UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Avaliação do potencial do extrato comercial de guaraná (*Paullinia cupana)* como alternativa preventiva frente à toxicidade induzida em por compostos carbonílicos

Leonardo da Silva Bittencourt

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SUMÁRIO

PARTE 1

Esta parte está constituída do resumo em língua portuguesa, inglesa (abstract), lista de abreviaturas, introdução da referida tese e os objetivos da mesma.

RESUMO

Os compostos carbonílicos (CC) são compostos altamente tóxicos. A exposição humana aos CC ocorre a partir da inalação de seus vapores, contato com a pele e ingestão através dos alimentos e geração endógena. Dentre estes CCs, podemos citar, acroleína (ACR), Glioxal (GO), Metil-Glioxal (MGO). O 2-propenal, também conhecido como acroleína (ACR). Este composto é um aldeído eletrofílico, α-β insaturado altamente reativo, incolor, que está presente no ambiente (presente no ar e na água devido à combustão incompleta de madeira, petróleo, carvão, plásticos, tabaco), em alimentos (frituras, bebidas alcoólicas), e ainda é um composto produzido endogenamente (ação de mieloperoxidases na degradação de treonina, degradação de drogas anti-câncer, lipoperoxidação de ácidos graxos poli-insaturados, especialmente no Sistema Nervoso Central- SNC).

Fortes evidências (tanto *in vitro* quanto *in vivo*) tem mostrado que a ACR gera forte estresse oxidativo (principalmente por depleção de glutationa - GSH), exercendo assim forte influência no início e progressão de doenças crônicas relacionadas ao estresse oxidativo, tais como câncer, doenças neurodegenerativas, aterosclerose, diabetes.

Dado a relevância do tema, recentemente a atenção da comunidade científica tem sido focada em entender como a ACR altera o estado-redox celular e principalmente procurar alternativas farmacológicas para atenuar a sua toxicidade. Dentre estas alternativas, estão os compostos com atividade antioxidante e os compostos com atividade diretamente quelante da acroleína, como compostos ricos em grupamentos tióis (SH), compostos nitrogenados (amino compostos) e compostos de ocorrência natural (ácido ascórbico, tocoferol e polifenóis).

O uso de plantas medicinais pela população mundial é muito significativo e neste cenário se encaixa o guaraná amazônico (*Paullinia cupana*, var. Sorbilis). O seu uso tem se disseminado mundialmente principalmente nas bebidas energéticas. Adicionalmente vários estudos produzidos independentemente por diversos grupos de pesquisa sugerem ter o guaraná ação: cardiotônica e cardioprotetora, energética, atividade anti-bacteriana, antioxidante, efeito anti-obesidade, termogênico, atividade contra lesões gástricas, quimiopreventivo, anti-depressivo e anti-mutagênico.

Nesta tese demonstramos pela primeira vez que: 1- o extrato de guaraná amazônico possui forte atividade antioxidante frente a várias espécies reativas; 2- atenua o decréscimo de viabilidade induzida por compostos carbonílicos (ACR, GO e MGO) aumentando a viabilidade celular e diminuindo o estresse oxidativo em experimentos com culturas de células SH-SY5Y; 3 – Exerce efeitos antioxidantes no soro de ratos sem exercer efeitos pro-oxidantes no sistema nervoso central e fígado quando administrado sozinho; 4 – Atenua de forma significativa a toxicidade e dano oxidativo induzidos pela acroleína em ratos, se mostrando como uma possível alternativa preventiva promissora para atenuar o dano oxidativo induzido compostos carbonílicos reativos.

ABSTRACT

The carbonyl compounds (CC) are highly toxic compounds. Human exposure to CC occurs from inhalation of vapors, skin contact and ingestion through food and endogenous generation. Among these CCs, such as, acrolein (ACR), Glyoxal (GO), Methyl Glyoxal (MGO). The 2-propenal, also known as acrolein (ACR) is an electrophilic, α-β unsaturated, highly, reactive aldehyde, which is present in the enviroment (in the air and the water due to the incomplete combustion of wood, petroleum, coal, plastics, tobacco), in foods (fried foods, alcoholic beverages), and produced endogenously (myeloperoxidase-induced degradation threonine, degradation of anti-cancer drugs, lipid peroxidation of polyunsaturated fatty acids, especially in the nervous system Central-CNS).

Strong evidence (both *in vitro* and *in vivo*) has shown that the ACR generates marked oxidative stress (mainly depletion of glutathione - GSH), thereby exerting strong influence on the onset and progression of chronic diseases associated with oxidative stress, such as cancer, neuro-degenerative diseases, atherosclerosis, diabetes.

Regarding the relevance, recently the attention of the scientific community has been focused on understanding how the ACR changes the cellular redox-state and mainly seek for pharmacological alternatives to mitigate its toxicity. Among these alternatives are compounds with antioxidant activity and compounds which can directly scavenge acrolein such as thiol (SH), nitrogen compounds (amino compounds) and naturally-occurring compounds (ascorbic acid, tocopherol and polyphenols).

The use of medicinal plants for the world's population is very significant, in this scenario we have the Amazon guarana (*Paullinia cupana* var. Sorbilis). Its use has spread worldwide mainly in energy drinks. Additionally several independente studies suggest guarana's action: cardiotonic and cardioprotective, energy, anti-bacterial activity, antioxidant, anti-obesity effect thermogenic, activity against gastric lesions, chemopreventive, anti depressant and anti-mutagenic.

In this study, we demonstrated for the first time: 1. Amazon guarana has strong antioxidant activity against several reactive species; 2 attenuates the decrease in cabonyl compounds-induced cell viability (ACR, GO, MGO) increasing cell viability and reducing oxidative stress; 3 - exerts antioxidant effects in the serum of rats without exerting pro-oxidant effects in the central nervous system and liver when administered alone; 4 - significantly attenuates the toxicity and oxidative damage induced by acrolein in rats showing a promising preventive alternative to alleviate the oxidative damage induced by reactive carbonyls.

LISTA DE ABREVIATURAS

2-DR – 2-Deoxy-D-Ribose
ACR – acroleína
AD – Alzheimer's Disease (Doença de Alzheimer)
ALT – Alanina Amino Transferase
ANVISA – Agência Nacional de Vigilância Sanitária
AST – Aspartato Amino Transferase
ATP – Trifosfato de adenosina
CAF – Cafeína
CAT – catalase
CC – Compostos carbonílicos
CNS – Central Nervous System
dG – Desoxiguanosina
EGCG – Epigalocatequina-3-galato
EROS – Espécies reativas de oxigênio
FDA – Food and Drug Administration
FRAP – Ferric Reducing Antioxidant Power assay
GGT – Gama Glutamil Transferase
GO – Glioxal
GSH – Reduced glutatione (Glutationa Reduzida)
HNE – 4-hidroxi-2-Nonenal
IL-6 – Interleucina 6
IL-8 – Interleucina 8
LDH – Lactato Desidrogenase
LPO - Lipoperoxidação
MDA – Malondialdeído
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC – N- Acetil-cisteína
SH – Grupamentos sulfidril
SNC – Sistema Nervoso Central
SOD – Super Óxido Dismutase
SRB – Sulforodamina-B
RE – Retículo endoplasmático
ROS – Reactive Oxygen Species
TAU – Proteína pertencente à classe das MAPS (proteínas associadas a microtúbulos)
TBARS – Ensaio para substâncias reativas ao ácido tiobarbitúrico
TNF-α – Fator de Necrose Tumoral Alfa

INTRODUÇÃO

A ACROLEÍNA

Os compostos carbonílicos (CC), aldeídos e cetonas podem ser emitidos diretamente para atmosfera através de fontes naturais ou antropogênicas. As fontes antropogênicas estão diretamente ligadas às atividades humanas, sendo assim os CC podem ser encontrados na queima de combustível, tabaco, gorduras animais e óleos vegetais, incineração de lixo entre outras (Gilbert et al, 2005). Como fontes naturais temos a queima espontânea de florestas, excrementos de animais e os gases vulcânicos, sendo que estes últimos contribuem com uma menor liberação destes compostos para a atmosfera (Andrade et al., 2002).

Acroleína (ACR) é um exemplo destes compostos carbonílicos, este aldeído é muito encontrado em alimentos fritos proveniente da desidratação do glicerol durante o processo de fritura e em bebidas alcoólicas como vodcas, vinhos, cervejas e cachaça podendo ser formada pela fermentação alcoólica (Ghilarducci & Tjeerdema, 1995). É diariamente introduzida no ambiente por diversas fontes como: queima de gasolina, fumaça de cigarro, aquecimento de gorduras, processos industriais e incêndio em vegetação (Kehrer & Biswal, 2000). Uma estimativa indica que a acroleína representa 5% do total de aldeídos poluentes do ar, sendo o formaldeído o maior contaminante dentre eles (Kehrer & Biswal, 2000). Industrialmente, a acroleína é usada como herbicida ou fungicida e pode ser usada também como um material de partida para a produção de ácido acrílico. A ACR existe naturalmente em alimentos e é formada durante a combustão de materiais orgânicos. ACR também é encontrada em todos os tipos de cigarros e em vapores provenientes da combustão de óleo de cozinha onde tem ocorrido graves exposições tóxicas aos seres humanos (Kehrer & Biswal, 2000). Este aldeído tem sido registrado como um dos componentes do fumo do cigarro responsável pelo mau hálito na boca do fumante.

Quimicamente, a ACR é um composto carbonílico α, β-aldeído insaturado, conhecido também como, aldeído acrílico, acrilaldeído ou 2-propenal. O 2-propenal é volátil e altamente inflamável sendo capaz de sofrer polimerização espontânea. É tóxica, seus vapores podem causar severas irritações respiratórias e oculares e se ingerida provoca náusea, vômito, colapso e coma

(Ghilarducci & Tjeerdema, 1995). Sua principal via de ataque são as mucosas do trato respiratório superior podendo produzir edema pulmonar em altas concentrações. O contato com acroleína líquida pode produzir necrose da pele ou dos olhos. A polimerização deste composto ocorre de forma violenta quando ocorre contato com materiais alcalinos ou ácidos. A dimerização da acroleína é muito lenta à temperatura ambiente, podendo tornar-se muito rápida a temperaturas elevadas (~90 º C).

Além disso, a ACR é gerada endogenamente através da degradação do aminoácido treonina via mielo-peroxidase, degradação da espermina mediada pela amino-oxidase (O'Brien et al., 2005), metabolismo de agentes alquilantes anti-cancer (Zhu et al., 2011) e lipoperoxidação de ácidos graxos poli-insaturados, principalmente no Sistema Nervoso Central (SNC) (Pan et al., 2005, Zhu et al., 2011). Dentre os aldeídos α, β insaturados, a ACR é detentora do título de maior eletrofilicidade, e a sua alta reatividade com nucleófilos biológicos que confere sua grande toxicidade (Bucham et al., 2008). Devido a sua solubilidade, a acroleína pode atravessar facilmente membranas celulares por difusão passiva (Stevens & Maier., 2008).

A sua toxicidade é causa principalmente através da alteração da estrutura de proteínas, ácidos nucléicos (DNA) através de reação direta, depletando glutationa (GSH), impondo forte pressão oxidativa intracelular, interferindo em todo estado-redox celular e sinalização mediada por estado redox (Zhu et al., 2011). No caso das proteínas, os sítios nucléofilos atacados diretamente pela acroleína são: os grupos sulfidril (SH) de cisteínas, ε-amino de lisinas e o anel imidazol da histidina. Dentre estes sítios, a ACR ataca preferencialmente os grupamentos sulfidril de cisteínas formando adutos de Michael, acreditando-se ser este o principal caminho por onde a ACR exerce sua toxicidade (Zhu et al., 2011).

Similar às proteínas, resíduos de desoxiguanosina (dG) no DNA servem como sítios nucleófilos para formação dos adutos α-hidroxi-PdG and γ-hidroxiPdG (forma cíclica e não cíclica do N-Propano desoxiguanosina respectivamente) após reação com ACR (Zhu et al., 2011). A alteração do DNA é precedente à distúrbios nos processos replicativos e transcricionais desta molécula, contribuindo desta forma como etiologia de muitas doenças. Para se ter uma idéia,

adutos ACR-desoxiguanosina foram bastante prevalentes em lesões de tecidos humanos e de roedores com grande potencial carcinogênico (Pan et al., 2016).

GSH é uma molécula antioxidante intracelular não enzimático fundamental envolvida na defesa antioxidante celular (Dickinson et al., 2002). A conjugação da ACR com GSH é considerada via principal para a detoxificação da ACR (Singh et al., 2015). ACR pode conduzir à depleção de GSH celular de um modo dependente da concentração, levando a efeitos prejudiciais para os seres humanos. Estudos sugerem que uma vez que o nível de GSH tenha atingido um certo limiar, grupos tiol proteicos podem ser progressivamente modificados e dar lugar a uma série de efeitos celulares/moleculares diretos, tais como a proliferação celular, apoptose e alterações na expressão do gene/proteína (Kehrer & Biswal, 2000).

Alguns estudos mostram a ACR como moduladora de vários fatores de transcrição tais como o fator nuclear kappa B (NF-kB) relacionado com cenário pró-inflamatório, proteína ativadora 1 (AP-1) e fator nuclear eritróide-2 (Nrf2), todos relacionados ao estado redox celular (Stevens & Maier., 2008; Kehrer & Biswal, 2000). Também é sugerido que a ação da ACR em várias vias de sinalização celular pode ainda ajudar a diminuir a proliferação celular e, provavelmente, aumentar a susceptibilidade das células à morte por apoptose e necrose. Evidências sugerem que o acúmulo crônico da ACR e de outros compostos carbonílicos, pode aumentar o estresse oxidativo, aumentando o dano e morte celulares, facilitando o início e progressão de doenças crônicas relacionadas ao estresse oxidativo, incluindo doenças neurogenerativas, principalmente Alzheimer, Câncer, aterosclerose (Zhu et al., 2011, Huang et al., 2013).

O ESTRESSE OXIDATIVO

Em altas concentrações, as ERO podem ser importantes mediadoras de danos a diversas estruturas celulares como lipídios, proteínas, ácidos nucléicos e carboidratos (Valko et al., 2006, 2007; Halliwell 2006), condição esta conhecida como estresse oxidativo. Em essência, o estresse oxidativo pode ser definido como um desbalanço grave entre a produção de espécies reativas e o sistema de defesa antioxidante (Halliwell, 2006). Entretanto, a natureza do dano celular induzido pelas ERO depende do local de sua formação (Goetz et al., 2008).

Estresse oxidativo pode ser resultado de:

1. Concentração diminuída de antioxidantes*:* Mutações que afetem a atividade das enzimas antioxidantes como CuZnSOD, MnSOD (Tatsch et al., 2012), ou GPx, ou toxinas que afetem o sistema de defesa antioxidante (xenobióticos que são metabolizados junto com GSH causando sua depleção). Deficiência nos minerais obtidos na dieta (Zn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Se) e ou antioxidantes obtidos também da dieta (Cerqueira et al., 2007);
2. Produção aumentada de espécies reativas: Exposição de células a altas concentrações de O_2 ou a outras moléculas que são por si próprias altamente reativas. A ativação excessiva dos sistemas existentes nas células (resposta inflamatória) que produzem naturalmente espécies reativas (Halliwell, 2006).

O dano oxidativo decorrente de estresse oxidativo inclui as seguintes consequências:

1. Adaptação da célula ou organismo através de uma sobre regulação (“up-regulation”) dos sistemas de defesa endógenos, que podem: (a) defender o organismo completamente; (b) proteger o organismo em parte e (c) superproteger (as células se tornam resistentes a altos níveis de estresse oxidativo) (Halliwell, 2006);

2. Injúria celular envolve dano oxidativo a todos os alvos celulares: lipídios, proteínas, ácidos nucléicos (principalmente o DNA) e carboidratos. O dano oxidativo pode ocorrer durante a fase de adaptação (item 1). Segundo Halliwell, 2006, *nem todo dano causado por estresse oxidativo é considerado dano oxidativo*; danos a biomoléculas podem ser causados por mudanças relacionadas ao estresse oxidativo, como alterações nos níveis de Ca^{2+} ou ativação de proteases.
3. Morte celular que pode ser desencadeada devido ao extenso dano ao DNA acionando mecanismos de morte celular. A pressão oxidativa extremamente alta pode causar dano generalizado à célula, provocando necrose.

PEROXIDAÇÃO LIPÍDICA

A membrana plasmática é basicamente formada por uma dupla camada lipídica no qual se encontram proteínas integrais e periféricas associadas a mesma. As ERO podem prejudicar a função das membranas pela oxidação dos ácidos graxos poliinsaturados presentes nos lipídios e indiretamente pela inibição da síntese dos lipídios, dessaturação de ácidos graxos ou ativação das lipases (Goetz et al.,2008).

Devido à sua abundância nas células e a susceptibilidade à oxidação pela presença de grupos metilênicos entre duplas ligações, os ácidos graxos poliinsaturados são, para os oxidantes, alvos mais prováveis do que o DNA (Loureiro, 2002). É estimado que aproximadamente 60 moléculas de ácido linoléico (18:2 $\Delta^{9,12}$) e 200 de ácido araquidônico (20:4 $\Delta^{5,8,11,14}$) (os ácidos graxos poliinsaturados mais abundantes em nossas células) são consumidas por oxidante que reage com a bicamada lipídica (Loureiro, 2002, Halliwell, 2006). A peroxidação lipídica se inicia pelo ataque à bicamada lipídica de qualquer espécie suficientemente reativa para abstrair um átomo de hidrogênio bis-alílico de um ácido graxo poliinsaturado. Foi verificado que espécies tais como $^{\bullet}OH$, $HO_2^{\bullet}$, $^{\bullet}NO_2$, $RO^{\bullet}$, $RO_2^{\bullet}$ podem realizar essa oxidação. Após iniciado, o processo se torna autocatalítico, levando à formação de hidroperóxidos e produtos secundários. É importante mencionar que a oxidação enzimática do ácido araquidônico, que ocorre durante a síntese de

eicosanóides, é uma importante fonte de espécies reativas de oxigênio. Além dos eicosanóides envolvidos na sinalização intra e intercelular, radicais de oxigênio e hidroperóxidos lipídicos são gerados durante as reações catalisadas por ciclooxigenases ou lipoxigenases (Loureiro et al., 2002).

Após a abstração do átomo de hidrogênio do ácido graxo poliinsaturado (LH) levando à formação de um radical lipídico centrado no carbono ($L^{\bullet}$), este é estabilizado por um rearranjo molecular, adquirindo a estrutura de dieno-conjugado. A adição extremamente rápida de uma molécula de oxigênio ao radical lipídico leva à formação do radical peroxila ($LOO^{\bullet}$) (Loureiro et al, 2002). Este é capaz de reagir com outro ácido graxo poliinsaturado, iniciando uma nova cadeia de oxidação a partir da formação de outro radical lipídico ($L^{\bullet}$) (fase de propagação). O radical $LOO^{\bullet}$ combina-se com o átomo de hidrogênio abstraído e forma um hidroperóxido lipídico (LOOH). Alternativamente, os radicais $LOO^{\bullet}$ podem formar peróxidos cíclicos pelo ataque a uma dupla ligação na mesma cadeia. Esses peróxidos cíclicos também podem propagar a peroxidação lipídica e, no caso da oxidação dos ácidos araquidônico, docosahexaenóico (22:6 $\Delta^{4,7,10,13,16,19}$) e eicosapentaenóico (20:5 $\Delta^{5,8,11,14,17}$), podem levar à formação de isoprostanos (Loureiro et al., 2002; Halliwell & Whiteman, 2004) que podem ser medidos no plasma e urina humanos (Halliwell & Whiteman, 2004).

Todas estas modificações oxidativas causam mudanças nas propriedades físicas e químicas das membranas, alterando sua fluidez e permeabilidade, com expansão do liquido intracelular e risco de ruptura tanto da membrana plasmática que circunda a célula quanto das membranas que delimitam as organelas. Estas rupturas podem levar a morte celular (Vasconcelos et al., 2007).

Decorrente dos processos de oxidação de lipídios de membrana, a maioria dos produtos formados são derivados do ácido graxo poliinssturado ω-6, como o malondialdeído (MDA) (70%), hexanal (15%), acroleína, glioxal, crotonaldeído, trans-2-nonenal, 4-oxo-2-nonenal, 4-hidroxi-2-nonenal (HNE). Os alcanais tais como o hexanal, são menos reativos e causam menos efeitos comparados aos aldeídos insaturados. Alquenais contendo duplas ligações entre carbonos

(C=C), tal como a acroleína, são mais reativos que os alcanais. Estas moléculas se tornam mais reativas se as ligações duplas ocorrem entre C2-C3, tornando o carbono C3 extremamente eletrofílico, podendo reagir com grupamentos nucleofilicos de proteínas, DNA e lipídios, danificando estas moléculas. (Ellis, 2007). A reatividade nestas regiões fica aumentada pela proximidade com o grupamento hidroxil em C4 que é sequestrador de elétrons e o grupo carbonil em C1. O HNE se enquadra neste tipo de situação. Os aldeídos extremamente reativos como o HNE, interagem diretamente com proteínas e membranas causando perda significativa de função de transportadores, enzimas, componentes sinalizadores, fatores de transcrição, proteínas do citoesqueleto e outras proteínas, tais como a TAU (Picklo et al., 2002). A acroleína também é citotóxica, e no caso de células nervosas, altera as concentrações de Ca^{2+} alterando desta forma, o transporte de Ca^{2+} e o transporte de glutamato.

O ESTRESSE OXIDATIVO E A ACROLEÍNA

O metabolismo aeróbico produz normalmente espécies reativas de oxigênio (ERO) que podem que podem danificar lipídios (lipoperoxidação - LPO), proteínas (carbonilação), carboidratos e ácidos nucleicos. Para evitar o dano oxidativo, as células possuem um sistema de defesa antioxidante enzimático e não enzimático. No entanto, a grande produção de ERO pode sobrecarregar esses mecanismos de defesa resultando em estresse oxidativo (Bierben et al., 2012). A ACR é considerada tanto um produto quanto um iniciador de LPO (Adams & Klaidman, 1993; Uchida et al., 1998) sendo considerada, assim, um agente indutor de estresse oxidativo. Estudos *in vitro* e *in vivo* demonstraram que a ACR pode causar danos oxidativos extensos, comprometendo a homeostase da membrana celular, integridade do DNA, induzir dano mitocondrial e apoptose (Moghe et al., 2015).

A exposição à ACR (0,1-5 μM) de linhagem de células da retina humana (ARPE 19) aumentou significativamente a pressão oxidativa através da diminuição GSH, enzimas anti-oxidantes (SOD e Glutationa-Peroxidase), e níveis totais e nucleares do fator de transcrição NRF2, coincidente com redução da função mitocondrial, sugerindo que ACR em danos na retina e degeneração macular relacionada com a idade. O SNC é particularmente vulnerável à acroleína devido abundância em ácidos graxos poliinstaturados e da falta de enzimas que metabolizam aldeídos reativos, portanto os neurônios são particularmente afetados pelo estresse oxidativo provocado pela ACR (Hamann & Shi, 2009). Diversos estudos demonstraram que a ACR é capaz de induzir estresse oxidativo e dano neuronal em diversos modelos experimentais, como porcos, roedores, células SHSY-5Y, células hipocampais HT-22, sugerindo que pode ser um fator de risco potencial para o desenvolvimento de doenças neurodegenerativas (Bittencourt, et al., 2014; Due et al., 2014; Shi et al., 2011; Zheng et al., 2013; Huang et al., 2013). Huang e colaboradores (Huang et al., 2013), mostraram em linhagem de células hipocampais HT-22 e em roedores Sprague-Dawley que a administração de acroleína de 25μM e 2,5 mg/kg/dia respectivamente induzem a hiperfosforilação da proteína associada a microtúbulos TAU, diminuem o

imunoconteúdo da enzima alfa-secretase ADAM-10 (A-desintegrina e metaloproteinase-10), aumentando o imunoconteúdo da beta-secretase (BACE-1), aumento de pressão oxidativa e diminuição da viabilidade celular. Em roedores Sprague Dawley, foi demonstrado que 2,5mg/kg/dia de ACR induzem astrogliose (aumento do imunoconteúdo de GFAP), picnose neuronal (atrofia neuronal), diminuição da atividade da enzima SOD no hipocampo destes animais, aumento do imunoconteúdo de RAGE, tau e BACE-1. Este estudo mostrou que a acroleína induz forte estresse oxidativo e inflamação no encéfalo dos animais, levando a importantes déficits cognitivos (Huang et al., 2013).

Foi demonstrado que a administração crônica de 3 mg/kg/dia de ACR induzem aumento da produção de MDA, diminuição de GSH e fortes alterações na homeostase energética em córtex de ratos Wistar (Rashedinia et al., 2013).

A exposição a ACR (1 a 5,2 nmol/mg de proteína) *in vitro* aumentou a agregação do neurofilamento-L que pode contribuir para neurodegeneração (Jeong & kang, 2008). Exposições a 1µM de ACR durante 4 horas resultou em aumento de permeabilidade da membrana ao iodeto de propídeo em modelo *ex vivo* de medula espinhal (Luo & Shi, 2004). Tanto *in vitro* quando *in vivo*, a acroleína induz diminuição da liberação de neurotransmissores devido a alteração estrutural e inibição da proteína relacionada a fusão membrana vesicular (NSF), da proteína associada a sinaptossomo 25 kDa (SNAP-25) e diminui a recaptação de neurotransmissores, devido a alteração no próprio neurotransmissor e em v-ATPases relacionadas com a internalização do mesmo nas vesículas sinápticas (Lopachin et al., 2007).

O dano oxidativo induzido pela ACR, vem acompanhado por disfunção mitocondrial e estresse de retículo. A mitocôndria é uma organela que possui papel dinâmico dentro das células, controlando a produção de ATP, promovendo β-oxidação, síntese de esteróides, controlando a homeostase do cálcio e sobrevivência celular. A ACR afeta de forma expressiva estas funções, marcadamente a apoptose. A apoptose induzida pela acroleína é geralmente induzida de forma caspase-dependente (células SHSY-5Y e A549) e caspase-independente (células CHO) (Dong et al., 2013; Roy et al., 2010; Tanel & Averill-Bates, 2005). Fora vias relacionadas à apoptose, a ACR afeta também a capacidade respiratória mitocondrial inibindo complexos I e II, piruvato

desidrogenase, alfa-ceto glutarato desidrogenase em concentrações a partir de 10µM, prejudicando todo o transporte de elétrons e produção de ATP. E inibições nestas enzimas-chave (devido ao sequestro do ácido lipóico promovido pela ACR), foram observadas em cérebros de pacientes com Alzheimer (Mackzurek., 2008).

O retículo endoplasmático (RE) é local de síntese e montagem de proteínas. Devido a formação de adutos proteicos pela ACR, levando alterações na estrutura e função, proteínas mal formadas se acumulam no RE gerando estresse de retículo, tornando este aldeído forte gerador de estresse de retículo. O estresse de retículo contribui para muitas situações patológicas incluindo, esteatose hepática (Vacaru et al., 2014), Doença de Alzheimer (Li et al., 2014), doenças cardiovasculares (Zhou & Tabas, 2013). Diversos estudos mostram que em concentrações que variam de 2 a 25µM, a ACR se mostrou capaz de disparar resposta relacionada ao estresse de retículo, aumentando a expressão de genes pró-inflamatórios como TNF-α, IL-6 e IL-8, demonstrando assim, propriedades pró-inflamatórias deste aldéido e seu papel em diversas doenças.

COMPOSTOS NATURAIS COMO SCAVENGERS ALTERNATIVOS DA ACROLEÍNA – O GUARANÁ AMAZÔNICO

Recentemente, compostos polifenólicos tem sido descritos como excelentes quelantes de compostos carbonílicos (GO, MGO e ACR). Dentre os principais estudados até agora, os mais promissores são: epicatequina, epigalocatequina, epicatequina-3-galato, epigalocatequina-3-galato (EGCG), teaflavina, teaflavina-3,3-digalato, cianomaclurina floretina, floridzina, pro-antocianidinas, antocianinas. Análises mais detalhadas da estrutura destas moléculas, revelaram que os anéis do tipo A B e C (figura 1) presentes na estrutura destas moléculas são os principais detentores de atividade quelante dos supra-citados aldeídos (Lo et al., 2006; Sang et al., 2007; Yamaguti-Sasaki et al., 2007; Beretta et al., 2008; Shao et al., 2008; Bittencourt et al., 2014).

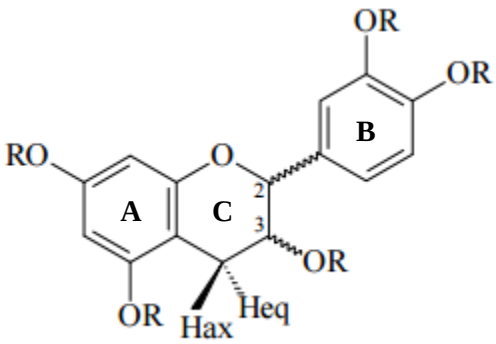

Figura 1. Molécula de epicatequina encontrada nas sementes de guarana. As letras **A**, **B** e **C** representam os anéis e a sigla OR significa oxigênio ligado a algum outro átomo, geralmente hidrogênios, formando grupamentos hidroxila, os prováveis responsáveis pela atividade *scavenger* da molécula. *Adaptado de Yamaguti-Sasaki et al., 2007.*

É sabido que a ACR reage com nucleófilos biológicos formando adutos de Michael, ou seja, uma molécula mista com menor reatividade. O mesmo mecanismo é proposto para polifenóis, onde o grupamento carbonila reage com os grupamentos hidroxila presentes nos carbonos 2, 3, 4 e 5 da molécula do polifenol gerando hemi-acetais (compostos oriundos da reação entre grupos OH e grupos carbonila) estáveis sem reatividade que podem sem ser degradados (Zhu et al., 2009) (Figura 2).

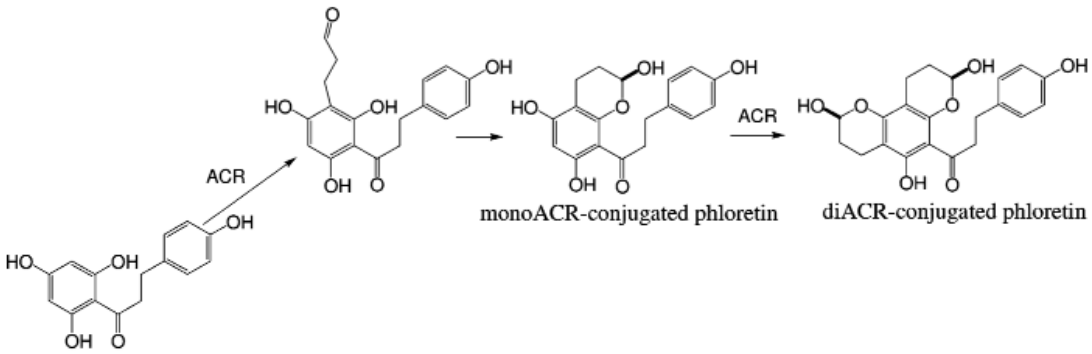

Figura 2. Mecanismo proposto da ligação de compostos fenólicos com a acroleína. *Adaptado de Zhu et al., 2009.*

O extrato de guaraná é um produto oriundo da Amazônia que atualmente é consumido como um suplemento alimentar aprovado pela Agência de Vigilância Sanitária (ANVISA-Ministério da Saúde).

Entretanto, segundo a medicina popular e os estudos realizados nas três últimas décadas mostraram que o guaraná possui compostos bioativos que agem no diretamente no metabolismo. As propriedades funcionais do guaraná (*Paullinia cupana* Mart. Sapindaceae) têm sido investigadas em estudos envolvendo cultura de células (Zeidán-Chuliá et al., 2013; Bittencourt et al., 2014), modelos experimentais animais e ensaios clínicos em seres humanos (Barbosa et. al., 2004). Tais propriedades estão baseadas nos compostos bioativos presentes na sua composição: metil xantinas, tais como a cafeína (Founi et al., 2007), teobromina (Belliardo et al., 1985; Salvadore et al., 1994), teofilina, taninos, saponinas, catequinas, epicatequinas, pró-antocianinas e outros compostos traço (Salvadore et al., 1994; Carlson & Tompson, 1998; Sombra et al., 2005; Pellozo et al., 2008).

A partir da década de 90 as investigações foram intensificadas e uma série de propriedades funcionais começaram a ser observadas a partir de estudos independentes: cardiotônica e cardioprotetora (Pontieri et al., 2007), atividade anti-agregadora de plaquetas (Bydlowski et al., 1988; Bydlowski et al., 1991; Ravi Subbiah & Yunker, 2008), energética (Bempong & Houghton, 1992), atividade anti-bacteriana (Basile et al., 2005; Yamaguti-Sasaki et al., 2007), melhoria cognitiva (Haskell et al., 2007; Kennedy et al., 2007), , efeito anti-obesidade, termogênico (Berube-Parent et al., 2005; Roberts et al., 2005; Ravi Subbiah et al., 2008), atividade

gastroprotetora (Campos et al., 2003), quimiopreventivo na hepatocarcinogênese (Fukumasu et al., 2006), anti-depressivo (Fonseca et al., 1994), efeito no metabolismo lipídico, através do aumento da lipólise por ativação dos receptores de adenosina, aumento da taxa de β-oxidação (Lima et al., 2005), anti-mutagênico (Fukumasu et al., 2008).

Com relação aos limites de segurança no consumo do guaraná, Mattei e colaboradores (1998) mostraram que o guaraná não exerceu efeitos tóxicos em ratos e camundongos tratados cronicamente. Fonseca e colaboradores (1994) relataram que na concentração de 30 mg/mL reduziu a sobrevivência de células bacterianas em 33%, mostrando efeito genotóxico da planta nesta concentração. Outro estudo também corroborou o trabalho dos autores citados anteriormente (Santa Maria et al., 1998). Recentemente, Zeidán-Chuliá et al (2013), mostraram que o guaraná em concentrações a partir de 2mg/mL, possui efeito citotóxico em linhagem SHSY-5Y; efeito este induzido através de excessiva atividade antioxidante do guaraná (estresse antioxidante).

Recentemente foi realizado um estudo epidemiológico em idosos ribeirinhos que vivem no Município de Maués, Amazonas que comparou o perfil epidemiológico e de alguns marcadores do metabolismo oxidativo entre idosos que consomem habitualmente guaraná e idosos que não consomem este produto. A investigação conduzida por Krewer e colaboradores (2010), observou que consumidores habituais do guaraná apresentavam menor prevalência de alguns distúrbios metabólicos como obesidade, menores níveis de pressão sanguínea e de LDL-colesterol, além de apresentarem também níveis plasmáticos menores de produtos avançados de oxidação de proteínas.

Até os dias de hoje, poucos estudos avaliaram as propriedades antioxidantes do guaraná, (Mattei et al., 1998; Basile et al., 2005; Yamaguti-Sasaki et al., 2007; Ushirobira et al., 2010; Zeidan-Chuliá et al., 2013; Bittencourt et al., 2014).

OBJETIVOS

Considerando todo o contexto apresentado na seção introdução, o objetivo central da presente tese foi: avaliar o potencial do extrato comercial de guaraná como alternativa preventiva frente à toxicidade induzida por compostos carbonílicos presentes na doença de Alzheimer. Sendo assim, o presente estudo apresentou os seguintes objetivos específicos:

1: Investigar as propriedades antioxidantes do guaraná frente a diversas fontes geradoras de radicais livres e espécies reativas (artigo 1);

2: Investigar as propriedades do guaraná na agregação do peptídeo beta-amilóide, anti-glicação mediada por MGO e GO e atenuadoras de citotoxicidade e estresse oxidativo mediada por ACR, GO e MGO em células SHSY-5Y (artigo 2);

PARTE 2

MATERIAIS, MÉTODOS E RESULTADOS

Nesta tese, os MATERIAIS E MÉTODOS, assim como os RESULTADOS, estão redigidos de forma detalhada nos 2 artigos científicos.

Estudo I

Capítulo I - Antioxidant profile characterization of a commercial *Paullinia cupana* (guarana) extracts

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Antioxidant Profile Characterization of a Commercial *Paullinia cupana* (Guarana) Extracts

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ABSTRACT

The Brazilian guarana has been used since pre-colombian times as a tonic aphrodisiac or stimulating beverage. However, the current literature about the antioxidant properties of guarana is restricted to a few studies and still remains poorly understood. In this study we showed the full guarana commercial extract redox properties against several radicals and reactive species since the overload of these molecules are involved in the pathogenesis of several diseases. All tested concentrations (1, 10, 100 and 1000 µg/mL) guarana extract presented high efficiency in quenching peroxyl radical (~1 µg/mL), malondialdehyde (60 µg/mL), hydroxyl radical (63 µg/mL) and nitric oxide radical (84.1 µg/mL) and chelating iron (46 µg/mL) at low IC_{50}. Since the reactive oxygen/nitrogen species and free radicals have pivotal roles in the initiation and/or progression in several diseases, such as neurodegenerative and cardiovascular diseases it is sovereign importance to know about the chemical profile and redox-active properties regarding medicinal plants candidate to being alternative forms in prevention or treatment of diseases. Thinking about this, our team provided the first milestone regarding the full redox profile of amazon *Paullinia cupana* (guarana) extracts.

1. Introduction

The Plantae Kingdom is considered one of the largest and diverse sources of bioactive molecules. Many plants used in folk medicine were the basis for the discovery and characterization of several drugs clinically used nowadays [1]. In fact, the worldwide use of folk medicinal plants is very significant data from the World Health Organization (WHO) show that about 80% of the world population uses herbal plants to relief diverse painful or unpleasant symptoms. In addition, several plants are usually consumed *in natura* or as a dietary supplement.

Guarana (*Paullinia cupana*), a rain forest bushy plant, from amazon basin cultured by its caffeine-polyphenol rich-seeds [2], which are the most physiologically active ingredient in many energy drinks. The US food and drug administration (FDA) [3] also consider guarana a safe dietary supplement. Despite of being considered a safe supplement there is little information available regarding it bioactive compounds and their biological properties. Although much of the guarana bioactivity is attributed to the caffeine content of the extract a growing scientific literature points out to several other biological active components. On the other hand, it has not been established whether or not these different properties are due to caffeine alone or to the other compounds present in guarana seeds. In a previous characterization study our team identified the catechin, epicatechin, epicatechin gallate as main polyphenol compounds (flavan-3-ols) present in the commercial guarana powder [4]. All of this polyphenols are known as powerful antioxidants. We may also consider the presence of a synergistic effect of all components of the extract it means the synergic effect between polyphenol content and xanthine content.

Previous studies have reported anti-bacterial, antioxidant, chemopreventive and anti-mutagenic activities for guarana extracts [4-10].

It is well known that oxidative or nitrosative stresses probably are pivotal components in the onset and progression of chronic diseases such as atherosclerosis, neurodegenerative disorders (Alzheimer Disease - AD, Parkinson Disease - PD) [11, 12] and diabetes [13]. In this context, supplementation with antioxidants such as vitamin E, ascorbic acid, omegas and glutathione precursors have been extensively studied along the last decades. In the flip side, due to an intrinsic capability of producing a variety of antioxidant compounds mixtures from its secondary metabolism natural functional foods have been considered a promising alternative in the modulation or attenuation of oxidative stress and its associated deleterious effects in chronic disease landscapes [14].

Taking into account that the range of guarana antioxidant properties remains to be investigated the aim of this work was to characterize the redox-active properties of this dietary supplement in an attempt to improve the knowledge about its antioxidant properties. In this intent, we performed some in vitro scavenging activity assays against different oxidants.

2. Experimental Methods

2.1 Chemicals

Guarana (*Paullinia cupana* Mart.) extract powder was obtained from Lifar Ltd. (Porto Alegre, RS, Brazil). Chemicals for oxidation of 2-deoxy-D-ribose assay: The 2-deoxy-D-ribose (31170-5G-F), Phosphoric Acid (W290017), phosphate buffered saline - PBS (P5368), iron II sulfate heptahydrate (215422), hydrogen peroxide (V000194), sodium hydroxide (V000101), 2-thiobarbituric acid (T5500). Chemicals for nitrite assay: Griess reagent (03553), sodium nitroprusside (71778), phosphate buffered saline (P5368). Chemicals for *in vitro* Thiobarbituric reactive substances assay (TBARS): Trichloroacetic acid (T6399), phosphate buffered saline (P5368), 2-thiobarbituric acid (T5500). Chemicals for total reactive antioxidant potential assay/total antioxidant reactivity (TRAP/TAR): Luminol (123072), AAPH (2,2′-Azobis (2-methylproprionamidine) dihydrochloride (440914)), phosphate buffered saline (P5368), chemicals for super-oxide dismutase like activity: (−)-epinephrine (E4250), catalase (C9322), glycine (410225). Chemicals for catalase-like activity: phosphate buffered saline (P5368), hydrogen peroxide (V000194).

Chemicals for ferric reducing antioxidant power assay (FRAP): 2.4.6-tris(2-piridyl)-s-triazine. Chemicals for ferrozine assay: 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (82959) were obtained from Sigma Chemical Co. (St. Luis, MO, USA). Caffeine were obtained from Sigma Chemical Co. (St. Luis, MO, USA).

Trolox 97% was purchased from ACROS-ORGANICS (New Jersey-USA). The egg yolks used on in vitro TBARS assay were obtained from local commercial establishments.

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2.2 Guarana Aqueous Extract Preparation, Polyphenol Assay and Chemical Characterization

The detailed chemical composition of guarana aqueous extracts and polyphenol identification assay was performed as previously described [4]. The preparation of guarana aqueous extracts was performed as previously described procedures [4, 15].

2.3 Guarana, Caffeine and TROLOX® Concentrations

Briefly, we added 50 mg of guarana powder to 10 mL PBS, vortexed vigorously and incubated to 37 °C for 15 minutes, the solution achieved 5 mg/mL final concentration. From this solution we performed serial dilutions to obtain the concentrations used in this study; 1, 10, 100 and 1000 µg/mL. The caffeine solution was prepared by dissolving 10 mg of caffeine in 10 mL PBS, vortexed and incubated to 37 °C for 15 minutes reaching a 1 mg/mL final concentration. From this, we performed a dilution to achieve 40 µg/mL. This concentration of caffeine was used because it equivalent to those found in the highest concentration of guarana extracts (1000 µg/mL). The TROLOX® final concentrations herein used was 200 nM according reference number [16].

2.4 Total Reactive Antioxidant Potential (TRAP) and Total Antioxidant Reactivity (TAR)

TRAP and TAR were used as an index of non-enzimatic antioxidant capacity and peroxyl scavenging activity of guarana extracts and caffeine. This assay is based on the quenching of luminol chemoluminescence (CL) of AAPH as the peroxyl radical generation source [4, 16-18]. The AAPH solution was prepared by adding 0.0542 g AAPH reagent to 20 mL of PBS pH 8.6 (120 mM AAPH final concentration) followed by 4 µL luminol 50 mM (0.01 mM final concentration) in the dark. The AAPH plus luminol are considered the radical generating system. We left this system to stabilize for additional 2 h before the first reading as previously validated [19]. The tested concentrations of guarana and caffeine were added to a mat 96/well microplate and the luminescence produced by the free radical production was quantified in a liquid scintillator counter (Wallac 1409, Perkin-Elmer, Boston, MA, USA) for 120 minutes.

Total antioxidant reactivity (TAR) were calculated as the ratio of the first reading in absence of samples (I0)/first reading of guarana and caffeine samples. It is important to highlight that TAR and TRAP are different evaluations obtained in the same experiment; TAR indicates the quality of the antioxidants present in the sample based on instant reactivity; TRAP indicates the amount and kinetic behavior of sample antioxidants. TROLOX® 200 nM final concentration was used as standard antioxidant. The results were calculated and expressed as percentage of area under curve (AUC) for TRAP and TAR.

2.5 Hydroxyl Radical-Scavenging Activity

The 2-DR oxidation assay is based on the capacity of a compound or mixture in inhibiting the oxidation of 2-deoxy-D-ribose (2-DR) by hydroxyl radicals. The 2-DR is incubated with a hydroxyl radical generation system, which produces malondialdehyde (MDA) end product. The mixture is then incubated with 2-thiobarbituric acid (TBA), which reacts with MDA and forms a chromophore quantifiable at 532 nm by spectrophotometry [16, 20]. The hydroxyl generating reactions consisted of H_2O_2 (100 µM final concentration), Fe^{2+} ($FeSO_4$ 6 µM final concentration) and 2-DR (5 mM final concentration) solutions in 20 mM PBS (pH 7.4). To measure guarana extracts and caffeine activity against hydroxyl radicals, such extracts were added to the system before H_2O_2 addition. Reactions were carried out for 15 minutes at room temperature and then stopped by the addition of 4% phosphoric acid (v/v). Thereafter, TBA 1% (0.3 g in 30 mL 50 mM NaOH v/v) was added the solutions were incubated for 15 minutes at 95 °C and then cooled at room temperature. The absorbance was measured at 532 nm and the results were expressed as percentage of MDA formed related to the system. Trolox 200 nM were used as standard antioxidant.

2.6 Nitric Oxide (NO) Scavenging Activity

In this assay, Sodium Nitroprusside (SNP) was used as nitric oxide (NO) generating system. Once generated, NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [21]. The reaction mixture (1 mL) containing both 11.11 mM SNP in 20 mM PBS was incubated with guarana and caffeine at 37 °C for 1 h. From this reaction mixture, aliquots of 0.1 mL were taken and mixed with 0.1 mL Griess reagent in a 96/well microplate. The absorbance was measured at 540 nm. Results were expressed as percentage of nitrite formed related to SNP alone. Trolox 200 nM was used as standard antioxidant.

2.7 In Vitro Thiobarbituric Acid Reactive Species (TBARS)

An adapted TBARS method was used to measure the antioxidant capacity guarana and caffeine using egg yolk homogenate as lipid-rich medium [22]. This method is based on measurement of the color produced during the reaction of thiobarbituric acid (TBA) with lipoperoxidation products, such as malondialdehyde and 4-hydroxynonenal [23]. Briefly, egg yolk was homogenized (1% w/v) in 20 mM PBS (pH 7.4) and sonicated at potency 4. 1 mL of homogenate was then homogenized with 0.1 mL of guarana and caffeine to achieve the tested concentrations. Lipid peroxidation was induced by addition of 0.1 mL of AAPH solution (120 mM). Control was just incubation medium without AAPH. Reactions were carried out for 30 minutes at 37 °C. After incubation, samples (0.5 mL) were homogenated with 0.5 mL of trichloroacetic acid (10% final concentration) and after centrifuged at 1200 g for 10 minutes. An aliquot of 0.5 mL from supernatant was mixed with 0.5 mL TBA (0.67%) and heated at 95 °C for 30 minutes. After cooling, 0.2 mL of the misture were added to 96/well microplate and the absorbance was measured at 532 nm. The results were expressed as percentage of MDA formed by AAPH alone (induced control). The TROLOX® 200 nM was used as standard antioxidant.

2.8 Determination of Superoxide Dismutase-Like Activity

The ability of guarana and caffeine to scavenge superoxide anion ("superoxide dismutase-like activity") was measured as previously described [24]. Guarana and caffeine were mixed with 190 µL glycine buffer (50 mM, pH 10.2) and 5 µL of native catalase 100 U/mL. Superoxide generation was initiated by addition of 5 µL of epinephrine 2 mM, and adrenochrome formation was monitored at 480 nm for 10 minutes (32 °C). Superoxide production was determined by monitoring the reaction curves of samples and measured as percentage of the rate of adrenaline auto-oxidation into adrenochrome [25]. TROLOX® 200 nM was used as standard antioxidant.

2.9 Determinantion of Catalase-Like Activity

The capacity of guarana and caffeine to degrade the hydrogen peroxide (H_2O_2) added in an incubation medium ("catalase-like activity") was measured as previously described [26]. H_2O_2 were diluted in 50 mM phosphate buffer (pH 7.4), to obtain a 1 mM final concentration was added to microplate 96/well with the guarana and caffeine already placed to achieve the tested concentrations.

The plate was then scanned in a spectrophotometric plate reader (SpectraMax 190. Molecular Devices) at 240 nm every 10 seconds for 5 minutes at 37 °C. Catalase-like activity was monitored based on the rate decomposition of H_2O_2. Data were expressed as percentage of the rate decomposition of hydrogen peroxide.

2.10 Ferric Reducing Antioxidant Power (FRAP)

This assay was used to verify the reductor potential of guarana extracts based on conversion of free ferric iron (Fe^{3+}) to ferrous (Fe^{2+}). Briefly, in dark room, we added 90 µL of diluted guarana extracts to 2.7 mL of FRAP reagent (25 mL 0.3 M acetate buffer pH 3.6, 2.5 mL 10 mM 2.4.6-tris(2-piridyl)-s-triazine and 20 mM ferric chloride). Afterwards, the mixture was homogenized and incubated to 37 °C during 30 minutes. The readings were performed at 595 nm and the FRAP reagent was used as a blank.

2.11 Chelating Activity on Ferrous Ions (Fe^{2+}) – Ferrozine Assay

Metal chelating activity was determined according to the method of Oke et al with some modifications. Briefly, the guarana (400 µL) was mixed with 50 µL $FeCl_2$ (2 mM) and the mixture was incubated at room temperature during 10 minutes. After, 200 µL of 5 mM ferrozine disodium salt (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4′′-disulfonic acid sodium salt) in each tube. Then the content was shaken vigorously and left standing at room temperature for another 10 min. After the mixture reached equilibrium, the volume was completed to 4 mL with absolute ethanol and the absorbance was then measured at wavelength 560 nm using a microplate spectrophotometer. The chelating activity was calculated as the percentage (%) of inhibition of ferrozine-Fe^{2+} complex formation determined as: 1 – (*A* sample/*A* control) × 100, where *A* control is the absorbance of the only ferrozin-Fe^{2+} complex, and *A* sample is the absorbance of the guarana extracts and ferrozin-Fe^{2+} mixture. EDTA was used as a standard positive control.

2.12 Statistical Analysis

All Biochemical data were first submitted to distribution analysis test (Kolmogorov-Smirnov) and parametric data were analyzed using the one-way ANOVA followed by Tukey's post hoc test. All data were analyzed with

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GraphPad Prism Software v.5.0 (GraphPad Software Inc, San Diego, CA, USA). Results were expressed as the mean ± SEM; p values were considered significant when $p < 0.05$.

3. Results and Discussion

3.1 Guarana Extracts Scavenging Activity against Peroxyl Radicals

A peroxyl generation system (AAPH) generates chemoluminiscence (CL) in constant rate and the effect of guarana in free radical CL is expressed as a percentage of area under the curve (AUC) over 120 minutes. The first evaluation of redox properties of guarana was through the TRAP/TAR assays. This in vitro method is suitable to evaluate the peroxyl scavenging activity present in many natural compounds [4, 7, 8, 16, 27, 28]. As presented in Fig. 1A, all tested guarana extract concentrations (1, 10, 100 and 1000 µg/mL) showed significant reduction of the AUC (area under curve) to 22.5, 2.17, 1.7 and 1.49% respectively, suggesting potent non-enzymatic antioxidant activities. Purified caffeine at equivalent concentrations also presented a minor, although significant, peroxyl scavenging activity (36% inhibition of luminescence) (Fig. 1A). Regarding TAR measurements, all the guarana concentrations tested also showed antioxidant capacity higher than purified caffeine (Fig. 2B).

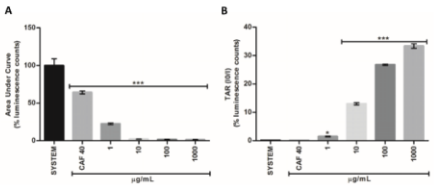

Fig. 1 TRAP and TAR analysis. (A) Total reactive antioxidant potential of different guarana concentrations and caffeine. (B) The TAR measurement was calculated by the ratio of CL intensity in the first reading in absence of extracts (I0)/CL intensity in the first reading in presence guarana and caffeine extracts and expressed as percentage. The vitamin E analogue Trolox® (200 nM) was used as standard antioxidant. The bars represent average ± SEM of three independent experiments. * $p < 0.05$, *** $p < 0.0001$. One-Way ANOVA followed by Tukey's post-hoc test

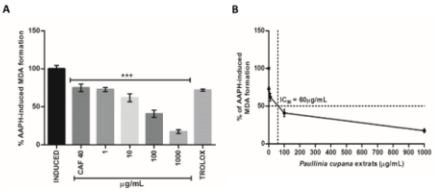

Fig. 2 (A) Percentage of AAPH-induced malondialdehyde (MDA) production. The lipid peroxidation was induced by AAPH free radical source in absence (induced control) and presence of guarana extracts and caffeine. Trolox® 200 nM was used as standard antioxidant. (B) Graphical showing of guarana extracts IC_{50} against peroxyl radicals. The bars represent average ± SEM of three independent experiments. *** $p<0.0001$. One-Way ANOVA followed by Tukey's post-hoc test

3.2 Guarana Prevents AAPH-Induced Lipid Peroxidation

Given the peroxyl scavenging activity of guarana extracts obtained from Fig. 1 experiments, we tested if it could also be able to attenuate the propagation of free radical chain reactions and damage to biomolecules. Then, the protective effects of guarana against AAPH mediated oxidative damage were assessed through measurement of MDA formation in a lipid-rich medium. All tested guarana concentrations (1-1000 µg/mL) were capable to protect lipids against peroxyl-induced damage by decreasing MDA formation in 27.2, 38.1, 59.2 and 82.7% respectively. The guarana extract concentration needed to decrease the MDA formation by 50% (IC_{50}) was 60 µg/mL (Fig. 2B). With a lesser effectivity, purified caffeine was also capable to protect by 25% the lipid environment against oxidative damage (Fig. 2A). Taken together, Figs. 1 and 2 showed that guarana extracts at low concentrations (from 1 µg/mL) are able to scavenge peroxyl radical and protect lipids from peroxidation (Fig. 2). This effect seems to be attributed to other compounds in a higher extent or to the combination of caffeine and the other components than the caffeine itself.

3.3 Guarana Inhibits Hydroxyl Radical Production by Reducing and Chelating Iron

To investigate the ability of guarana to scavenge hydroxyl radicals, we performed the in vitro 2-DR oxidation through Fenton Reaction. Guarana extract concentrations ranging from 1 to 1000 µg/mL were able to inhibit hydroxyl radical production in 41.1, 44, 54.2 and 82.3% of the control, respectively (Fig. 3A). Caffeine was also able to decrease 2-DR oxidation (28.87% of the control), but again, not as efficient as guarana extracts (Fig. 3A). The IC_{50} herein found for guarana extract was 63 µg/mL (Fig. 3B), indicating that guarana extract is able to decrease hydroxyl production at lower concentrations than caffeine (Fig. 3).

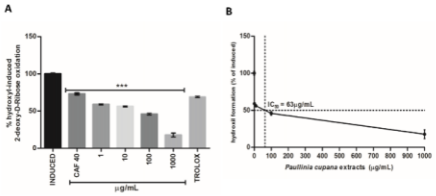

Fig. 3 (A) Hydroxyl-scavenging activity was measured by using hydroxyl-mediated 2-deoxy-D-ribose (2-DR) oxidative degradation. The induced control is MDA production from 2-DR oxidation with $FeSO_4$ and H_2O_2 alone. Other groups represent MDA production by Trolox 200 nM was used as standard antioxidant. (B) Graphic of the guarana IC_{50}. Bars represent average ± SEM of three independent experiments. *** $p < 0.0001$. One-Way ANOVA followed by Tukey's post-hoc test

The redactor potential of guarana extracts was assessed by FRAP assay. Guarana extracts were very efficient in reducing iron (Fe^{3+} to Fe^{2+}) in all tested concentrations. The concentrations of 1 and 10 µg/mL are capable to reduce iron but not as efficient as TROLOX®, while 100 µg/mL showed similar reducing potential to trolox. The higher concentration exhibited iron reducing capacity significantly increased compared to TROLOX®. Caffeine was not able to reduce iron indicating that probably this compound does not possess such property and the phenolic content is the main responsible for such effect (Fig. 4).

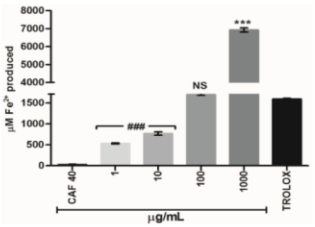

Fig. 4 Iron reducing activity was evaluated through the FRAP assay. The amount of reduced iron was calculated through Fe_2SO_4 standard curve. Bars represent average ± SEM of three independent experiments. ### $p < 0.0001$ indicating less efficiency than Trolox®. NS (Non-Significant related to Trolox®), indicating similar efficiency to Trolox®. *** $p < 0.0001$ indicating higher efficiency than Trolox®. One-Way ANOVA followed by Tukey's post hoc test

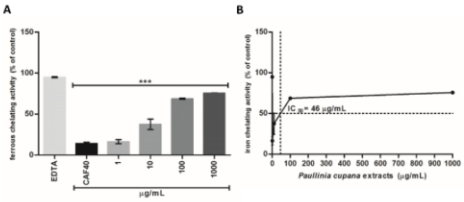

Fig. 5 Chelating activity evaluated by Ferrozine assay. **(A)** The ability of guarana extrats in prevent the Fe^{2+}-ferrozine complex formation, thus decreasing the red color compared to control. **(B)** Graphic of IC_{50}. Bars represent average ± SEM of three independent experiments. *** $p < 0.0001$. One-Way ANOVA followed by Tukey's post-hoc test

It is well known that iron is required to oxygen transport, cellular respiration and enzymatic activity such as catalase. Free Fe^{2+} is also capable to trigger the Fenton reaction thus generating hydroxyl radicals, leading to great oxidative damage. The ferrous-chelating activity is

considered a suitable indicator in pro-oxidant scenarios where the ferrous specie is an important redox-active catalyst. The Ferrozine assay allows this quantification by the formation of complexes with Fe^{2+}, yielding an intense red chromophore quantifiable by spectrophotometry. However in the presence of chelating agents such as guarana the complex formation is prevented resulting in a decrease in the red color. The measurement of color reduction therefore allows for estimation of the metal chelating activity of the tested extracts. Here we observed for the first time that guarana extract was able to chelate ferrous ions efficiently in all tested concentrations as may be seen in Fig. 5B. The purified caffeine also demonstrated ability as an iron chelator but not as efficient as the guarana extracts showing that this alkaloid alone is not the main responsible for such property.

3.4 Guarana Capacity to Scavenge the Nitric Oxide (NO·) Radical

In order to evaluate the effect of guarana extracts against the nitrogen oxidative specie NO, we quantified nitrite accumulation from the spontaneous degradation of Sodium Nitroprusside (SNP) to NO through Griess reaction. As shown in Fig. 6A, all tested guarana extract concentrations were able to significantly inhibit nitrite accumulation in 23.1, 42.92, 51.8 and 58.7% compared to SNP controls, respectively, yielding an 84.1 µg/mL as IC_{50} (Fig. 6B). Purified caffeine also decreased nitrite production, although with a lesser efficacy than guarana (37.3% inhibition).

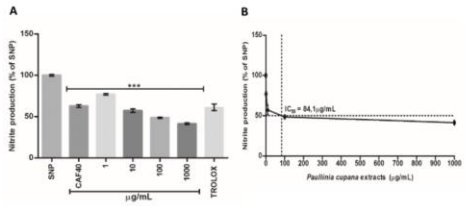

Fig. 6 (A) Nitric oxide was generated from decomposition of sodium nitroprusside (SNP) generating nitrite ions, which were measured by the Griess reaction in 96 well microplate. Nitrite production by SNP alone was compared to nitrite production by SNP in the presence of the tested guarana concentrations and 40 µg/mL caffeine. Trolox was used as standard antioxidant. (B) Graphic of IC 50. Bars represent average ± SEM of three independent experiments. *** $p < 0.0001$. One-Way ANOVA followed by Tukey's post-hoc test

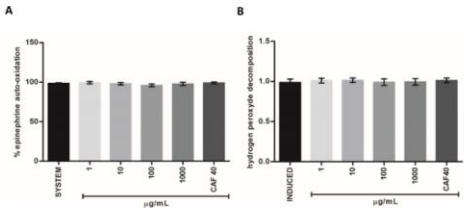

Fig. 7 SOD-like and CAT-like activities. (A) Superoxide dismutase-like (SOD-like) activity was determined by following formation of adrenochrome at 480 nm in absence and presence of guarana and caffeine. (B) CAT-like activity was measured in a phosphate reaction buffer (50 mM) with H_2O_2 with and without guaraná or caffeine. The experiments were performed in triplicate and bars represent average ± SEM of three different experiments. Statistical analysis was performed by One-way ANOVA followed by Tukey's post hoc test

3.5 SOD and CAT Like Activities

To assess the scavenging activity of guarana extracts against superoxide anions (SOD-like activity), we quantified the inhibition of superoxide-dependent adrenaline auto-oxidation to adrenochrome. Moreover, we also tested the ability of guarana extract to decompose hydrogen peroxide in vitro (CAT-like activity). Fig. 7A and B respectively, show that all tested concentrations of guarana extract did not presented any SOD nor CAT like activities.

The growing body of evidence has increasingly placed oxidative stress as pivotal condition for the onset and progression of various pathological conditions such as neurodegenerative and cardiovascular diseases. The plant kingdom is considered a powerful laboratory synthesis of bioactive molecules waiting to being studied from chemical to biological properties. Many plants have increasing attention from the scientific community, which is always in a constant search for properties of main bioactive compounds mainly antioxidant, anti-inflammatory, inhibiting enzymes, etc. This is the case of the Amazon guarana, where studies about it bioactive properties and mechanisms of action were intensified from 2007. But it antioxidant activity remained without the due highlight with few published studies. Regarding this our team provided for the first time the full redox active profile of the Brazilian guarana (*Paullinia cupana*).

It is well known that the peroxyl radical is the responsible for the propagation phase in the lipid peroxidation reaction, contributing to the increased damage to biological membranes and cell injury caused by oxidative species [29]. Taken together, both the Fig. 1A and B suggest that the effect of guarana extracts on non-enzimatic peroxyl scavenging activity is likely attributed to both caffeine and other xanthine and/or polyphenols present and already characterized in guarana extracts [4].

The lipid peroxidation generates as end-products aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), both known as dangerous molecules due to their reactivity [29-31]. These aldehydes can react with proteins modifying their structure and function, with DNA bases generating mutagenic lesions [29] and modifying cellular functions. MDA and 4-HNE are involved in several pathologies such as cancer, neurodegenerative and cardiovascular diseases [31-33]. Lipid peroxides reacts with iron ions generating even more peroxyl and hydroxyl radicals [29]. In this context it is suitable to accept that blocking lipoperoxidation could be an interesting mechanism of guarana antioxidant activity in biological systems.

Hydroxyl is one of the most reactive and damaging radical to the cells. Due to high oxidant power, hydroxyl radicals, attacks DNA molecules, causing strand breakage, contributing to mutagenesis and cytotoxicity [16, 29, 34]. In addition, hydroxyl radicals attack lipids damaging cell membranes increasing lipid peroxidation and cell oxidative damage.

It is well known that iron is required to oxygen transport, cellular respiration and enzymatic activity, such as catalase. Free Fe^{2+} is also capable to trigger the Fenton reaction, thus generating hydroxyl radicals, leading to great oxidative damage. The ferrous-chelating activity is considered a suitable indicator in pro-oxidant scenarios where the ferrous specie is an important redox-active catalyst. The Ferrozine assay allows this quantification by the formation of complexes with Fe^{2+}, yielding an intense red chromophore quantifiable by spectrophotometry. However in the presence of chelating agents, such as guarana the complex formation is prevented, resulting in a decrease in the red color. The measurement of color reduction therefore allows for estimation of the metal chelating activity of the tested extracts. Here we observed for the first time that guarana extract was able to chelate ferrous ions efficiently in all tested concentrations as may be seen in Fig. 5B. The purified caffeine also demonstrated ability as an iron chelator but not as efficient as the guarana extracts showing that this alkaloid alone is not the main responsible for such property.

Several evidences have shown that catechin, epicatechin, epicatechin galate, phenolic compounds present in guarana extracts [35], can reduce or chelate transition metals, as copper and iron, which are known to their harbor redox active properties [36]. In addition, an impairment in brain iron homeostasis is a key factor to early neuropathological events in Alzheimer's disease (AD), including oxidative stress, inflammatory processes, amyloid deposition, tau phosphorylation and neuronal cell cycle regulatory failure, leading to apoptosis [37]. This result suggests that guarana extract may be a useful resource for the prevention of iron metabolism impairment.

NO is a key modulating agent present in acute and chronic inflammation an important component in several pathological conditions, such as neurodegenerative, cardiovascular and pulmonary diseases. It is also an important messenger in Central Nervous System (CNS). In chronic inflammation NO may reach high concentrations thus interacting with superoxide anion, generating a potent oxidizing agent, peroxynitrite ($ONOO^-$). This radical can damage proteins, DNA and lipids, compromising the cellular integrity.

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Several studies have shown the anti-inflammatory benefits of natural compounds present in foods such as black tea, green tea, strawberry and coffee [38-41]. Our group also recently showed evidences about the protective effects of guarana extracts against SNP-mediated cytotoxicity in NIH-3T3 fibroblasts [15]. Another study reported the anti-allergic effects

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of guarana extracts in passive cutaneous anaphylaxis and mast degranulation models [42]. Taking into account the aforementioned results guarana extracts could be a promising alternative in preventing or/and controlling diseases where inflammation is a prevalent feature.

4. Conclusion

In this study, we provided the first milestone about the full redox-active profile of a commercial guarana powder. A growing number of evidences in literature supports that the antioxidant capacity, at low doses, of natural compounds is wide, but the most articles uses isolated methods, such as DPPH, to address this question. It is important to emphasize that oxidative stress is not caused by a unique radical molecule, but a combination of several molecules with differing chemical reactivity, stability and diffusion potentials. Based in this fact, it is more accurate to assess the antioxidant capacity of a natural compound in different contexts, e.g. considering several reactive species and radicals.

Here in we observed that all tested guarana extract concentrations displayed high antioxidant activity against several free radical sources. Such activity may be attributed to it polyphenol and xanthines content, as showed in Figs. 1-6, where caffeine (the main xanthine present in guarana) also presented antioxidant activity. However, our results suggest that caffeine content alone could not fully explain the extension of the observed antioxidant effects of guarana.

Commonly, the polyphenols are the main molecules responsible for the most of antioxidant activity in natural products. The major class of polyphenols found in guarana extracts is the flavan-3-ols (catechins), such as catechin, epicatechin and epicatechin gallate. Such molecules are described as the main responsible for the antioxidant properties found in several natural products. However, the caffeine content also can count to the total antioxidant and we cannot exclude it participation in the overall antioxidant effect of the extract. Our research group has been successful in demonstrating the effectiveness of natural compounds in inhibiting or preventing oxidative stress in diverse pathological scenarios such as cancer, Alzheimer and diabetes models. In addition, our group also has successfully characterized the redox-active properties of other natural compounds such as usnic acid, *Passiflora manicata*, *Hyptis pectinata*, *Lypia sidoides*, *Remirea maritima* extracts in a scenario where the natural products are gaining even more notoriety and attention of scientific community.

In this study, we observed that guarana extracts exerted antioxidant activity mainly against peroxyl free radicals, the harmful hydroxyl, by scavenging the own radical or reducing and chelating iron and thus preventing the Fenton Chemistry and also decreasing levels of nitric oxide whereas none effect on hydrogen peroxide or superoxide anion was observed. This reveals some important antioxidant mechanism whereby guarana supplements could be beneficial in preventing oxidative stress, a key event in the onset and progression of a variety of diseases.

We are aware that this study is limited to characterization of the redox profile in-vitro, but the first step towards the knowledge of the properties of a particular extract, in this case the guarana extracts is it complete chemical characterization that consist in the identification of main extract bioactive compounds already published in reference number 4, complete redox-active profile based in chemical reactions, enzyme inhibitory assays, etc. Regarding this the mentioned manuscript explored the complete redox-active profile of amazon guarana.

After this step, we set out to study biological properties, which are used experimental models (tissues, cells or animal models) where our group also showed for the first time, some biological properties as, anti-aggregating and protective effects of guarana against amyloid-beta peptide and several toxic aldehydes that occur in brains with Alzheimer's in an in vitro model of neuronal cells.

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Estudo II

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Guarana (*Paullinia cupana* Mart.) Prevents β-Amyloid Aggregation, Generation of Advanced Glycation-end Products (AGEs), and Acrolein-Induced Cytotoxicity on Human Neuronal-Like Cells

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Advanced glycation end-products (AGEs) are considered potent molecules capable of promoting neuronal cell death and participating in the development of neurodegenerative disorders such as Alzheimer's disease (AD). Previous studies have shown that AGEs exacerbate β-amyloid (Aβ) aggregation and AGE-related cross-links are also detected in senile plaques. Acrolein (ACR) is an α, β-unsaturated aldehyde found in the environment and thermally processed foods, which can additionally be generated through endogenous metabolism. The role of ACR in AD is widely accepted in the literature. Guarana (*Paullinia cupana* Mart.) is popularly consumed by the population in Brazil, mainly for its stimulant activity. In the present study, we showed that guarana (10, 100, and 1000 μg/mL) is able to prevent protein glycation, β-amyloid aggregation, *in vitro* methylglyoxal, glyoxal, and ACR (20 μM)-induced toxicity on neuronal-like cells (SH-SY5Y). Since these are considered typical AD pathological hallmarks, we propose that guarana may deserve further research as a potential therapeutic agent in such a neurodegenerative disease. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: Alzheimer's disease; natural products; neurodegenerative diseases; neuroprotection; polyphenols; caffeine.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease worldwide. Its incidence rate doubles every 5 years after the age of 65, with a prevalence of 5% after 65 years of age that increases to about 30% in people aged 85 years or older (Querfurth and LaFerla, 2010; Albarracin *et al.*, 2012; Galimberti and Scarpini, 2012). AD is a progressive neurodegenerative process characterized by the presence of senile plaques, neurofibrillary tangles (due to hyperphosphorilation of Tau), and neuronal loss, especially in the cerebral cortex and hippocampus (Yankner, 1996; LaFerla and Oddo, 2005). Deposition of β-amyloid (Aβ) peptide, a 39-43-amino acid peptide derived from the transmembrane amyloid precursor protein, is found in extracellular senile plaque cores and associated with neurodegeneration in later stages of AD (Loewen and Feany, 2010).

Glycation reaction represents a post-translational modification process between free reducing sugars and free amino groups in many proteins. Advanced glycation end-products (AGEs) are irreversible adducts of the Maillard reaction which are known to accumulate in the brain during the course of aging (Edison *et al.*, 2008). In fact, several reports showed the presence of activated microglial cells, Aβ deposition, and increased AGEs levels in the brains of AD individuals, suggesting their pathological role in this context of neurodegeneration (Hickman *et al.*, 2008; Krautwald *et al.*, 2011).

Acrolein (ACR) is a highly electrophilic α, β-unsaturated aldehyde formed during combustion of organic materials, including engine exhaust, wood, tobacco, over-heated cooking oils, and alcohol consumption (Srivastava *et al.*, 2011). It has already been shown the association of ACR-induced oxidative damage and neurodegenerative diseases, especially with AD (Bradley *et al.*, 2010; Dang *et al.*, 2010a, 2010b, 2011). ACR induces hyperphosphorylation of microtubule-associated protein tau (Kuhla *et al.*, 2007) and promotes Aβ aggregation in senile plaques (Seidler and Squire, 2005), directly linking ACR to the pathogenesis of AD (Singh *et al.*, 2010). With growing evidences associating ACR to human diseases, strategies to eliminate its hazardous impacts are of great relevance. One potential strategy is the use of reactive scavengers to directly trap ACR and AGEs (Zhu *et al.*, 2009; Wang *et al.*, 2012; Zhu *et al.*, 2011).

Considering that oxidative metabolic reactions and their by-products have been implicated in the pathogenesis of AD, a number of natural antioxidants (e.g. Ginkgo biloba flavonoids, isoflavones) are considered as potential therapies for the disease due to their neuroprotective

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effects *in vitro* and/or *in vivo* (Praticò, 2008; Zhao and Zhao, 2012).

Guarana (*Paullinia cupana*) is a plant from the Amazon basin with high content of polyphenols and caffeine. It is traditionally consumed by indigenous communities of the Amazon region, and its seeds are used to produce guarana powder (Woods, 2012). Even though Guarana powder is mainly consumed as stimulant, both antibacterial and antioxidant activities have been reported in the literature (Basile *et al*., 2005; Dalonso and Petkowicz, 2012). Furthermore, our group has recently demonstrated strong antioxidant properties of guarana *in vitro*, in human neuronal-like cells (SH-SY5Y) (Zeidán-Chuliá *et al*., 2013a) and fibroblasts (NIH-3T3) (Bittencourt *et al*., 2013).

In the present study, we aimed to evaluate the potential effect of guarana extract against amyloid-β 1-42 aggregation, protein glycation, as well as methylglyoxal (MGO)-, glyoxal (GO)-, and ACR-induced cytotoxicity in SH-SY5Y cells.

MATERIALS AND METHODS

Chemicals. Guarana (*P. cupana* Mart.) powder was obtained from Lifar Ltd. (Porto Alegre, RS, Brazil). Liquid chromatography (LC)-grade acetonitrile and methanol (Tedia, Fairfield, OH, USA), acetic acid (Cromoline, São Paulo, Brazil), ethyl acetate (Scientific HEXIS S/A. Mexico), and purified water (Milli-Q™ system, Millipore, Bedford, MA, USA) were used for mobile phase preparation. Caffeine anhydrous (99%), (+)-catechin hydrate (99%), (−)-epicatechin (90%), theobromine (99%), theophylline anhydrous (99%), amyloid-β peptide 1-42, ACR, GO, and MGO were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Polyphenol assay. The LC analysis of Guaraná (*P. cupana* Mart.) powder was performed as described by Sousa *et al.* (2011), using a Shimadzu Prominence equipment (Kyoto, Japan) coupled to a SPD-20A UV/VIS detector. The stationary phase was a Kromasil RP-18 column (Eternity-5-C18 250 × 4.6 mm i.d., 5 µm particle size) guarded by a Phenomenex precolumn (2 × 4 mm i.d., 10 µm particle size). The mobile phase consisted of water: acetonitrile:methanol:ethyl acetate:acetic acid (89:6:1:3:1). The flow rate was 1.0 mL/min, and the injection volume was 20 µL. The detection wavelength was 274 nm, and the analysis was carried out at room temperature. Caffeine anhydrous (99%), (+)-catechin hydrate (99%), (−)-epicatechin (90%), theobromine (99%), and theophylline anhydrous (99%) were used as external standards. They were dissolved in HCl 0.1 M: methanol (77:33, *v/v*) and diluted to obtain the concentrations between 0.1 and 20.0 µg/mL. These solutions were filtered through a 0.45-mm membrane filter (Millipore, HVLP). The linear equations were $y = 60984x + 13333$ ($r^2 = 0.9994$), $y = 49426x + 8691$ ($r^2 = 0.9998$), $y = 25872x + 94$ ($r^2 = 0.9996$), $y = 12769x + 306$ ($r^2 = 0.9999$), and $y = 12721x + 947$ ($r^2 = 0.9998$) for theobromine, theophylline, caffeine, catechin, and epicatechin, respectively.

Sample preparation for HPLC analysis. The Guarana powder (100 mg) was dispersed in 10.0 mL of purified water, shaken for 5 min and incubated at 37 °C during 15 min. From this dispersion, an aliquot of 1.0 mL was diluted to 10.0 mL with a mixture of HCl 0.1 M:methanol (77:33, *v/v*). The resulting solution was filtered through a 0.45-mm membrane (Millipore, HVLP) and analyzed by LC, taking into account the residual moisture content of the Guarana powder. The moisture content was determined by the loss on drying method described in the Farmacopeia Brasileira (2010).

Cell culture. The human neuroblatoma cell line (SH-SY5Y) was obtained from the Rio de Janeiro Cell Bank (BCRJ), Rio de Janeiro, Brazil and cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM of glutamine, 0.28 mg/mL of gentamicin, and 250 µg of amphotericin B in a 5% CO_2 humidified incubator at 37 °C as previously described (da Frota Junior *et al*., 2011; Rabelo *et al*., 2012). Cells were cultured until they reached a confluence of 80–90% and then trypsinized. Approximately one half of volume from the old cell culture medium was replaced with fresh 10% FBS-DMEM: F12 medium 24 h after trypsinization. Thereafter, 2×10^4 cells per well were seeded in 96-well plates to proceed with the experiments. All treatments (24 h) were performed when cells reached a confluence of approximately 75%.

Treatments. The guarana stock solution is prepared by dissolving it in a culture medium and incubating at 37 °C for 15 min. Thereafter, the solution is filtered with a syringe filter (0.22) µm before every cell treatment (Bittencourt *et al*., 2013; Zeidán-Chuliá *et al*., 2013a). A stock solution 10 mg/mL is prepared, and then serial dilutions are performed to achieve the different concentrations used in the present study. The caffeine stock solution was prepared by dissolving it in DMEM-F12 medium at a final concentration of 1 mg/mL, and thus filtered in a syringe filter (0.22 µm) and kept at 4 °C. From this stock solution, we diluted to achieve the 40 µg/mL work solution.

In order to study the effects of guarana against ACR and AGE-induced toxicity, concentrations of 10, 100, and 1000 µg/mL of the drug were tested. Briefly, ACR solutions were prepared by diluting in DMEM-F12 medium with 10% FBS to a final concentration of 20 µM. This concentration was selected according to the results observed in a dose–response curve performed to determine LD_{50}.

MGO and GO solutions were prepared by diluting in the DMEM-F12 medium with 10% FBS to final concentrations of 350 and 600 µM, respectively.

Determination of cellular viability by the sulphorodamine B (SRB) assay. After treatments with ACR, MGO, or GO for 24 h, cell viability was determined by the SRB assay as previously described (Vichai and Kirtikara, 2006). The SRB is a red dye that in acid conditions binds to basic aminoacids and dissociates in basic conditions. The SRB assay is based in protein quantification which is assumed to be proportional to the number of viable cells. Briefly, cells were seeded into a 96-well plate at a density of

2×10^4 cells per well in growth medium and cultured to about 75% confluence, prior to the initiation of experimental treatment. After drug treatments, the medium was removed and the cells were fixed for 1 h with trichloroacetic acid (TCA) 10% at 4 °C (100 µL/well). TCA was removed, and the fixed cells were carefully washed three times with distilled water. Cells let dry in room temperature, and 100 µL of 0.4% SRB was added to each well followed by 15-min incubation at room temperature. After incubation, SRB was removed, and the plate was washed with 1% acetic acid. Finally, the incorporated SRB was solubilized in Tris-base pH 10.5, and the absorbance was read with a microplate reader in 515 nm. Results were expressed as percentage relative to control.

Determination of amyloid fibril formation by the thioflavin T assay. In order to determine amyloid fibril formation, 10 µL of the incubated Aβ 1-42 with or without guarana was periodically added to 190 µL of 5 µM ThT in 50 mM phosphate buffer, pH 7.4. Fluorescence was measured in a 96-well black plate by using a microplate reader with excitation at 450 nm and emission at 482 nm, as previously described (Wang *et al.*, 2012) with slight modifications. Phosphate buffer with guarana was used as experimental control. Results were expressed as relative fluorescence units determined by a time scan after subtracting the fluorescence contribution from the control solution and guarana solutions when incubated alone.

Protein glycation and AGE production. Bovine serum albumin (BSA) solutions were made in the 50 mM PBS with 0.02% sodium azide to prevent degradation. Fructose, glucose, GO, and MGO were prepared in 0.02% sodium azide. Aliquots of BSA, sugars, and AGEs were added to 5-cm screw capped test tubes to give final concentrations of 7 mg/mL BSA, 25 mM glucose, and 25 mM fructose in the physiological range (Vinson and Howard, 1996) and 6 mM to each AGE. Usually, the concentrations of reactive aldehydes such as GO and MGO can reach a concentration of ~6 mM due to oxidative insults. Incubations were done in quadruplicate, and the tubes were degassed with nitrogen before being placed in a constant temperature bath at 37 °C for 7 days.

Fluorescence of samples was measured at the excitation and emission maxima of 350 and 450 nm, respectively.

Total reactive antioxidant potential. The total reactive antioxidant potential (TRAP) was used as an index of non-enzymatic antioxidant capacity of guarana extracts used in this study. Briefly, this assay is based on the peroxyl radical generated by AAPH solution, 2,2′-azobis(2-amidinopropane), with luminol quenching by sample compounds. The system contained AAPH (10 mM) and luminol (35 µM) dissolved in 0.1 M glycine buffer (pH 8.6). After 2 h of stabilization at room temperature, guarana was added in different concentrations to the system in order to determine its antioxidant potential. Results are expressed as the area under the curve (AUC) of each guarana concentration and compared to system AUC (Dresch *et al.*, 2009). In SH-SY5Y cells, TRAP was determined after cell treatment with guarana extracts in different concentrations for 24 h. After this, the culture medium was removed; the cells were washed in PBS buffer and then scraped. Cell lysates were prepared in phosphate buffer pH 7.4.

Measurement of intracellular reactive oxygen species (ROS) production by the dichlorofluorescein diacetate (DCF-DA) assay. Intracellular ROS production was measured by the DCF-DA assay. This non-fluorescent dye freely permeates into cells, where it de-esterifies to form the ionized free acid (dichlorofluorescein) and reacts with ROS to form the fluorescent 2,7 DCF (Nijmeh *et al.*, 2010). Cells were pre-incubated in DMEM supplemented with 1% FBS for 1 h at 37 °C. Then, the cells were washed with PBS and loaded with 100-µM H_2DCF-DA in 1% FBS DMEM-F12 for 2 h at 37 °C. Thereafter, cells were again washed with PBS and then treated with GO, MGO, and ACR for 2 and 4 h, respectively. Fluorescence was analyzed using a microplate reader at excitation and emission wavelengths of 485 and 532 nm, respectively. Results were expressed as arbitrary fluorescence of ROS.

Statistical analysis. All experiments were repeated at least three independent times. Data were presented as mean ± S.E.M. Statistics were analyzed with one-way ANOVA followed by Tukey's post hoc test (Prism Graph Pad 5.0 software). Statistical difference was considered when p-value <0.05.

RESULTS

Guarana composition

Identification of caffeine, theobromine, (+)-catechin, and (−)-epicatechin in the guarana powder was possible through liquid chromatographic separation. The composition of the guarana powder used for the present study was: caffeine (34.19 ± 1.26 mg/g), theobromine (0.14 ± 0.01 mg/g), (+)-catechin (3.76 ± 0.12 mg/g), and (−)-epicatechin (4.05 ± 0.16 mg/g) (Table 1 and Fig. 1).

Guarana exert antioxidant properties *in vitro*

Guarana at concentrations of 10, 100, and 1000 µg/mL, alone or in SH-SY5Y cells, was able to decrease the luminescence counts (area under curve, AUC) when compared to the system AUC ($p < 0.0001$) (Fig. 2); an effect that was also observed with 40 µg/mL of caffeine

Table 1. Composition of guarana powder (µg/100 g DW). *$n = 3$; S.D. = standard deviation; Rt = retention time. Different letters in the same column indicate significant differences between treatments

No. of peak	Polyphenol	Rt (min)	Concentrations (mg/g) ± S.D.*
1	Theobromine	3.2	0.14 ± 0.01[c]
2	Caffeine	5.0	34.19 ± 1.26[a]
3	(+)-Catechin	8.7	3.76 ± 0.12[b]
4	(−)-Epicatechin	10.4	4.05 ± 0.16[b]
Total			42.15 ± 1.53

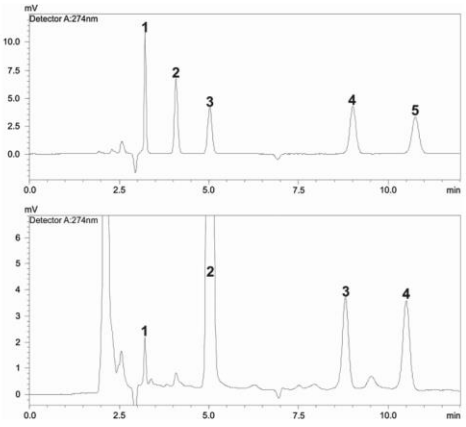

Figure 1. Guarana composition by HPLC. **(A):** Chromatographic profile of polyphenols standards: (1) theobromine, (2) theophylline, (3) caffeine, (4) (+)-catechin, and (5) (−)-epicatechin. **(B):** Chromatographic profile of the polyphenols found in the guarana powder: (1) theobromine, (2) caffeine, (3) (+)-catechin, and (4) (−)-epicatechin at 274 nm.

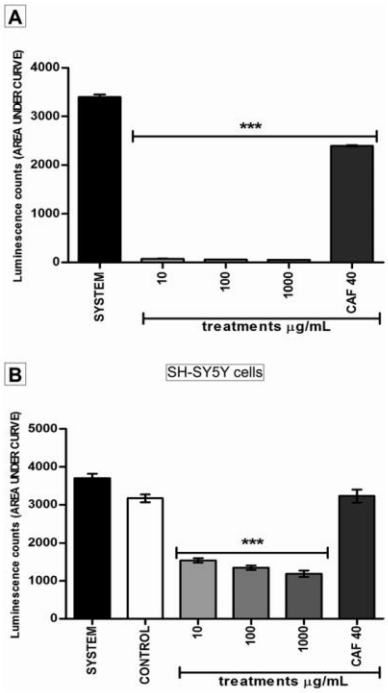

Figure 2. *In vitro* effect of guarana on total radical-trapping antioxidant potential (TRAP). **(A):** The bars represent mean ± S.E.M of three experiments, where black bar represents the system and gray bars represent guarana and caffeine without cells compared to system group. **(B):** Black bar represent the system, white bar represents cellular control (untreated cells), and gray bars represents guarana and caffeine added to cells. $*p < 0.05$ and $***p < 0.0001$.

(Fig. 2A) but not in cells (Fig. 2B). These results demonstrate the antioxidant potential of guarana *in vitro*.

Guarana prevents Aβ aggregation

In order to elucidate the effect of guarana on Aβ aggregation, the ThT fluorescence assay was used to monitor fibrilization of Aβ 1-42. As expected, in the absence of the guarana, Aβ 1-42 displayed a 100% of fibrilization. However, in the presence of 100 and 1000 µg/mL of guarana, there was a significant decrease in the aggregation rate from 100% to 29 and 86%, respectively ($p < 0.01$ and 0.0001, respectively) (Fig. 3).

Guarana inhibits glucose/fructose, GO, and MGO-mediated albumin glycation

Results from the glycation assay showed that glucose and fructose-mediated glycation is inhibited in the

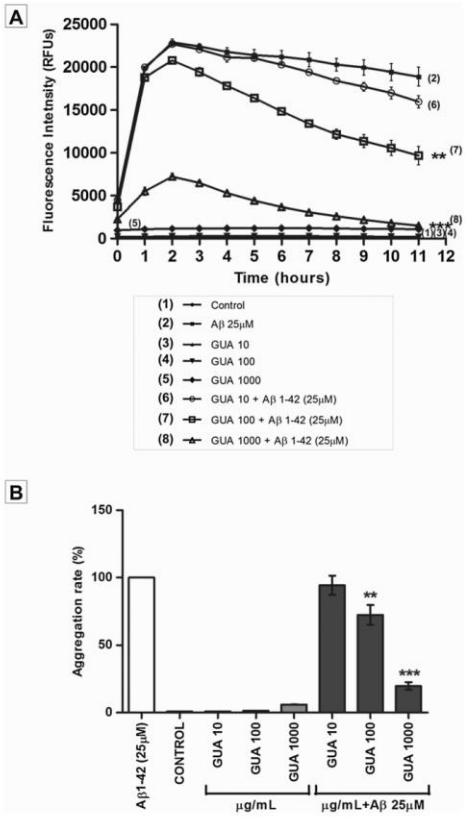

Figure 3. Kinetic of Aβ aggregation. **(A):** Aβ42 (25 µM) aggregation and **(B):** aggregation rate monitored by ThT fluorescence in the absence and presence of 10, 100, and 1000 µg/mL guarana. Fluorescence intensity was measured at an excitation wavelength of 450 nm and an emission wavelength of 482 nm. $**p < 0.001$ and $***p < 0.0001$ relative to Aβ alone. Data are expressed as mean ± S.E.M.

presence of all tested concentrations of guarana (10, 100, and 1000 μg/mL) to 35.9, 11.6, and 5.83%, respectively ($p < 0.0001$ to all concentrations) (Fig. 4A). A similar inhibitory effect, regarding GO and MGO-mediated glycation was observed. Concentrations of 10, 100, and 1000 μg/mL inhibited GO-mediated glycation to values of 67.4, 57.8, and 36.3% ($p < 0.0001$), respectively. Same guarana concentrations inhibited MGO-mediated glycation to values of 68.9, 59.2, and 46.9% ($p < 0.0001$) (Fig. 4B and 4C). Same effects were observed for 40 μg/mL of caffeine (Fig. 4).

Guarana prevents GO and MGO-induced cytotoxicity on human neuronal-like cells

To evaluate whether guarana could protect against AGE-induced cytotoxicity, we used the SH-SY5Y cell line as *in vitro* model, and both GO and MGO as AGEs. After 24 h with 600 μM og GO and 350 μM of MGO, we observed that concentrations of 100 and 1000 μg/mL were able to prevent GO and MGO-induced cytotoxicity ($p < 0.05$ and 0.0001, respectively) (Fig. 4A and 4B). Such effect was not observed when cells were treated with AGEs and 40 μg/mL of caffeine (Fig. 4).

Guarana prevents ACR-induced cytoxicity on human neuronal-like cells

In order to determine whether guarana could protect against oxidative stress-induced cell death, the SH-SY5Y cell line was utilized as an *in vitro* model and ACR as pro-oxidant insult. The results showed that all tested concentrations of ACR significantly reduced the viability of SH-SY5Y cells when exposed for 24 h (Fig. 5A and 5B). Considering these results and the concentrations of ACR already found in both hippocampus and amygdale

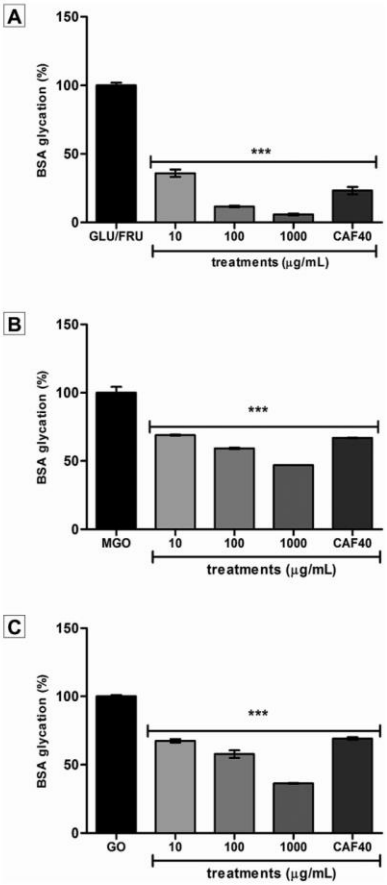

Figure 4. Glycation assay. Albumin plus 25 mM fructose and glucose, MGO, and GO were taken as 100% glycation (black bars). **(A):** Guarana extracts inhibit glucose/fructose, **(B)**: MGO- and **(C)**: GO- mediated glycation. ***$p < 0.0001$ relative to 100% glycation. Data are expressed as mean ± S.E.M.

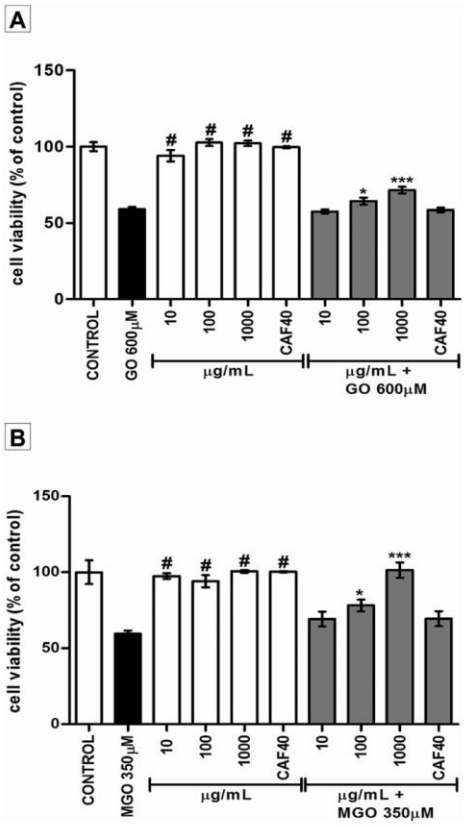

Figure 5. Effect of guarana on GO- and MGO-induced cytotoxicity. **(A):** Cell viability of cells co-treated with GO and guarana. **(B):** Cell viability of cells co-treated with MGO and guarana. *$p < 0.05$ and *$p < 0.0001$ related to GO and MGO alone. # Non-significant when compared to control (untreated cells). Data are expressed as mean ± S.E.M.

from AD patient's brains (Singh *et al.*, 2010), we chose 20 μM (~LD_{50}) as the concentration of ACR to be tested with guarana *in vitro* (Fig. 6). After 24 h of ACR exposure, alone or in combination with guarana, we observed that only 100 μg/mL and 1000 μg/mL of guarana significantly protected against ACR-induced cell death ($p < 0.0001$) (Fig. 7A), which is consistent with the changes of morphology seen by phase-contrast microscopy and its prevention when cells were co-exposed to both ACR and guarana (Fig. 7B).

Guarana decreases ACR-induced intracellular ROS production

It is well known that ACR-induced cellular toxicity is mediated by ROS production and oxidative stress-induced damage (Tomitori *et al.*, 2012). Thus, we investigated whether guarana could prevent ACR-induced intracellular ROS production in SH-SY5Y cells and could account for the cytoprotective actions (Fig. 8). Our results showed that 20 μM of ACR increased the intracellular ROS production when cells were exposed for 2 and 4 h. However, both 100 and 1000 μg/mL of guarana were able to significantly reduce ACR-induced production of ROS ($p < 0.0001$) to levels similar to those observed in control cells (Fig. 8A and 8B). These effects were not observed when cells were treated with ACR and 40 μg/mL of caffeine (Fig. 8).

DISCUSSION

Natural products with antioxidant properties (*in vitro* or *in vivo*) to be used in the context of AD have attracted considerable attention from the scientific community (Zhao and Zhao, 2012). Our own group, for instance, has successfully proposed and demonstrated the potential use of natural antioxidants in other pathological scenarios, such as cancer or even periodontal inflammation (Zanotto-Filho *et al.*, 2012; Zeidán-Chuliá *et al.* 2012, 2013b, 2014a, 2014b).

Since pre-Colombian times, guarana (*P. cupana*) extract (a native plant found in the central Amazon basin) has been used in traditional medicine as tonic/stimulant, aphrodisiac, and protector of the gastrointestinal tract (Ângelo *et al.*, 2008). To date, the available literature about guarana is very limited (e.g. 116 results in Pubmed with '*P. cupana*' as keyword) but it has already been reported the antioxidant properties (Matter *et al.*, 1998; Basile *et al.*, 2005; Bittencourt *et al.*, 2013; Zeidán-Chuliá *et al.*. 2013a), antibacterial and anti-platelet aggregatory activities (Basile *et al.*, 2005; Ravi Subbiah and Yunker, 2008), promotion of short-term weight and fat loss (Boozer *et al.*, 2001), as well as antimutagenic and anticarcinogenic activities (Fukumasu *et al.*, 2011) of guarana extract. In another study in humans, guarana was shown to improve secondary memory performance and increase alert and content mood ratings, effects that were not attributed to its caffeine content alone (Haskell *et al.*, 2007). These therapeutical properties of guarana

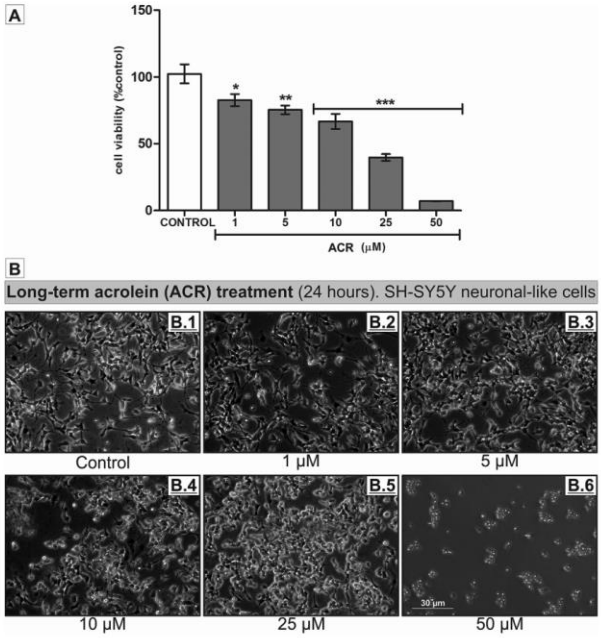

Figure 6. Effect of ACR on SH-SY5Y cellular viability. **(A):** Cell viability measured by SRB assay in the presence of ACR **(B):** Phase contrast micrographs (200×) of SH-SY5Y cells treated for 24 h with ACR. Data are expressed as mean ± S.E.M. *$p < 0.05$, ** $p < 0.001$, ***$p < 0.0001$.

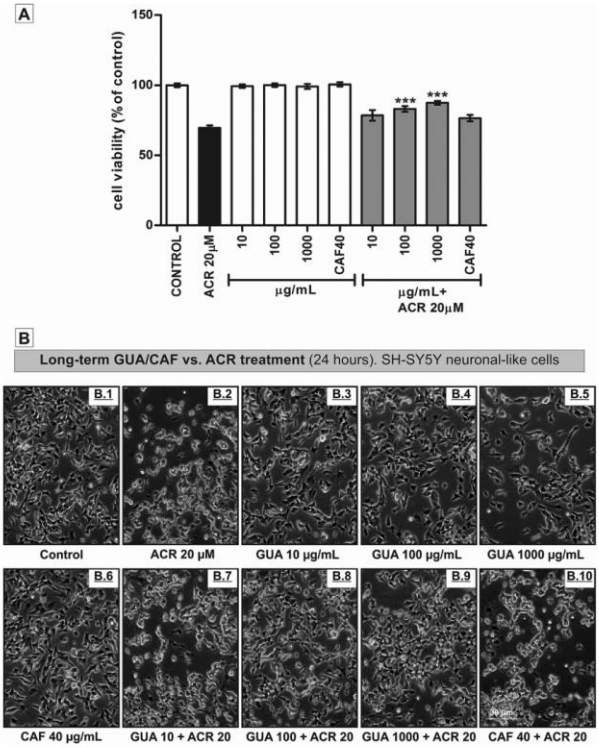

Figure 7. Effect of guarana on ACR-induced neurotoxicity. **(A):** Concentrations of guarana and caffeine alone (white bars) and co-treatments with ACR (gray bars). **(B):** Phase contrast micrographs (200×) of SHSY-5Y cells treated for 24 h with guarana/caffeine and 20 μM ACR for 24 h. $***p < 0.0001$.

can be attributed to either one or several of its major components; for example, caffeine, theobromine, theophylline, catechins, or epicatechins among other compounds in trace concentrations (Fukumasu *et al.*, 2006). With all, nothing is known about the potential effects of guarana effects over these typical hallmarks of AD, and no *in vitro* testing has ever been performed to date. We herein explored radical quenching potential, Aβ 42 fibril formation, protein glycation, and the cytoprotective actions against an ACR and AGEs-induced toxicity of guarana *in vitro*. To achieve these goals, we used the human SH-SY5Y cell line as *in vitro* model because of its well-characterized neuron-like properties (Cheung *et al.*, 2009; da Frota Junior *et al.*, 2011; Navarra *et al.*, 2010; Nicolini *et al.*, 1998; Pahlman *et al.*, 1984; Rabelo *et al.*, 2012; Yu *et al.*, 2011; Zeidán-Chuliá *et al.*, 2013a).

LC analysis of the guarana powder demonstrated that caffeine content (34.19 ± 1.26 mg/g) was actually lower than the one declared by the supplier (40 mg/g). This difference may be related to the spectrophotometric method utilized by the supplier, which is not specific for caffeine determination but usually applied to quantify total methylxanthines. However, the amounts we report in the present study are consistent with those found by Carlson and Thompson (1998) and Sousa et al. (2010) describing a range of caffeine content of 24–45 mg/g in commercial guarana powders. Since caffeine is a major compound in guarana powder with described therapeutic effects in the context of Alzheimer's disease, it was then used as a positive control for the experiments (Arendash and Cao, 2010; Prasanthi *et al.*, 2010). Recently, our group demonstrated that concentrations of both caffeine (0.125–2 mg/mL) and guarana powder from the same manufactures (3.125–50 mg/mL) were able to exert concentration-dependent nonenzymatic antioxidant potential, decreased the basal levels of free radical generation, and reduced both superoxide dismutase and catalase activities on human neuronal SH-SY5Y cells (Zeidán-Chuliá *et al.*, 2013a). We here observed that lower concentrations of guarana still maintained its antioxidant properties *in vitro* (Fig. 2), results that are consistent with our previous study in this same cell model (Zeidán-Chuliá *et al.*, 2013a) but also, with other reports that described similar properties of guarana (Basile *et al.*, 2005; Bittencourt *et al.*, 2013). Catechin and epicatechin, in addition to caffeine, are compounds found in guarana (Subbiah and Yunker, 2008; Zeidán-Chuliá *et al.*, 2013a) and could account for the antioxidant effect we here describe *in vitro*.

High free radical and ROS production have been described as a pivotal event in AD that exacerbates Aβ

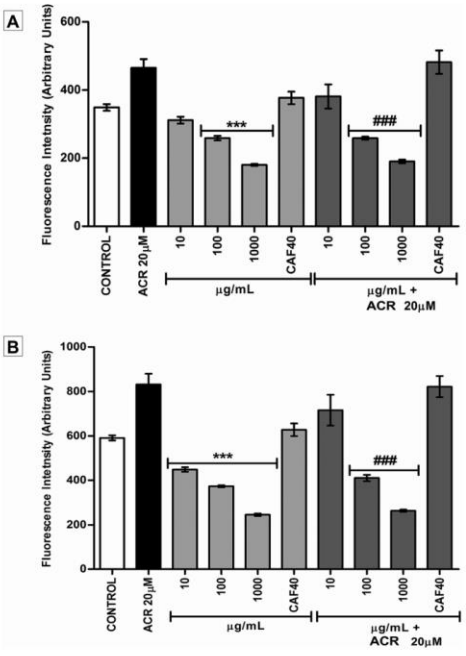

Figure 8. Effects of guarana on ACR-induced ROS production. **(A):** Fluorescence measured of SH-SY5Y cells treated for 2 h with guarana and caffeine alone (gray bars), and co-incubation with ACR (dark gray bars). **(B):** Fluorescence measured of SHSY-5Y cells treated for 4 h with guarana and caffeine alone (gray bars), and co-incubation with ACR (dark gray bars). $***p < 0.0001$ vs. untreated control cells, $###p < 0.0001$ vs. ACR-treated cells in the absence of guarana or caffeine. Data are expressed as mean ± S.E.M.

aggregation and contributes to neuronal dysfunction. Therefore, we evaluated the effect of guarana in such a context and found that 100 and 1000 μg/mL of drug are able to prevent prevents Aβ aggregation and fibrilization (Fig. 3). Our results are consistent with data from Toda *et al.* (2011) where procyanidins (compounds also present in guarana) were potent suppressor of Aβ aggregation, able to suppress Aβ42 aggregation and dissociated Aβ42 aggregates in a dose-dependent manner. In general, other natural polyphenols have been shown to act on different amyloidogenic proteins by inhibiting amyloid formation (Ferreira *et al.*, 2012).

Aberrant cerebral glucose metabolism, an invariant pathophysiological feature of AD, is well accepted as a critical contributor to the pathogenesis of the disease (Chen and Zhong, 2013). In an elegant study by Byun et al., it was highlighted the critical relevance of AGE-albumin in AD, since it is the most abundant form of brain AGEs. It is synthesized by microglia, released into extracellular space, and its rate of synthesis is significantly elevated in the human brains of AD patients when compared to controls (Byun *et al.*, 2012). In other words, strategies with the aim of inhibiting AGEs, generated through glycation, may represent a potent preventive factor in the development of AD. In the present study, we found that concentrations of 10, 100, and 1000 μg/mL guarana are able to inhibit glucose/fructose, MGO, and GO-mediated albumin glycation. In this case, the amount of caffeine present in guarana could also account for this effect (Fig. 4). Other groups have already reported natural anti-glycation substances (e.g. from herbs and spices) (Dearlove *et al.*, 2008); but to the best of our knowledge, this is the first time described for the amazon guarana (*P. cupana* Mart.). This could be a critical positive factor if guarana is considered as a potential therapeutic agent for AD since Aβ is a suitable substrate for glycation, producing one form of the AGEs (Yan *et al.*, 1996), and it has already been hypothesized that glycated Aβ *in vitro* could exacerbate the neurotoxicity of Aβ.

In conclusion, the present study shows *in vitro* evidence that guarana could be a promising alternative for prevention and/or treatment of AD.

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Conflict of Interest

None declared.

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PARTE 3

Esta parte é constituída pela discussão dos dados obtidos nesta tese com suporte da literatura especializada.

DISCUSSÃO

Os avanços na área biomédica proporcionaram um aumento na expectativa de vida humana, de aproximadamente 49 anos para mais de 70 anos. Desta forma, uma parcela crescente da população alcança a idade na qual as doenças neurodegenerativas tornam-se comuns. Dentre estas, as demências têm crescido a níveis alarmantes, sendo a DA responsável entre 60% a 80% dos casos (Alzheimer's Association, 2015).

A doença de Alzheimer (DA) é a mais comum forma de doença neurodegenerativa relacionada à idade e constitui um dos mais devastadores diagnósticos que um paciente e seus familiares podem receber. É uma doença até o momento incurável marcada por uma deterioração gradual ou progressiva da função intelectual, um declínio acentuado da capacidade de realizar atividades do cotidiano, com alterações na personalidade e no comportamento, provocando um comprometimento da memória, afazia (distúrbio da linguagem no qual o paciente deixa de falar), déficits visuais, espaciais (delírios, alucinações e desorientação ambiental) e comprometendo a capacidade de fazer cálculos e abstrações. As alterações da personalidade são um achado frequente e que indicam a terapia a ser administrada ao paciente. Os pacientes se tornam cada vez mais passivos e mais agressivos na demonstração de emoções além de menos espontâneos (Alzheimer's Association, 2015).

Cerca de 99% do casos da DA são esporádicos (*Late-onset Sporadic Alzheimer's Disease – LOAD)*, onde o maior risco é o envelhecimento, seguido pela DA de origem familiar (*Early Onset* Alzheimer's Disease – *EODA*) que somam < 1% dos casos (Alzheimer's Association, 2015).

A Alzheimer´s Association publicou em 2015 algumas informações importantes e que muitas contribuições trouxeram para a compreensão da DA:

- Estima-se que cerca de 5,4 milhões de Norte Americanos em todas as idades são portadores da DA em 2015, destes, 5,2 milhões possuem mais de 65 anos de

idade, configurando a maior prevalência da DA esporádica e 200 mil indivíduos abaixo de 65 anos, caracterizando a DA familiar.

- Segundo a Alzheimer's Association, a faixa etária de incidência da DA é de 13% em idosos na faixa de 65 anos ou superior e acima de 85 anos a doença atinge 45% na população americana em geral (Alzheimer's Association, 2015). Isto se traduz em uma proporção preocupante onde uma em cada oito pessoas acima dos 65 anos ou quase uma em cada duas pessoas acima dos 85 anos seja portadora da DA (Alzheimer's Association, 2015).
- Sob o aspecto econômico, foi estimado um custo anual superior a 200 bilhões de dólares em 2012 e uma projeção de cerca de 1,1 trilhão de dólares em 2050 somente nos Estados Unidos (Alzheimer's Association, 2015).

K. Ziegler-graham et al. (2008) afirmaram em trabalho realizado sobre a prevalência da Doença de Alzheimer em diversos países do mundo (Estados Unidos, Canadá e Europa), que o risco de DA cresce exponencialmente com a idade, duplicando em aproximadamente 5 ou 6 anos depois. Embora as formas das curvas de incidência sejam semelhantes, existe uma considerável variação na incidência absoluta das taxas em todo o mundo (K. Ziegler-graham et al., 2008). No Brasil, a incidência e a prevalência da DA seguem as estimativas mundiais, estimando-se que até 2025 em torno de 1,2 milhões de pessoas desenvolverão a DA (Aprahamian et al., 2009).

Dentre os diversos eventos moleculares que estão associados ao início e progressão da doença de Alzheimer, está o estresse oxidativo. Por outro lado, o SNC também está particularmente exposto ao estresse oxidativo pelo fato de: 1) utilizar grandes quantidades de oxigênio (cerca de 20% do oxigênio consumido), 2) possuir grande quantidade de ácidos graxos poliinsaturados, 3) grande quantidade de metais de transição e 4) uma relativa escassez de defesas antioxidantes (Smith et al, 2007; Pocernich et al., 2011a). Cabe enfatizar que déficits cognitivos associados a patologias neurodegenerativas, como a DA, também estão intimamente associados ao estresse oxidativo (Pocernich et al., 2011a; Williams & Spencer, 2011).

Como estratégias para prevenção e tratamento da DA estão incluídas: terapia estimulatória através de exercícios físicos, tarefas cognitivas, música e socialização; e a farmacoterapia que inclui: inibidores da acetil colinesterase (Akasofu et al., 2008; Hansen et al., 2008), drogas anti-hipertensivas (Ohrui et al., 2004; Sink & Leng, 2007), drogas anti-inflamatórias (Viad et al, 2008; Walker & Lue., 2009), inibidores da secretase (Hussain et al., 2007; Fleisher et al., 2008), drogas para resistência à insulina (Reger et al., 2008; Neumann et al., 2008), fator neurotrófico derivado do cérebro (BDNF) (Li et al., 2009; Nagahara et al., 2009), imunização (Holmes et al., 2008; Vellas et al., 2009).

O uso de "adjuntos" complementares, como extratos de plantas, suplementos a base de ervas varia de acordo com as diferenças culturais. Na medicina ortodoxa ocidental, em contraste com a oriental (a medicina chinesa, por exemplo), propriedades farmacológicas de plantas relacionadas à melhoria da memória/aprendizagem são muito pouco investigadas, com exceção dos gincolídeos, cuja ação neuroprotetora e atividades colinérgicas foram consideradas relevantes e comprovadamente eficazes (Trevisan & Macedo, 2003; Wollen, 2010). Por muito tempo, os alimentos funcionais têm se mostrado como alternativas promissoras na modulação ou na diminuição do estresse oxidativo e seus efeitos deletérios, especialmente na DA (Pocernich et al, 2011b).

Os componentes celulares não são protegidos totalmente por antioxidantes endógenos, e é bem estabelecido que antioxidantes obtidos da dieta são indispensáveis para a defesa apropriada contra oxidação e, portanto, têm importante papel na manutenção da saúde. Deste modo, as plantas medicinais e os alimentos têm sido considerados como importantes recursos para a obtenção de compostos antioxidantes não enzimáticos desde os primórdios da humanidade até os nossos dias.

O Reino Plantae, além de ser o maior reservatório de moléculas orgânicas conhecido, é um poderoso laboratório de síntese. A partir de plantas utilizadas pelo conhecimento popular, foram descobertos diversos medicamentos empregados até os nossos dias pela medicina. Até hoje diversas moléculas com estrutura complexa, dependem de síntese biológica, pois a síntese em laboratório ou não é possível, ou é economicamente inviável; por isso plantas são utilizadas como

matéria-prima para a fabricação de medicamentos (Alves & Silva, 2002). Assim, o uso de plantas medicinais pela população mundial é muito significativo. Dados da Organização Mundial da Saúde (OMS) mostram que cerca de 80% da população do planeta faz uso de algum tipo de planta na busca de alívio de alguma sintomatologia dolorosa ou desagradável. Desse total, apenas 30% ocorre por indicação médica (Alves & Silva, 2002).

Além das propriedades fitoterapêuticas de algumas plantas, os alimentos consumidos na dieta também são fonte de compostos secundários ativos que atuam sobre a homeostase corporal. Os benefícios para a saúde associados ao consumo de frutas e hortaliças que foram descritos a partir de evidências epidemiológicas, clínicas e experimentais devem-se, em parte, à presença de antioxidantes nestes alimentos, desta forma, é de grande importância a busca por novas fontes de compostos bioativos exógenos, que podem ser tanto de origem natural quando sintética (Barreira et al., 2008).

Muitas moléculas provenientes dos alimentos além da atividade antioxidante podem apresentar outras propriedades importantes na regulação bioquímica e fisiológica do organismo como: atividade anti-inflamatória, anti-agregadora de plaquetas, anti-proliferativa, moduladora da função endócrina, e dos mecanismos de apoptose, etc. Além do mais, grande parte dos alimentos apresenta mais de um tipo de compostos bioativos, que no seu conjunto podem agregar diversas propriedades biológicas importantes (Barreira et al., 2008).

Este é o caso do guaraná (*Paullinia cupana*) de origem amazônica. O seu uso tem se disseminado mundialmente principalmente nas bebidas energéticas. Adicionalmente um conjunto de estudos produzidos independentemente por diversos grupos de pesquisa sugerem ter o guaraná ação: cardiotônica e cardioprotetora, atividade antiplaquetária, energética, atividade anti-bacteriana, antioxidante (ainda muito pouco estudado), efeito anti-obesidade, termogênico e no aumento da taxa metabólica, atividade contra lesões gástricas, quimiopreventivo, anti-depressivo, efeito no metabolismo lipídico, anti-mutagênico, anti-tumoral.

Os efeitos antioxidantes do guaraná também são muito pouco documentados (Mattei et al., 1998; Basile et al, 2005; Majhenic et al., 2007) portanto investigações adicionais sobre os efeitos antioxidantes deste alimento no SNC também são necessários.

Apesar do uso crescente do guaraná como aditivo alimentar, os efeitos comportamentais, de melhoria cognitiva (melhoria da memória, atenção, velocidade de raciocínio, diminuição da fadiga mental) e neuroquímica deste produto natural têm recebido pouca atenção ultimamente. Uma busca detalhada encontrou poucos estudos (Espindola et al., 1997; Otobone et al., 2005; Otobone et al., 2007; Antonelli-Ushirobira, et al, 2010; Scholey et al 2013).

E somente um estudo avaliou a eficiência do extrato de guaraná como alternativa protetora frente ao desafio com compostos carbonílicos como MGO, GO e ACR (Bittencourt et al., 2014) em modelo celular SHSY-5Y. Neste estudo, os autores constaram pela primeira vez que o guaraná diminuiu o estresse oxidativo provocado pelo MGO, GO e ACR, aumentando a viabilidade celular. Os autores também comprovaram pela primeira vez que o extrato de guaraná amazônico foi capaz de diminuir o efeito glicante destes compostos carbonílicos (Bittencourt et al., 2014).

Uma das novas estratégias para evitar a toxicicidade da ACR, incluem os polifenóis como compostos "emergentes" para tal finalidade. Desta forma, estudos destas moléculas podem prover mais informação para facilitar o entendimento da interação entre estes compostos e a cascata de intermediários formados durante o processo de lipoperoxidação bem como os seus produtos finais (aldeídos α, β-insaturados). Ansari et al, 2008 descrveram que o pycnogenol (PYC), um composto polifenólico sintético derivado de *Pinus maritma*, é capaz de diminuir a toxicidade mediada pela acroleína em células SH-SY5Y. Neste estudo, os níveis de GSH, produção de espécies reativas (ERO) e a atividade da NAPDH oxidase foram modulados de forma dose-resposta dependente pelo PYC, resultando em maior viabilidade celular, diminuição de nitração/oxidação (carbonilação e 3-nitrotirosina) e inibição da formação da produção de produtos de peroxidação (Ansari et al., 2008). Os extratos de *Bancopa mineira* (BM), também se mostraram efetivos contra a toxicicidade induzida pela ACR em culturas celulares. Além de diminuir a geração de ERO, influenciou o potencial de membrana mitocondrial e também modulou a expressão de diversos proteínas envolvidas na regulação do estado redox celular, entre elas Nfκ-B, Sirt-1, ERK1/2 e p66Shc, levando a diminuição da toxicidade induzida pela ACR (Sing et al., 2010). Além disso, extratos de *Scutellaria baicalensis* diminuiu a toxicicidade induzida por ACR em células

endoteliais através do aumento de GSH e aumentando a expressão de muitas enzimas relacionadas ao metabolismo de GSH (Zhang et al. 2011).

Neste trabalho de tese, demonstramos pela primeira vez os efeitos protetores dos extratos oriundos de sementes do guaraná amazônico em células SHSY-5Y, onde o extrato foi capaz de atenuar a citotoxicidade mediada por ACR através da diminuição da geração de ERO a partir de 2 horas de tratamento, aumentando desta forma a viabilidade das células. (Bittencourt, et al., 2014 – estudo II). Sabe-se que compostos com atividade antioxidante são excelentes candidatos para prevenir/atenuar os efeitos da acroleína e outros compostos carbonílicos, seja sequestrando diretamente o aldeído, diminuindo o estresse oxidativo provocado por estes ou modulando o sistema de defesa antioxidante. Neste contexto, o guaraná apresenta forte atividade antioxidante, como evidenciado pelos estudos I e II desta tese, sendo capaz de atenuar a toxicidade induzida por compostos carbonílicos (ACR, GO e MGO), quelando diretamente os radicais gerados pela ação destes (estudo II) e provavelmente quelando diretamente os aldeídos. Estes efeitos provavelmente se devem ao conteúdo fenólico (catequinas, epicatequina, epicatequina-galato) e metilxantinas presentes nas sementes da planta. Para dar maior suporte a estas afirmações, os compostos fenólicos presentes nesta planta e em muitas outras, foram isolados e também tem sido avaliados com relação aos seus efeitos contra a toxicidade induzida por ACR. A catequina mais abundante encontrada no chá verde, epigalo-catequina-3-galato (EGCG) inibiu a ligação de ACR com desoxiguanosina em condições oxidativas (Nath et al., 2010). O robinetinidol-epigalocatequina-3-galato (REO), isolada de *Acacia mearnsii*, diminuiu o imunoconteúdo de caspase-3 e suprimiu a fosfo-JNK todos mediados por ACR em células SHSY-5Y, sugerindo atividade protetora contra efeitos pró-apoptóticos de ACR (Huang et al., 2010). Visto que o guaraná possui diversas catequinas em seu conteúdo fenólico (Yamaguti-Sasaki et al., 2007; Bittencourt et al., 2014), seria razoável hipotetizar que o mesmo possa inibir caspases, modular potencial de membrana mitocondrial entre outros mecanismos.

Seguindo esta linha de pensamentos, outros estudos abordando outros compostos fenólicos também se mostraram eficientes contra o estresse oxidativo induzido pela acroleína. O resveratrol aumentou a eficácia fagocítica de células RPE em toxicidade aguda induzida por acroleína e

peróxido de hidrogênio (Liu et al., 2007). O hidroxitirosol, um composto fenólico abundante no óleo de oliva, também se mostrou eficiente em atenuar o dano oxidativo e disfunção mitocondrial mediado pela ACR. Neste estudo os autores postularam que os principais efeitos se dão por dimunuição da pressão oxidativa, modulação de Nrf2 e de PPARGC1α (coativador do receptor para proliferação peroxissomal) (Liu et al., 2007).

In vivo, foi demonstrado em modelo de ratos que o chá verde atenua dano pulmonar agudo induzido pela fumaça de óleos ferventes através da diminuição da pressão oxidativa e diminuição da síntese de proteínas pró-apoptóticas. Como já citado na introdução desta tese, a acroleína é o principal aldeído constituinte da fumaça de óleos ferventes (Yang et al., 2009). Levando em consideração a nossa exposição ambiental, estima-se que os seres humanos estão expostos de aproximadamente 2,5 a 7mg/kg de ACR por dia (Huang et al., 2013). No estudo III desta tese, ratos Wistar foram submetidos a administração crônica de 2,5mg/kg de ACR durante 8 semanas. Isto levou ao aumento de peroxidação lipídica, diminuição de GSH, diminuição de SH em fígado e SNC, prevalência de comportamentos semelhantes a ansiedade/depressão e diminuição da atividade locomotora, todos estes sintomas são prevalentes na doença de Alzheimer (Alzheimer's Association, 2015). Doses maiores de ACR não foram apresentadas na tese pelo fato do aldeído em questão aumentar significativamente a mortalidade dos animais. Outro estudo com ratos, utilizado a mesma dose de ACR também mostrou que este aldeído induz estresse oxidativo diminuindo a atividade da SOD, induzindo atrofia neuronal e astrogliose e modula positivamente enzimas relacionadas a produção do peptídeo beta-amilóide, prejudicando desta forma aspectos cognitivos importantes dos animais (Huang et al., 2013).

O guaraná possui várias classes de compostos naturais (Yamaguti-Sasaki et al., 2007), que segundo a literatura citada, possuem grande potencial frente à toxicidade induzida pela ACR, mas com diferentes reatividades frente ao referido composto. Esta eficiência poderia acontecer desde a interação direta com a ACR até a modulação dos sistemas de defesa enzimáticos e não enzimáticos intracelulares envolvidos nas cascatas de dano mediado pela ACR. Neste trabalho podemos sugerir que uma explicação possível para o aumento da GSH e SH seriam que o extrato de guaraná induziu diminuição direta da pressão oxidativa excessiva provocada ACR, através do

sequestro das espécies reativas decorrentes da ação deste aldeído. Ainda o extrato de guaraná poderia modular a atividade de enzimas relacionadas ao metabolismo de GSH, aumentando a conjugação da GSH com ACR, ou a atividade de enzimas presentes no metabolismo de compostos carbonílicos. Além disso, os compostos fenólicos e metil xantinas presentes no guaraná poderiam interagir diretamente com acroleína formando hemiacetais.

O potencial do extrato de guaraná como neutralizador da ACR poderia representar uma alternativa bastante promissora na prevenção de doenças onde a ACR e seus efeitos sejam fatores chave para a sua iniciação e progresso.

CONCLUSÕES FINAIS

A partir dos resultados obtidos no presente trabalho, foi possível concluir:

In vitro

1) O extrato comercial de guaraná foi capaz de quelar o radical peroxil, hidroxil e oxido nítrico, reduzir a produção de MDA em sistemas *in vitro* com baixa IC 50;
2) O extrato comercial de guaraná tem potencial para reduzir e quelar ferro com baixa IC 50;
3) O extrato comercial de guaraná se mostrou efetivo em diminuir a glicação mediada por frutose, glicose, MGO e GO em um sistema semelhante ao soro humano;
4) A exposição de células SH-SY5Y a ACR provocou perda de viabilidade e aumento de estresse oxidativo em curto espaço de tempo, levando estas células a apoptose após 24 horas;
5) A exposição de células SH-SY5Y ao extrato de guaraná sozinho não induziu perda de viabilidade nas células SH, mas induziu a queda na produção de espécies reativas;
6) O tratamento com extrato comercial de guaraná atenuou a queda de viabilidade e estresse oxidativo mediado pela acroleína.

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