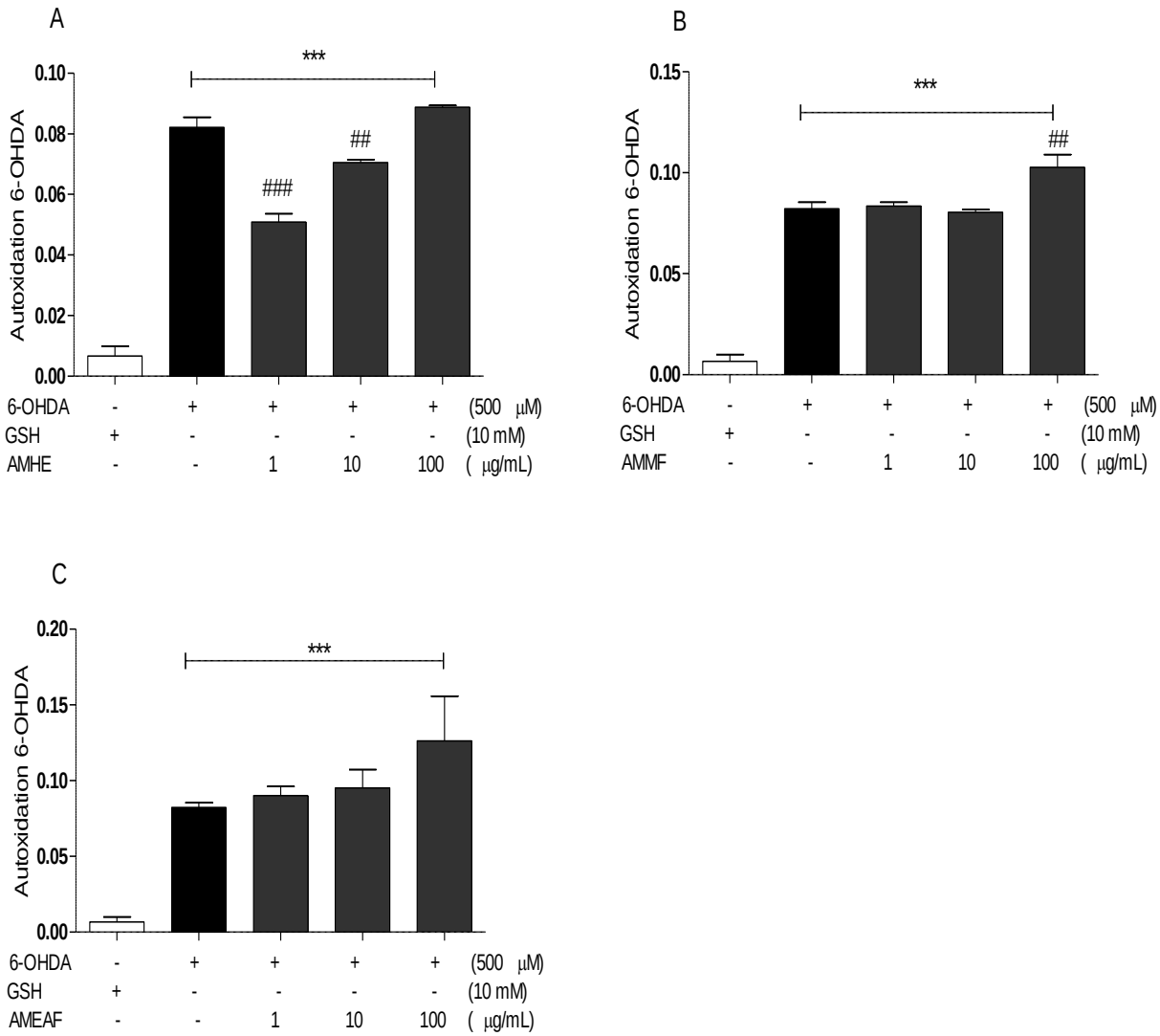


Figure 3. Effects of 6-OHDA autoxidation in the presence of *A. microcarpum* and GSH. The autoxidation of 6-OHDA (500 µM) was followed spectrophotometrically by monitoring the formation of p-quinone at 490 nm. The cuvette holder was thermostatically maintained at 37°C. Then, the autoxidation was initiated with addition of 5 µL of stock solution of 6-OHDA (100 mM) at a final concentration of 0.5 mM. The monitoring of the corresponding kinetics was immediately initiated and maintained for the subsequent 3 min. GSH 10 mM was used as positive control. Data are expressed as percentage of the untreated control ± SEM (n = 3). ***$p<0.0001$ as compared to GSH control. ##$p<0.001$ and ###$p<0.0001$ as compared to 6-OHDA group.

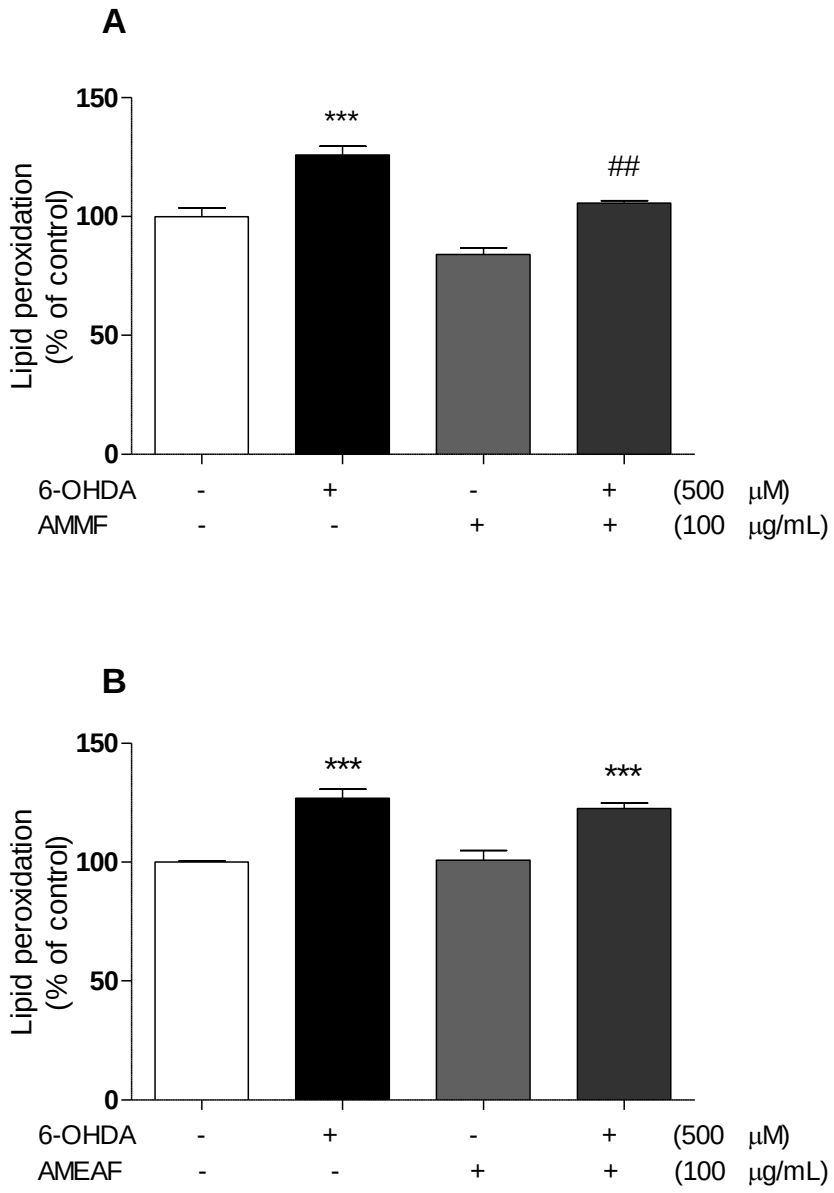


Figure 4. Effects of *A. microcarpum* on 6-OHDA (500 µM) induced lipid peroxidation (LPO) in cortical slices. Cortical slices were incubated with (A) AMMF and (B) AMEAF in presence / absence of 6-OHDA for two hours, and lipid peroxidation was evaluated by formation of TBARS at 532 nm. Data are expressed as percentage of the untreated control ± SEM (n = 3). ***p< 0.0001 as compared to control. ##p< 0.001 as compared to 6-OHDA group.

Table 1. Activity of antioxidant enzymes in cortical slices submitted to treatment with the neurotoxin 6-OHDA and *A. microcarpum* methanolic fraction.

	GST (mg/mU protein)	**GPx (mg/mU protein)**	**TRx (mg/mU protein)**
Control	241.2 ± 8.49	41.37 ± 5.01	2.375 ± 0.342
6-OHDA 500 µM	406.4 ± 75.89*	23.82 ± 2.07*	4.675 ± 0.608*
AMMF 100 mg/mL	186.2 ± 42.13$^{\#}$	51.01 ± 4.04	2.459 ± 0.137$^{\#\#}$
AMMF 100 mg/mL + 6-OHDA 500 µM	251.5 ± 36.48$^{\#}$	46.29 ± 3.20$^{\#\#}$	3.164 ± 0.496$^{\#}$

Data are expressed as percentage of the untreated control ± SEM *$p < 0.05$ in relation to control group #$p < 0.05$; ##$p < 0.001$ in relation to 6-OHDA group.

Table 2. Effect of treatment with 6-OHDA and *A. microcarpum* on intracellular redox homeostasis.

	GSH (% of control)	**GSSG (% of control)**	**Total Glutathione (% of control)**	**GSH/GSSG (% of control)**
Control	88.05 ± 6.73	90.60 ± 5.48	100.0 ± 10.66	100.0 ± 2.51
6-OHDA 500 µM	87.49 ± 5.41	61.61 ± 12.87*	66.56 ± 11.34*	168.1 ± 19.64**
AMMF 100 mg/Ml	136.7 ± 6.54**	117.2 ± 10.04##	129.7 ± 4.18##	120.7 ± 9.21##
AMMF 100 mg/mL + 6-OHDA 500 µM	111.4 ± 13.28	111.9 ± 3.17##	111.8 ± 4.96#	99.61 ± 8.99##

Data are expressed as percentage of the untreated control ± SEM *p < 0.05; **p < 0.001 as different from control group. #p< 0.05; ##p < 0.001 when compared to 6-OHDA group.

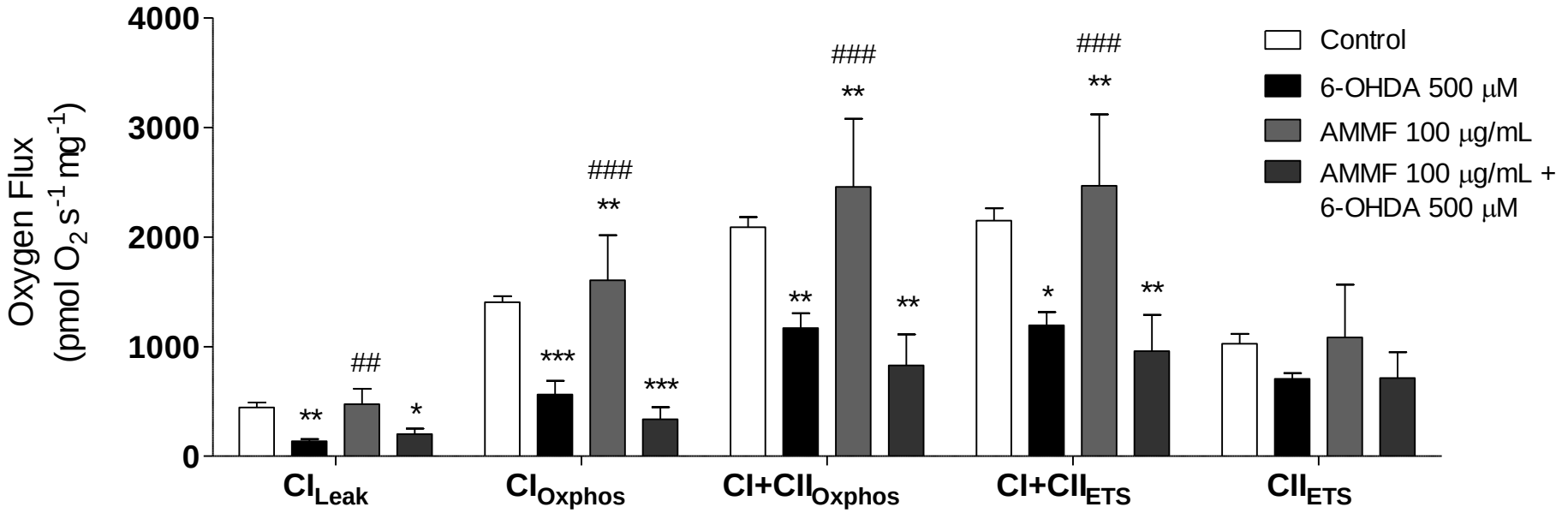
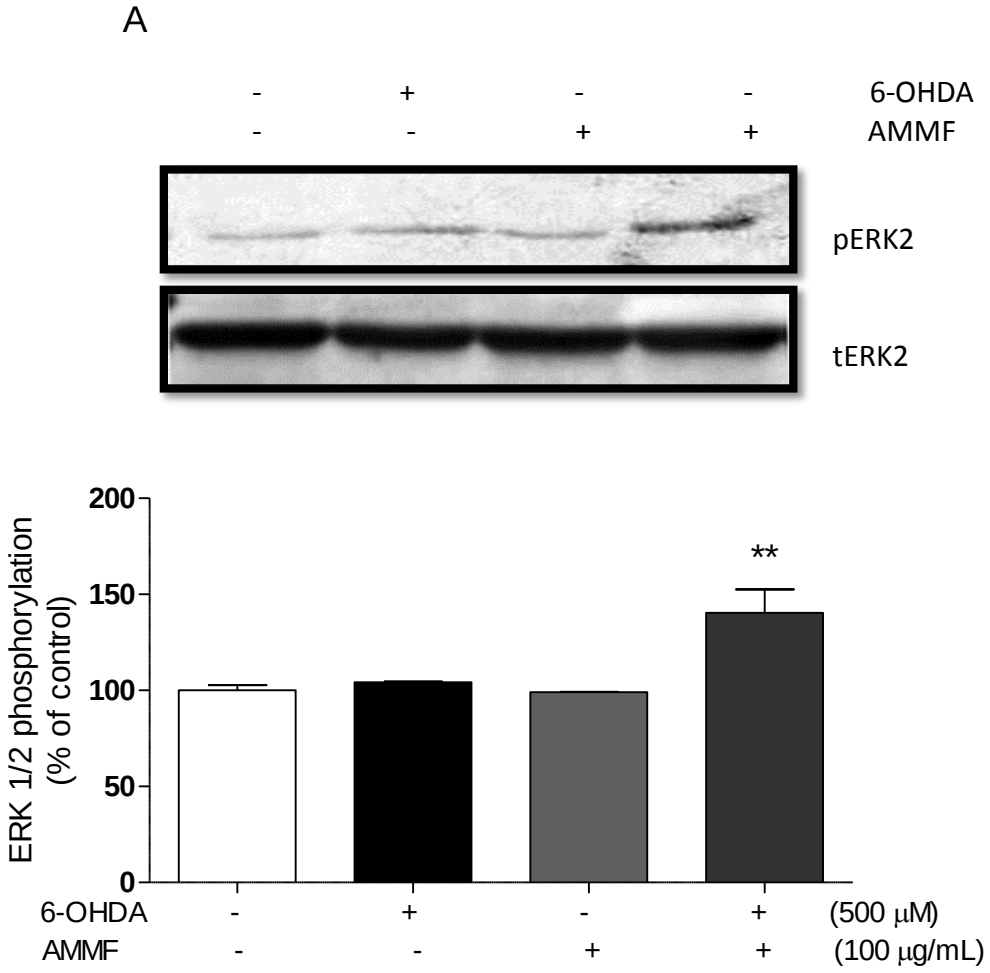
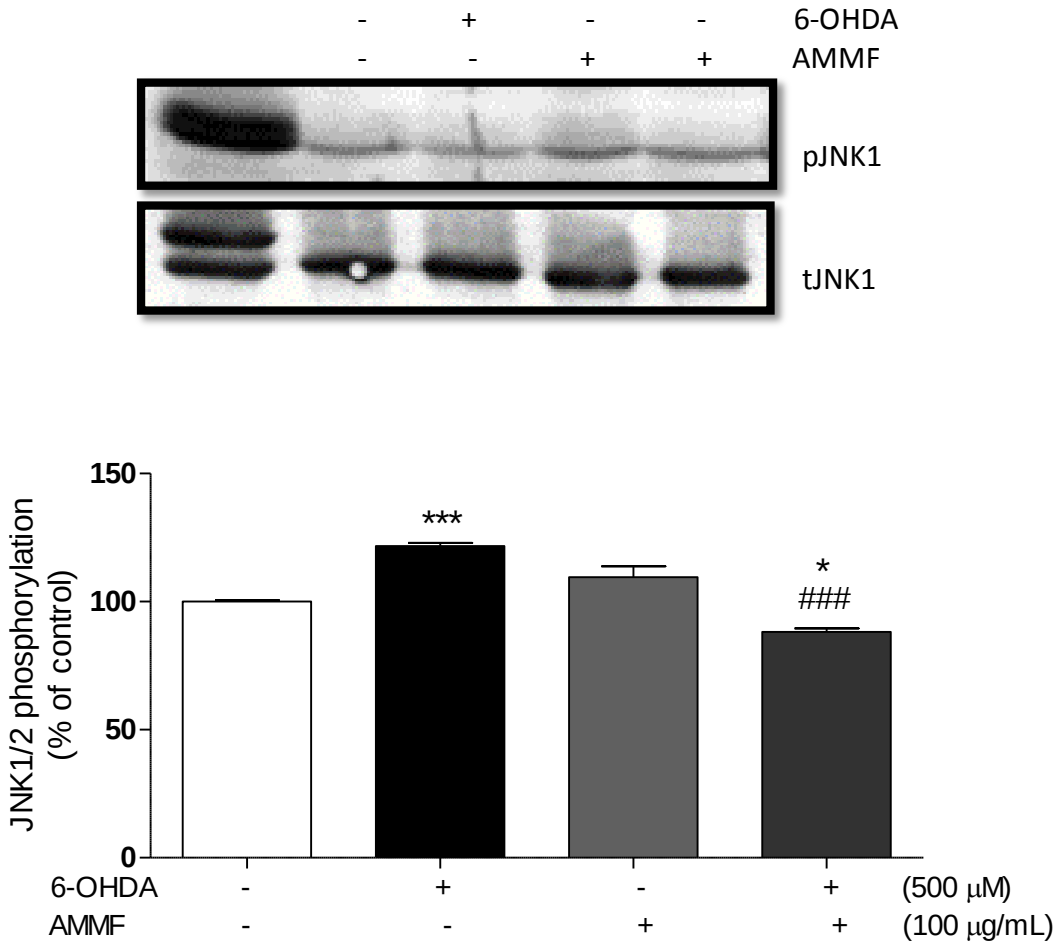


Figure 5. O2 flux measured in mitochondria of cortex homogenate exposed to 6-OHDA and/or *A. microcarpum*. Mitochondrial function are presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in presence of glutamate + malate (CI_{LEAK}), + ADP (CI_{OXPHOS}), + succinate (CII_{OXPHOS}), + FCCP ($CI+II_{ETS}$), + rotenone (CII_{ETS}). Antimycin A was used to correct for residual O_2 consumption. Results are means ± S.E.M. for 4 different preparations. *$p < 0.05$; **$p < 0.001$ and *** $p < 0.0001$ different from control group. ##$p < 0.001$ and ###$p < 0.0001$ when compared to 6-OHDA group.


A
- + - - 6-OHDA
- - + + AMMF
pERK2
tERK2
ERK 1/2 phosphorylation (% of control)
200
150
100
50
0
**
6-OHDA - + - + (500 μM)
AMMF - - + + (100 μg/mL)

B


- + - - 6-OHDA
- - + + AMMF
pJNK1
tJNK1
JNK1/2 phosphorylation (% of control)
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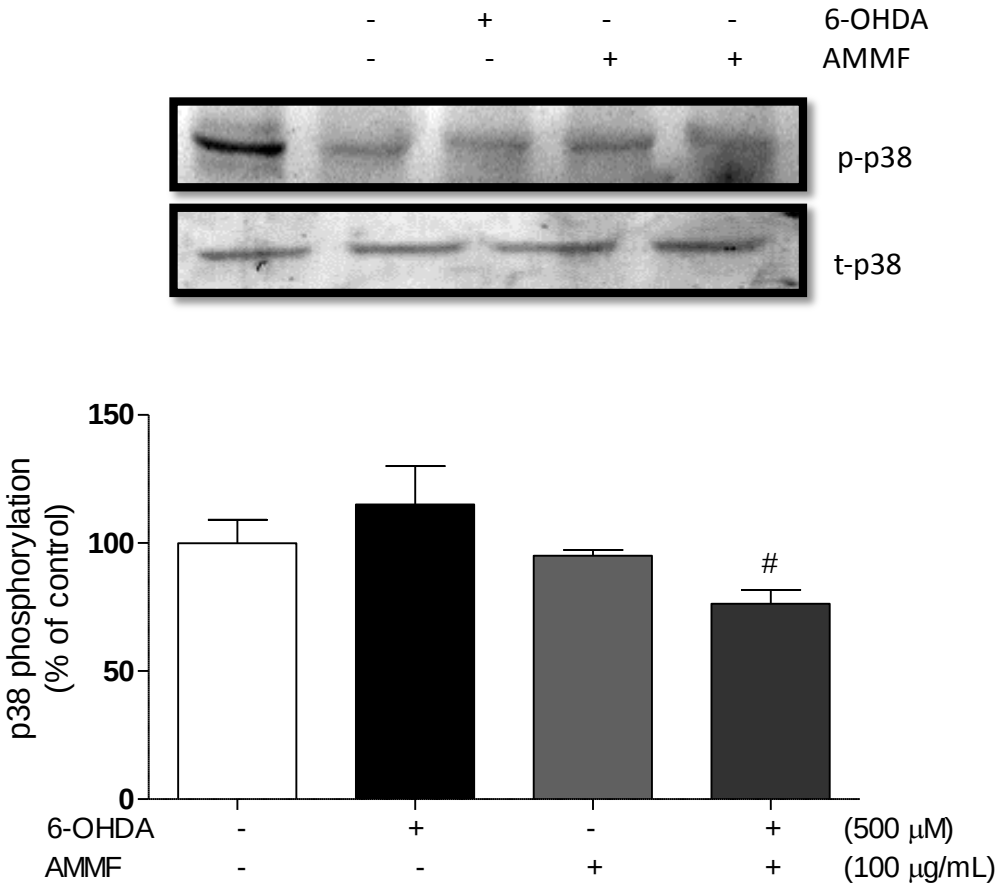
6-OHDA - + - + (500 μM)
AMMF - - + + (100 μg/mL)

C


- + - - 6-OHDA
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p-p38
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150
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p38 phosphorylation
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AMMF - - + + (100 μg/mL)

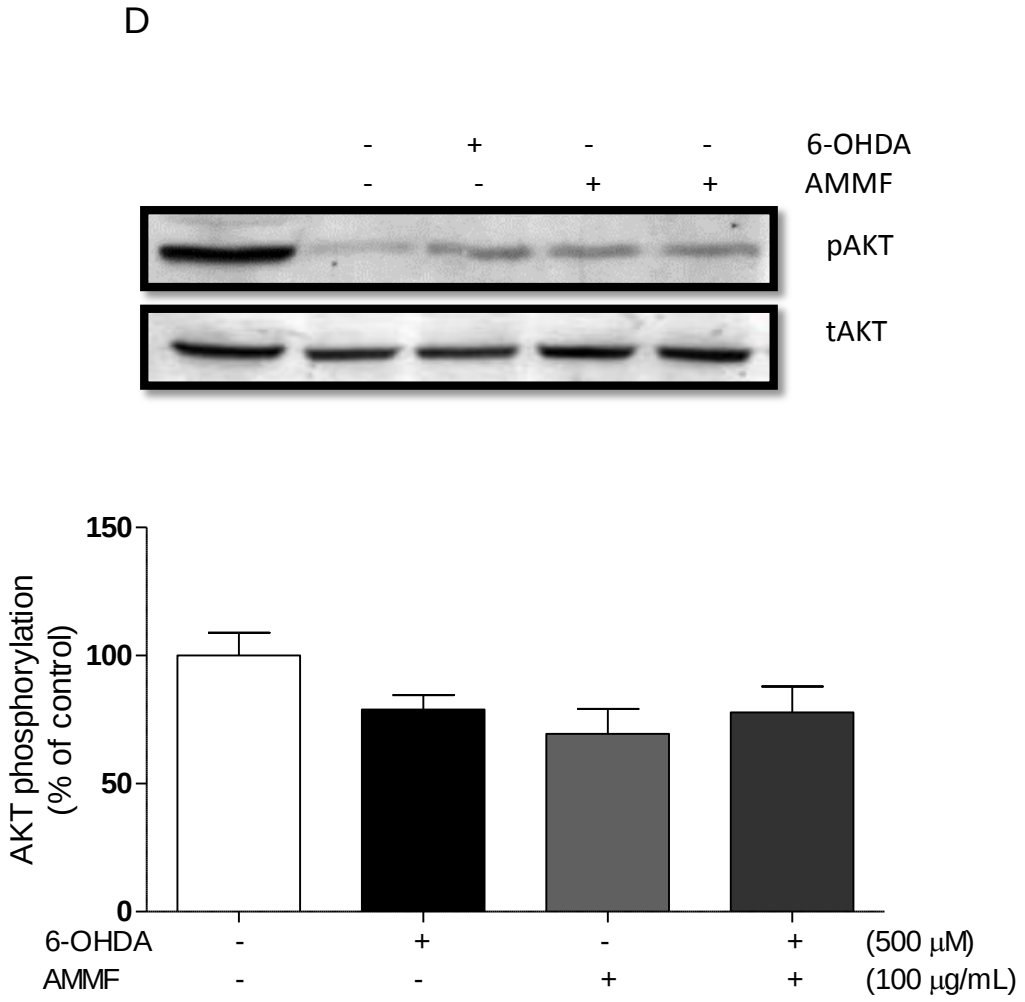

Figure 6. Modulation of p38MAPK, ERK1/2 and JNK1/2 and AKT phosphorylation in response to treatment with *A. microcarpum* and 6-OHDA in cortical slices after 2h. Proteins were separated by SDS–PAGE and transferred to nitrocellulose membrane. Total content and phosphorylation of proteins were detected by specific antibodies and the reaction was developed by ECL. (A) Western blotting of phosphorylated and total forms of ERK1/2 and the quantitative analysis of its phosphorylation after 2 h of treatment. (B) Representative Western blot of phosphorylated and total forms of JNK1 after 2 h treatment. (C) Western blotting of phosphorylated and total forms of $p38^{MAPK}$ and the quantitative analysis of its phosphorylation after 2 h of treatment. (D) Western blotting of phosphorylated and total forms of AKT after 2 h treatment. The data are expressed as fold increase related to control group and represent mean ± SEM of four to six experiments. Statistical analysis was performed by ANOVA, followed by the Newmann-Keuls test. *$p < 0.05$; **$p < 0.001$ and *** $p < 0.0001$ different from control group. #$p < 0.05$; ##$p < 0.001$ and ###$p < 0.0001$ when compared to 6-OHDA group.

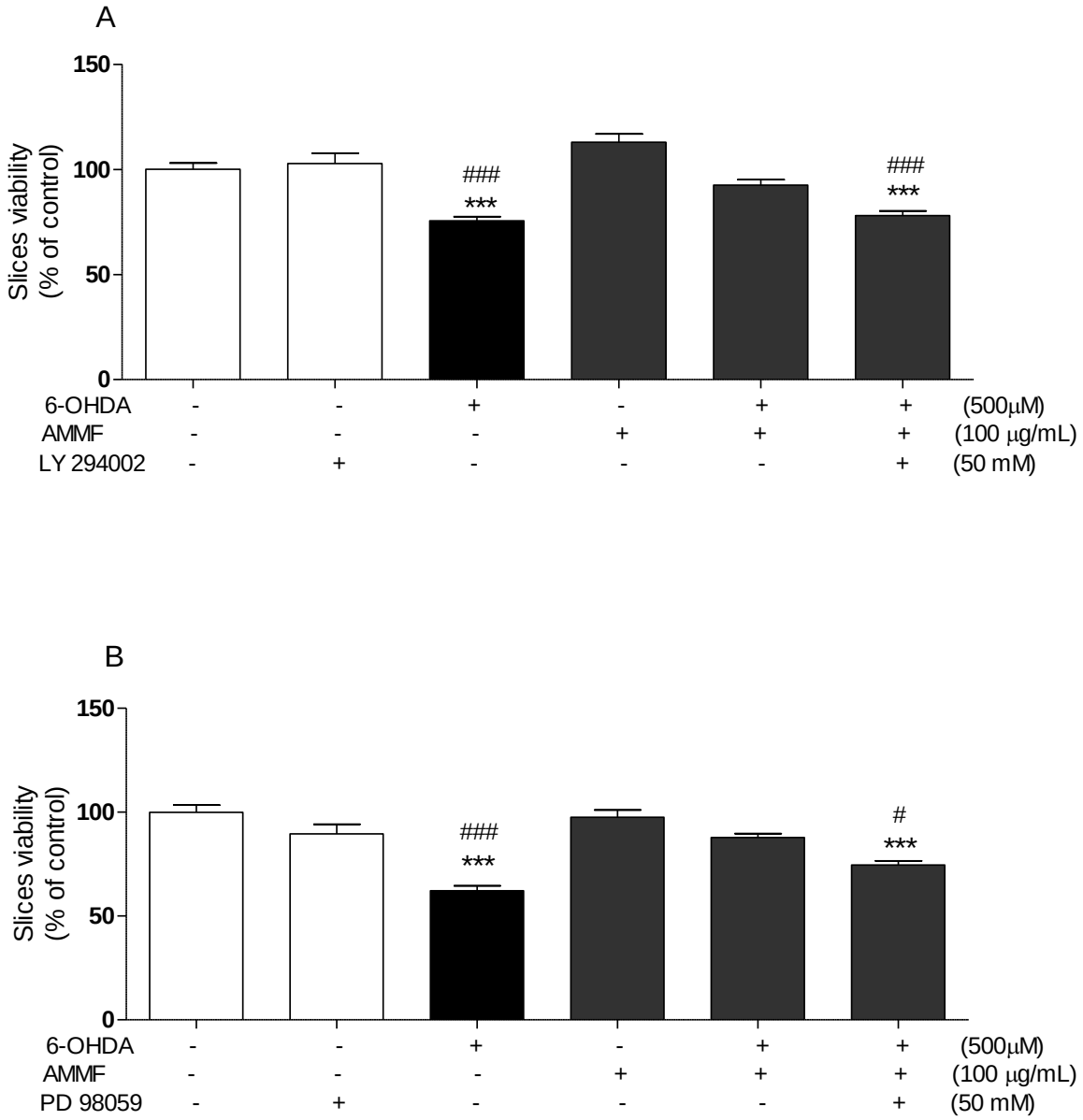

Figure 7. Effects of *A. microcarpum* and 6-OHDA on the viability of cortical slices in the presence of PD (98059) and LY (294002) inhibitors. Inhibition of (A) ERK1/2 and (B) Pi3k/Akt pathways increases the cytotoxicity of 6-OHDA on cortical slices. The slices were incubated with AMMF in the presence or absence of 6-OHDA (500 µM) during 2 h. Data are expressed as percentage of the untreated control ± SE (n = 3). ***p< 0.0001 as compared to control. #p<0.05; ###p< 0.0001 as compared to control treated with inhibitor.

4. CONCLUSÕES

Com base nos resultados obtidos neste trabalho, chegamos as seguintes conclusões:

- ✓ AMMF apresentou melhor efeito protetor quando comparado ao extrato hidroalcoólico e a fração acetato de etila;
- ✓ AMMF não reverteu a inibição da respiração mitocondrial;
- ✓ AMMF não impediu a autoxidação da 6-OHDA;
- ✓ AMMF apresentou efeito antioxidante protegendo o quadro de estresse oxidativo promovido pelo 6-OHDA;
- ✓ ERK e AKT parecem estar implicadas no efeito protetor da fração metanólica de *A. microcarpum* sobre a toxicidasde da 6-OHDA.

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