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THALLITA KELLY RABELO

CARACTERIZAÇÃO REDOX-ATIVA DO ÁCIDO ÚSNICO E SEU EFEITO CITOTÓXICO EM CÉLULAS SH-SY5Y

ARACAJU
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Dissertação apresentada ao Núcleo de Pós-graduação em Medicina da Universidade Federal de Sergipe como requesito parcial à obtenção do grau de Mestre em Ciências da Saúde.

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DEDICATÓRIA

Dedico esta dissertação aos meus maiores mestres, Manoel (Pitito) e Maria Aparecida (Cida) meus pais, pelo exemplo de vida, moral e perseverança.

Ao meu esposo João Paulo, por todo o conhecimento compartilhado, incentivo e amor.

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Não é o mais forte que sobrevive, nem o mais inteligente, mas o que melhor se adapta às mudanças.

(Charles Darwin)

RESUMO

RABELO, Thallita Kelly. Caracterização redox-ativa do ácido úsnico e seu efeito citotóxico em células SH-SY5Y. Dissertação (Mestrado em Ciências da Saúde) UFS/SE, Aracaju, 2012. O ácido úsnico (AU) é um dos mais comuns e abundantes metabólitos secundários liquênicos com potencial aplicação terapêutica. As propriedades anti-inflamatória e antitumoral já foram relatadas e extratos de liquens enriquecidos com ácido úsnico, são amplamente utilizados para tratar diversas doenças na medicina popular. Entretanto, um crescente número de estudos tem sugerido que o AU apresenta propriedades pró-oxidantes em sistemas biológicos, as quais podem induzir dano celular mediado por espécies reativas. Baseado nesses dados, primeiro foi realizado a avaliação in silico das interações do AU com genes / proteínas e compostos importantes para o equilíbrio celular redox. Além disso, analisamos as propriedades redox-ativa do AU contra diferentes espécies reativas (SR) geradas in vitro, e seu efeito em células neuronais SH-SY5Y na presença de peróxido de hidrogênio (H_2O_2), já que nenhum dado neurotoxicológico in vitro tem sido relatado até agora. O índice de potencial antioxidante reativo total (TRAP) mostrou uma capacidade antioxidante significativa do AU a 20 µg/mL; AU também foi eficaz contra os radicais hidroxila e reduziu a formação do óxido nítrico. No entanto, a lipoperoxidação in vitro foi induzida pelo AU, e a viabilidade celular foi diminuída ao longo de 24 horas de tratamento, de acordo com ensaio de MTT (brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio) e análise morfológica. Além disso, o AU não protegeu a célula contra a morte celular induzida pelo H_2O_2. O ensaio DCFH-DA (2'7' diacetato de diclorofluoresceína) mostrou que o tratamento de AU (20 µg/mL) por 1 hora e (2 ng/mL a 20 µg/mL) durante 4 e 24 horas, aumentou a produção basal de espécies reativas e quando as células foram co-tratadas com o H_2O_2, o AU parece potencializar o H_2O_2 induzindo a produção de espécies reativas. Nossos resultados sugerem que o AU exibe variáveis propriedades redox-ativas, atuando como um agente antioxidante e pró-oxidante, de acordo com as diferentes condições do sistema e / ou ambiente celular. Estas propriedades pró-oxidantes em células SH-SY5Y podem ser responsáveis por possíveis efeitos neurotóxicos do AU.

Palavras chave: ácido úsnico; radicais livres; estresse oxidativo; viabilidade celular; células SH-SY5Y.

ABSTRACT

Usnic acid (UA) is the most common and abundant lichenic secondary metabolite with potential therapeutic application. Anti-inflammatory and antitumour properties have already been reported and UA-enriched extracts are widely used to treat several diseases in the folk medicine. On the other hand, a growing body of evidence has suggested that UA present pro-oxidant properties, which might induce cellular damage mediated by reactive species. Based on this data, first we performed in silico evaluation of UA interactions with genes/proteins and important compounds for cellular redox balance. Then, we assessed UA redox properties against different reactive species (RS) generated in vitro, and evaluated its action on SH-SY5Y neuronal-like cells subjected to hydrogen peroxide (H_2O_2) treatment, since no in vitro neurotoxicological data has been reported so far. Total reactive antioxidant potential index (TRAP) showed a significant antioxidant capacity of UA at 20 µg/mL; UA was also effective against hydroxyl radicals and reduced nitric oxid formation. However, in vitro lipoperoxidation was enhanced by UA, and cell viability was decreased along 24 hours of treatment, according to MTT assay (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and morphological analysis. Moreover, UA did not display protective effects against H_2O_2-induced cell death in any case. The DCFH- DA (2',7'-dichlorfluorescein-diacetate) based assay indicated that UA enhanced basal reactive species production at 20 µg/mL for 1 hour and from 2 ng/mL to 20 µg/mL for 4 and 24 hours. In addition, UA appears to potentiate H_2O_2-induced reactive species production. Our results suggest that UA displays variable redox-active properties, acting either as antioxidant or pro-oxidant agent according to different system conditions and/or cellular environment. These pro-oxidant properties in SH-SY5Y might be responsible by potential neurotoxic effects of UA.

Keywords: Usnic acid; Free radicals; Cellular viability; Oxidative stress; SH-SY5Y cells.

LISTA DE FIGURAS

CAPÍTULO II

CAPÍTULO III

LISTA DE ABREVIATURAS E SIGLAS

AAPH	2,2-azobis62-metilpropionamida
AU	Ácido úsnico
CAT	Catalase
COX	Cicloxigenase
DCFH	Diclorofluoresceína
DCFH-DA	2'7'Diacetato de Diclorofluoresceína
DMSO	Dimetilsufóxido
DR	Deoxiribose
EROs	Espécies Reativas de Oxigênio
ERNs	Espécies Reativas de Nitrogênio
GPx	Glutationa Peroxidase
GSR	Glutationa Redutase
GSH	glutationa reduzida
GSSG	Glutationa Oxidada
$HO^{\bullet}$	Radical Hidroxila
IκB	Enzima kinase
LPO	Lipoperoxidação
MDA	Malondialdeído
MTT	Brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio
Mn SOD	Superóxido Dimutase Dependente de Manganês
$NADP^{+}$	Nicotinamida Adenina Dinucleotídeo Fosfato, forma oxidada
NADPH	Nicotinamida Adenina Dinucleotídeo Fosfato, forma reduzida
NFκB	Fator nuclear κB
PG	Prostaglandina
SNP	Nitroprussiato de Sódio
SOD	Superóxido Dismutase
TAR	Reatividade Antioxidante Total
TBA	Ácido Tiobarbitúrico
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TRAP	Potencial Antioxidante Reativo Total
TROLOX	Ácido 6 – hidróxi-2,5,7,8 – tetrametilchroman-2-carboxílico

SUMÁRIO

CAPÍTULO I

INTRODUÇÃO E OBJETIVOS

1. INTRODUÇÃO

Parte significativa da população brasileira utiliza uma grande variedade de produtos naturais no tratamento de doenças crônicas e agudas. Isso se deve a fatores como baixo poder aquisitivo e acesso limitado aos serviços de saúde, associados à disposição de uma rica e variada flora regional de fácil acesso para uso medicinal (SANTOS et al., 2008). Segundo dados da Organização Mundial da Saúde (OMS), atualmente, 80% da população dos países em desenvolvimento utilizam-se de práticas medicinais tradicionais sendo que 85% dessas práticas envolvem o uso de produtos naturais (ALMEIDA et al., 2006).

Neste contexto, compostos naturais isolados de algas, fungos, macrofungos, liquens e plantas superiores constituem importantes fontes de pesquisa de moléculas bioativas, seja através do uso direto dos metabólitos secundários, ou empregando compostos derivados da biossíntese, com o intuito de aumentar a efetividade ou absorção do composto bioativo (HOSTETTMANN, WOLFENDER, RODRIGUEZ, 1997).

O uso de liquens na medicina popular ocorre em vários continentes, sendo utilizado no tratamento de micoses, bronquite, hemorróidas, disenteria, alívio da febre, como anti-séptico para ferimentos e como droga hemostática (GÜLÇIN et al., 2002; INGÓLFSDÓTTIR, 2002). A atividade biológica exibida por algumas espécies de liquens se deve ao fato de tais espécies produzirem metabólitos secundários com possíveis propriedades terapêuticas (INGÓLFSDÓTTIR, 2002).

De todos os metabólitos secundários liquênicos, o ácido úsnico é o mais estudado, apresentando diversas atividades biológicas, tais como propriedades antibióticas, antivirais, antiproliferativas, anti-inflamatórias, antitumorais e gastroprotetoras (RIBEIRO-COSTA et al., 2004; De CARVALHO et al., 2005; ODABASOGLU et al., 2006).

No entanto, apesar de apresentar uma variedade de atividades biológicas, o ácido úsnico inibe a função mitocondrial dos hepatócitos, levando a um aumento da produção de espécies reativas de oxigênio, induzindo assim, o estresse oxidativo. (HAN et al, 2004; JOSEPH et al, 2009). Embora os mecanismos de toxicidade do ácido úsnico sejam pouco conhecidos, Kohlhardt-floehr e colaboradores (2010) descreveram que esse composto pode induzir dano (pró-oxidante) e ao mesmo tempo proteger (antioxidante)

uma linhagen de linfócitos T humanos (jurkat-cells) contra um dano oxidativo (radiação UV-B).

Estudos realizados por Moure e colaboradores (2005) mostraram que, potentes antioxidantes podem auto-oxidar e gerar substâncias reativas e, portanto, também atuarem como pró-oxidantes, dependendo da composição do sistema.

As propriedades biológicas exercidas pelo ácido úsnico possivelmente podem estar associadas a sua ação pró-oxidante e antioxidante generalizada. A atranorina, outro metabólito secundário estudado pelo nosso grupo, além de apresentar um duplo comportamento atuando como pro-oxidante e antioxidante frente a diferentes espécies reativas, foi capaz de reverter danos celulares induzidos pelo peróxido de hidrogênio apresentando assim, efeito citoprotetor (MELO et al., 2011). Essas atividades biológicas exercidas por estes metabólitos se devem ao fato dessas substâncias serem compostos fenólicos que podem ajudar a combater o estresse oxidativo atuando como um antioxidante, e ao mesmo tempo desequilibrar o estado redox celular no sentido pró-oxidante, dependendo das condições específicas, ou seja, ambiente celular, tipo de espécies reativas e quantidade da droga (ODABASOGLU et al., 2006; HALLIWELL, 2008).

Sendo assim, se faz necessária uma melhor compreensão sobre as características redox-ativas do ácido úsnico. Além disso, apesar de ser um composto extensivamente estudado, não foram encontrados relatos na literatura da sua ação em células neuronais. Para isso, utilizamos uma linhagem de neuroblastoma humano - SH-SY5Y, que tem sido usada como modelo celular in vitro para o estudo das propriedades neuronais e de possíveis mecanismos de neurotoxicidade. Substâncias que atuam como pró-oxidante e antioxidante e que induzem a inibição da formação de neuritos em células SH-SY5Y, podem ser considerada possíveis indicadores de neurotoxicidade (NOSTRANDT, EHRICH, 1992; TOSETTI, TAGLIETTI, TOSELLI, 1998; UBERTI et al, 2002).

A presente dissertação está dividida em capítulos, sendo o primeiro deles destinado à introdução e objetivos, cuja finalidade foi fazer uma abordagem geral do tema, assim como determinar os objetivos a serem alcançados; o segundo capítulo compreende a revisão bibliográfica dos assuntos abordados ao longo da elaboração do trabalho; e por fim, o terceiro capítulo apresenta o artigo gerado no decorrer da pesquisa, de acordo com os objetivos estabelecidos.

1.1. OBJETIVOS

1.1.1. Objetivo Geral

Determinar o perfil redox-ativo do ácido úsnico frente à diferentes espécies reativas geradas in vitro e o seu efeito na viabilidade de uma linhagem celular derivada de neuroblastomas humanos (SH-SY5Y).

1.1.2. Objetivos Específicos

- Determinar in silico as possíveis interações do ácido úsnico com genes/proteínas e compostos envolvidos no balanço redox celular;
- Avaliar o Potencial Reativo Antioxidante Total (TRAP) e a Reatividade Antioxidante Total (TAR) do ácido úsnico in vitro;
- Observar a atividade "scavenger" do ácido úsnico in vitro, frente ao radical hidroxila;
- Verificar a atividade "scavenger" in vitro do ácido úsnico, frente ao radical óxido nítrico;
- Avaliar a capacidade do ácido úsnico em prevenir ou potencializar a lipoperoxidação causada por radicais livres in vitro;
- Determinar atividade "scavenger" in vitro do ácido úsnico frente ao radical superóxido;
- Verificar a habilidade do ácido úsnico em degradar o peróxido de hidrogênio in vitro;
- Avaliar o efeito do ácido úsnico na viabilidade das células SH-SY5Y;
- Verificar a capacidade do ácido úsnico em modificar os níveis basais de espécies reativas de oxigênio em células SH-SY5Y.

CAPÍTULO II

FUNDAMENTAÇÃO TEÓRICA

2. FUDAMENTAÇÃO TEÓRICA

2.1. Liquens e Substâncias Liquênicas

As interações entre algas fotoautotróficas (fotobiontes) e fungos heterotróficos (micobiontes), que começou cerca de 551 a 600 milhões anos atrás e levou à formação de associações simbióticas auto-sustentáveis, são chamadas de liquens (YUAN, XIAO, TAYLOR, 2005). Essa disposição simbiótica entre os organismos é indubitavelmente bem sucedida e proporcionou aos líquens a capacidade de serem organismos dominantes em ambientes caracterizados por extremas condições ecológicas, possibilitando assim, que liquens sejam encontrados desde os trópicos até as regiões polares, compreendendo cerca de 8% da superfície da terra (ENGEL et al., 2007; BUCAR et al., 2004). Além disso, o controle de divisão celular e regulação da viabilidade que o organismo micobionte presente no liquen exerce sobre o organismo fotobionte é decorrente da produção de substâncias biologicamente ativas conhecidas como metabólitos primários e secundários, sendo estes, responsáveis pela maioria das propriedades biológicas dos liquens (FASHELT, 1994).

As substâncias que derivam do metabolismo primário são: carboidratos, carotenóides e vitaminas, aminoácidos e proteínas. Estas substâncias, ligadas à parede celular e ao protoplasto, são frequentemente solúveis em água e extraídas com água quente. Estes compostos são sintetizados pelas células das hifas, sendo posteriormente utilizados como mecanismo de defesa frente a infecções externas (NASH, 1996).

Os metabólitos secundários resultantes de rotas biossintéticas são os ácidos graxos, compostos aromáticos (depsídeos e depsidonas), derivados fenólicos, meta-depsídeos, dibenzofuranos, ácidos úsnicos, cromonas, xantonas, antraquinonas, terpenos e derivados do ácido pulvínico (HONDA, VILELAS, 1998).

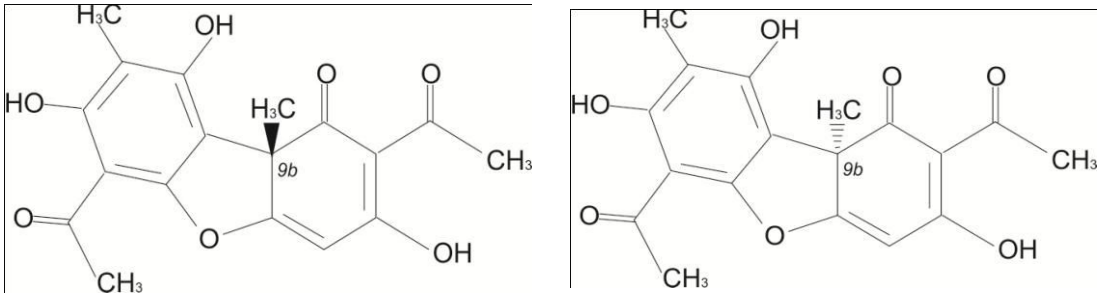
São conhecidos cerca de 630 metabólitos secundários. A maioria é produzida unicamente pelos liquens e uma pequena minoria, em torno de 50 a 60, é produzida por fungos de vida livre e plantas superiores. Os depsídeos, depsidonas, dibenzofuranos, ácidos úsnicos e a depsona são derivados fenólicos encontrados exclusivamente em liquens (MÜLLER, 2001). O ácido úsnico, além de ser considerado um dos mais importantes metabólitos liquênicos biologicamente ativos é o mais estudado do ponto de vista farmacológico, quanto a sua ação antibiótica, antiviral, antiproliferativa, antitumoral, anti-inflamatória e analgésica (INGÓLFSDÓTTIR, 2002).

2.2. Ácido Úsnico

Desde o seu primeiro isolamento em 1844, data da origem da química orgânica e fitoquímica, o ácido úsnico [2,6-diacetil-7,9-dihidroxi-8,9b-dimetil-1,3-(2H,9H)-dibenzofurano, $C_{18}H_{16}O_7$,], tornou-se um dos metabólitos liquênicos mais extensivamente estudados e um dos poucos comercialmente disponíveis (INGÓLFSDÓTTIR, 2002).

Encontrado abundantemente nos gêneros Usnea, Cladonia, Cetraria, Ramalina e Parmelia (VENKATARAMANA, KRISHNA, 1992; MÜLLER, 2001; COCCHIETTO et al., 2002; INGÓLFSDÓTTIR, 2002), o ácido úsnico caracteriza-se por ser uma substância de baixo peso molecular (344,32 g) e pigmentação amarela, insolúvel em água e em glicerol, parcialmente solúvel em etanol e facilmente solúvel em éter, acetona, clorofórmio e acetato de etila (KRISTMUNDSDÓTTIR et al., 2002).

O ponto de fusão dos seus cristais é em torno de 203°C; seu caráter hidrofóbico é devido à presença dos três grupos cetônicos e do anel furano que liga os dois anéis aromáticos existentes em sua estrutura e a seu efeito citotóxico é atribuído aos grupamentos β-tricetona presentes na molécula (SHIBATA, 1964; CAMPANELLA et al., 2002). Ocorre na natureza em duas formas enantioméricas (+) e (-), as quais diferem na orientação do grupo metila localizado na posição 9b. (Figura 1) (COCCHIETTO et al., 2002).



(+) (-)

Figura 1. Estrutura molecular plana dos enantiómeros (+) ácido úsnico e (-) ácido úsnico mostrando o centro estereogênico 9b (COCCHIETTO et al., 2002).

2.3. Atividades biológicas do ácido úsnico

2.3.1. Antimicrobiana e antiviral

Nas décadas de 40 e 50 diversos compostos liquênicos foram relatados como sendo ativos contra várias linhagens de bacilos da tuberculose e organismos Gram-positivos. Dentre estes compostos, o ácido úsnico foi o mais potente agente antibiótico (MÜLLER, 2001). Ingólfsdóttir e colaboradores (1998), estudando a atividade antimicrobiana de cinco derivados de ácido úsnico, relataram que o ácido úsnico foi o mais ativo contra Mycobacterium aureum. Estes resultados estão de acordo com Venkataramana e Krishna (1992), os quais reportaram que ambas formas enantioméricas do ácido úsnico inibiram, o crescimento in vitro de M. turberculosis e M. tufu (modelo de M. leprae) com uma concentração relativamente baixa.

Da mesma forma, Weckesser e colaboradores (2007) relataram que o ácido úsnico inibiu o crescimento de outras bactérias dos gêneros Streptococae e Corynebactéria, assim como de microorganismos aeróbios e anaeróbios, sendo também considerado um agente seletivo contra o Streptococcus mutans (NÉRIS, 2007).

Em estudos realizados por Campanella e colaboradores (2002), o ácido úsnico inibiu a replicação do DNA viral, através de ação indireta na transcrição do RNA do poliomavírus in vitro das células 3T6 de linhagem de fibroblastos de camundongos.

2.3.2. Anti-inflamatória, antinociceptiva e antipirética

No estudo da atividade anti-inflamatória do (+) ácido úsnico isolado de Roccella montagnei, em ratos, Vinjayakumar e colaboradores (2000) demostraram que esse metabólito secundário reduziu de forma significativa à formação do edema de pata induzido pela carrageninas, sugerindo que este composto apresenta uma atividade comparada ao ácido acetil salicílico.

Jin, Li e He (2008) mostraram que o mecanismo molecular responsável pelo efeito anti-inflamatório do ácido úsnico em macrófagos RAW264.7, é devido à diminuição dos níveis do fator de necrose tumoral - alfa (TNF-α) e inibição da produção de óxido nítrico, possivelmente através da supressão da translocação do fator nuclear kappa B p65 e degradação da enzima kinase I-κB.

O ácido úsnico obtido da Usnea diffracta reduziu de forma dose-dependente, as contorções abdominais induzidas pelo ácido acético, mostrando-se sugestivo no tratamento da dor e confirmando sua atividade antinociceptiva (YAMAMOTO et al.,

1998). Em outros estudos também utilizando o ácido úsnico isolado da Usnea diffracta, esse composto apresentou ação antipirética e antinociceptiva, atuando contra hipertemia induzida por lipopolissacarídeos em camundongos (OKUYAMA et al., 1995; MÜLLER, 2001).

2.3.3. Gastroprotetora

Estudos recentes indicam que o ácido único isolado da Usnea longissima proporcionou um maior efeito protetor no tratamento de lesões gástricas induzidas pela indometacina, quando comparado com a ranitidina, que é um bloqueador de receptores H_2. Esse efeito gastroprotetor do ácido úsnico pode ser atribuído ao efeito redutor contra o dano oxidativo e o seu efeito inibitório na infiltração neutrofílica em estômago de ratos (ODABASOGLU et al., 2006). Além disso, Safak e colaboradores (2009) estudaram a atividade in vitro do ácido úsnico contra o Helicobacter pylori. Os resultados revelaram que o uso do ácido úsnico pode ser benéfico no tratamento de úlceras através da erradicação do H. pylori, particularmente de cepas resistentes a claritromicina.

2.3.4. Antitumoral, antiproliferativa e citotóxica

A atividade antitumoral do ácido úsnico foi primeiramente relatada há mais de três décadas. Em 1975 foi reportada a atividade inibitória do ácido úsnico contra o carcinoma de Lewis (TAKAI, UEHARA, BEISLER, 1979). Além disso, o ácido úsnico apresentou uma atividade antiproliferativa frente a células neoplásicas, K-562 (adenocarcinoma de endométrio) e HEC-50 (eritroleucemia) (CARDARELLI et al., 1997). Em estudos realizados por Bézivin e colaboradores (2004), o ácido úsnico induziu apoptose em células L1210.

Na pesquisa desenvolvida por Santos e colaboradores (2006) foi estudada a atividade antitumoral in vivo do ácido úsnico em sua forma livre e nanoencapsulada frente ao Sarcoma 180. Neste estudo obteve-se um aumento de inibição tumoral pelo ácido úsnico em nanocápsulas (68%) quando comparado com a forma livre (43%).

O (+)-ácido úsnico extraído da Cladonia arbuscula e (-)-ácido úsnico dan Alectoria ochroleuca apresentaram um efeito inibitório sobre o crescimento e a proliferação de duas linhagens de células humanas, T-47D (câncer de mama) e Capan-2 (câncer pancreático) com alteração do potencial de membrana mitocondrial. Além disso, não foi notada diferença entre os dois enantiômeros (EINARSDÓTTIR et al., 2010).

Recentemente, foram testados os efeitos citotóxicos e antiproliferativo do ácido úsnico e mais três metabólitos secundários de liquens (atranorina, ácido girofórico e parientina) em nove linhagens de células de câncer humano: A2780 (carcinoma de ovário), HeLa (adenocarcinoma cervical), MCF-7 (adenocarcinoma de mama), SK- BR-3 (adenocarcinoma de mama), HT-29 (adenocarcimona de cólon), HCT-116 p53(+/+) (carcinoma de cólon humano de tipo selvagem), HCT-116 p53(-/-) (carcinoma de cólon humano p53-nulo), HL-60 (leucemia promielocítica) e Jurkat (leucemia de células T). De todos os metabólitos testados o ácido úsnico e a atranorina reduziram significativamente a viabilidade celular e induziram apoptose (BAČKOROVÁ et al., 2011).

2.4. Neuroblastoma humano SH-SY5Y

Neuroblastoma é uma doença embrionária maligna, originada a partir do sistema nervoso simpático, sendo considerado um dos tumores sólidos extracranianos mais comuns, responsável por 15% das mortes na infância (MARIS, 2005). A principal característica clínica desse tumor é a heterogeneidade, pois pode apresentar regressão espontânea, maturar transformando-se em ganglioneuroblastoma benigno ou apresentar rápida progressão tumoral e não responder a qualquer tipo de tratamento levando ao óbito (THIELE, 1998).

O neuroblastoma SH-SY5Y é uma sublinhagem clonada três vezes do neuroblastoma SK-N-SH, procedente de uma biopsia de medula óssea de um paciente com neuroblastoma de origem ganglionar simpático adrenérgico nos anos 70 (BIEDLER, HELSON, SPENGLER, 1973).

A linhagem SK-N-SH contém três diferentes tipos de fenótipo: Neuroblastica (Tipo N), Schwannian (tipo S) e intermediário (Tipo I). As células do tipo N, são geralmente pequenas e pouco aderentes a um substrato, crescem formando aglomerados (pseudogânglia) e com processos neuriticos bastante curtos. Em contraste, as células do tipo S aderem firmemente ao substrato, são grandes e achatadas e têm uma duração limitada na cultura, ou seja, apresenta uma inibição do crescimento por contato. As células do tipo I (intermediário), assim chamada, pois apresentam características das tipo N e S, morfologicamente são células pequenas, achatadas, com ou sem processos neuríticos e com moderada aderência. Este tipo de célula foi reconhecida como um neuroblastoma de células tronco maligno devido a sua auto-renovação e diferenciação bidirecional (ROSS et al., 1995; ROSS, SPENGLER, 2004).

A linhagem de neuroblastomas humanos (SH-SY5Y) exibe propriedades bioquímicas e funcionais de neurônios humanos e tem sido usada como modelo celular in vitro para estudo das propriedades neuronais e de possíveis mecanismos de neurotoxicidade (HONG-RONG, LIN-SEM, GUO-YI, 2010; CHEUNG et al., 2009).

2.5. Espécies reativas, modulação da proliferação celular e morte celular

Um radical livre pode ser definido como qualquer espécie química (seja um átomo, um metal de transição ou uma molécula) capaz de ter existência independente e que contém um ou mais elétrons desemparelhados no seu orbital molecular externo. Quimicamente, esta valência livre torna a molécula extremamente reativa e, fisicamente, este elétron desemparelhado torna a molécula paramagnética (HALLIWELL, GUTTERIDGE, 2007). Outra característica importante dos radicais livres é sua capacidade de sustentar reações em cadeia, onde uma molécula reduzida perde seu elétron para o radical livre, e aquela reduzida passa a ser um novo radical, podendo reagir com outro composto químico sucessivamente (HALLIWELL, 2006).

Os radicais livres que possuem o elétron desemparelhado centrado nos átomos de oxigênio ou nitrogênio são denominados respectivamente, espécies reativas de oxigênio (EROs) ou espécies reativas de nitrogênio (ERNs) (HALLIWELL, 2001).

As EROs são representadas por radicais livres como o ânion radical superóxido ($O_2^{\bullet -}$), o radical hidroxila ($^{\bullet}OH$) e por outras espécies não radicalares, tais como, o peróxido de hidrogênio (H_2O_2), o oxigênio singleto ($^1O_2$) e o ácido hipocloroso (HClO) (HALLIWELL, 2001). As ERNs são representadas pelo óxido nítrico (NO•), um radical livre pouco reativo, que em concentrações iguais ou superiores a 1 μM inibe a enzima mitocondrial citocromo c oxidase, consequentemente aumentando a formação de $O^{2\bullet -}$ que reage com o próprio $NO^{\bullet}$ gerando o peroxinitrito ($ONOO^-$), radical altamente reativo e de alto potencial danoso para biomoléculas, especialmente proteínas através de modificações no aminoácido tirosina (PACHER, BECKMAN, LIAUDET, 2007).

Espécies reativas de oxigênio e nitrogênio (EROs e ERNs) são geradas intracelularmente e liberadas no espaço extracelular por neutrófilos e macrófagos residentes nos tecidos, por enzimas associadas a membranas, tais como, a NADPH oxidase, mieloperoxidase, xantina oxidase e óxido nítrico sintase (GENESTRA, 2007; VALKO et al., 2007).

Durante muito tempo acreditou-se que as EROs e ERNs, agiam como indutores de dano oxidativo a biomoléculas e isto estaria intimamente associado à etiologia e/ou

patogênese de diversas doenças, incluindo vários tipos de câncer, doenças cardiovasculares, neurodegenerativas, úlcera gástrica e problemas associados ao envelhecimento (OZDEMIR et al, 2009; DREHER, JUNOD, 1996; CAI, HARRISON, 2000; HALLIWELL, 2001; LIMA, MELO, LIMA, 2002). Entretanto, estudos recentes têm demonstrado que em condições fisiológicas as EROs e ERNs desempenham um papel molecular de regulação e sinalização intracelular (STONE, YANG, 2006). Todavia, quando a taxa de produção das EROs e ERNs aumenta em relação às defesas celulares, estabelece uma quadro de estresse oxidativo e nitrosativo, onde diversas proteínas e vias de sinalização são estimuladas/inibidas mediante resposta ao estresse (KEFALOYIANNI, GAITANAKI, BEIS, 2006).

A ativação das vias de sinalização redox em ambientes pró-oxidantes pode participar do processo de modulação da proliferação e morte celular dependendo do tipo de célula e da duração do estímulo pró-oxidante (STONE, YANG, 2006).

2.6. Lipoperoxidação

As espécies reativas podem agir em diversas biomoléculas, todavia a membrana lipídica é uma das mais atingidas em decorrência da oxidação dos ácidos graxos poli-insaturados, que induz perturbações estruturais nas membranas alterando a sua integridade, fluidez e permeabilidade, podendo inclusive, levar à lise celular (NIKI, 2009).

A lipoperoxidação (LPO) é uma reação em cadeia de ácidos graxos poli-insaturados mediada por radicais livres e iniciada por processos ambos enzimáticos e não-enzimáticos nas membranas celulares, representada pelas etapas de iniciação, propagação e terminação. Estas etapas estão apresentadas nas reações seguintes, onde L representa o lipídio (MARNETT, PLASTARAS, 2001).

Iniciação $LH + OH^{\bullet}$ (ou LH) $\longrightarrow L^{\bullet} + H_2O$ (ou LOH)

Propagação $L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$

Propagação $LH + LOO^{\bullet} \longrightarrow L^{\bullet} + LOOH$

Propagação $LOOH \xrightarrow{Fe^{2+}} LO^{\bullet} + OH^{\bullet}$

Propagação $LOOH \xrightarrow{Fe^{3+}} LOO^{\bullet} + H^{+}$

Terminação $LOO^{\bullet} + L^{\bullet} \longrightarrow LOOL$

Terminação $LOO^{\bullet} + LOO^{\bullet} \longrightarrow LOOL + O_2$

O radical hidroxila $OH^{\bullet}$ ou radical alcoxila $LO^{\bullet}$, sequestra o hidrogênio do ácido graxo poli-insaturado (LH) da membrana celular levando a formação de $L^{\bullet}$ (radical lipídico). Na primeira equação de propagação, o $L^{\bullet}$ reage rapidamente com o oxigênio molecular O_2, resultando na formação do $LOO^{\bullet}$ (radical peroxila), que sequestra um novo hidrogênio do ácido graxo poli-insaturado, formando novamente o $L^{\bullet}$ e o hidroperóxidos lipídicos LOOH que podem ser catalisado por metais de transição como o cobre e ferro, levando à formação de radicais altamente reativos, alcoxila ($LO^{\bullet}$) e peroxila ($LOO^{\bullet}$). O término da lipoperoxidação ocorre quando os radicais ($L^{\bullet}$ e $LOO^{\bullet}$) produzidos nas etapas anteriores propagam-se até destruírem a si próprios (BUETTNER, 1993).

A LPO in vivo tem sido implicada com mecanismos subjacentes em numerosas desordens e doenças cardiovasculares, câncer, distúrbios neurológicos, e processo envelhecimento. Torna-se extremamente importante avaliar a atividade antioxidante de compostos exógenos para utilizá-los como profiláticos destas patologias, ou mesmo como adjuvantes em procedimentos terapêuticos (AMAROWICZ et al., 2004).

2.7. Sistema de defesa antioxidante

Todos os sistemas biológicos expostos ao oxigênio sofrem degradação peroxidativa. Para manter suas propriedades físicas, químicas e funcionais, esses sistemas são protegidos por antioxidantes (HALLIWELL, GUTTERIDGE, 1999). Antioxidantes podem ser definidos como substâncias que, quando presentes em baixas concentrações comparadas com os extratos oxidáveis, previnem a oxidação destes (ARUOMA, 2003). Do ponto de vista biológico, antioxidantes são definidos como compostos que protegem o sistema biológico contra os efeitos deletérios dos processos ou das reações que levam à oxidação e nitração de macromoléculas ou estruturas celulares (MONTEIRO, 2006). No entanto, durante a atividade de um antioxidante pode ocorrer a formação de sub-produtos com atividade oxidante. Sendo assim, substâncias antioxidantes podem direta ou indiretamente apresentar propriedades pró-oxidantes (HALLIWELL, 2000).

Os sistemas antioxidantes presentes no nosso organismo podem ser de origem endógena agindo enzimaticamente, como a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), ou não-enzimaticamente, como a glutationa reduzida (GSH) (NORDBERG, ARNÉR, 2001). Além destes antioxidantes produzidos no nosso organismo, existem os oriundos da dieta, tais como α-tocoferol (vitamina E),

β-caroteno (pró-vitamina A), ascorbato (vitamina C) e compostos fenólicos (ácidos fenólicos e flavonoides) (BARREIROS, DAVID, DAVID, 2006).

As enzimas citadas são tidas como defesas antioxidantes primárias, ou seja, agem diretamente sobre a molécula do radical livre, antes que este possa oxidar uma biomolécula (NORDBERG e ARNÉR, 2001). A SOD apresenta quatro classes: Mn-SOD (localizada na matriz mitocondrial), Cu, Zn-SOD (citosólica), Ni-SOD e SOD extracelular. Todas estas formas de SOD catalisam a reação de dismutação do radical $O_2^{\bullet-}$, transformando-o em H_2O_2 e O_2, como pode ser verificada na seguinte reação:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2\ H^+ \longrightarrow H_2O_2 + O_2$$

Embora a remoção do $O_2^{\bullet-}$ previna a formação de EROs e ERNs, o H_2O_2 liberado através da dismutação do $O_2^{\bullet-}$ deve ser eliminado pelas enzimas catalase, glutationa peroxidase e também por outras peroxidases para impedi-lo de reagir com metais de transição e gerar o $OH^{\bullet}$ (HALLIWELL, GUTTERIDGE, 2007).

A CAT, que se encontra predominantemente nos peroxissomos, é uma hemeproteína que catalisa a conversão do H_2O_2, gerado na reação acima, em água e oxigênio por meio da reação:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

A GPx, encontrada no citosol, mitocôndrias e meio extracelular, tem a função de converter o H_2O_2 em água e hidróxidos orgânicos (LOOH) em álcoois (CHANCE, SIES, BOVERIS, 1979; SHAN, AW, JONES, 1990).

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + 2\ H_2O$$

$$LOOH + 2\ GSH \longrightarrow GSSG + H_2O + LOH$$

A GPx junto com a enzima glutationa redutase, participa de um ciclo redox, onde a GSH é usada, pela GPx, para transformar H_2O_2 em água; e NADPH é utilizado pela GSR para reduzir a GSSG, na primeira reação, novamente em GSH (CHANCE, SIES, BOVERIS, 1979; GILBERT, 1990).

$$GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+$$

A GSH, um antioxidante não enzimático, é um tripeptídio formado através de resíduos de glicina, glutamato e cisteína. A sua capacidade redutora é determinada via

presença do grupamento tiólico (-SH) da cisteína. A GSH pode ser considerada um dos agentes mais importantes do sistema de defesa antioxidante da célula, protegendo-a contra a lesão resultante da exposição a agentes como íons ferro (GALLEANO, PUNTARULO, 1995).

CAPÍTULO III

Redox characterization of usnic acid and its cytotoxic effect on human neuron-like cells (SH-SY5Y)

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Redox characterization of usnic acid and its cytotoxic effect on human neuron-like cells (SH-SY5Y)

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ABSTRACT

Usnic acid (UA) is the most common and abundant lichenic secondary metabolite with potential therapeutic application. Anti-inflammatory and antitumour properties have already been reported and UA-enriched extracts are widely used to treat several diseases in the folk medicine. First, we performed *in silico* evaluation of UA interactions with genes/proteins and important compounds for cellular redox balance and NO pathway. Then, we assessed UA redox properties against different reactive species (RS) generated *in vitro*, and evaluated its action on SH-SY5Y neuronal like cells upon hydrogen peroxide (H_2O_2), since no *in vitro* neurotoxicological data has been reported so far. Total reactive antioxidant potential index (TRAP) showed a significant antioxidant capacity of UA at the highest tested concentration; UA was also effective against hydroxyl radicals and reduced the formation of nitric oxide. *In vitro*, lipoperoxidation was enhanced by UA and changed the cellular viability at highest concentration of 20 μg/mL for 1 and 4 h, as well as 2 and 20 μg/mL for 24 h of treatment, according to MTT reduction assay. Moreover, UA did not display protective effects against H_2O_2-induced cell death in any case. Evaluation of intracellular RS production by the DCFH-based assay indicated that UA was able to induce changes in basal RS production at concentration of 20 μg/mL for 1 h and from 2 ng/mL to 20 μg/mL for 4 and 24 h. In conclusion, UA could display variable redox-active properties, according to different system conditions and/or cellular environment. Moreover, our results suggest that potential neurotoxicological effects of UA should be further studied by additional approaches; for instance, *in vivo* and clinical studies.

1. Introduction

Therapeutic properties displayed by some species of lichens are related to the production of secondary metabolites such as depsides, depsidones, dibenzofuranes, xanthenes, anthraquinones, aliphatic acids, atranorin and usnic acid (Ingólfsdóttir, 2002). Usnic acid (UA) is one of the most abundant secondary lichen metabolites and has been extensively studied. It has several biological activities, such as antibiotic (Cocchietto et al., 2002), antiviral (Campanella et al., 2002; Scirpa et al., 1999), analgesic and antipyretic (Okuyama et al., 1995), as well as anti-inflammatory properties (Vijayakumar et al., 2000). UA was reported to induce apoptosis of murine leukemia L1210 cells in a dose- and time-dependent manner (Bezivin et al., 2004), and exhibited anti-proliferative action against MCF7 breast cancer cells (Mayer et al., 2005). These results highlighted UA as a potentially new precursor for novel chemotherapic agents.

Already, UA has shown a dualistic effect *in vivo* when compared to *in vitro* data. For instance, it exerts a significant gastroprotective effect in indomethacin-induced gastric ulcer rats, by reducing the formation of reactive species, and therefore, oxidative damage (Odabasoglu et al., 2006); but also it has shown to be a potent hepatotoxic agent that disrupts electron transport in mitochondria, inducing oxidative stress in cells (Han et al., 2004; Joseph et al., 2009). However, there exist many controversies about the toxic activities undertaken by this compound. All in all, better comprehension about UA-induced cellular mechanism would significantly contribute towards a safer therapeutic use.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the pathogenesis of numerous diseases such as cancer, inflammatory diseases and neurodegenerative disorders (Seifried et al., 2007). Under normal physiological conditions, ROS/RNS participate as intracellular messengers and regulatory molecules. They are tightly regulated by balancing systems formed

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by different antioxidants, antioxidant enzymes, and proteins (Kowaltowski et al., 2009). Non-enzymatic antioxidants coming from diet or other processes regulate oxidative and nitrosative reactions in the body, which prevents oxidative stress by removing both ROS and RNS (Maes et al., 2011).

Main actions of secondary metabolites in biological systems have also been linked to their redox properties. Our group has been studying the redox properties of atranorin, another lichenic secondary metabolite with potential therapeutic activity, similar to that exerted by UA. In that study, atranorin increased lipoperoxidation and displayed general antioxidant activity (Melo et al., 2011). The potential health-promoting effects of naturally occurring compounds are traditionally ascribed to a general antioxidant action (Aravindaram and Yang, 2010). In fact, the properties of UA are generally associated to its widespread antioxidant action, found in most phenolic compounds, since many therapeutic properties attributed to lichen extracts (and to UA itself) are intimately associated to oxidative stress; together with an unbalanced free radical production, mutagenicity, and inflammation (Halliwell and Gutteridge, 2007). However, only few works have studied the potential pro- or antioxidant properties of UA (Carlos et al., 2009; Jayaprakasha and Rao, 2000; Toledo Marante et al., 2003; Valencia-Islas et al., 2007). Interestingly, a number of phenolic compounds and other naturally derived substances, that were initially observed to act as antioxidants in mammalian cells, have later been described to unbalance the cellular redox state towards pro-oxidant states, depending on specific conditions (i.e., higher or lower drug concentrations) (Halliwell, 2008).

Since there are some studies showing that UA prevents oxidative stress by removing both ROS and RNS, we decide to deeper characterize *in silico* the general landscape of interactions of UA with genes/proteins, and compounds generally involved in both redox and NO pathways, by using system biology tools. Thereafter, we performed an *in vitro* characterization of the redox properties of UA against different reactive species, and evaluated its potential effects on cellular viability by using SH-SY5Y cells neuronal like cells since no *in vitro* neurotoxicological reports have been published yet.

2. Materials and methods

2.1. Chemicals

AAPH (2,2′-Azobis(2-methylpropionamidine)dihydrochloride), Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), 2-deoxyribose, glycine, Griess reagent, SNP (sodium nitroprusside), TBA (2-thiobarbituric acid), (4,6-dihydroxypyrimidine-2-thiol), H_2O_2 (hydrogen peroxide), adrenaline, catalase, SOD (superoxide dismutase), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMSO (Dimethyl sulfoxide), DCFH-DA (2′,7′-dichlorohydrofluorescein diacetate) and (+)-Usnic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP, Brazil) and from Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, Brazil). The rest of reagents used in this study were of either analytical or HPLC grade. For each assay, UA (20 mg mL^{-1}) was dissolved in DMSO (100%) and serial dilutions were obtained from this stock solution. Therefore, at the highest concentration of UA in these assays (20 μg mL^{-1}), concentration of the vehicle DMSO would correspond to 0.1%.

2.2. Interaction networks of compounds and gene/proteins

In order to develop a model network for gene/protein and UA interaction, we first selected a number of gene/proteins involved in redox and NO-related pathways and then, by using STITCH 2.0 (Kuhn et al., 2008, 2010) we screened the possible protein–protein and protein-compound interactions based on experimental knowledge and database (confident score = 0.4, medium). A list with gene symbols and Ensembl protein IDs is additionally provided (Supplementary Table 1).

The network connected 61 proteins and 5 compounds together, based on their possible interaction through "activation", "catalysis", "binding", "inhibition", and "reaction"; giving rise to a model for UA interactions through redox/NO pathways (MUA network).

2.3. Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR)

Total reactive antioxidant potential (TRAP) is utilized to estimate the non-enzymatic antioxidant capacity of samples *in vitro*. This method is based on the quenching of luminol-enhanced chemiluminescence (CL) derived from the thermolysis of AAPH as the free radical source (Lissi et al., 1992). Briefly, we prepared AAPH solution, added luminol (AAPH + luminol, radical generating system) and then, we waited for the system to stabilize for 2 h before the first reading. Different concentrations of UA were added and the luminescence produced by the free radical reaction was quantified in a liquid scintillator counter (Wallac 1409, Perkin–Elmer, Boston, MA, USA) for 60 min. The results were transformed in percentage and area under curve (AUC), and calculated by software (GraphPad software.®San Diego, CA; version 5.00) as previously described (Dresch et al., 2009).

Total antioxidant reactivity (TAR) was analyzed by using the same samples utilized for TRAP readings. TAR results were calculated as the ratio of light intensity in absence of samples (I_0)/light intensity right after UA addition. Although TAR and TRAP evaluations are obtained in the same experiment, they represent different observations, since the TAR is more related to the antioxidant quality (reactivity, the scavenging capacity in a short-term period) and TRAP is more related to the antioxidant amount and kinetic behavior (Lissi et al., 1995).

2.4. Hydroxyl radical-scavenging activity

The formation of ·OH (hydroxyl radical) from Fenton reaction was quantified by using the 2-deoxyribose oxidative degradation assay. The principle of the assay is the incubation of 2-deoxyribose together with a hydroxyl radical generation system, which produces malondialdehyde (MDA). This system is then incubated with 2-thiobarbituric acid (TBA), which reacts with MDA and forms a chromophore quantifiable by spectrophotometry (Lopes et al., 1999). Briefly, typical reactions were started by the addition of Fe^{2+} ($FeSO_4$ 6 μM final concentration) to solutions containing 5 mM 2-deoxyribose, 100 mM H_2O_2 and 20 mM phosphate buffer (pH 7.2).

To measure UA antioxidant activity against hydroxyl radicals, different concentrations of UA were added to the system before Fe^{2+} addition. Reactions were carried out for 15 min at room temperature and were stopped by the addition of 4% phosphoric acid (v/v) followed by 1% TBA (w/v, in 50 mM NaOH). Solutions were boiled for 15 min at 95 °C, and then cooled at room temperature. The absorbance was measured at 532 nm and results were expressed as percentage of TBARS formed.

2.5. Nitric oxide (NO·) scavenging activity

Nitric oxide was generated from spontaneous decomposition of sodium nitroprusside in 20 mM phosphate buffer (pH 7.4). Once it is generated, NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (Basu and Hazra,

2006). The reaction mixture (1 mL) containing both 10 mM sodium nitroprusside (SNP) in phosphate buffer (pH 7.4) and UA at different concentrations were incubated at 37 °C for 1 h. An aliquot of 0.5 mL was taken and homogenized with 0.5 mL Griess reagent. The absorbance of chromophore was measured at 540 nm. Results were expressed as percentage of nitrite formed by SNP alone.

2.6. Thiobarbituric acid reactive species (TBARS)

Thiobarbituric acid-reactive substances (TBARS) assay was employed to quantify lipid peroxidation (Draper and Hadley, 1990), and an adapted TBARS method was used to measure the antioxidant capacity of UA using egg yolk homogenate as lipid rich substrate (Melo et al., 2011). The principle of the method is based on spectrophotometric measurement of the color produced during the reaction of thiobarbituric acid (TBA) with lipoperoxidation products, such as malondialdehyde and 4-hydroxynonenal (Mansour and Mossa, 2009). Briefly, egg yolk was homogenized (1% w/v) in 20 mM phosphate buffer (pH 7.4), 1 mL of homogenate was sonicated and then homogenized with 0.1 mL of UA at different concentrations. Lipid peroxidation was induced by addition of 0.1 mL of AAPH solution (0.12 M). Control was just incubation medium without AAPH. Reactions were carried out for 30 min at 37 °C. Samples (0.5 mL) were centrifuged with 0.5 mL of trichloroacetic acid (15%) at 1200*g* for 10 min. An aliquot of 0.5 mL from supernatant was mixed with 0.5 mL TBA (0.67%) and heated at 95 °C for 30 min. After cooling, sample's absorbance was measured by using a spectrophotometer (UV-1800 Shimadzu) at 532 nm. The results were expressed as percentage of TBARS formed by AAPH alone (induced control).

2.7. Determination of superoxide dismutase-like activity (SOD)

The ability of UA to scavenge superoxide anion ("superoxide dismutase-like activity" or "SOD-like activity") was measured as previously described (Misra and Fridovich, 1972). UA was mixed with 200 μl glycine buffer (50 mM, pH 10.2) and 5 μl of native catalase 100 U/mL. Superoxide generation was initiated by addition of adrenaline 2 mM, and adrenochrome formation was monitored at 480 nm for 5 min (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 (Bannister and Calabrese, 1987).

2.8. Determination of catalase-like activity (CAT)

The capacity of UA to degrade the hydrogen peroxide (H_2O_2) added in an incubation medium ("catalase-like activity" or "CAT-like activity") was measured as previously described (Aebi, 1984). Briefly, H_2O_2 diluted in 0.02 M phosphate buffer (pH 7.0), to obtain a 5 mM final concentration, was added to microplate wells, in which different concentrations of UA were already placed. The plate was then scanned in a spectrophotometric plate reader (SpectraMax 190. Molecular Devices) at 240 nm every 15 s for 5 min 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 H_2O_2.

2.9. Cell culture

Exponential growing human neuroblastoma cell line SH-SY5Y, obtained from Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, Brazil), were maintained in a mixture 1:1 of Ham's F12 and Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM of glutamine, 0.28 μg/μL of gentamicin and 250 μg of amphotericin B, in a humidified atmosphere of 5% of CO_2 in air at 37 °C. Cell medium was replaced each 2 days and cells were sub-cultured once they reached 90% confluence. All treatments were performed when cells were 70–90% confluence.

2.10. MTT assay

After treating with either different concentrations of UA alone or in the presence of H_2O_2 1 mM for 1, 4, and 24 h SH-SY5Y cells viability was quantified by the MTT assay as previously described (Gelain and Moreira, 2008). This method is based on the ability of viable cells to reduce MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/mL) was added to the incubation medium in the wells at a final concentration of 0.2 mg/mL. The cells were left for 45 min at 37 °C in a humidified 5% CO_2 atmosphere.

Culture medium was then removed and plates were shaken with DMSO for 30 min. Optical density of each well was measured at 550 nm (test) and 690 nm. H_2O_2 1 mM was used as positive control for the assay. Data were expressed as percentage of the formazan formation in untreated cells (control). Qualitative morphology of the cell cultures was also evaluated by phase-contrast light microscopy (Nikon Eclipse TE 300).

2.11. DCFH-DA assay

Intracellular reactive species production was determined by the DCFH-DA assay, as described by (Wang and Joseph, 1999). In the presence of reactive species (RS), DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) can be used as an index to quantify the overall ROS in cells. Briefly, 2×10^4 SH-SY5Y cells were seeded in 96-well plates and 100 μM DCFH-DA dissolved in medium containing 1% FBS was added to each well and incubated for 2 h to allow cellular incorporation. After, the medium was discarded and cells were treated with different concentrations of UA alone or in the presence of H_2O_2 (1 mM) and DCF fluorescence was read at 37 °C during 1, 4 and 24 h in a fluorescence plate reader (Spectra Max M2, Molecular Devices, USA) with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm. The results were expressed as percentage DCF fluorescence. Hydrogen peroxide (1 mM) was used as positive control for intracellular reactive species production.

2.12. Statistical analysis

The *in vitro* procedures were carried out with $n = 3$ (i.e., 3 vials per group) while cell culture experiments were performed with $n = 6$ (i.e., 6 wells per group). Experiments were repeated four different times, and the results were expressed as mean ± standard error of the mean (SEM) of four independent experiments. The differences among data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. In all cases differences were considered significant if $p < 0.05$. Data analyses were performed using the (GraphPad software.®San Diego, CA; version 5.00).

3. Results

In silico analysis gave rise to a network where exactly 61 proteins and 5 compounds where interconnected ("MUA network") (Fig. 1A). Based on experimental data and database, UA directly interacts with hydroxyl radicals (Fig. 1F) and through them, with antioxidant enzymes (Fig. 1E), such as SOD1, SOD3, CAT, GPX1, and GPX2. UA interaction with hydroxyl radicals interconnects it with NO and proteins involved in NO biosynthesis (Fig. 1C); for in-

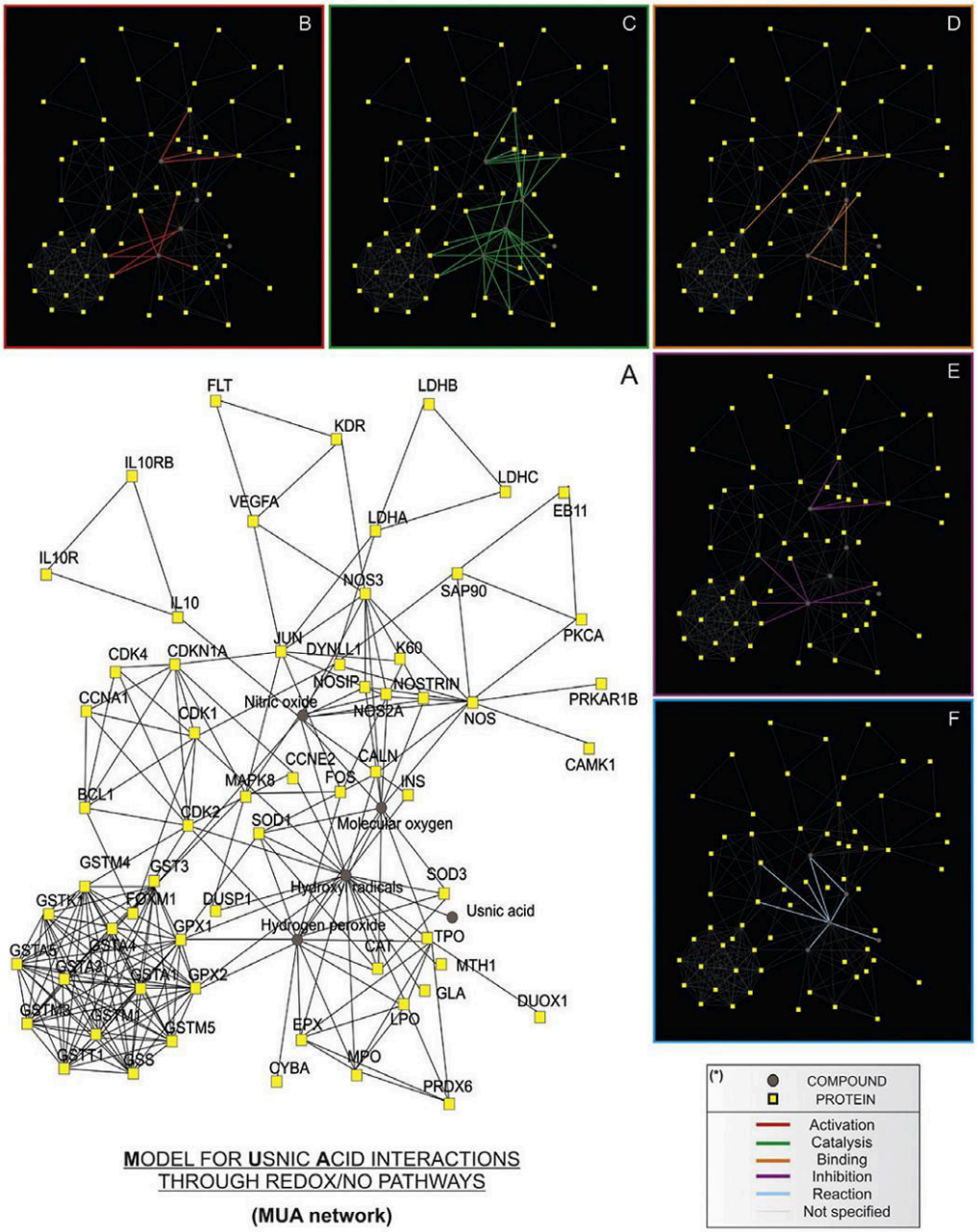

Fig. 1. Model for UA interactions through redox and NO pathways (A) *In silico* analysis based on experimental data and database gave rise to a network where exactly 61 proteins and 5 compounds where interconnected ("MUA" network). (B) Landscape of protein–protein and protein–compound interactions through activation processes. (C) Landscape of protein–protein and protein–compound interactions through catalysis processes. (D) Landscape of protein–protein and protein–compound interactions through binding processes. (E) Landscape of protein–protein and protein–compound interactions through inhibition processes. (F) Landscape of protein–protein and protein–compound interactions through diverse reactions.

stance, Nostrin, which is known to modulate nitric oxide release (Zimmermann et al., 2002). Landscape of protein–protein and protein-compound interactions through either activation or binding processes are additionally provided (Fig. 1B and D, respectively).

Redox properties of UA were first evaluated (TRAP and TAR assays) by using a method based on the quenching of luminol-enhanced chemiluminescence (CL) of AAPH. At 20 μg/mL of UA, we observed an antioxidant capacity of UA by TRAP assay (Fig. 2A). Usnic acid at 20 μg μg/mL also showed significant antioxidant capacity according to TAR measurements (Fig. 2B). In both methods, this concentration was able to efficiently reduce AAPH-induced CL, indicating peroxyl/alkoxyl non-enzymatic scavenging activity.

The 2-deoxyribose degradation assay was employed to investigate the ability of UA to scavenge *in vitro*-generated hydroxyl radicals by Fenton reaction. Fig. 3A shows that concentrations of UA from 2 μg μg/mL to 20 μg μg/mL inhibited hydroxyl radical-induced deoxyribose degradation, indicating an antioxidant effect against hydroxyl radicals. The capacity of UA to scavenge NO was measured by quantifying the production of nitrite derived from sodium nitroprusside (SNP) by the Griess reaction. Fig. 3B shows that all concentrations of UA tested (2 ng/mL to 20 μg/mL) significantly decreased SNP-derived nitrite formation.

The protective effect of UA against oxidative damage to lipids was measured by quantifying TBARS generated by AAPH in a

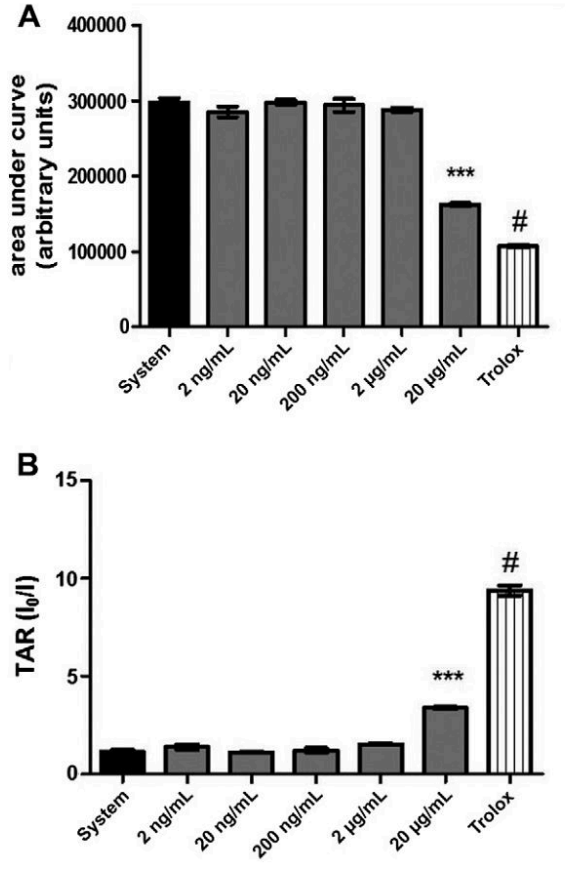

Fig. 2. TRAP and TAR measurements. (A) The total reactive antioxidant potential (TRAP) of UA at different concentrations. A free radical source (AAPH) generating system produces peroxyl/alcoxyl radicals at a constant rate, and the effect of different concentrations of UA on free radical-induced chemiluminescence is measured as area under a curve for 60 min. (B) Total antioxidant reactivity (TAR) values are calculated as the ratio of light intensity in absence of samples (I_0)/light intensity right after UA addition (I) and expressed as percent of inhibition. All groups denote samples in the presence of AAPH. Trolox (75 µg/ml) was used as standard antioxidant. The experiments were performed in triplicate, and bars represent mean ± SEM of four different experiments. ***$p < 0.0001$ (1-way ANOVA followed by Tukey's *post hoc* test).

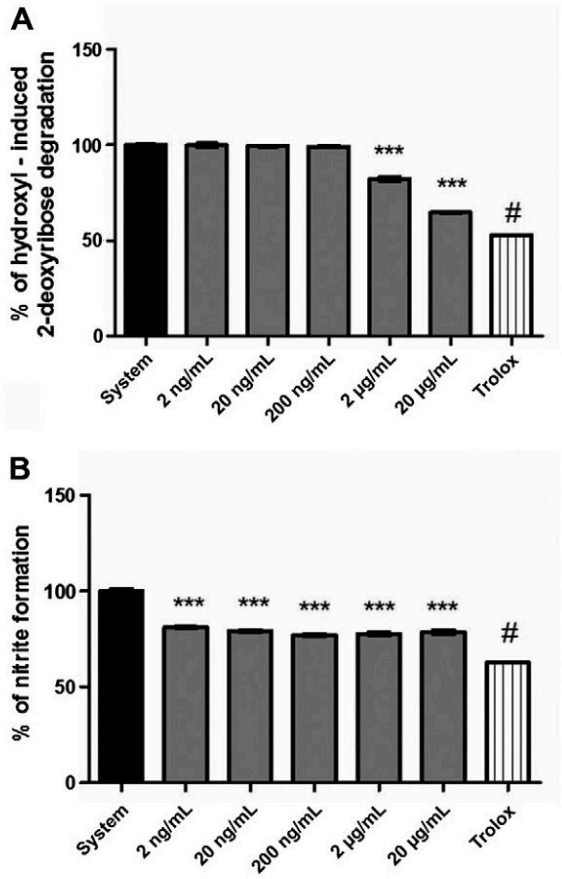

Fig. 3. Hydroxyl and nitric oxide (NO) scavenging activities assays. (A) Hydroxyl radical-scavenging activity was quantified by using hydroxyl-mediated 2-deoxyribose oxidative degradation *in vitro*, which produces malondialdehyde (MDA) by condensation with 2-thiobarbituric acid (TBA). System is MDA production from 2-deoxyribose degradation with $FeSO_4$ and H_2O_2 alone (hydroxyl generating system). Other groups denote MDA production by $FeSO_4$ and H_2O_2 in the presence of different concentrations of UA. (B) NO scavenging assay. Nitric oxide was generated from spontaneous decomposition of sodium nitroprusside (SNP) in the presence of oxygen, producing nitrite ions, which were measured by the Griess reaction. Nitrite production by SNP alone was compared to nitrite production by SNP in the presence of different concentrations of UA. Trolox was used as standard antioxidant in both assays. The experiments were performed in triplicate, and bars represent mean ± SEM of four different experiments. ***$p < 0.0001$. One-way ANOVA followed by Tukey's *post hoc* test was applied to all data.

lipid-rich incubation medium. All the tested concentrations (2 ng/mL–20 µg/mL) of UA increased the AAPH-induced lipoperoxidation (Fig. 4), indicating that UA is either an enhancer of lipid peroxidation or somehow, it may facilitate chain reactions involved in the propagation of lipid peroxides.

The activity of UA against superoxide anions (SOD-like activity) was quantified by the inhibition of superoxide-dependent adrenaline auto-oxidation to adrenochrome. Moreover, we also assessed the ability of UA to decompose H_2O_2 *in vitro* (CAT-like activity). Respectively, Fig. 5A and B show that all tested concentrations of UA did not exert any significant variation in CAT and SOD-like activities.

In order to ellucidate the potential effects that UA may exert in a cellular system (challenged by a pro-oxidant agent), we chose SH-SY5Y cells as *in vitro* neurotoxicological model. Phase contrast microscopy showed that cells treated with the highest concentration of UA (20 µg/mL) for 1 and 4 h, induced changes in their neuronal-like morphology (Fig. 6). At 1 h of treatment with 20 µg/mL of UA, neuritic processes seem to be stopped (Fig. 6a7) and cells looked more rounded. When treatment reached 4 h of incubation at the same concentration, cells already seem to lose their viability and start to detach from the culture plate (Fig. 6b7). In the presence of H_2O_2 (1 mM) together with UA (20 µg/mL), cells detached and seemed to lose their viability already at 1 h of treatment (Fig. 6a14). Interestingly, such effect looked stronger in comparison to cells that were treated only with 20 µg/mL of UA (Fig. 6a7). Moreover, even though clear morphological effects and potential lost of viability were only detectable at the highest concentration of UA (20 µg/mL) when treatment lasted 4 h (Fig. 6b7), 4 h of H_2O_2 and UA co-treatment showed an UA dose-dependent cellular detachment in comparison to those cells treated with 1 mM of H_2O_2 alone (positive control) (Fig 6b8–b12). Strongest effect appeared at the highest concentration of UA (20 µg/mL) together with H_2O_2 (1 mM) (Fig. 6b12). For studying long-term UA effect on the cells, treatments were performed (in the presence of 1% of FBS) for 24 h. Results showed signs of cell detachment and lose of viability when cells were incubated with 2 µg/mL of UA (24 h) (Fig. 7f), and such effect was significantly stronger with 20 µg/mL of UA, with virtually 100% of the cells detached from the plate (Fig. 7g). 24 h co-treatment of UA together with 1 mM of H_2O_2 resulted in complete lack of cellular viability at any concentration

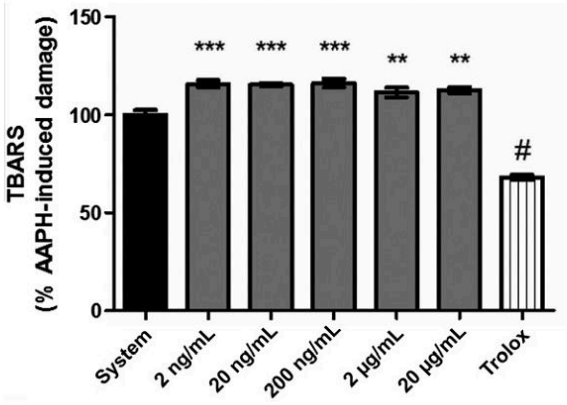

Fig. 4. Production of Thiobarbituric acid-reactive substances (TBARS) *in vitro*. A lipid-rich system was incubated with a free radical source (AAPH) and the effect of different concentrations of UA on the lipoperoxidation was measured by quantifying TBARS. Trolox (75 µg/ml) was used as standard antioxidant. The experiments were performed in triplicate, and bars represent mean ± SEM of four different experiments. $^{**}p < 0.001$, $^{***}p < 0.0001$ (1-way ANOVA followed by Tukey's *post hoc* test).

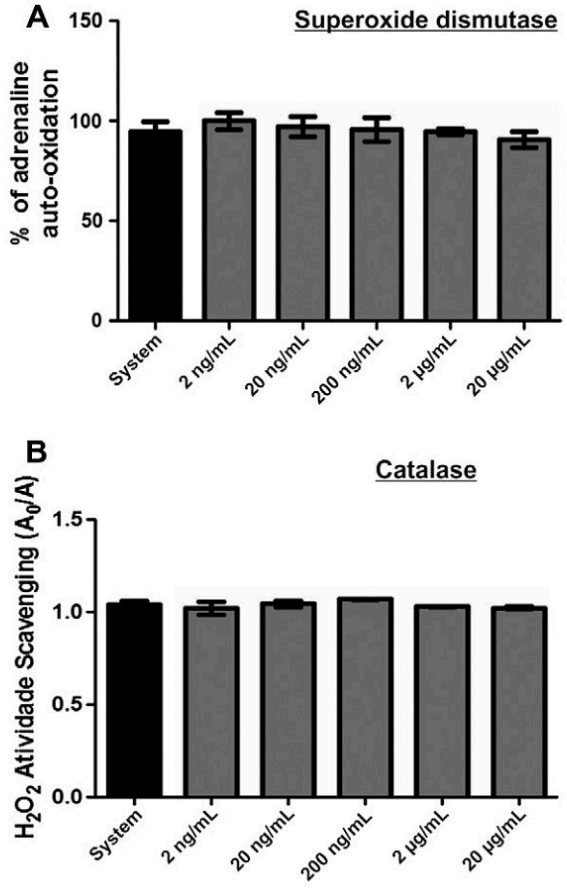

Fig. 5. SOD-like and CAT-like activities. (A) Superoxide dismutase-like (SOD-like) activity was determined by following formation of adrenochrome in a SOD reaction buffer containing native catalase and adrenaline O_2^- generator group). (B) Catalase-like (CAT-like) activity was measured in a catalase reaction buffer with H_2O_2. The experiments were performed in triplicate, and bars represent mean ± SEM of four different experiments. One-way ANOVA followed by Tukey's *post hoc* test was applied to all data.

of UA (Fig. 7j–n). These results were confirmed when MMT assays showed that cells treated for 1 and 4 h with the highest concentration of UA (20 µg/mL) cellular viability was reduced in a 41.3% and 88.67% respectively in relation to control (100%) (Fig. 8A and B). During long-term treatments of 24 h, with concentrations of 2 µg/mL and 20 µg/mL, cellular viability was reduced in a 41.9% and 95.23% respectively, in relation to control (100%) (Fig. 8C).

Moreover, UA did not display protective effects in any case after 1, 4 or 24 h of treatment against 1 mM H_2O_2-induced cell death, which on the contrary, was able to significantly decrease cellular viability in a 88.4%, 92.06% and 95.96% respectively, in relation to control (100%) (Fig. 8A, B and C). This result suggests that UA is not able to prevent oxidative-mediated H_2O_2-induced cell death at any concentration.

Next, we evaluated if UA was able to induce intracellular ROS production in SH-SY5Y cells by the DCFH-DA assay. As shown in Fig. 9A, SH-SY5Y cells treated with UA (20 µg/mL) for 1 h increased in 76.73% when compared to control cells. After 4 and 24 h of treatment, levels of intracellular ROS were enhanced at any tested concentration of UA (Fig. 9B and C). Respectively: 2 ng/mL (44.99–62.71%), 20 ng/mL (60.63–78.20%), 200 ng/mL (79.56–90.32%), 2 µg/mL (53.69–64.60%) and 20 µg/mL (106.07%–122.70%).

These results suggest that UA is able to trigger ROS production in SH-SY5Y cells and therefore, to induce an oxidative stress scenario. Besides, we also evaluated the effect on intracellular ROS production when cells were co-treated with UA together with H_2O_2 (1 mM) for 1, 4, and 24 h. At any concentration, UA appears to potentiate the effect of H_2O_2 when it comes to ROS production (Fig. 9A, B and C).

4. Discussion

Several studies have shown that redox activity associated with natural antioxidants is attributed to total content of phenolic compounds (Halliwell, 2008; Rice-Evans et al., 1995; Scalbert et al., 2005). Moreover, antioxidant and pro-oxidant biochemical agents have presented important role to design strategies for prevention and/or management of oxidative damage. Here, we aimed to characterize the redox properties of UA by using different approaches, in order to understand possible interactions of such compound with different types of reactive species.

The potential of UA scavenging peroxyl radicals, by using TRAP/TAR assays, indicated a significant antioxidant capacity at the highest tested concentration. Moreover, UA was capable to quench hydroxyl radicals. Hydroxyl radical has a high oxidant power and it is probably the most reactive radical (Pastor et al., 2000). It is able to join DNA nucleotides and cause strand breakage, which contributes to carcinogenesis, mutagenesis, and cytotoxicity (Manian et al., 2008). The hydroxyl radical-scavenging capacity of any compound is directly related to its antioxidant activity (Babu et al., 2001). In addition, NO is an important mediator of acute and chronic inflammation. NO stimulates cyclooxygenase (COX) activity resulting in unbalanced production of pro-inflammatory prostaglandins (PG) (Salvemini et al., 1993). Other mechanism by which NO may modulate inflammatory processes is through its interaction with the Rel/nuclear transcription factor kappaB (NF-kB) family of transcription factors (Laroux et al., 2001). At high concentrations, NO may interact with superoxide radicals generating peroxynitrite ($ONOO^-$), a potent oxidizing molecule capable of eliciting damage to proteins, lipids and DNA, which modulates COX (Zhang et al., 1994; Mollace et al., 2005; Halliwell and Gutteridge, 2007). Here, we observed that UA is able to reduce the production of nitrite, indicating a potential role as NO-scavenging agent and consequently, it may limit the action of these reactive species in biological systems.

ROS and RNS have also been related to act as pro-inflammatory signals *in vivo* by stimulating the activation of TNF-α, IL-1β and IL-

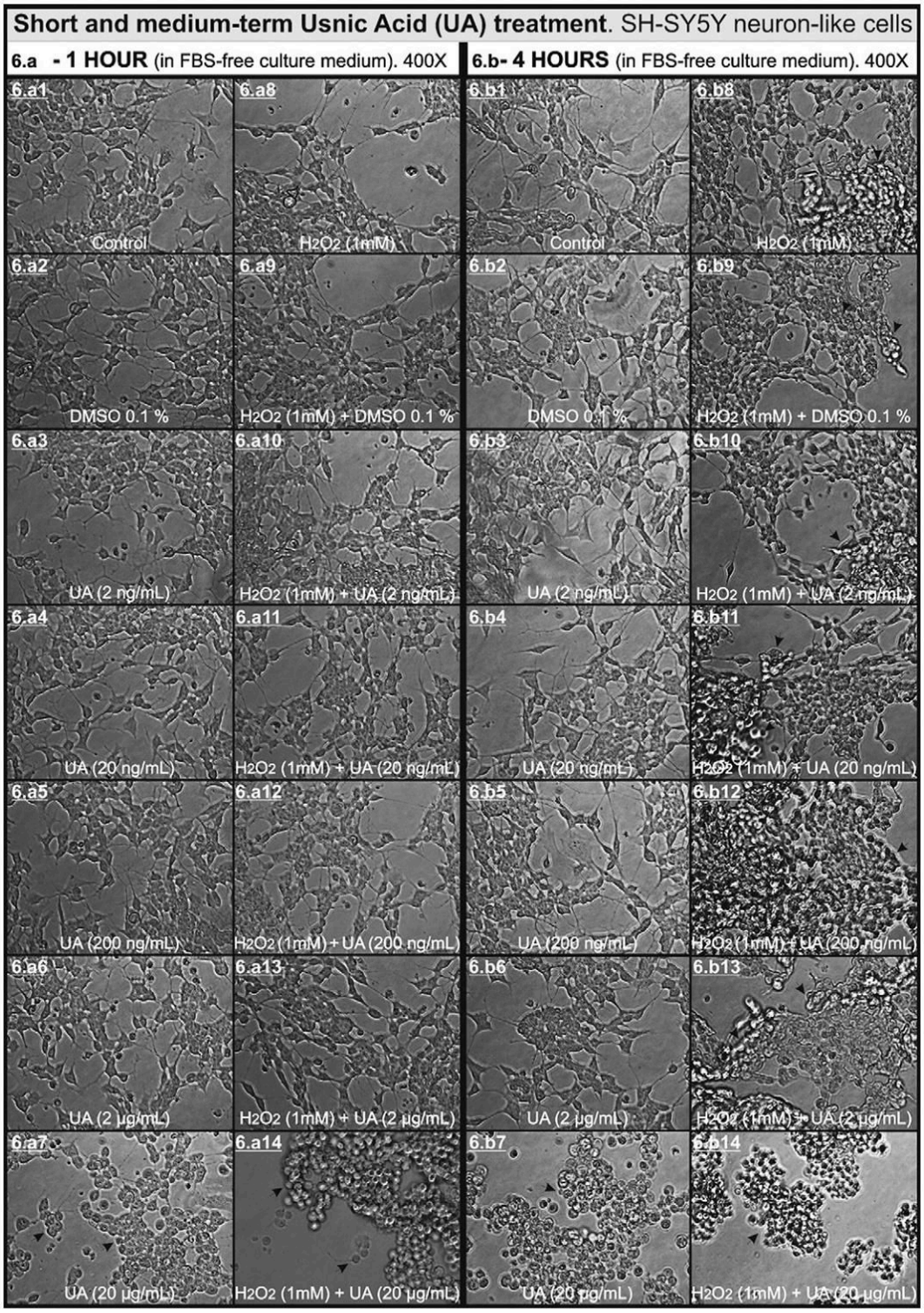

Fig. 6. Morphological effects of short and medium-term UA treatment on SH-SY5Y cells. Phase contrast microscopy of SH-SY5Y cells after 1 (A) and 4 h (B) treatment with different concentrations of UA, in the absence or presence of H_2O_2 (1 mM). Photomicrographies representative of three independent experiments.

6 genes through activation of the redox-sensitive transcription factor NF-kB (Beauparlant and Hiscott, 1996). Thus, the anti-inflammatory and analgesic activities exerted by UA could also be a consequence of its NO-scavenging activity, which may prevent free radical-induced NF-kB activation and consequent pro-inflammatory cytokine production; a cycle that would perpetuate inflammatory processes.

On the other hand, UA presented a pro-oxidant capacity in a lipid-rich system, enhancing TBARS formation induced by AAPH incubation. Lipid peroxidation has been shown to induce disturbance of membrane organization, functional loss as well as modification of proteins and DNA bases (Niki, 2009). Usnic acid is a lipophilic weak acid that can diffuse through mitochondrial membranes and cause proton leaking (uncoupling) (Joseph et al., 2009).

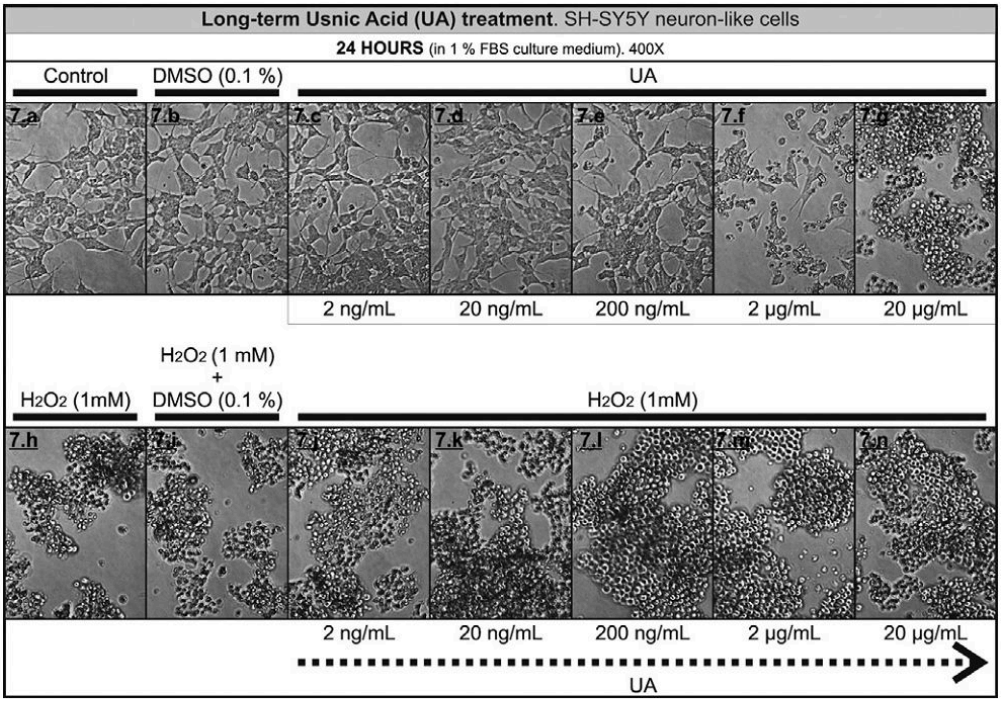

Fig. 7. Morphological effects of long-term UA treatment on SH-SY5Y cells. Phase contrast microscopy of SH-SY5Y cells after 24 h treatment with different concentrations of UA, in the absence or presence of H_2O_2 (1 mM). Photomicrographies representative of three independent experiments.

The uncoupling of mitochondrial oxidative phosphorylation by UA is similar to that observed by the classical uncoupler, 2,4-dinitrophenol (DNP). In mouse liver mitochondria, high doses of DNP resulted in increased lipid peroxidation due to higher oxidative stress (Futakawa et al., 2006). Odabasoglu et al. (2006) showed an antioxidant effect of UA when used against indomethacin-induced gastric ulcers in rats. They observed a significant inhibition of reactive species formation, a decrease in lipid peroxidation and increase of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase.

Although, the ability of compounds to quench hydroxyl radicals is generally related to the prevention of lipid peroxidation propagation (Shukla et al., 2009), the capacity of free radical scavenging by antioxidants does not necessarily correlate with the capability to inhibit lipid peroxidation. It is well-described that lipid peroxidation may occur not only through free radical-mediated oxidations but also upon enzymatic oxidation as well as radical-independent non-enzymatic oxidations. Lipid peroxidation leads to cytotoxycity, excepting for those cases of sublethal concentrations where the result could be either diverse cellular adaptive responses or even up-regulation of antioxidant enzymes with increased tolerance to following oxidative stress. All this together, provides putative scenarios that support our results in SH-SY5Y cells (Niki et al., 2005; Niki, 2009). Besides, studies concerning the hepatotoxic effect of (+) UA in isolated rat hepatocytes and liver mitochondria using carbon tetrachloride (CCl_4), as the reference hepatotoxin, showed that UA seems to trigger the same cytotoxic mechanisms as CCl_4, increasing the production of MDA, a marker for lipid peroxidation (Pramyothin et al., 2004).

Then, we could speculate that pro-oxidant actions of UA observed in mitochondria and hepatocytes could be a result of the capacity that UA has to enhance free radical-induced lipoperoxidation; similarly to what we observed in our AAPH-generating lipoperoxidation system.

Usnic acid has several interesting biological properties. For instance, it is used in pharmaceutical preparations against infections, bacterial eczema, mastitis, furunculosis and polydermy (Cocchietto et al., 2002). Thus, novel natural compounds isolated from lichens may represent a source of novel substances with selective biological action. The fact that UA is a non-genotoxic antineoplastic agent that works in a p53-independent manner (Yanmamoto et al., 1995) makes it a potential candidate for novel cancer therapies. However, to the best of our knowledge, nothing is known about UA effects in any *in vitro* neurotoxicological model. For this purpose, we decide to use SH-SY5Y cell line as well-characterized and accepted neuronal-like *in vitro* model because of their neuron-like properties, their capability to undergo neurite outgrowth as well as morphological changes, induced by oxidative stress (Cheung et al., 2009; Frota Junior et al., 2011; Navarra et al., 2010; Nicolini et al., 1998; Pahlman et al., 1984; Yu et al., 2011).

Overproduction of ROS can cause severe impairment of cellular functions. RS are involved in apoptotic mechanisms and may contribute to the apoptotic process found in several diseases (Jung et al., 2007). Our results have shown, that UA induced changes in their neuronal-like morphology, as well as, seems to have stopped the neuritic processes. The morphological changes, mainly in neurite outgrowth in SH-SY5Y, can be induced by increased intracellular ROS production (Jung et al., 2007). In addition, UA decreased cell viability with consequent cell death. This loss of cellular viability induced by UA can be due to increase the ROS.

Through DCFH-DA assay, our results showed that UA significantly increased intracellular ROS production when cells were treated for 1, 4 and 24 h, respectively. The advantage of this assay is that one could use intact living present a relevant redox-active action, acting as either pro-oxidant or antioxidant agent, depending on the specific radical that is being generated and the microenvironment provided by the system. As a matter of fact this dualistic effect is already known for some antioxidants that are able to autooxidize and therefore, to both generate reactive substances and act as pro-oxidants; strongly depending on the system composition (Moure et al., 2001). For instance, our laboratory has extensively characterized an apparently unexpected pro-oxidant effect of vita-

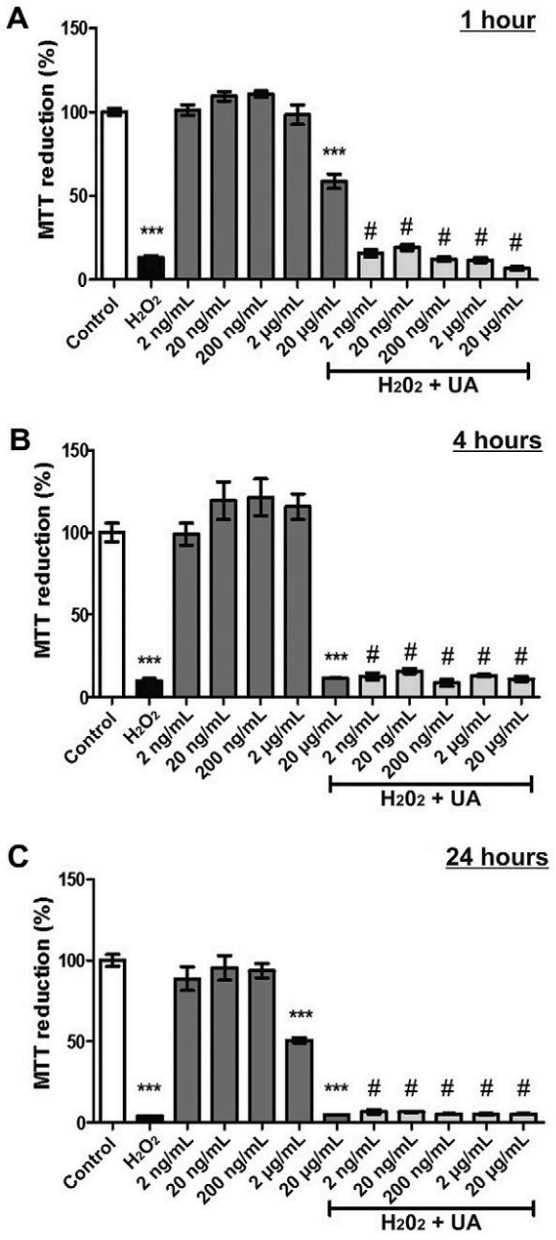

Fig. 8. MTT assay. Cultured SH-SY5Y cells were incubated with different concentrations of UA during 1 h (A), 4 h (B), and 24 h (C) at 37 °C in humidified 5% CO_2. MTT solution (sterile stock solution of 5 mg/mL) was added to the incubation medium in the wells at a final concentration of 0.2 mg/mL and incubated for 45 min. The medium was then replaced for DMSO and the optical density of each well was measured at 550 nm (test) and 690 nm (reference). H_2O_2 1 mM was used as positive control for cell death. Data were expressed as percentage of the formazan formation in untreated cells (control). Bars represent mean ± SEM of data from four different experiments (six wells per group in each independent experiment). Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{**}p < 0.001$, $^{***}p < 0.0001$. # Different from control.

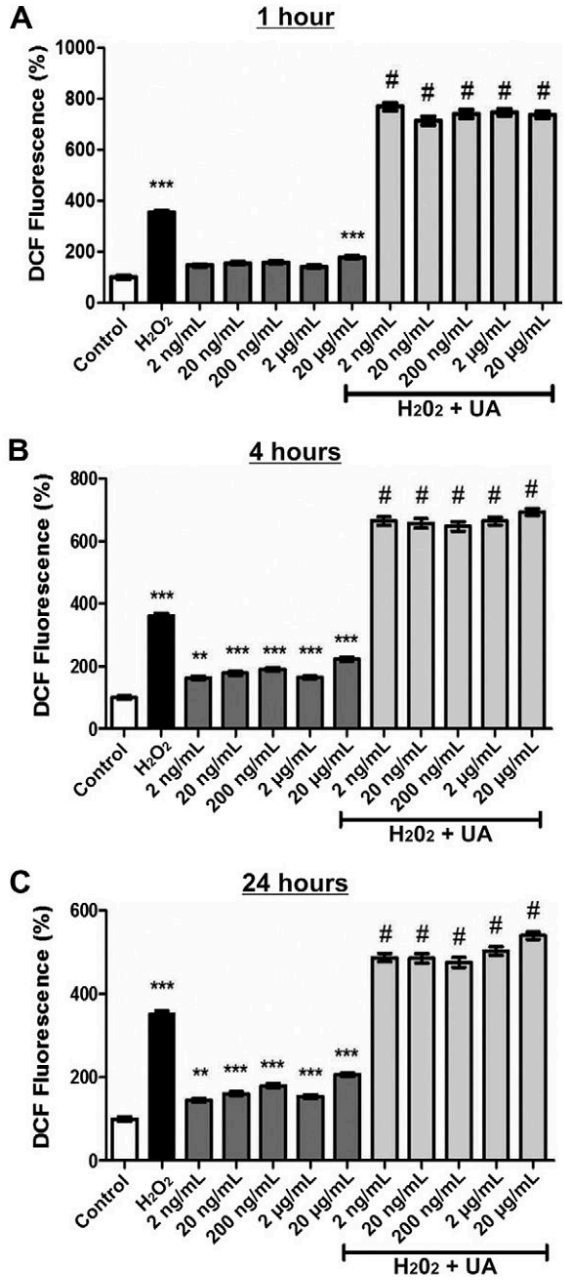

Fig. 9. Intracellular reactive species production (DCFH assay). Cultured SH-SY5Y cells were seeded in 96-well plates and 100 µM DCFH-DA dissolved in medium containing 1% FBS was added to each well and incubated for 2 h. Medium was then discarded and cells were treated with different concentrations of UA alone or in the presence of H_2O_2 (1 mM) and DCF fluorescence was read at 37 °C during 1 h (A), 4 h (B), and 24 h (C) (endpoint) in a fluorescence plate reader (Spectra Max M2, Molecular Devices, USA) with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm. Bars represent mean ± SEM of data from four different experiments (six wells per group in each independent experiment). $^{**}p < 0.001$, $^{***}p < 0.0001$ (1-way ANOVA followed by Tukey's *post hoc* test). # Different from control.

min A (and related carotenoids) both *in vitro* and *in vivo* at initially therapeutic conditions (Dal-Pizzol et al., 2000; Gelain et al., 2006, 2008; Klamt et al., 2003).

Our results suggest that UA displays variable redox-active properties, acting an antioxidant e pro-oxidant agent, according to different system conditions and/or cellular environment. These pro-oxidant properties in biological systems might be responsible by potential neurotoxicological effects of UA. Data presented in this work, may contribute to elucidation of the bifunctional behaviour and neurotoxicological actions of UA in biological systems.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tiv.2011.12.003.

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4. CONCLUSÕES

Conclui-se que o AU é uma molécula redox-ativa, atuando como antioxidante geral em ensaios como o TRAP/TAR e apresenta uma atividade "*scavenger*" inibidora/degradativa frente ao radical hidroxila e óxido nítrico. Por outro lado, o AU pode atuar como uma molécula pró-oxidante promovendo à auto-oxidação de sistemas lipídicos. Além disso, esse metabólito secundário é capaz de interagir com diversos genes/proteínas e compostos que estão relacionados com o balanço celular redox.

As alterações morfológicas induzidas pelo AU nas células SH-SY5Y, assim como perda da viabilidade celular é mediada pelo aumento da produção basal de espécies reativas.

De uma forma geral, o AU apresenta uma atividade antioxidante frente a diferentes espécies reativas in vitro. Entretanto, em uma linhagem celular derivada de neuroblastomas humanos (SH-SY5Y), esse composto apresenta propriedades pró-oxidantes, possivelmente sendo responsáveis pelos efeitos citotóxicos.

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ANEXOS

MATERIAL SUPLEMENTAR

List of Genes/Proteins			
Gene Symbol	**ENSEMBL PROTEIN ID**	**Gene Symbol**	**ENSEMBL PROTEIN ID**
GSTM4	ENSP00000358845	DUOX1	ENSP00000373689
FOS	ENSP00000306245	GSTM3	ENSP00000354357
DUSP1	ENSP00000239223	CAT	ENSP00000241052
GSTA1	ENSP00000335620	KDR	ENSP00000370742
GSTA5	ENSP00000360028	CDKN1A	ENSP00000362816
NOSTRIN	ENSP00000318921	NOS2A	ENSP00000327251
JUN	ENSP00000314522	K60	ENSP00000306512
PKCA	ENSP00000284384	DYNLL1	ENSP00000242577
PRDX6	ENSP00000342026	EPX	ENSP00000225371
SOD3	ENSP00000226877	LPO	ENSP00000374227
CDK4	ENSP00000257904	FOXM1	ENSP00000342307
EB11	ENSP00000263269	SOD1	ENSP00000270142
CYBA	ENSP00000261623	LDHA	ENSP00000227157
GPX1	ENSP00000373481	NOS3	ENSP00000297494
MTH1	ENSP00000339503	GPX2	ENSP00000374265
GSTT1	ENSP00000248935	IL10R	ENSP00000227752
GSTM5	ENSP00000358827	GSTK1	ENSP00000367415
IL10RB	ENSP00000290200	CDK1	ENSP00000362917
CCNE2	ENSP00000309181	MPO	ENSP00000344419
VEGFA	ENSP00000361099	GST3	ENSP00000196968
NOSIP	ENSP00000343497	GLA	ENSP00000218516
CDK2	ENSP00000266970	LDHC	ENSP00000280704
GSTA4	ENSP00000360002	INS	ENSP00000370731
NOS	ENSP00000320758	GSTM1	ENSP00000358838
CAMK1	ENSP00000256460	BCL1	ENSP00000227507
LDHB	ENSP00000229319	GSS	ENSP00000216951
TPO	ENSP00000329869	SAP90	ENSP00000293813
FLT	ENSP00000282397	MAPK8	ENSP00000363291
IL10	ENSP00000356066	CCNA1	ENSP00000255465
CALN	ENSP00000320580	PRKAR1B	ENSP00000353415
GSTA3	ENSP00000211122		

List of Compounds	
COMPOUND NAME	**CID**
Nitric Oxide	CID000145068
Usnic Acid	CID000005646
Hydrogen Peroxide	CID000000784
Hydroxyl Radicals	CID000000961
Molecular Oxygen	CID000000977