INSTITUTE OF BASIC HEALTH SCIENCES

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DOCTOR OF PHILOSOPHY THESIS

ANTIOXIDANT AND NEUROPROTECTIVE PROPERTIES OF TRICHILIA CATIGUA (CATUABA) AGAINST ISCHEMIA-REPERFUSION AND PRO-OXIDANTS AGENTS IN RAT HIPPOCAMPAL SLICES

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Porto Alegre, RS, Brazil

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FEDERAL UNIVERSITY OF RIO GRANDE DO SUL

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BY

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2013

"A vida não é fácil, mas também, não é difícil. Se você se esforça de todo seu coração, você conseguirá".

"The life is not easy, but at the same time, is not difficult. If you try with all your heart, you will get it".

"La vie n´est pas facile, mais, n´est pas aussi difficile. Si vous vous efforcez de tout votre coeur, vous reussirez".

Jean Paul Kamdem

DEDICATION

- My sweet wife **Agrippine Sidoine Kamdem** who has abandoned everything because of me, and whose love and encouragement allowed me to finish this journey;

- My Mother, **Marie Mbouche** for all your sacrifice for me;

- Dra. Roseane **Cardoso Marchiori** for your unconditional support.

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Above all, I owe it all to Almighty God for granting me the wisdom, health and strength to undertake this research task and enabling me to its completion.

Presentation

The present thesis is organized in three parts, in accordance with the rules of the Postgraduate Program in Biological Sciences: Biochemistry, of the Federal University of Rio Grande do Sul (UFRGS). It is presented as follow:

Part I: Abstract (written both in English and Portuguese), Introduction and Objectives.

Part II: Results, presented as scientific articles. Each article represents one chapter.

Part III: Discussion, Conclusion, Perspectives and References. The discussion section is a general interpretation of the results obtained from different works (Chapters). The conclusion is an overview of each chapter and the perspectives are related to open questions resulting from the results obtained in this thesis. The reference list is a combination of citations from Parts I (Introduction) and III (Discussion). However, the references of Part II are already at the end of each chapter.

The works presented in this thesis have been performed at the Federal University of Santa Maria, Postgraduate Program in Biological Sciences: Biochemical Toxicology Unit, under the co-supervision of Prof. Dr. João Batista Teixeira da ROCHA.

Table of Contents

LIST OF FIGURES

Introdução

Chapter 1

Chapter 2

Chapter 3

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

PART I

Where the introduction is presented and the objectives are defined.

ABSTRACT

Medicinal plants have been shown to have beneficial effects against oxidative stress-induced pathophysiology of various diseases including brain ischemia-reperfusion (I/R). *Trichilia catigua*, popularly known in Brazil as "catuaba", is widely used as a neurostimulant and aphrodisiac. Infusions of the bark are popularly used in folk medicine against sexual weakness, exhaustion, insomnia, stress, memory and central nervous systems disabilities. However, the involvement of antioxidant ability of *T. catigua* in its pharmacological properties especially in the management of neurological-related diseases is scanty in the literature. In this context, the first part of this study investigated the potential antioxidant activity of *T. catigua* using chemical and biological models. As a result, we have demonstrated that ethanolic extract and different fractions from the stem bark of *T. catigua* scavenged the 1,1-diphenyl-2-picrylhydrazyl **(**DPPH) radical, and inhibited the formation of thiobarbituric acid reactive substances (TBARS) caused by Fe^{2+} in rat's brain homogenates. However, ethanolic extract exhibited the highest antioxidant activity. In addition, ethanolic extract inhibited Ca^{2+}-induced reactive oxygen/nitrogen species (ROS/RNS) generation and caused a decrease in the mitochondrial membrane potential ($\Delta\Psi m$) only at high concentrations. On the basis of the aforementioned results, we hypothesized that ethanolic extract from *T. catigua* may at least, markedly reduce oxidative damage induced by *in vitro* I/R in rat hippocampal slices through attenuation of ROS/RNS production. Thus, the second part of this study investigated the protective effects of ethanolic extract of *T. catigua* against oxidative damage induced by I/R in rat hippocampal slices. *T. catigua* prevents hippocampal slices from the deleterious effects caused by I/R, by increasing mitochondrial viability, which was associated with decreased lactate dehydrogenase (LDH) leakage in the incubation medium; by decreasing DCFH oxidation in the medium, and increasing non-protein thiols (NPSH) content in slices homogenates. In contrast, *T. catigua* could not protect slices from I/R when it was added to the medium after ischemic insult, suggesting that it can only be used as preventive and not as curative agent against brain damage. Taking that alteration in learning and memory function are common consequences of a wide variety of toxic insults and disease states, the third part of this study was undertaken to determine whether *T. catigua* offered neuroprotection against oxidative stress induced by different pro-oxidants. Exposure of rat hippocampal slices for 1 h to hydrogen peroxide (H_2O_2), sodium nitroprusside (SNP) and 3-nitropropionic acid (3-NPA) decreased mitochondrial activity, increased ROS/RNS in the incubation medium and caused TBARS formation in rat hippocampal slices homogenates. These deleterious effects were significantly attenuated by pre-treatment of slices with ethanolic extract of *T. catigua*. Overall, our data showed that the use of *T. catigua* extract may be beneficial in preventing neurological disorders associated with oxidative stress, and that its beneficial effects seems to be related at least, in part, to its antioxidant activity, which can be attributed to its polyphenolic content.

Keywords: Catuaba, ischemia-reperfusion, *Trichilia catigua*, antioxidante activity, oxidative stress, pro-oxidants.

RESUMO

Plantas medicinais apresentam efeitos benéficos contra a patofisiologia de várias doenças induzida pelo estresse oxidativo incluindo isquemia-reperfusão (I/R). *Trichilia catigua*, popularmente conhecida no Brasil como "catuaba", é amplamente utilizada como um neuroestimulante e afrodisíaco. Infusões da casca são popularmente utilizadas na medicina popular contra debilidade sexual, cansaço, insônia, estresse e deficiências relacionadas à memória e sistema nervoso central. Porém, o envolvimento da atividade antioxidante de *T. catigua* em suas propriedades farmacológicas especialmente em relação ao sistema nervoso ainda é escasso na literatura. Sendo assim, a primeira parte deste estudo investigou o pontencial antioxidante de *T. catigua* usando modelos químicos e biológicos. Como resultado, foi demonstrado que o extrato etanólico e diferentes frações da casca de *T. catigua* eliminaram o radical 1,1-difenil-2-picrilhidrazila **(**DPPH), e inibiram a geração de substâncias reativas ao ácido tiobarbitúrico (TBARS) causadas pelo Fe^{2+} em homogenatos dos cérebros de rato. O extrato etanólico apresentou a maior atividade antioxidante. Além disso, o extrato etanólico inibiu a produção de espécies reativas de oxigênio/nitrogênio (EROS/ERNS) induzidas pelo Ca^{2+} e diminuiu o potencial de membrana ($\Delta\Psi m$) mitocondrial nas maiores concentrações. Com base nos resultados acima, nós hipotetizamos que o extrato etanólico de *T. catigua* pode, pelo menos, reduzir consideravelmente os danos oxidativos induzidos pela isquemia reperfusão (I/R) em fatias de hipocampo de rato através da atenuação da produção de EROS/ERNS. Baseado nisso, a segunda parte deste estudo investigou o efeito protetor do extrato etanólico de *T. catigua* contra os danos oxidativos induzidos por I/R em fatias de hipocampo de ratos. Como resultado foi demonstrado que *T. catigua* previniu os efeitos deletérios causados por I/R nas fatias de hipocampo, através do aumento da viabilidade mitocondrial, o qual foi associado com o decréscimo na liberação de lactato desidrogenase (LDH) no meio de incubação; pelo decréscimo da oxidação de DCFH no meio; e aumento do conteúdo de tióis não proteicos (NPSH) em fatias homogeneizadas. No entanto, *T. catigua* não foi capaz de proteger as fatias da I/R quando adicionadas ao meio após da injúria isquêmica, sendo assim, sugerindo que ela possa ser usada somente como preventiva e não como agente curativo frente ao dano cerebral. Uma vez que alterações de aprendizado e memória são consequências comuns a uma variedade de doenças e agressões tóxicas, a terceira parte deste estudo concentrou-se em determinar se *T. catigua* ofereceria neuroproteção contra o estresse oxidativo induzido por diferentes pro-oxidantes. Os resultados indicaram que a exposição de fatias de hipocampo de rato por 1h ao peróxido de hidrogênio (H_2O_2), nitroprussiato de sódio (NPS) e ácido 3-nitropropiônico (3-ANP) diminui a atividade mitocondrial; aumentou a geração de ROS/RNS no meio de incubação e causou a formação de TBARS nas fatias homogeneizadas. A diminuição destes efeitos deletérios foi significativa quando as fatias foram pré-tratadas com o extrato etanólico de *T. catigua*. Em conclusão, nossos resultados demonstraram que o uso do extrato de *T. catigua* pode ser benéfico na prevenção de desordens neurológicas associadas ao estresse oxidativo, e que seus efeitos benéficos parecem estar associados, pelo menos em parte, a sua atividade antioxidante, que, por sua vez, podem ser atribuídas ao conteúdo polifenólico da planta.

Palavras-chaves: Catuaba, isquemia reperfusão, *Trichilia catigua*, atividade antioxidante, estresse oxidativo, pró-oxidantes.

LIST OF ABBREVIATIONS

Ca^{2+}: Calcium ion

CAT: Catalase

DCFH-DA: 2',7'-Dichlorofluorescein Diacetate (DCFH-DA)

DCFH: 2',7'-Dichlorofluorescein

DPPH: 1,1-diphenyl-2- picrylhydrazyl

GPx: Glutathione Peroxidase

H_2O_2: Hydrogen Peroxide

HPLC-DAD: High Performance Liquid Chromatography coupled to Diode Array Detector

I/R: Ischemia-Reperfusion

LDH: Lactate Dehydrogenase

LPO: Lipid Peroxidation

METC: Mitochondrial Electron Transport Chain

NADPH: Nicotinamide Adenine Dinucleotide Phosphate (reduced form)

$NADP^+$: Nicotinamide Adenine Dinucleotide Phosphate (oxidized form)

NADH: Nicotinamide Adenine Dinucleotide (reduced form)

NAD^+: Nicotinamide Adenine Dinucleotide (oxidized form)

NO_2^-: Nitrogen dioxide

NO/$^{\cdot}$ON: Nitric oxide

NPSH: Non Protein Thiol

OGD: Oxygen and Glucose Deprivation

$ONOO^-$: Peroxynitrite

ONOOH: Peroxynitrous acid

$OH^{\cdot}$/$^{\cdot}$OH: Hydrogen Peroxide

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SNP: Sodium Nitroprusside

SOD: Superoxide Dismutase

TBARS: Thiobarbituric Acid Reactive Substances

XO: Xanthine oxidase

2,4-DNP: 2,4-Dinitrophenol

3-NPA: 3- Nitropropionic Acid

ΔΨm: Mitochondrial Membrane Potential

1. INTRODUÇÃO

1.1. Os radicais livres no sistema fisiológico

Os radicais livres podem ser definidos como moléculas ou fragmentos moleculares que contenham um ou mais elétrons desemparelhados nas orbitais atômicos ou moleculares (Halliwell and Gutteridge, 1999; Gilbert, 2000). Este elétron desemparelhado geralmente dá um considerável grau de reatividade para o radical livre. As espécies de radicais livres incluem as espécies reativas de oxigênio (EROs) e as espécies reativas de nitrogênio (ERN). Os radicais livres de oxigênio e nitrogênio podem ser convertidos em outras espécies reativas não radicalares tais como o peróxido de hidrogênio (H_2O_2), o ácido hipocloroso (HOCl), o ácido hipobromoso (HOBr) e o peroxinítrito ($ONOO^-$). Assim, os EROs e ERNs incluem espécies radicalares e não radicalares. Espécies reativas de nitrogênio em sistemas biológicos incluem principalmente o óxido nítrico ($NO^{\cdot}$) e o dióxido de nitrogénio ($^{\cdot}NO_2$), enquanto que, as principais EROs geradas em sistemas biológicos são o ânion superóxido (O_2-), o peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($OH^{\cdot}$).

As EROs e ERN são continuamente gerados como subprodutos da respiração aeróbica e de vários outros processos catabólicos e anabólicos (Halliwell, 1991; Kehrer et al., 2013), mas, são subsequentemente transformado e desintoxicado. As principais reações da produção dos radicais livres de oxigênio e de nitrogênio no sistema biológico estão ilustradas na Figura 1.

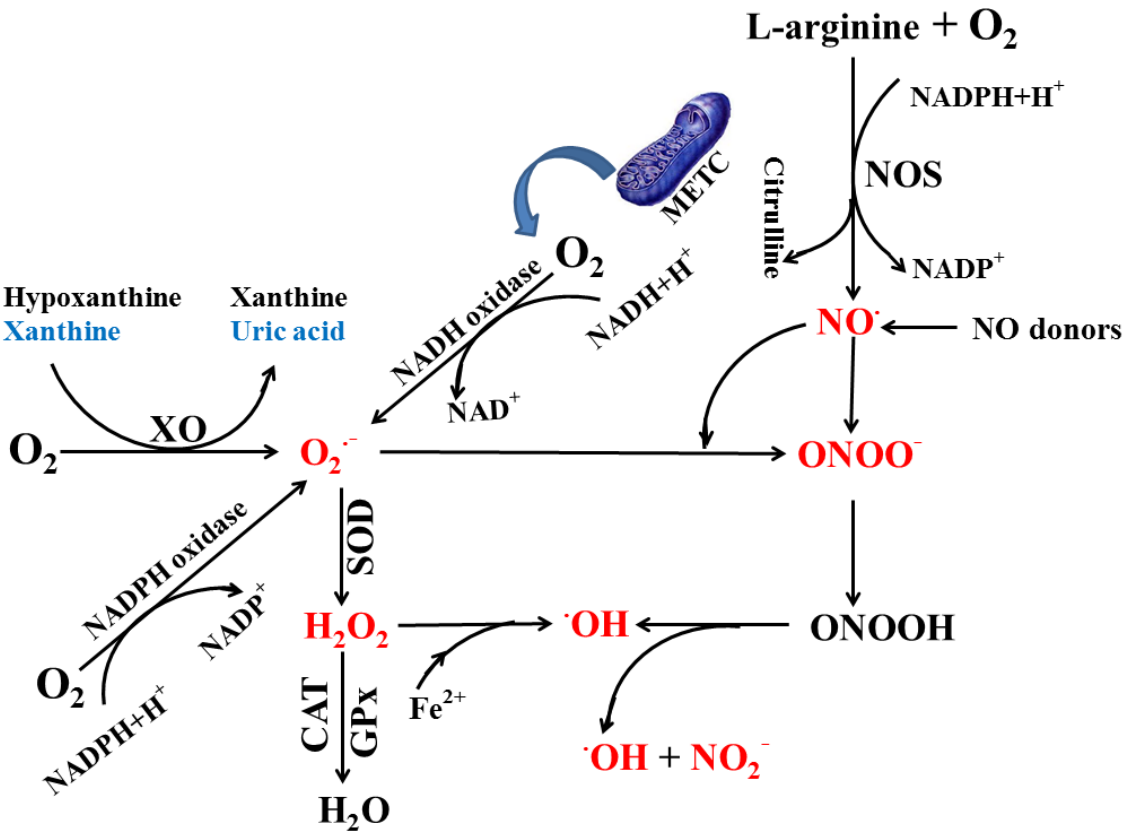


Figura 1: As principais reações da produção dos radicais livres de oxigênio e de nitrogênio no sistema biológico. Em vermelho, a geração das EROs e ERN, e em azul, o substrato e o produto. Adaptado de Fang et al. (2002).

Além do metabolismo normal, as EROs e ERN podem também ser produzidas em resposta a diferentes estímulos ambientais, tais como a radiação ionizante, UV, toxinas etc.

Tradicionalmente vistos como agentes nocivos, as EROs/ERN exercem também um papel importante na modulação de vários processos biológicos, incluindo a sinalização celular, a proliferação e a diferenciação (Finkel, 2011; Murphy et al., 2011). Este paradoxo aparente delineia as EROs/ERN como moléculas de dupla face (Valko et al., 2006; Pala and Tabakçioglu, 2007; Dickinson and Chang, 2011). Os efeitos benéficos das EROs/ERN ocorrem em concentrações relativamente baixas ou moderadas. De particular relevância, as EROs produzidas por células do sistema imunológico (neutrófilos e macrófagos) durante o processo de explosão respiratória podem combater os agentes infecciosos (Freitas et al., 2010). Da mesma forma, os níveis fisiológicos do óxido nítrico (ON) produzidos pelas células endoteliais são essenciais para a regulação

do relaxamento e proliferação de células vasculares de músculo liso, adesão de leucócitos, agregação plaquetária, trombose vascular e hemodinâmica (Ignarro et al., 1999). Além disso, o óxido nítrico (ON) produzido pelos neurónios serve como um neurotransmissor (Freidovich 1999).

Em contraste, a produção excessiva das EROs/ERN podem ocorrer quando a sua produção no sistema excede a capacidade do sistema antioxidante (enzimático e não enzimático) para neutralizá-las e eliminá-las. O excesso das EROs/ERNs pode causar a peroxidação lipídica, danos às mitocôndrias, proteínas e ácidos nucleicos (Figura 2), comprometendo seu funcionamento (Cooke et al., 2003; Evans et al., 2004; Filipcik et al., 2006; ChakravartiandChakravarti, 2007). Os efeitos deletérios das EROs e ERNs nos sistemas biológicos são denominados de estresse oxidativo e nitrosativo, respectivamente (Kovacic and Jacintho, 2001; Ridnour et al., 2005).

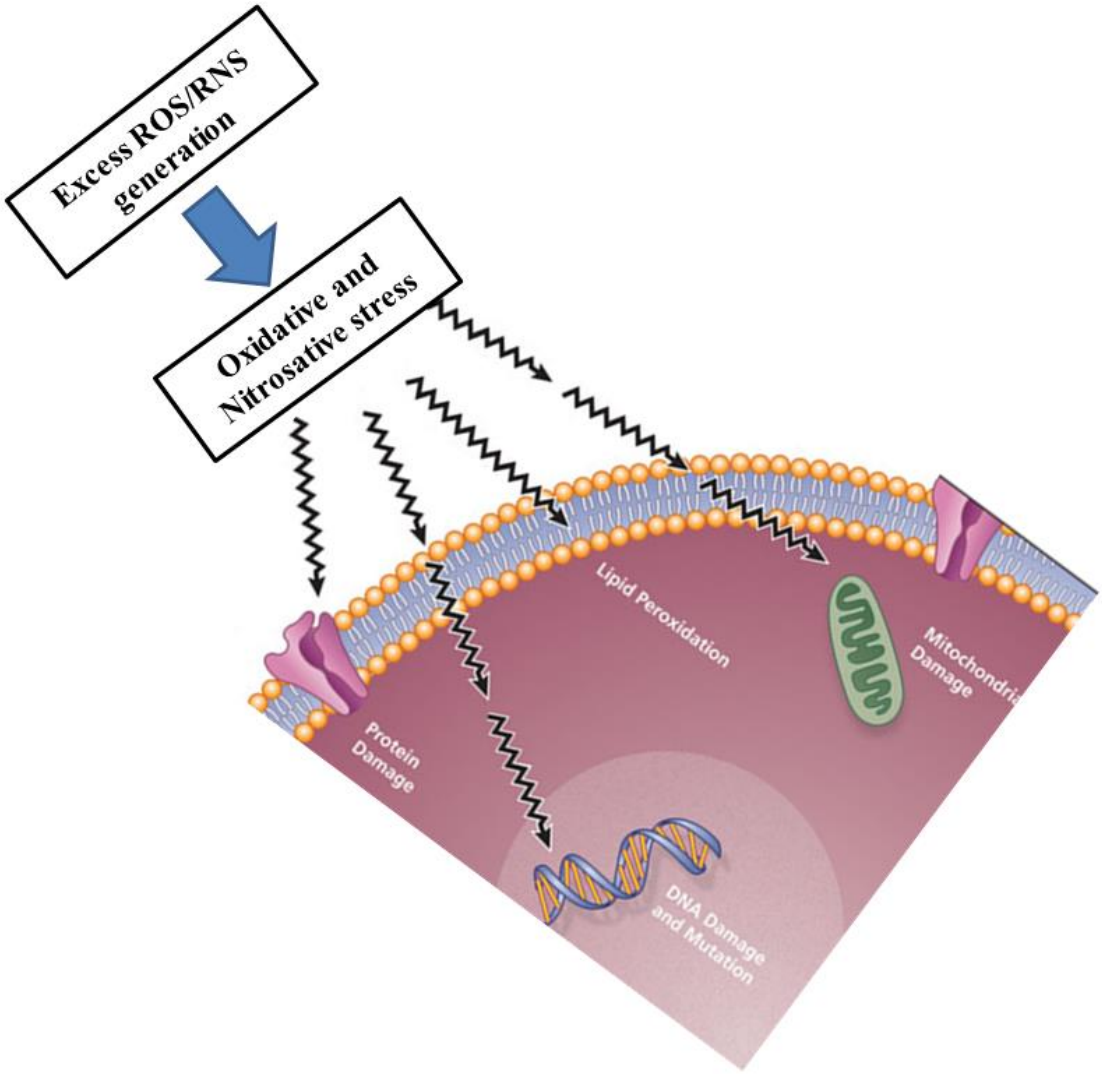


Figura 2: Danos celulares causados pelo excesso das espécies reativas de oxigênio (EROs) e as espécies reativas de nitrogênio (ERNs).

I.2. Estresse oxidativo e nitrosativo

O estresse oxidativo resulta das reações metabólicas que utilizam o oxigênio, porém, as ERNs reagem conjuntamente com as EROs para causar o estresse nitrosativo. As EROs/ERNs são o resultado dos processos que ocorrem naturalmente, tais como o metabolismo de oxigênio e processos inflamatórios. Por exemplo, quando as células usam o oxigênio para gerar energia, os radicais livres são gerados como consequência da produção da ATP pela mitocôndria. Estas espécies radicalares podem interagir, formando outras espécies mais reativas, tais como os radicais hidroxil e peroxinítrito (um produto da reação entre o ânion superóxido e óxido nítrico) (ver Figura 1). Devido

a isso, o estresse oxidativo/nitrosativo está diretamente relacionado a várias doenças, bem como no processo de envelhecimento.

I.3. Desordens neurológicas

O estresse oxidativo/nitrosativo está envolvido na patofisiologia de várias perturbações neurológicas, tais como as doenças de Alzheimer, Parkinson, Huntington e a isquemia cerebral (Halliwell, 2006; Chen, 2011; Quintanillaet al., 2012; Perfeito et al., 2012). O cérebro é particularmente vulnerável aos radicais livres, principalmente os radicais de oxigênio, isto por que: (i) consome cerca de 20% de oxigênio e 25% de glicose, e representa apenas 2% do peso corporal total, (ii) possui escassez relativa de enzimas antioxidantes quando comparada com outros órgãos, (iii) tem níveis elevados de metais de transição, (iv) e é rico em ácidos graxos poli-insaturados, que são particularmente sensíveis ao ataque dos radicais livres (Halliwell, 2006; Belanger et al., 2011; Friedman, 2011). Portanto, o foco deste estudo foi a isquemia cerebral, considerando que se trata-se de uma das mais importantes causas de morte no mundo inteiro (Rosamond et al., 2007; Kleinschnitz and Plesnila, 2012; Wu and Grotta 2013).

I.3.1. Isquemia cerebral

A isquemia pode ser dividida em dois tipos: isquêmica e hemorrágica (Sims and Muydermanet al., 2010). Acidentes vasculares cerebrais isquêmicos são mais prevalentes do que hemorrágicas, tornando-se aproximadamente 87% de todos os casos, e tem sido o foco da maioria dos estudos farmacológicos (Rosamondet al., 2007). Porém, este estudo teve como alvo o acidente vascular cerebral isquêmico ou isquemia cerebral.

I.3.1.1. Isquemia e reperfusão (I/R) cerebral

A isquemia cerebral pode ser definida como qualquer estado fisiopatológico em que o fluxo sanguíneo cerebral de toda ou qualquer parte do cérebro é insuficiente para atender às demandas metabólicas do cérebro. Existem quatro causas da isquemia cerebral que são:

- A trombose (isto é, a obstrução de vaso sanguíneo por um coágulo sanguíneo formado localmente),

- A embolia (ou seja, a obstrução devido a um êmbolo de outras partes do corpo) (Donnanet al., 2008),

- A hipoperfusão sistêmica (isto é, a diminuição geral no fornecimento de sangue, como por exemplo, em estado de choque) (Shuaib and Hachinski, 1991),

- A trombose venos (Stam, 2005).

Cada uma destas causas provoca vários processos conhecidos como "cascata isquêmica", que se refere a uma série de reações bioquímicas provocadas no cérebro depois de alguns segundos a alguns minutos, após a redução do fluxo sanguíneo ou isquemia. Por exemplo, os neurônios isquêmicos podem despolarizar devido à falta de fornecimento da energia, e da liberação de potássio e do glutamato no espaço extracelular. Na região do núcleo ("core"), ou seja, a área do cérebro afetada pelo insulto isquêmico, a maioria das células neuronais isquêmicas morrem imediatamente, devido à ação dos metabólitos produzidos durante e após a oclusão do vaso ou evento isquêmico (Figura 3). Todavia, nas regiões da penumbra (onde alguma perfusão é preservada) as células podem se repolarizar, mas à custa de consumo de energia adicional (Dirnagl et al., 1999).

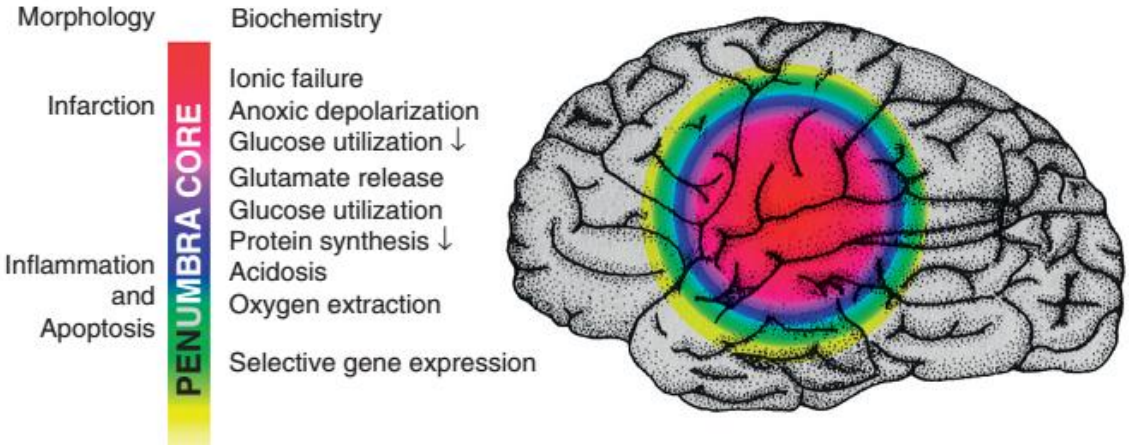


Figura 3: A penumbra isquêmica. A região do cérebro de baixa perfusão em que as células que perderam o seu potencial de membrana ("core") está rodeado por uma área na qual a perfusão intermediária prevalece ("penumbra"). Existem limites de perfusão abaixo dos quais certas funções bioquímicas estão impedidas (código de cores de escala). De Dirnaglet al. (1999).

A cascata isquêmica na região do núcleo (“core”) é um fenômeno que depende do tempo. Ele pode continuar durante uma ou duas horas, mas também pode ser estendido para alguns dias, mesmo após o restabelecimento do fluxo sanguíneo (Figura 4) (Dirnaglet al., 1999; Endres et al., 2009). Os mecanismos de lesão isquêmica incluem a excitotoxicidade, a despolarização, o estresse oxidativo, a inflamação e a apoptose (Ozbalet al., 2008; Candelario-Jalil, 2009; Yousuf et al., 2009). Os principais mecanismos da região do núcleo incluem a excitotoxicidade e a despolarização (Figura 4), que danificam irreversivelmente as células neuronais. Ao contrário, na penumbra ocorre o estresse oxidativo, a inflamação e a apoptose (Figura 4) (Dirnaglet al., 1999; Doyle et al., 2008).

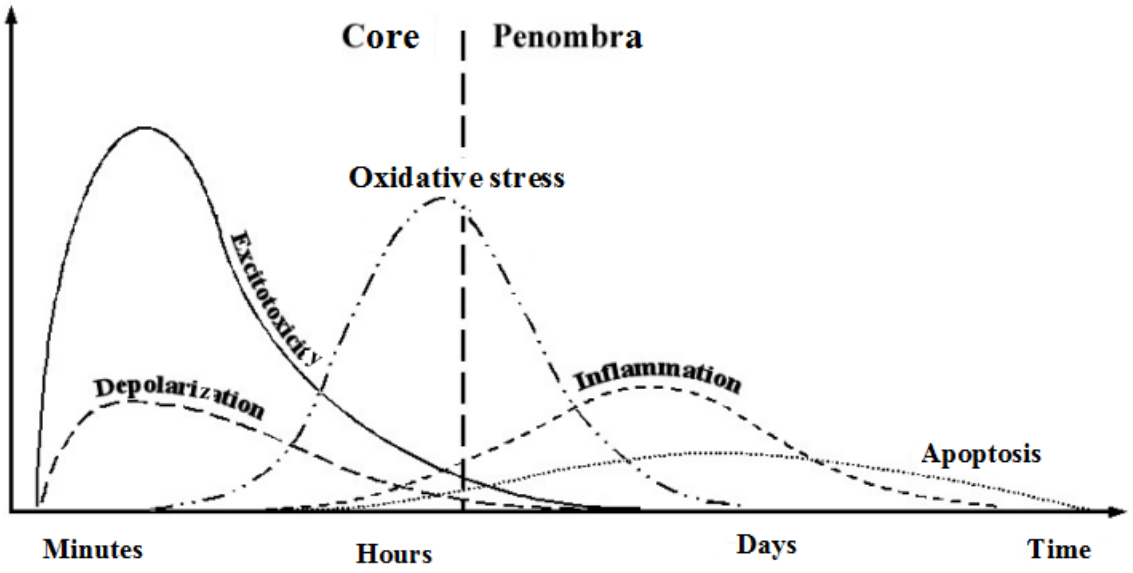
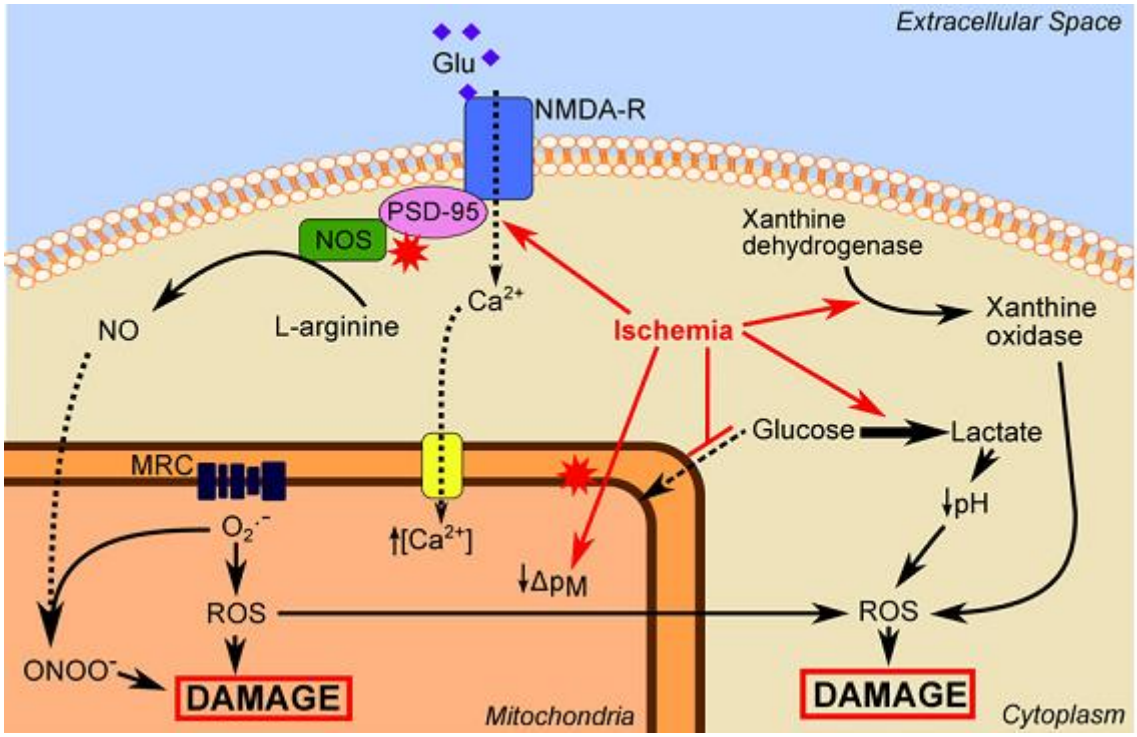


Figura 4: Cinética dos mecanismos envolvidos na isquemia cerebral. De Dirnagl et al. (1999).

A reperfusão precoce ou reoxigenação é o alvo principal da maior parte das intervenções experimentais, para tornar as células na penumbra resistente à morte celular (Dirnaglet al., 1999), uma vez que oferece substrato para numerosas reações de oxidação enzimáticas (Chan, 1994; 2001). Paradoxalmente, a restauração do fluxo sanguíneo cerebral provoca mais danos ao cérebro isquêmico (Frantsevaet al., 2001; Tsubota et al., 2010). Portanto, a procura dos agentes neuroprotetores que podem efetivamente inibir, retardar, impedir ou proteger o cérebro contra os danos cerebrais causadas pela isquemia reperfusão (I/R) são de grande interesse.

1.3.1.2. Fisiopatologia da isquemia e reperfusão cerebral

Nas condições fisiológicas, o oxigênio e a glicose são essenciais para manter as funções cerebrais. Durante a isquemia cerebral, o oxigênio e a glicose fornecidos ao cérebro são significativamente reduzidos, conduzindo a um bloqueio da fosforilação oxidativa, e consequentemente uma redução na síntese de ATP (Erenciska and silver, 1989; Martin et al., 1994; Manzanero et al., 2013). Várias excelentes revisões têm descrito de maneira detalhada os mecanismos fisiopatológicos envolvidos na I/R (White et al., 2000; Deb et al., 2010; Bretón and Rodríguez, 2012; Manzanero et al., 2013; Sanderson et al., 2013). A Figura 5 apresenta uma vista geral simplificada do envolvimento da produção das EROs/ERNs no mecanismo fisiopatológico da I/R.



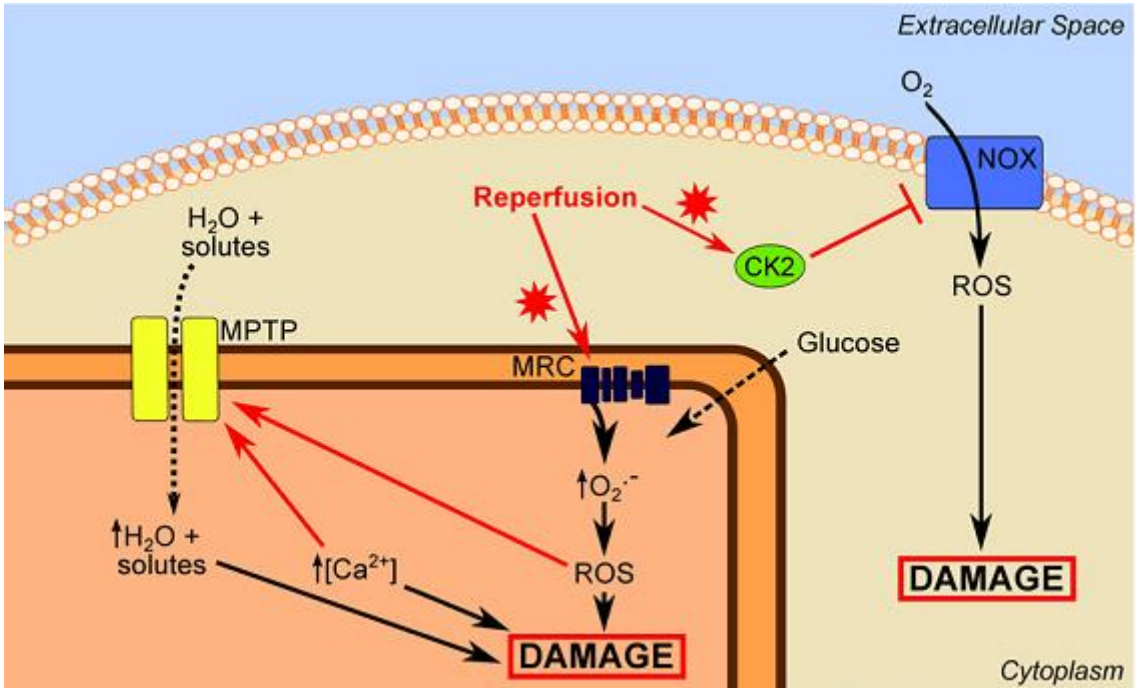


Figura 5: Visão geral simplificada da geração das EROs durante a isquemia (**parte de cima**) e reperfusão (**parte de baixo**) cerebral. Durante a isquemia ocorre uma redução significativa de oxigênio para o cérebro, levando a um bloqueio da fosforilação oxidativa e, consequentemente, uma redução na síntese de ATP. Como primeira consequência, as células neuronais fermentam a glicose para o lactato.

Por causa da queda da ATP, as bombas iônicas dependentes de energia param de funcionar, permitindo o influxo do cálcio que, consequentemente, faz com que ocorra a despolarização neuronal. Devido ao aumento da concentração intracelular de cálcio, o glutamato, liberado no espaço extracelular ativa o receptor NMDA (NMDA-R), resultando a um aumento do fluxo de cálcio e subsequentemente da densidade da proteína pós-sináptica (PSD-95), mediada pela ativação de óxido nítrico sintase neuronal (nNOS), que gera o óxido nítrico (NO), a partir da L-arginina. Nestas condições, a xantina desidrogenase é convertida em xantina oxidase, contribuindo ao aumento da produção das EROs. A cascata de eventos iniciada durante a isquemia é agravada durante a reperfusão ou reoxigenação. A presença do oxigênio reativa a cadeia respiratória mitocondrial (MRC), que resulta na produção do ânion superóxido e, consequentemente, a geração das EROs. Isto permite a entrada da água e dos solutos do citoplasma para a mitocôndria, resultando no inchaço mitocondrial. Sob esta condição, a

expressão da Caseína quinase 2 (CK2), um inibidor da NADPH-oxidase (NOX) é reduzida, contribuindo para a ativação da NOX, o que resulta na geração das EROs. Além disso, o ROS ainda pode ser produzido durante a reperfusão, através da ação de NOX. Modificado de Manzaneroet al. (2013).

I.3.2. Modelo experimental *in vitro* da I/R

A privação do oxigênio e da glicose (OGD) seguida da reoxigenação representa um modelo *in vitro* válido para o estudo das respostas celulares fisiopatológicos a I/R (Yin et al., 2002; Pugliese et al., 2006; Cimarosti and Henley, 2008; Dixon et al., 2009; Sun et al., 2010). A privação do oxigênio e glicose especialmente nas fatias de hipocampo reproduzem vários estados patológicos induzidos pela falta de energia cerebral, uma vez que ela pode manter a mesma composição de células semelhante ao que ocorre nos danos cerebrais (Taylor et al., 1995).

I.4. Agentes pro-oxidantes

Os pró-oxidantes são os produtos químicos que induzem o estresse oxidativo pela geração das EROs/ERNs ou pela inibição do sistema antioxidante (Puglia and Powell, 1984). Alguns pró-oxidantes neurotóxicos tais como o peróxido de hidrogênio (H_2O_2), o nitroprussiato de sódio (SNP) e o ácido 3-nitropropiónico (3-NPA), são amplamente utilizados na literatura para induzir o estresse oxidativo através diversos mecanismos, e para estudar os efeitos protetores dos compostos e/ou extratos de plantas com atividade antioxidante.

I.4.1. Peróxido de hidrogénio (H_2O_2)

O peróxido de hidrogênio tem sido envolvido em desordens neurodegenerativas tais como a doença de Alzheimer (Simonian and Coyle, 1996; Tabneret al., 2005; Fang et al., 2012). O H_2O_2 exerce a sua neurotoxicidade principalmente pela formação do radical hidroxil através da reação de Fenton. Embora, a depleção dos níveis de GSH e a ruptura da homeostase do cálcio possam também contribuir ao efeito tóxico do H_2O_2 (Farberet al., 1990; Rimpler et al., 1999).

1.4.2. Nitroprussiato de sódio (SNP)

Em vários estudos *in vitro* e *in vivo* têm sido demonstrados que o nitroprussiato de sódio (SNP), um doador do óxido nítrico (NO), pode causar o estresse oxidativo e a citotoxicidade pela libertação do cianeto, do ferro e do óxido nítrico que pode reagir com o ânion superóxido formando o peroxinítrito (Arnold et al., 1984; Pryor and Squadrito, 1995). Tem sido relatado que o NO está envolvido na fisiopatologia de várias doenças, incluindo a I/R, doenças de Alzheimer e de Parkinson (Puzzoet al., 2006; Aquilano et al., 2008).

I.4.3. Ácido 3-nitropropiónico (3-NPA)

O ácido 3-nitropropiónico induz a neurotoxicidade *in vitro* e *in vivo* pela inibição irreversível da atividade do succinato desidrogenase (SDH), uma enzima do complexo II mitocondrial, responsável da oxidação do succinato o fumarato no ciclo de Krebs e do transporte subsequente dos elétrons na fosforilação oxidativa (Coles et al., 1979). Ele é utilizado como uma ferramenta para estudar os mecanismos envolvidos na doença de Huntington (DH), uma vez que ela produz em animais, alterações comportamentais, bioquímicas e morfológicas semelhantes às que ocorrem em pacientes com a DH (Kumar and Kumar, 2009; 2010;Túnez et al., 2010; Wu et al., 2010; Menze et al., 2012).

I.5. Os compostos sintéticos contra os produtos naturais na terapia da isquemia e reperfusão cerebral

Estudos sobre a busca de drogas neuroprotetoras para acidente vascular cerebral isquêmico estão em andamento (O'Collins et al., 2006). O objetivo da neuroproteção é de interferir nos eventos da cascata isquêmica, visando um ou mais mecanismos de dano, bloqueando assim, os processos patológicos e prevenindo a morte neuronal na penumbra isquêmica (O'Collins et al., 2006; Wu and Grotta, 2013).

Vários compostos sintéticos (ebselen, disseleneto de difenila, disufenton de sódio, etc) com uma variedade de propriedades farmacológicas, têm sido relatados de reduzir o volume de enfarto na isquemia cerebral em modelos *in vivo* e *in vitro*. No entanto, apesar de seus efeitos benéficos em modelos experimentais, pouco tem sido alcançado em trazê-los para as aplicações de rotina clínicas (Gladstone et al., 2002; Rahman et al., 2005; Fatahzadeh and Glick, 2006; O'Collins et al., 2006; Durukan and Tatlisumak, 2007; Shuaib et al., 2007). Além disso, estes compostos são geralmente associados à

efeitos secundários ou tóxicos (Nogueira and Rocha, 2011). Portanto, a busca dos produtos naturais pode dar esperança na prevenção e/ou no tratamento da isquemia cerebral.

Produtos naturais derivados das ervas são geralmente considerados seguros com poucos ou sem efeitos colaterais. Eles são baratos e de fácil acesso. As plantas medicinais têm gerado um interesse considerável na prevenção, proteção e/ou no tratamento de várias doenças associadas ao estresse oxidativo, e algumas delas têm constituído uma nova direção na descoberta de novas drogas (Bastianetto and Quirion, 2002; Wu et al., 2010; Kim et al., 2012).

1.5.1. *Trichilia catigua*

Popularmente conhecida como catuaba, catiguá vermelho, pau ervilha e catuaba do Norte (Garcez et al., 1997), *Trichilia catigua* (Meliaceae, Figura 6) é uma planta nativa do Brasil, e se encontra também na Argentina, Paraguai e Bolívia. Ela é amplamente utilizada como neurostimulante, afrodisíaco, purgante e no tratamento do reumatismo (Garcez et al., 1997; Kletter et al., 2004). A infusão de suas cascas é usada na medicina popular como um tônico para o tratamento da neurastenia (fadiga, estresse, impotência, déficits de memória) (Pizzolatti et al., 2002; Viana et al., 2009; Mendes, 2011).

Figura 6: *Trichilia catigua*

No Brasil, diferentes gêneros e famílias são popularmente conhecidos como "catuaba", tais como *Anemopaegma* (Bignoniaceae), *Erythroxylum* (Erythroxylaceae), *Illex* (Aquifoliaceae), *Micropholis* (Sapotaceae), *Secondatia* (Apocynaceae), *Tetragastris* (Burserеceae), *Trichilia* (Meliaceae). Isto é devido às identificações errôneas destas plantas (Marques, 1998), uma vez que todas elas são utilizadas com a mesma finalidade médica, apesar de terem diferentes constituintes. De acordo com a Farmacopéia

Brasileira (1926), a espécie registrada como “catuaba” verdadeira, para fins médicos é *Anemopaegma arvense* (Veil.) Stellfeld (Bignoniaceae). Marques (1998) descreveu as diferenças entre as espécies conhecidas como “catuaba” e concluiu que a principal espécie comercialmente disponível no Brasil como “catuaba” é a *T. catigua*. A mesma conclusão foi alcançada por Kletter et al. (2004) e por Daolio et al. (2008).

1.5.1.1. Constituentes fitoquímicos

O extrato de casca da *T. catigua* contém um número de produtos químicos bioativos com alta concentração de polifenóis (Pizzolatti et al., 2002; Beltrame et al, 2006; Resende 2011), bem como alcalóide tropano (Kletter et al, 2004). Fenilpropanoídicos (Pizzolatti et al., 2002; Beltrame et al., 2006; Tang et al., 2007; Resende et al., 2011), e lignanas (Pizzolatti et al., 2002) são os principais metabólitos secundários encontrados na *T. catigua*. Flavaligninas (fenilpropanóides epicatequinas-substituídos), tal como cinchonainas Ia e Ib, sesquiterpenos (Garcez et al., 1997), alguns γ-lactonas, e esteróis (Pizzolatti et al., 2004) foram isolados a partir desta planta. Além disso, cinchonain Ic, cinchonain Id, catiguanina A e catiguanina B também foram isolados (Tang et al., 2007). Mais recentemente, Resende et al. (2011) isolaram apocinina E que é um novo fenilpropanóide substituído flavan-3-ol, em conjunto com a epicatequina, procianidina B2, procianidina B4, procianidina C1, cinchonain Ia, cinchonain Ib, cinchonain IIb e cinchonain IIa a partir da casca de *T. catigua*. A cromatografia líquida de alta performancia (HPLC) do extrato de casca da *T. catigua* revelou que a planta contém quercetina, rutina, ácido caféico e ácido rosmarínico, entre outros compostos (Kamdem et al., 2012a, b). Todos estes compostos têm exibido uma variedade de propriedades farmacológicas incluindo a atividade antioxidante (Tang et al., 2007; Resende et al., 2011).

1.5.1.2. Propiedades farmacológicas

Os extratos da casca da *T. catigua* apresentam um amplo espectro de atividades farmacológicas. Alguns estudos farmacológicos com a casca da *T. catigua* relataram propriedades antioxidantes (Brighente et al., 2007; Kamdem et al., 2012a), anti-microbianas (Pizzolatti et al., 2002), antinociceptivas (Viana et al., 2009), antidepressivas (Campos et al., 2005) e anti-inflamatórias (Campos et al., 2005). Estudos anteriores sobre *T. catigua* indicaram que a planta induziu relaxamento no

corpo cavernoso de coelhos (Antunes et al., 2001), que é um passo fundamental na ereção peniana.

2. OBJETIVOS GERAIS

Os objetivos deste estudo foram avaliar *in vitro*, a atividade antioxidante de *Trichilia catigua*, bem como seus potenciais efeitos neuroprotetores em fatias de hipocampo de ratos expostos à privação de oxigênio e glicose ou a diferentes pró-oxidantes.

Os objetivos específicos aparecem na introdução de cada capítulo da parte II desta tese.

3. MATERIAS E MÉTODOS

Esta seção já está incorporada em cada capítulo da parte II da presente tese.

PART II

Where the results are presented by chapter

Chapter I

IN VITRO ANTIOXIDANT ACTIVITY OF STEM BARK OF TRICHILIA CATIGUA ADR. JUSS.

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Original research paper

In vitro antioxidant activity of stem bark of *Trichilia catigua* Adr. Juss

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Antioxidant activity of the ethanolic extract and fractions from the stem bark of *T. catigua* was investigated. IC_{50} (for DPPH scavenging) by *T. catigua* varied from 9.17 ± 0.63 to 76.42 ± 5.87 μg mL^{-1} and total phenolic content varied from 345.63 ± 41.08 to 601.27 ± 42.59 μg GAE g^{-1} of dry extract. Fe^{2+}-induced lipid peroxidation was significantly reduced by the ethanolic extract and fractions. Mitochondrial Ca^{2+}-induced dichlorofluorescein oxidation was significantly reduced by the ethanolic extract in a concentration-dependent manner. Ethanolic extract reduced mitochondrial $\Delta\psi_m$ only at high concentrations (40–100 μg mL^{-1}), which indicates that its toxicity does not overlap with its antioxidant effects. Results suggest involvement of antioxidant activities of *T. catigua* in its pharmacological properties.

Keywords: Trichilia catigua (*Meliaceae*), antioxidant, flavonoids, phenolics, reactive oxygen species, oxidative stress

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Many plants contain substantial amounts of antioxidants such as vitamins C and E, carotenoids, flavonoids and tannins that can scavenge free radicals from the human body (1). Since ancient times, a high percentage of the populations of many developed countries have been using medicinal plants in the treatment of different pathologies, including neurodegenerative diseases in which free radical assaults are implicated in their etiology.

Trichilia catigua, is a plant used in Brazil as an aphrodisiac and neurostimulant. It exhibits a variety of pharmacological properties, including antidepressive and anti-inflammatory ones, and its use has been reported to be safe with no known side effects or toxicity in healthy human volunteers (2). Phytochemical reports on *T. catigua* indicated that the plant contains omega-phenyl alkanes, omega-phenyl alkanoic acids, omega-phenyl--gamma-lactones, alkyl-gamma-lactones, alkenyl-gamma-lactones, fatty acids, β-sitosterol, stigmasterol, campesterol, epicatechin, cinchonains (Ia, Ib, IIa, IIb), catiguanins A and B, procyanidins B2 and C1, tannins and a mixture of flavalignans (3, 4).

It is of particular pharmacological significance that many pathological processes in which *T. catigua* exerts its beneficial action can be associated with overproduction of reactive oxygen species (ROS) which can impair energy metabolism *via* oxidative changes in key mitochondrial components (5).

Considering the fact that *T. catigua* has been widely employed empirically in folkloric medicine in the management of free radical related diseases, and that there is little information in the literature about the potential antioxidant properties of *T. catigua*, we investigated whether the ethanolic extract and fractions of different polarities extracted from the stem bark of *T. catigua* exhibited *in vitro* antioxidant activity using chemical and biological models.

EXPERIMENTAL

Chemicals

All chemicals used, including solvents, were of analytical grade. 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Folin Ciocalteu's phenol reagent, malonaldehyde bis-(dimethyl acetal) (MDA), thiobarbituric acid, sodium dodecyl sulfate, ascorbic acid, 2',7'-dichlorofluorescein diacetate (DCFH-DA), Tris-HCl, ethylene glycol tetraacetic acid (EGTA), quercetin, rutin, chlorogenic acid and gallic acid were purchased from Sigma Chemical Co. (USA). 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), ferrous sulfate, mannitol and sucrose were obtained from Vetec (Brazil).

Plant collection and separation of the different fractions

Extract of *Trichilia catigua* bark was obtained from Ely Martins (Ribeirão Preto, São Paulo, Brazil), in 2007, registered under the number CAT-i0922 (Farm. Resp.: Ely Ap. Ramos Martins). The stem bark powder of *T. catigua* (100 g) was macerated at room temperature with 70 % ethanol and extracted for a week. On the seventh day, the combined ethanolic extract was filtered and the solvent was fully evaporated under reduced pres-

sure to give a brown solid (11.61 g). This was divided into two parts and one part was suspended in water and partitioned successively with dichloromethane, ethyl acetate and *n*-butanol (3 × 50 mL for each solvent). Dichloromethane was added to one part of the extract (1 : 1, *V*/*V*) and the mixture was allowed to remain at room temperature for 15 min. The solution was decanted and the solvent was evaporated to obtain the dichloromethane fraction (CH_2Cl_2, 1.98 g). The other fractions (ethyl acetate, AcOEt) and butanolic fraction (*n*-BuOH) were processed as described for the dichloromethane fraction and the quantities obtained after evaporation were 1.05 g and 1.52 g, respectively.

In this procedure, the extract was suspended 3 times with each solvent (3 × 50 mL). The fractions and EtOH extract were then diluted in ethanol in order to prepare different concentrations (10, 40, 100 and 400 μg mL^{-1}). *T. catigua* is normally used as a tea; consequently, hot and cold water extracts from *T. catigua* were tested to compare their antioxidant capacities with the ethanolic extract and fractions.

Animals

Male Wistar rats, weighing 270–320 g and aged from 2.5 to 3.5 months, from our own breeding colony (Animal House-holding, UFSM, Brazil) were kept in cages with free access to food and water in a room with controlled temperature (22 ± 3 °C) and in 12 h light/dark cycle. The protocol of this study has been approved by the Brazilian Association for Laboratory animal Science (COBEA).

Antioxidant assays

The free radical scavenging activity of the *T. catigua* extract was measured with the stable radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) in terms of hydrogen-donating or radicals scavenging activity. A solution of DPPH (0.3 mmol L^{-1}) in ethanol was prepared, and 100 μL of this solution was added to 20 μL of each fraction and ethanolic extract at different concentrations (10, 40, 100 and 400 μg mL^{-1}). Ethanol and ascorbic acid, at the same concentrations used for fractions and ethanolic extract, were used as negative and positive controls, respectively. After 30 minutes, absorbances were measured at 518 nm in an ELISA plate reader (TP-Reader, Brazil).

Analysis of phenolics

For the determination of total phenolic content, samples of the extract/fraction (10–400 μg mL^{-1}) were added to a test tube and the volume was adjusted to 1.4 mL with distilled water. Then, 0.2 mL of 10 % Folin-Ciocalteu reagent (diluted 1 : 1 with water) and 0.4 mL of sodium carbonate solution (7.5 %) were added sequentially to the test tube. The tubes were then incubated for 40 min at 45 °C and the absorbance was measured at 725 nm in a spectrophotometer (SP-2000UV, Biospectro, Brazil). The standard curve was prepared using 0, 1, 2.5, 5, 10 and 15 μg mL^{-1} solutions of gallic acid (0.1 mg mL^{-1}). Total phenol value was calculated and expressed as the microgram gallic acid equivalent (μg GAE g^{-1}) of dry extract.

In vitro *Fe^{2+}-induced lipid peroxidation in the brain*

Rats were decapitated; whole brain was dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 10 mmol L^{-1} Tris–HCl, pH 7.4 (1/10, mass/volume). The homogenate was centrifuged for 10 min at 3600 × *g* to yield a pellet, which was discarded, and a low-speed supernatant (S1) was used for the thiobarbituric acid reactive substances (TBARS) assay.

Aliquots of the brain homogenate and the pro-oxidant agent (10 μmol L^{-1} $FeSO_4$) were incubated for 1 h at 37 °C in the presence or absence of the *T. catigua* extract (10–20 μg mL^{-1}). To the reaction mixture, 8.5 % sodium dodecyl sulfate (SDS), acetic acid/HCl (pH 3.4) and 0.6 % thiobarbituric acid (TBA) were subsequently added and the mixture was incubated at 100 °C for 1 h. Lipid peroxidation (LPO) was measured by TBARS formation as described by Puntel *et al*. (6). Color was read at 532 nm using an ELISA plate reader. Standard curve of malondialdehyde (MDA) was used to quantify TBARS production in brain homogenates.

Quantification of phenolics and flavonoids by HPLC-DAD

The phenolics and flavonoids in the extract were quantified by reverse phase chromatographic analysis by the method described by Laghari *et al*. (7), with slight modifications. Reverse phase chromatographic analysis was carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5-μm diameter particles. The mobile phase was water containing 2 % acetic acid (A) and methanol (B), and the composition gradient was: 5 % (B) for 2 min; 25 % (B) until 10 min; 40, 50, 60, 70 and 80 % (B) every 10 min. All samples and the mobile phase were filtered through a 0.45-μm membrane filter (Millipore, USA) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031–0.250 mg mL^{-1} quercetin and rutin, and 0.006–0.250 mg mL^{-1} for gallic and chlorogenic acids. Quantification was carried out by integration of the peaks using the external standard method, at 257 nm for gallic acid, 325 nm for chlorogenic acid and 365 for quercetin and rutin. The flow rate was 0.8 mL min^{-1} and the injection volume was 40 μL. Chromatographic peaks were confirmed by comparing their retention time and diode-array-UV spectra with those of the reference standards. All chromatography operations were carried out at ambient temperature and in triplicate.

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated as previously described by Puntel *et al*. (8), with some modifications. The livers were rapidly removed (within 1 min) and immersed in ice-cold "isolation buffer I" containing in mmol L^{-1}: 225 manitol, 75 sucrose, 1 K^+-EGTA and 10 K^+-HEPES, pH 7.2. The tissue was minced using surgical scissors and then extensively washed. The tissue was then homogenized in a power-driven, tight-fitting Potter-Elvehjem (Reviglass, Brazil) homogenizer with a teflon pestle. The resulting suspension was centrifuged for 7 min at 2,000 × *g* in a Hitachi CR 21E centrifuge (Japan). The supernatant was centrifuged again for 10 min at 12,000 × *g*. The pellet was resuspended in "isolation buffer II" containing in mmol L^{-1}: 225 manitol, 75 sucrose, 1 K^+-EGTA (ethyleneglycol tetraacetic acid) and 10 K^+-HEPES [4-(2-hydroxyethyl)-1-piperazine ethane-

sulfonic acid], pH 7.2, and recentrifuged at 12,000 × g for 10 min. The supernatant was decanted, and the final pellet was gently washed and resuspended in respiration buffer containing in mmol L^{-1}: 100 sucrose, 65 KCl, 10 K^+-HEPES and 0.05 EGTA, pH 7.2, to a protein concentration of 0.6 mg mL^{-1}.

Determination of reactive oxygen species (ROS)

ROS production in isolated mitochondria was measured using a 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence probe. Mitochondrial suspensions (0.25 mg mL^{-1}) in respiration buffer containing 100 mmol L^{-1} sucrose, 65 mmol L^{-1} KCl, 10 mmol L^{-1} K^+-HEPES and 50 μmol L^{-1} EGTA, pH 7.2, were incubated with 10, 40, 100 μg mL^{-1} of the ethanolic extract *T. catigua* in the presence or absence of $CaCl_2$ (80 μmol L^{-1}) (13). Then, 3.33 μmol L^{-1} of DCFH-DA was added to the solution. The formation of the oxidized fluorescent derivative 2′,7′-dichlorofluorescain (DCF) was monitored using a spectrofluorimeter (Shimadzu RF-5301, Japan) with excitation and emission wavelengths of 488 and 525 nm, respectively, and with slit widths of 1.5 nm.

Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was estimated by fluorescence changes of safranine (5 μmol L^{-1}) recorded by a RF-5301 Shimadzu spectrofluorimeter operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 3 nm. Values of mitochondrial membrane potential ($\Delta\psi_m$) were expressed as the percent of control.

Protein estimation

Protein concentration was measured by the method of Lowry *et al*. (9), using bovine serum albumin (BSA) as a standard.

Statistical analysis

Results were expressed as mean ± SEM (standard error of mean). One-way or two-way ANOVA followed by Duncan's multiple range tests were utilized to evaluate the differences between the groups when appropriate. The data of cold and hot water extracts were compared using *t*-test. Pearson's correlation coefficient was calculated to determine the relationship between two variables.

RESULTS AND DISCUSSION

Various extracts from the stem barks of *T. catigua* scavenged DPPH radical in a concentration-dependent manner (Fig. 1a,b), which can be mediated by the different polyphenolic components found in these extracts. The total phenolic content of different crude extracts from *T. catigua* is given in Table I. The concentration varied from 345.63 μg GAE g^{-1} (in butanolic fraction) to 601.27 μg GAE g^{-1} (in ethyl acetate fraction) of plant

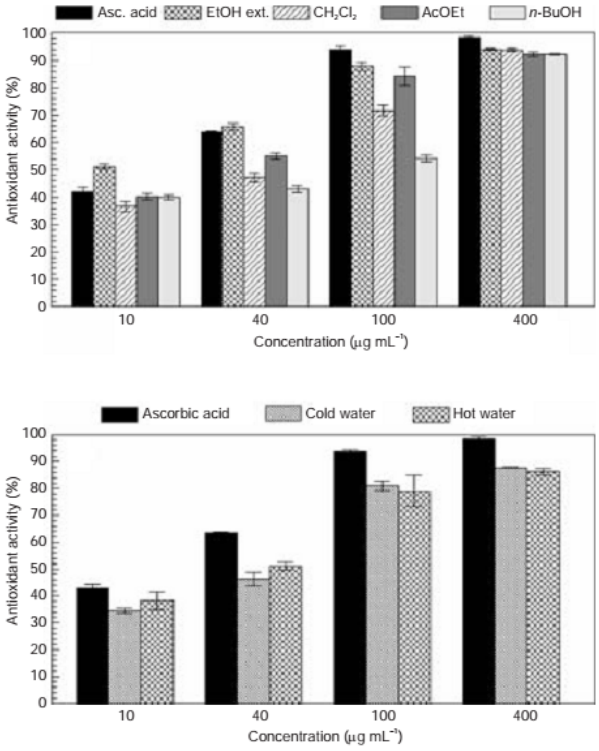

Fig. 1. Quenching of DPPH color by extracts from the stem barks of *T. catigua vs*. asorbic acid: a) ethanolic, ethyl acetate, dichloromethane and butanolic, b) aqueous (cold and hot water extracts). Mean ± SEM, n = 3–4 independent experiments.

extract. Surprisingly, we observed that the highest content of total phenol in ethyl acetate fraction did not correlate with the highest antioxidant activity evaluated by the DPPH assay. The effectiveness order of IC_{50} (the extract concentration required to inhibit 50 % of DPPH radical) for decolorizing DPPH was: EtOH extract (9.17 μg mL^{-1}) > AcOEt (30.28 μg mL^{-1}) > CH_2Cl_2 (42.42 μg mL^{-1}) > *n*-BuOH (76.35 μg mL^{-1}) (Table I).

Table I. Phenolics and flavonoids from different fractions of T. catigua *stem barks and their* IC_{50} *values (DPPH)*

	Reference (ascorbic acid)	EtOH extract	CH_2Cl_2	AcOEt	*n*-BuOH
Total phenolics (μg GAE g^{-1})	–	443.87 ± 22.23	594.03 ± 31.32	601.27 ± 42.59	345.63 ± 41.08
Gallic acid (mg g^{-1})	–	16.04 ± 1.68	1.90 ± 0.19	25.40 ± 0.30	0.90 ± 0.10
Chlorogenic acid (mg g^{-1})	–	27.30 ± 0.20	5.10 ± 0.30	14.90 ± 0.40	1.70 ± 0.30
Rutin (mg g^{-1})	–	7.90 ± 0.20	–	10.50 ± 0.20	2.80 ± 0.10
Quercetin (mg g^{-1})	–	14.2 ± 0.10	1.10 ± 0.20	23.70 ± 0.50	0.70 ± 0.40
IC_{50} (μg mL^{-1})	20.72 ± 1.30[a]	9.17 ± 0.63[a]	42.42 ± 4.92[a]	30.29 ± 1.37[a]	76.35 ± 5.92[a]

Mean ± SEM, n = 3–4.
[a] Significantly different from ascorbic acid (reference) (t-test; $p < 0.05$).

Several authors (10, 11) have described a positive correlation between phenolic content and antioxidant activity using similar assay systems. However, we have not observed such type of correlation. This could be explained by the fact that factors other than total phenolics can play a major role in the antioxidant activity of these extracts. Boligon *et al.* (12) and Kiliçgün and Altiner (13) found no correlation between phenolic content and antioxidant activity measured by various methods, either.

All the extracts exhibited a significant inhibitory effect on Fe^{2+}-induced TBARS production in brain homogenates ($p < 0.05$) and at 10 μg mL^{-1} a maximal inhibitory effect was attained for all the fractions (Fig. 2a). Similarly to what was observed with extracts obtained with organic solvents, cold and hot water extracts of *T. catigua* significantly inhibited Fe^{2+}-induced TBARS production in brain homogenates in a concentration-dependent manner (Fig. 2b) ($p < 0.05$).

Free Fe^{2+} can induce neurotoxicity *via* stimulation of the Fenton reaction and its levels are increased in some degenerative diseases. *T. catigua* extracts inhibited Fe^{2+}-induced lipid peroxidation in brain homogenates and this antioxidant effect can, at least partly, be associated with iron chelation. In fact, the chelating effects of some plant extracts could be attributed to the presence of flavonoids, which are well known to be chelator compounds. *T. catigua* extracts possess flavonoids, among which are quercetin and rutin (Fig. 3, Table I) that may form redox inactive complexes with Fe^{2+}, rendering this pro-oxi-

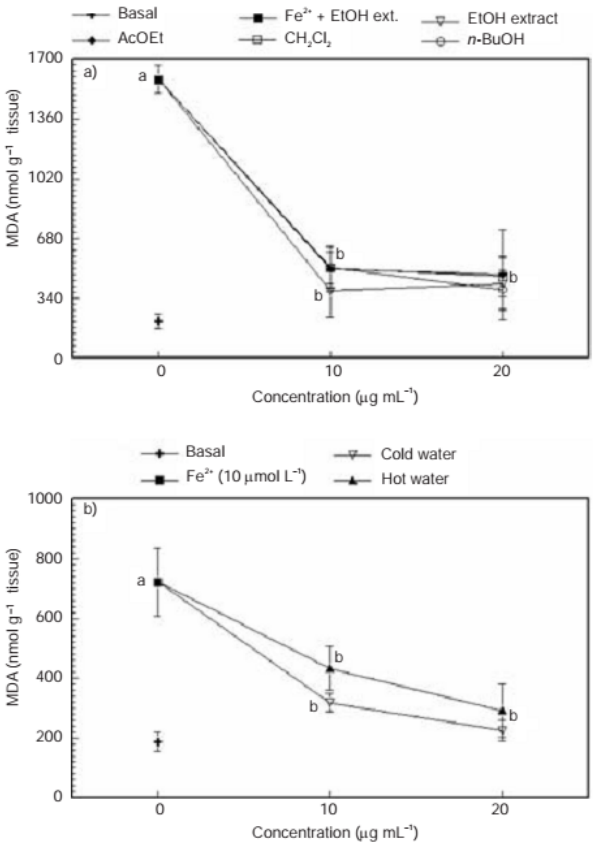

Fig. 2. Effects of (a) crude extracts and (b) aqueous extracts from the stem bark of *T. catigua* on Fe^{2+} (10 μmol L^{-1})-induced TBARS production in brain homogenates. The samples were incubated for 1 h with Fe^{2+} in the presence or absence of plant extracts (basal). Mean ± SEM, n = 3–4 independent experiments. Significant difference: a) $p < 0.05$ *vs*. basal, b) $p < 0.05$ *vs*. Fe^{2+} + ethanol (used as solvent), $p < 0.05$ *vs*. Fe^{2+} alone.

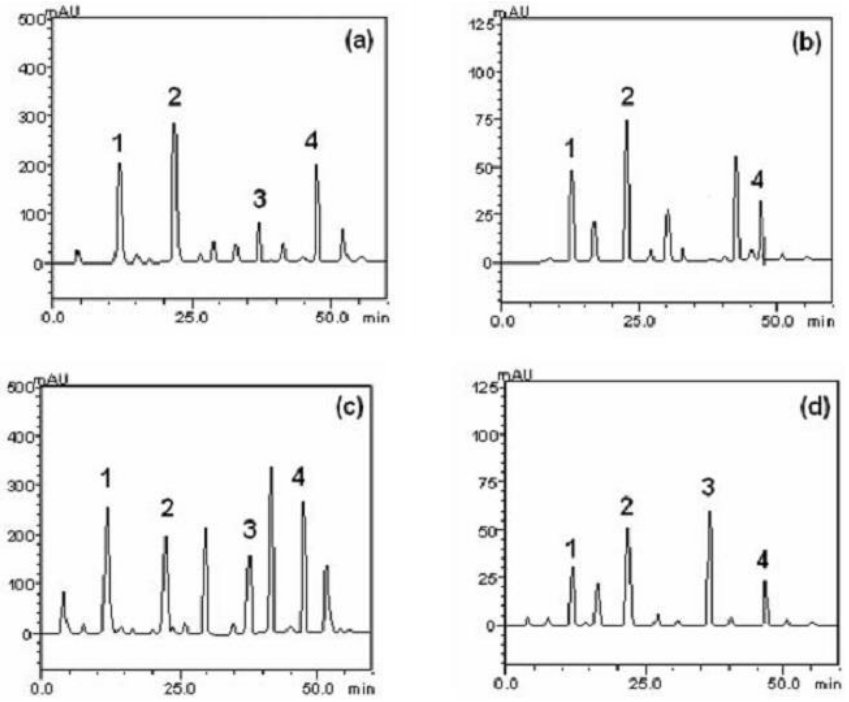

Fig. 3. High performance liquid chromatographic profile of phenolics and flavonoids in: a) ethanolic, b) dichloromethane, c) ethyl acetate, d) butanolic extract of *T. catigua*. Gallic acid (peak 1), chlorogenic acid (peak 2), rutin (peak 3) and quercetin (peak 4).

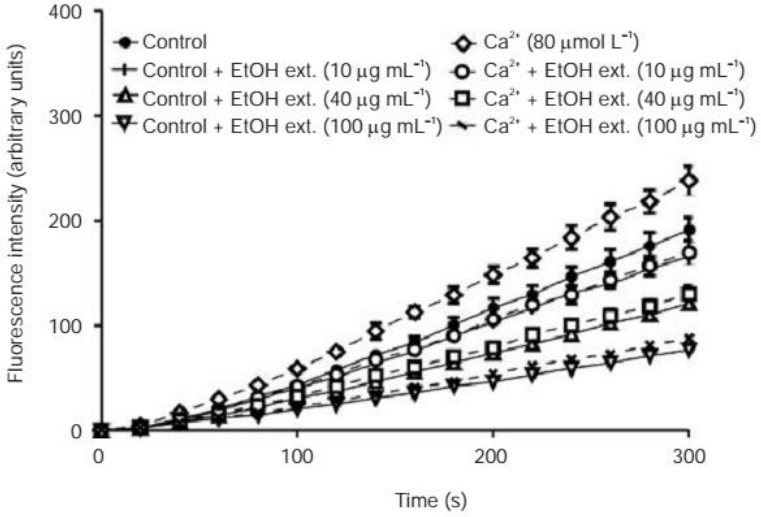

Fig. 4. Effect of calcium and ethanolic extract of *T. catigua* on rat liver mitochondrial DCFH oxidation. Mitochondria (0.25 mg protein mL^{-1}) were suspended in respiration buffer and mitochondrial ROS generation was determined by monitoring the fluorescence of DFCH oxidation (emission at 525 nm with excitation at 488 nm. Mean ± SEM, n = 4 independent measurements.

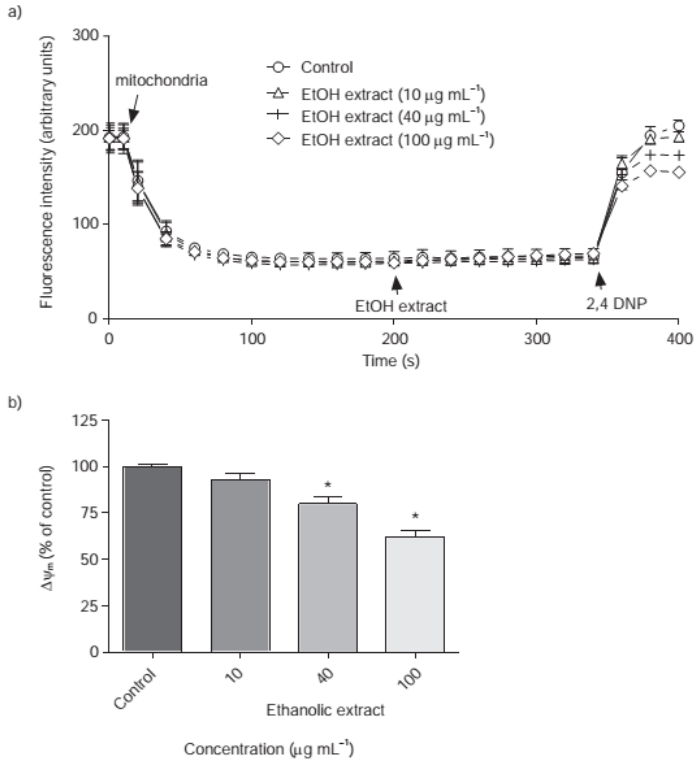

Fig. 5. Effect of ethanolic extract of *T. catigua* on mitochondrial membrane potential. Isolated rat liver mitochondria (0.6 mg mL^{-1}) were incubated in standard medium and the $\Delta\psi_m$ was monitored as described in experimental session. a) Effect of ethanolic extract (10–100 μg mL^{-1}) on mitochondrial membrane potential; b) values of $\Delta\psi_m$ after adding the mitochondrial uncoupler 2,4-dinitrophenol (2,4-DNP). The mitochondria (0.6 mg mL^{-1}), ethanolic extract or 2,4-DNP were added where indicated by arrows. Experiments were performed three times using independent mitochondrial preparation. Mean ± SEM, $n = 3$. * Significant difference *vs*. control: $p < 0.05$.

dant unavailable for Fenton reaction. Accordingly, quercetin and its glycoside form, rutin, effectively block Fe^{2+}-induced TBARS production in brain homogenates (14).

Mitochondrial oxidation of DCFH was stimulated by Ca^{2+} (80 μmol L^{-1}) and the ethanolic extract of *T. catigua* prevented ROS production stimulated by Ca^{2+} in a concentration-dependent fashion (Fig. 4). Substantial evidence in the literature has indicated that Ca^{2+} can increase mitochondrial oxidative stress (15). In line with this, Ca^{2+} increased the rate of DCFH oxidation compared to the control. Interestingly, the production of ROS induced by Ca^{2+} in the presence of ethanolic extract was not significantly different from those produced during basal conditions (control). This suggests that ROS production induced by Ca^{2+} was fully suppressed by the ethanolic extract, which is in accord with previous data from our laboratory indicating that quercetin, quercitrin and rutin protected brain mitochondria from Ca^{2+}-induced oxidative stress (16).

Ethanolic extract of *T. catigua* at high concentrations (40–100 μg mL^{-1}), produced a decrease in $\Delta\psi_m$ (~20 % and ~ 38 % depolarization, respectively) compared to the control

($p < 0.05$) whereas no effect was observed at 10 μg mL^{-1} (Figs. 5a,b). However, a partial decrease in $\Delta\psi_m$ can be associated with cardioprotection, which may be related to a reduction in mitochondrial ROS production (17). Consequently, the *in vitro* decrease in mitochondrial ROS production by *T. catigua* can be related to the partial depolarization of mitochondria.

CONCLUSIONS

Crude extracts from the stem bark of *T. catigua* have *in vitro* antioxidant activity in different chemical and biological models, which can be, in part, attributed to flavonoids and phenolic compounds present in the plant extracts. Taken together, our results indicate that *T. catigua* has promising compounds to be tested not only as potential antioxidant drugs for the treatment of diseases resulting from oxidative stress, but also for the use in different fields such as pharmaceuticals and cosmetics.

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S A Ž E T A K

In vitro antioksidativni učinak kore stabljike *Trichilia catigua* Adr. Juss

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U radu je opisano ispitivanje antioksidativnog učinka etanolnog ekstrakta i pojedinih frakcija kore stabljike *T. catigua*. IC_{50} (za DPPH test) varirao je između 9,17 ± 0,63 i 76,42 ± 5,87 μg mL^{-1}, a ukupni sadržaj fenola od 345,63 ± 41,08 i 601,27 ± 42,59 μg GAE po gramu suhog ekstrakta. Etanolni ekstrakt i frakcije značajno su reducirale Fe^{2+}-induciranu lipidnu peroksidaciju. Nadalje, reducirana je oksidacija diklorfluoresceina inducirana ionima kalcija u mitohondrijima, a redukcija je ovisila o dozi etanolnog ekstrakta.

Etanolni ekstrakt smanjio je mitohondrijsku $\Delta\psi_m$ samo pri visokim koncentracijama ($40 \pm 100\ \mu g\ mL^{-1}$), što ukazuje da se toksičnost ne preklapa s antioksidativnim učinkom. Rezultati pokazuju da u farmakološko djelovanje *T. catigua* treba uključiti i antioksidativni učinak.

Ključne riječi: *Trichilia catigua* (*Meliaceae*), antioksidans, flavonoidi, fenoli, reaktivne kisikove specije, oksidativni stres

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Chapter II

CATUABA (TRICHILIA CATIGUA) PREVENTS AGAINST OXIDATIVE DAMAGE INDUCED BY IN VITRO ISCHEMIA–REPERFUSION IN RAT HIPPOCAMPAL SLICES

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ORIGINAL PAPER

Catuaba (*Trichilia catigua*) Prevents Against Oxidative Damage Induced by In Vitro Ischemia–Reperfusion in Rat Hippocampal Slices

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Abstract Oxidative stress is implicated in brain damage associated with ischemia–reperfusion. Natural antioxidants found in some plants used in folk medicine have been indicated as potential neuroprotective agents. Here we investigated whether *Trichilia catigua*, a traditional Brazilian herbal medicine alleged to exhibit a variety of neuropharmacological properties (antidepressant, anti-neurasthenic, anti-inflammatory etc.), could have neuroprotective properties in rat hippocampal slices subjected to 2 h oxygen and glucose deprivation (OGD) followed by 1 h reperfusion. Ischemia–reperfusion (I/R) significantly decreased mitochondrial viability, increased dichlorofluorescein oxidation above control both in the incubation medium and slices homogenates, increased lactate dehydrogenase into the incubation medium and decreased non-protein thiols. *T. catigua* (40–100 μg/mL) protected slices from the deleterious effects of OGD when present before OGD and during the reperfusion periods. Oxidative stress in the medium was also determined under different conditions and the results demonstrated that *T. catigua* could not protect slices from I/R when it was added to the medium after ischemic insult. Although the translation to a real in vivo situation of I/R is difficult to be done, the results indicated that *T. catigua* should be used as preventive and not as a curative agent against brain damage.

Keywords *Trichilia catigua* · Hippocampus · Neuroprotection · Antioxidant · Oxidative stress

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Introduction

Cerebral ischemia caused by a reduction of oxygen and glucose supply to brain cells is associated with oxidative stress after blood reperfusion. The overproduction of free radicals/ROS normally causes mitochondrial dysfunction associated with mitochondrial Ca^{2+} overload and ATP depletion, which can secondarily lead to extensive lipid, protein and DNA molecules damage [1–6].

The cascade of neurotoxic events triggered by ischemia–reperfusion (I/R) can culminate in neural cell loss. Oxygen deprivation activates hypoxia inducing factor, a transcription factor that activates the expression of a number of genes involved in the adaptation of tissues to hypoxia [7, 8]. Furthermore, the xanthine dehydrogenase/xanthine oxidase conversion that occurs during reperfusion can

increase the oxidative stress in brain and consequently cause neuronal damage [9–14].

Recently, there has been an increased interest in natural antioxidants as potential remedies against ROS-mediated neurotoxicity associated with I/R in animal models [15–20]. As a rule, natural products empirically used in folk medicine are considered safe to human [21], whereas synthetics antioxidants can have unwanted side effects [22]. Of neuropharmacological significance, natural products or bioactive components with antioxidant [23, 24], anti-inflammatory [17] and neuroprotective [25–27] properties have been reported to exhibit preventive or therapeutic effects on experimental models of brain oxidative stress.

Trichilia catigua, a native plant from Brazil (also found in Argentina, Paraguay and Bolivia), member of the family of Meliaceae, is widely used as a neurostimulant and aphrodisiac. Moreover, it is used in folk medicine as a tonic for the treatment of neurasthenia (fatigue, stress, impotence, deficits of memory) [28, 29]. Accordingly, *T. catigua* crude extracts have also been reported to relax the corpus cavernosum [30] and to exhibit antioxidant [31], anti-microbial [32], antinociceptive [28], antidepressant [33–35] and anti-inflammatory [36] properties.

Although *T. catigua* has beneficial neurobehavioral effects in different experimental models, there are no available studies about the potential neuroprotective effect of *T. catigua* against cerebral ischemia. Thus, the present study was undertaken to investigate the effect of *T. catigua* on cerebral I/R-induced neuronal cell damage in rat hippocampal slices.

Materials and Methods

Chemicals

All chemicals used including solvent, were of analytical grade. 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), Tris–HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), dibasic phosphate potassium and monobasic phosphate potassium were obtained from Vetec (Rio de Janeiro, RJ, Brazil).

Plant Collection and Preparation

Extract of *Trichilia catigua* bark was purchased from Ely Martins (Ribeirão Preto, São Paulo, Brazil) in 2007, registered under the number CAT- i0922 (Farm. Resp.: Ely Ap. Ramos Martins). The powder of stem bark of *T. catigua* (100 g) was macerated at room temperature with ethanol 70 % and extracted for 1 week. On seventh day, the combined ethanolic extract was filtered and the solvent was fully evaporated under reduced pressure to give a brown solid (11.61 g) that was suspended in water and used in the experiments with hippocampal slices.

Quantification of Phenolics and Flavonoids Compounds by High Performance Liquid Chromatography (HPLC–DAD)

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 µm diameter particles; the mobile phase was water containing 2 % acetic acid (A) and methanol (B), and the composition gradient was: 5 % (B) for 2 min and changed to obtain 25, 40, 50, 60, 70 and 100 % (B) at 10, 20, 30, 40, 50 and 65 min, respectively, following by the method described by Laghari et al. [37] with slight modifications. The extract of *T. catigua* was analyzed, at a concentration of 5 mg/mL. The presence of phenolic acids (gallic and chlorogenic acids) and flavonoids (quercetin and rutin) was investigated. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.8 mL/min, injection volume 40 µL and the wavelength were 257 nm for gallic acid, 325 nm for chlorogenic acid, and 365 nm for quercetin and rutin. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031–0.250 mg/mL for quercetin and rutin; and 0.006–0.250 mg/mL for gallic and chlorogenic acids. All chromatography operations were carried out at ambient temperature and in triplicate.

Animals

Male Wistar rats weighing 270–320 g and with age from 2.5 to 3.5 months from our own breeding colony (Animal House-holding, UFSM, Brazil) were kept in cages with free access to foods and water in a room with controlled temperature (22 ± 3 °C) and in 12 h light/dark cycle. The protocol of this study has been approved by the guidelines of the Brazilian association for laboratory animal science (COBEA) of the Federal University of Santa Maria, Brazil (23081.002435/2007-16).

Oxygen-Glucose Deprivation and Treatment

Animals were killed by decapitation, the hippocampi were quickly dissected out and transverse sections (400 µm) were prepared using a McIlwain tissue chopper (Campden instruments). Hippocampal slices were divided into two equal sets (non-OGD and OGD-groups), and pre-treated for 30 min in the presence or absence of *T. catigua* (10–100 µg/

Table 1 Study design

Groups	Pre-treatment (0–30 min)	Ischemia (OGD) (30–150 min)	Reperfusion (150–210 min)
Control (Ctrl)	None	None	Yes
Ctrl-treated (10–100 μg/mL)	Yes	None	Yes
Ischemia (OGD)	None	Yes	Yes
OGD-treated (10–100 μg/mL)	Yes	Yes	Yes

mL) in an artificial cerebrospinal fluid (aCSF) containing (in mM): 120 NaCl, 0.5 KCl, 35 $NaHCO_3$, 1.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 Na_2HPO4, 10 D-glucose (pH 7.4) [38]. Control and OGD experiments were run concomitantly using three slices from the same animal per group in each plate. A total of 24 hippocampal slices from the same animal was used for one experiment. The study design is summarized in Table 1.

After pre-treatment with *T. catigua* (10–100 μg/mL), the medium in the OGD-groups was replaced with another aCSF free glucose. To mimic ischemic conditions, OGD slices were incubated at 37 °C in a chamber containing an anaerobic gas mixture (95 % Argon, 5 % CO_2) for 2 h (OGD period). Whereas control slices were incubated for 2 h at 37 °C with 95 % O_2/5 % CO_2. After OGD period, the medium from both control and OGD slices were removed and the two groups (non-OGD and OGD-groups) received medium with glucose. Slices were then incubated at 37 °C for 1 h (reperfusion period) in an incubator in the presence or absence of *T. catigua* (10–100 μg/mL).

Assessment of Mitochondrial Viability and Cellular Damage: MTT and LDH Assays

Mitochondrial viability assay was performed by the colorimetric 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. After 2 h OGD followed by 1 h reperfusion, 10 μL of MTT (5 mg/mL, for a final concentration of 50 μg/mL) was added and the plates were incubated for an additional 30 min at 37 °C. Only viable slices can reduce MTT into a purple formazan product that is soluble in dimethyl sulfoxide (DMSO) [39]. The optical density was measured using microplate reader (TP-Reader, Brazil) at 540 and 630 nm, and the net A540–A630 was taken as an index of cell viability [40]. After reperfusion, OGD-induced neuronal injury was quantified by the amount of Lactate Dehydrogenase (LDH) released in the incubation solution [41]. LDH activity was measured spectrophotometrically at 340 nm (Shimadzu Comp., UV-2450) using a commercial kit (LDH Liquiform, Labtest, Brazil).

Reactive Oxygen Species (ROS) Measurement

Levels of cellular oxidative stress were measured by using the fluorescent probes 2′,7′-dichlorofluorescein diacetate (DCFH-DA). At the end of OGD and reperfusion, 5 μM DCFH-DA was added to supernatants and kept in the dark. Samples were read after 1 h by measuring the formation of the fluorescence product of DCFH oxidation (i.e., DCF). Slices of each sample were homogenized and an aliquot was used in order to quantify ROS production in the slice homogenates. The DCF fluorescence was monitored using excitation and emission wavelengths of 488 and 525 nm, respectively (spectrofluorophotometer, Shimadzu RF-5301) as described by Wagner et al. [25].

Potential Prevention or Remediation by T. catigua of Ischemia/Reperfusion (I/R)—Induced Oxidative Stress

In view of the fact that the presence of *T. catigua* in the pre-incubation and reperfusion periods afforded protection against I/R, we decided to study whether the extract could prevent (i.e., present during only pre-incubation (PRE group)) or remedy the effect of ischemia (i.e., present only during reperfusion (REP group)) on DCFH oxidation determined in the incubation medium. For this set of experiment, only the concentration of 40 μg/mL was used. In addition, *T. catigua* was also present only during OGD (OGD group), present during pre-incubation + OGD (PREOGD group), pre-incubation + reperfusion (PREREP group), as described below.

Different conditions	*T. catigua* added to the medium		
	Pre-incubation (30 min)	OGD (2 h)	Reperfusion (1 h)
Control	−	No	− −
Ischemia (OGD)	−	Yes	− −
PRE	+	Yes	− −
OGD	−	Yes	+ −
REP	−	Yes	− +
PREOGD	+	Yes	+ −
PREREP	+	Yes	− +

+ indicate the presence of *T. catigua* in the incubation medium, − indicate the absence of *T. catigua* in the incubation medium. Apart from the control slices, the other groups were subjected to OGD as described in study design (see Table 1)

Tissue Homogenization and Non-protein Thiols (NPSH) Assays

Hippocampal slices from each sample were homogenized in 200 µL of 10 mM Tris–HCl, pH 7.4 and 10 µL of this was used for the assay. NPSH was determined by the method of Ellman [42] with slight modifications. Briefly, a total of 50 µL of tissue homogenates was precipitated with 50 µL of 10 % TCA (1:1 v/v) and then, the samples were subjected to centrifugation at 3,000×*g* at 4 °C for 10 min. After the centrifugation, the protein pellet was discarded and free -SH groups were determined in the clear supernatant. The assay mixture contained 170 µL of potassium phosphate buffer (1 M, pH 7.4), 20 µL of clear supernatant and 10 µL DTNB (10 mM). The yellow color that developed was read at 412 nm in ELISA microplate reader (TP-Reader, Brazil). The NPSH levels was calculated as µmol NPSH/mg of protein and the protein concentrations were determined by the method of Bradford [43] using bovine serum albumin (BSA) as standard.

Statistical Analysis

Values were expressed as mean ± SEM (standard error of mean). Unless otherwise stated, data were analyzed using two-way ANOVA and main effect of ischemia (OGD), *T. catigua* extract concentrations and interaction extract concentrations OGD versus *T. catigua* extract concentrations is presented only in the figure legends (when they were significant). Data of ROS production evaluated in the incubation medium under different conditions of treatment with plant extract were analyzed using one-way ANOVA followed by Duncan′s test. The results were considered statistically significant for $p < 0.05$.

Results

HPLC Analysis

The flavonoids (quercetin and rutin) and phenolics acids (gallic and chlorogenic acids) were identified by comparisons to the retention times and UV spectra of authentic standards analyzed under identical analytical conditions. The chromatogram of the barks of *T. catigua* extract revealed the presence of the gallic acid (retention time, $t_R = 13.05$ min, peak 1) chlorogenic acid ($t_R = 22.67$ min, peak 2), rutin ($t_R = 38.17$ min, peak 3) and quercetin ($t_R = 47.02$ min, peak 4) (Fig. 1). The quantification of quercetin, rutin, gallic acid and chlorogenic acid by HPLC–DAD is presented in Table 2. The results with ethanolic extract suspended in water were very similar to those published recently by Kamdem et al. [44] using a similar extraction with ethanol, excepting that the dried extract was suspended in ethanol.

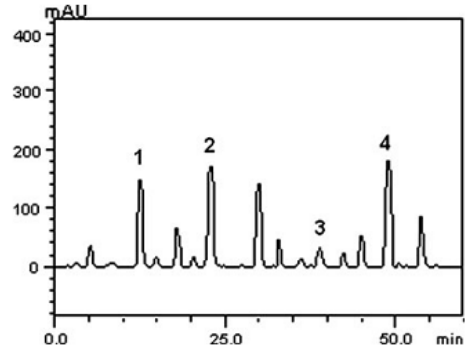

Fig. 1 High performance liquid chromatography phenolics and flavonoids profile of ethanolic extract of the bark of *T. catigua* extract. Gallic acid (peak 1), chlorogenic acid (peak 2), rutin (peak 3) and quercetin (peak 4). The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–500 nm). Calibration curve for gallic acid: Y = 32761x + 1189.3 (r = 0.9989); chlorogenic acid: Y = 47609x + 1394.5 (r = 0.9978); rutin: Y = 37421x + 1046.7 (r = 0.9999) and quercetin: Y = 41805x + 1241.0 (r = 0.9976). All chromatography operations were carried out at ambient temperature and in triplicate

Table 2 Quantification of some phenolics and flavonoids from the barks of *T. catigua* by HPLC–DAD

Compounds	*T. catigua* (mg/g)
Gallic acid	13.69 ± 0.2
Chlorogenic acid	14.13 ± 0.1
Rutin	3.85 ± 0.4
Quercetin	16.27 ± 0.3

T. catigua Protects Hippocampal Mitochondria from OGD

Figure 2 shows that *T. catigua* when it was present during pre-incubation and reperfusion periods significantly protected against OGD-induced mitochondrial damage. Apparently, *T. catigua* extract by itself did not have any significant effect on mitochondrial viability (Fig. 2, non-OGD). On the other hand, when hippocampal slices were subjected to 2 h OGD followed by 1 h of reperfusion, there was a 52.3 % decrease in cell viability when compared to control ($p < 0.001$). However, pre-incubation of slices with *T. catigua* extract before OGD period and also during the reperfusion period protected hippocampal slices from OGD-induced mitochondrial damage assessed by MTT reduction. No significant difference was observed when the

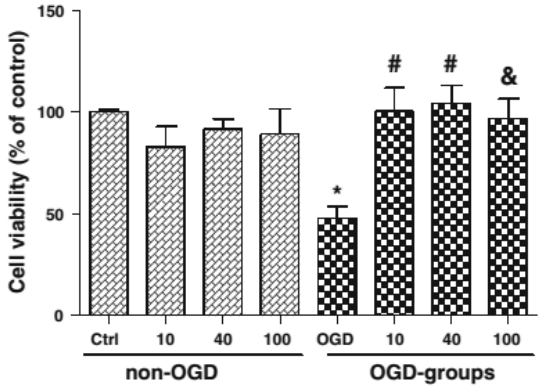

Fig. 2 Effects of different concentrations of *T. catigua* extract on mitochondrial viability. Results are expressed as percentages of the control (*Ctrl*). Values shows mean ± SEM for four independent experiments performed in duplicate. (*) represent significant difference from untreated slices (control) at $p < 0.001$; (#) and (&) represent significant difference from slices subjected to OGD at $p < 0.001$ and 0.01 respectively. Two-way ANOVA yielded a significant interaction between OGD condition versus plant extract concentration. As can be observed, OGD decreased MTT reduction and this was blunted by *T. catigua* extract

mitochondrial viability from non-OGD versus OGD-treated taken at the same concentration was compared.

Effect of *T. catigua* Extract on ROS Production

OGD caused a significant increase in DCF fluorescence in the medium when compared to the fluorescence found in the medium obtained from control slices (Fig. 3a; $p < 0.001$). The presence of *T. catigua* extract (10–100 µg/mL) during the pre-incubation prior to OGD period and also during reperfusion significantly attenuated the increase in DCF fluorescence in a concentration-dependent manner. Additionally, *T. catigua* at the highest concentration tested (100 µg/mL) significantly reduced ROS production in the medium obtained from slices maintained under basal condition ($p < 0.001$).

Similar to the effects observed in the incubation medium, *T. catigua* crude extract (40–100 µg/ml) present during pre-incubation before OGD period and also during reperfusion significantly decreased DCF Fluorescence in slice homogenates. In control slices, plant extract did not modify DCFH oxidation rate (Fig. 3b).

Comparative Potential Remediation or Protection by T. catigua Treatment Against Brain Ischemia

To further investigate the full potential curative or protective effect of *T. catigua*, ROS production was evaluated in the incubation medium after different conditions of treatment with plant extract. Here we have used only the

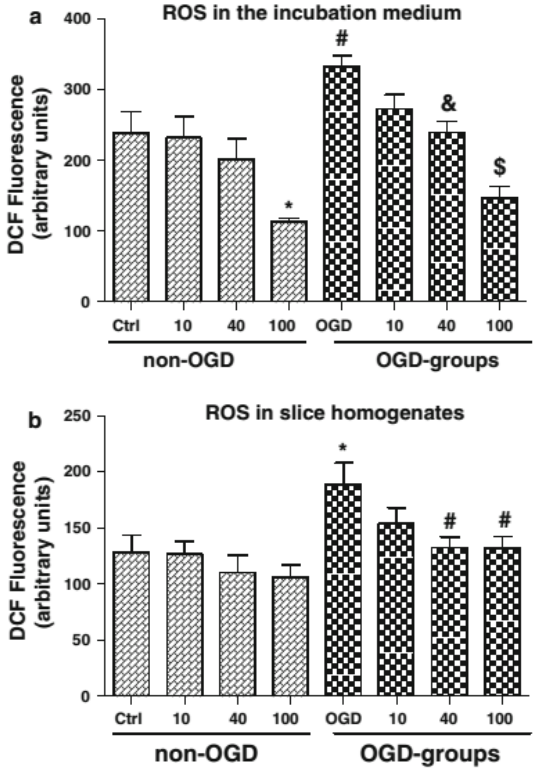

Fig. 3 **a** Effect of *T. catigua* extract on OGD-induced ROS production in the incubation medium. Data of ROS levels are presented as fluorescence intensity emission. 5 µM DCFH-DA was added to the incubation medium after 1 h of reperfusion, and DFC fluorescence was measured 1 h after incubation. Data are expressed as mean ± SEM of four independent experiments. (*) and (#) represent significant difference from untreated slices (control) at $p < 0.001$; (&) and ($) represent significant difference from slices subjected to OGD at $p < 0.05$ and 0.001 respectively. Two-way ANOVA indicated a significant main effect of ischemia condition and of plant extract concentration. As can be observed in the figure, OGD increased DFCH oxidation, whereas plant extract caused a decrease in oxidative stress. **b** Effect of *T. catigua* extract on OGD-induced ROS production in slices homogenates. Data are expressed as mean ± SEM of four independent experiments. After 2 h of OGD followed by 1 h of reperfusion, slices were homogenized and suspended in aCSF buffer and 5 µM of DCFH-DA was added to the solution. The formation of the oxidized fluorescent derivative (DCF) versus time was expressed in *bars*. (*) represent significant difference from untreated slices (control) at $p < 0.05$; (#) represent significant difference from slices subjected to OGD at $p < 0.05$. Two way ANOVA of data from Fig. 3b yielded a significant main effect of ischemia condition and a significant interaction between this factor and *T. catigua* concentration. As can be observed, OGD (I/R) increase DCFH oxidation, whereas plant extract blunted the increase in oxidative stress production

concentration of 40 µg/mL (which presented a significant protection against OGD, Figs. 2, 3a, b). We decided to determine the ROS in the medium because we realized that

Table 3 Effect of *T. catigua* on ROS production evaluated in the incubation medium under different conditions

Different conditions	*T. catigua* (40 µg/mL) added in the medium			ROS production (a.u)
	Pre-incubation	OGD	Reperfusion	
Control	−	−	−	256.83 ± 13.65
Ischemia (OGD)	−	−	−	390.76 ± 8.29*
PRE	+	−	−	290.68 ± 11.83#
OGD	−	+	−	280.20 ± 12.19#
REP	−	−	+	448.08 ± 24.52*
PREOGD	+	+	−	254.05 ± 18.52#
PREREP	+	−	+	300.80 ± 7.34#

The results are the mean ± SEM of 4 independent experiments. + indicate the presence of *T. catigua*; − indicate the absence of *T. catigua*. Apart from the control slices, the other groups were subjected to OGD as described in study design (see Table 1). * $p < 0.001$ represent significant difference versus control (without extract), # $p < 0.001$ versus OGD (without extract) as determined by one-way ANOVA followed by Duncan's multiple test

it most likely reflect differences in intracellular oxidative stress. In addition, by evaluating it in the incubation medium, the results can be more consistent, since there is no manipulation of slices. The results indicated that *T. catigua* extract did not protect slices when it was added to the incubation medium after ischemic insult (REP group only) when compared to OGD ($p > 0.05$, Table 3). In fact, when present only during reperfusion, 40 µg/mL of *T. catigua* has a tendency to increase DCFH oxidation (Table 3). As observed in Table 3, *T. catigua* protect hippocampal slices from I/R-induced oxidative stress, when the extract was present during pre-incubation and reperfusion (PREREP group). Here we have observed that *T. catigua* extracts present only during the OGD period (OGD group) or during the pre-incubation plus OGD period (PREOGD group) also caused a reduction in the oxidative stress induced by I/R (Table 3).

Effect of *T. catigua* Extract on Slices Integrity

The influence of OGD and/or *T. catigua* on the release of LDH from hippocampal slices is presented in Fig. 4. OGD increased significantly the amount of LDH released in the medium when compared with control slices ($p < 0.01$). However, *T. catigua* (40–100 µg/mL) present during pre-incubation before OGD period and also during the incubation period upon reperfusion markedly attenuated LDH release in slices either under the influence of OGD treatment. The effect of *T. catigua* against I/R-induced LDH release was concentration dependent and 10 µg/mL of *T. catigua* did not protect against OGD-induced neurotoxicity.

Effect of *Trichilia catigua* on Non-protein Thiol (NPSH) Contents

NPSH was significantly decreased after 2 h of OGD followed by 1 h reperfusion in comparison with the levels found in control slices (Fig. 5, $p < 0.05$). The presence of *T. catigua* extract (10–100 µg/mL) during the pre-incubation prior to OGD period and also during the incubation upon reperfusion significantly increased NPSH level in a concentration-dependent manner. Apart of concentration 100 µg/mL, no significant difference was observed when NPSH levels from non-OGD versus OGD slices treated with 10 and 40 µg/mL were compared.

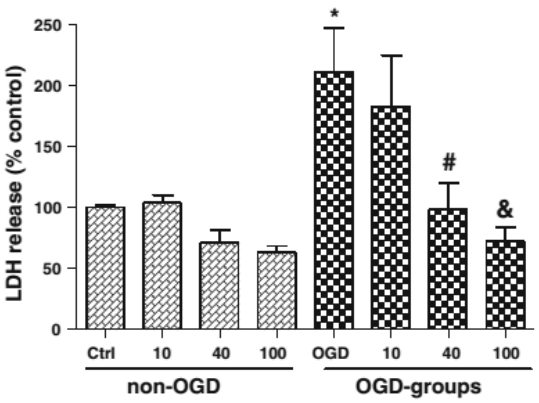

Fig. 4 Effect of *T. catigua* extract on OGD-induced LDH release. Results are expressed as percentages of the control. Columns represent mean ± SEM for four independent experiments. (*) represent significant difference from untreated slices (control) at $p < 0.01$; (#) and (&) represent significant difference from slices subjected to OGD at $p < 0.01$ and $p < 0.001$ respectively. LDH release in the presence of *T. catigua* extract under ischemic conditions (OGD-treated slices) was not significantly different from LDH released under basal condition (non-OGD), when the same concentration of plant extract was compared between control and OGD groups. Two way ANOVA yielded a significant main effect of ischemia condition (i.e., OGD followed by I/R were associated with an increase in LDH release) and a significant ischemia condition vs plant extract concentrations because *T. catigua* concentrations reduced the release of LDH only after I/R and not under basal condition (non-OGD groups)

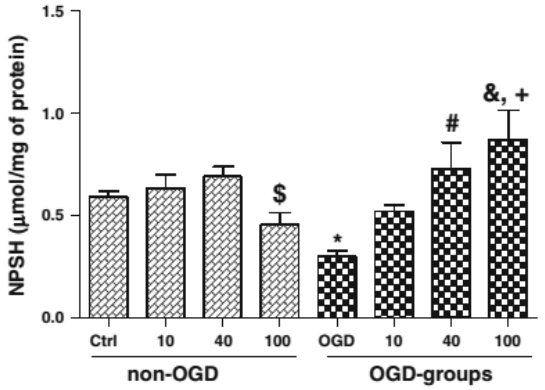

Fig. 5 Effect of *Trichilia catigua* extract on NPSH contents in rat hippocampal slices after 2 h of OGD followed by 1 h of reperfusion. Results are expressed as mean ± SEM for four independent experiments. (*) represent significant difference from untreated slices (control) at $p < 0.05$; (#) and (&) represent significant difference from slices subjected to OGD at $p < 0.01$ and $p < 0.001$ respectively; $ represent significant difference from non-OGD-treated (100 µg/mL); $^{+}p < 0.05$ versus non-OGD at 100 µg/mL. Apart of concentration 100 µg/mL, no significant difference was observed when comparing the same *T. catigua* concentrations on NPSH levels from non-OGD versus OGD-groups. Two way ANOVA indicated a significant interaction between ischemia condition and *T. catigua* extract concentration. As it can be seen in figure, under basal condition the highest plant extract concentration decrease hippocampal slice NPSH levels, whereas after I/R (OGD-groups) *T. catigua* extract caused a concentration dependent increase in NPSH

Discussion

Development of protective agents from traditional herb medicine has been considered a promising strategy to potentially treat cerebral ischemia injury and related neurodegenerative diseases [18, 27]. In this context, the neuroprotective effect of *T. catigua* extract was assessed in model of in vitro ischemia in rat hippocampal slices which is widely used in the literature to study the neuronal damage associated with I/R [45–47]. To decrease ischemia–reperfusion (I/R) injury, some studies tested the drug only before ischemia (i.e., for prevention) or after reperfusion (i.e., for remedy), but others have given the drug for both ischemia and reperfusion [40, 48–50]. In our study, we used *T. catigua* (10–100 µg/mL) before ischemia and during reperfusion. Our aim was to determine a possible prophylactic effect before ischemic insult and to detect the protective effect on immediate damage that will occur after reperfusion. Our results indicate that *T. catigua* (40–100 µg/mL) protected slices from the deleterious effects of OGD when present before OGD and during the reperfusion periods. To further investigate its potential protective effect, *T. catigua* was added to the medium during only pre-incubation (PRE), OGD, reperfusion (REP) and pre-incubation + OGD (PREOGD). Interestingly, in all these conditions tested, *T. catigua* was able to protect slices from ischemic insult, excepting when it was added to the medium during only reperfusion period (REP) (Table 3).

Consistent with previous studies, our data demonstrate that the exposure of hippocampal slices to OGD followed by reperfusion, resulted in increased LDH release both in the incubation medium and in slices homogenates when compared to control, which is a consequence of cell damage or death [41, 51]. This cell damage is related to the high vulnerability of hippocampus to oxidative stress induced by OGD [52, 53]. In fact, the results obtained here demonstrated an increase in ROS generation caused by OGD and *T. catigua* extract could not protect slices from OGD when it was added to the incubation medium only after ischemic insult (REP). But, slices were protected from ischemic insult when they were pre-treated with *T. catigua* before or during exposure to OGD. This result suggest that, *T. catigua* may be used preventively against neurotoxic effect of ischemic insult (and perhaps against other neuropathologic conditions), but not as remedy for the treatment of brain ischemia.

ROS are normally produced during normal metabolism, however, under pathologic conditions such as I/R, their overproduction lead to oxidative stress, causing cell damage to nervous tissue, which can lead to proteins and DNA oxidation, promoting chain reactions of membrane lipid peroxidation, and/or alterations in membrane fluidity [4, 5, 54–56]. Mitochondrion is the main source of ROS production in the cell and mitochondrial damage caused by I/R condition can lead to an increase in ROS generation [3, 5, 57–59]. Consequently, the results presented here suggest that OGD and reperfusion-induced neurotoxicity in hippocampal slices is, at least in part, a result of increased free radical production, and that the neuroprotective effect of *T. catigua* is linked to its ability to counteract ROS cytotoxicity.

The overproduction of ROS can be detoxified by endogenous antioxidants, causing their cellular stores to be depleted [52]. Glutathione, which is the most important intracellular non-protein thiol (NPSH), has a crucial role as a ROS scavenger [60, 61]. In the current work, NPSH was significantly reduced due to I/R and *T. catigua* (40–100 µg/mL) present before ischemia and during reperfusion periods significantly elevated NPSH up to control levels in a concentration-dependent manner.

One has to bear in mind that crude extracts are complex mixtures of compounds, in which only one or a few may be relevant for the observed activity. Therefore, the protective effect of *T. catigua* may be the result of the synergic action of compounds from this plant extract. Following this line of evidence, when the compounds catiguanin A and cinchonain Ia (among other compounds) isolated from the bark of *T. catigua* were tested for their neurotrophic

activities (i.e., ability to induce neurite outgrowth and to attenuate serum withdrawal-induced cytotoxicity) by using PC12 cells, both compounds had no effect on PC12 cells and NGF (Nerve Growth Factor)-mediated PC12 cells [62]. Although we have not isolated the compounds responsible for the neuroprotective activity, we speculate that it may be related to the flavonoids and phenolic compounds in the plant extract [31, 62–65] (Fig. 1; Table 2).

In conclusion, *T. catigua* offered significant neuroprotection in cerebral I/R in in vitro model. This neuroprotective effect may be attributed to the reduction in ROS generation, which was associated with a reduction in LDH release, preservation of slices reducing ability of MTT and NPSH slices levels. Additional work is required to better understand the mechanism by which *T. catigua* exerts its neuroprotection. Nevertheless, effects reported here are consistent with the medicinal use of this plant in traditional Brazilian medicine. However, *T. catigua* should be considered as preventive and not as curative agent against neuropathological situations (including ischemic insult and perhaps other neurological conditions associated with oxidative stress).

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Conflict of interest The authors declare no conflict of interest with any person or other organization.

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Chapter III

TRICHILIA CATIGUA (CATUABA) BARK EXTRACT EXERTS NEUROPROTECTION AGAINST OSIDATIVE STRESS INDUCED BY DIFFERENT NEUROTOXIC AGENTS IN RAT HIPPOCAMPAL SLICES

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Trichilia catigua (Catuaba) Bark extract exerts Neuroprotection against Oxidative Stress induced by different Neurotoxic agents in Rat Hippocampal Slices

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Abstract

Plant extracts have been reported to prevent various diseases associated with oxidative stress. *Trichilia catigua*, a traditional Brazilian herbal medicine, exhibits beneficial behavioral effects in experimental models of neuropathologies and protects rat hippocampal slices from oxidative stress induced by ischemia-reperfusion injury. In the present study, we investigated the protective effects of *T. catigua* against hydrogen peroxide (H_2O_2)-, sodium nitroprusside (SNP)-, and 3-nitropropionic acid (3-NPA)-induced neurotoxicity in rat hippocampal slices. Exposure of rat hippocampal slices to H_2O_2, SNP or 3-NPA (150-500 μM) for 1 h caused significant decrease in cellular viability (evaluated by MTT reduction), increased reactive oxygen/nitrogen species in the incubation medium as well as lipid peroxidation in slices homogenates. Pre-treatment of slices with *T. catigua* (10-100 μg/mL) for 30 min significantly attenuated the toxic effects of pro-oxidants. Phytochemical profile of *T. catigua* determined by high performance liquid chromatography (HPLC-DAD) indicated the presence of phenolic and flavonoid compounds. These antioxidant compounds can be involved in *T. catigua* neuroprotective effects. Consequently, *T. catigua* antioxidative properties may be useful in the prevention of cellular damage triggered by oxidative stress found in acute and chronic neuropathological situations.

Keywords: Catuaba; hippocampal slices; oxidative damage; polyphenol; *Trichilia catigua.*

1. Introduction

Oxidative stress is an imbalance between the production of reactive oxygen/nitrogen species (ROS/RNS) and endogenous antioxidants defenses. It has been implicated in the pathophysiology of several neurodegenerative disorders (ex. Alzheimer´s disease, Parkinson´s disease, Huntington´s disease, amyotrophic lateral sclerosis and ischemia-reperfusion) (Emerit et al., 2004; Qureshi et al., 2004; Mariani et al., 2005; Reynolds et al., 2007; Tsang and Chung, 2009; Melo et al., 2011) which can be associated with progressive loss of neurons, and cognitive performance (Coyle and Puttfarcken, 1993; Olanow, 1993; Sen and Chakraborty, 2011). Different mechanisms have been implicated in the pathogenesis of these diseases such as "mitochondrial oxidative stress" and "inflammatory oxidative conditions" (Hirsch et al., 2005; Trushina and McMurray, 2007; Amor et al., 2010; Taylor et al., 2013).

Hydrogen peroxide (H_2O_2), sodium nitroprusside (SNP) and 3-nitropropionic acid (3-NPA) are extensively used in the literature to trigger oxidative stress (Zhang and Zhao, 2003; Ou et al., 2010; Túnez et al., 2010; Sani et al., 2011). H_2O_2 is a highly diffusible ROS molecule formed during normal metabolism. In the presence of transition metals such as iron (II), H_2O_2 can be transformed into hydroxyl radicals, which initiates oxidative damage. SNP can cause oxidative stress and cytotoxicity either by releasing cyanide, iron and nitric oxide (NO) which can generate peroxynitrite radical (Boullerne et al., 1999; Broderick et al., 2007; Cardaci et al., 2008). Peroxynitrite can cause protein nitration and together with iron trigger lipid peroxidation (Ischiropoulos et al., 1992). 3-NPA, a rarely distributed plant and fungal neurotoxin, is an irreversible inhibitor of the mitochondrial complex II succinate dehydrogenase (SDH), which can induce neuronal degeneration *in vitro* and *in vivo* (Wiegand et al., 1999; Huang et al., 2006).

Accordingly, it has been reported that treatment with 3-NPA causes anatomical and neurological changes similar to those present in Huntington´s disease patients (Beal et al., 1993; Brouillet et al., 2005; Tasset et al., 2009; Túnez et al., 2010).

Search for natural products as potential useful exogenous or as stimulating of the endogenous cellular antioxidant defense mechanisms is gaining much interest. One of such plants is *Trichilia catigua*, commonly known as "catuaba" or "catiguá". *T. catigua* is found in the South America (Brazil, Argentina, Paraguay and Bolivia) and is widely used as a neurostimulant, anti-neurasthenic and aphrodisiac. In effect, *T. catigua* exhibits a variety of beneficial behavioral effects in models of depression and nociception (Campos et al., 2005; Viana et al., 2009; Chassot et al., 2011; Taciany et al., 2012) and it protects rat hippocampal slices from oxidative stress induced by ischemia-reperfusion injury (Kamdem et al., 2012b).

Considering the importance of oxidative stress in the pathogenesis of various diseases of the central nervous system (CNS) and the potential of plant extracts in preventing and/or treating such diseases, the present study was undertaken to determine whether *T. catigua* offered neuroprotection against H_2O_2-, SNP- and 3-NPA-induced neurotoxicity in hippocampal slices from rats. Furthermore, antioxidant phytochemicals from plant extracts that could be involved in the neuroprotection of *T. catigua* against these neurotoxic agents were investigated.

2. Materials and Methods

2.1. Chemicals

All chemicals including solvents were of analytical grade. Sodium nitroprossude (SNP), 3-Nitropropionic acid (3-NPA), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2´,7´-dichlorofluorescein diacetate (DCFH-DA) and malonaldehydebis-(dimethyl acetal) (MDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) and thiobarbituric acid (TBA) were purchased from vetec (Rio de Janeiro, RJ, Brazil).

2.2. Plant collection and preparation

T. catigua bark extract was purchased from Ely Martins (Ribeirão Preto, São Paulo, Brazil) in 2007, registered under the number CAT- i0922 (Farm. Resp.: Ely Ap. Ramos Martins). The powder of stem bark of *T. catigua* (100 g) was macerated at room temperature with 70 % ethanol and extracted for a week. On the 7^{th} day, the combined ethanolic extract was filtered and the solvent was fully evaporated under reduced pressure to give a brown solid (11.61 g) that was suspended in water and used in the experiments.

2.3. Quantification of phenolics and flavonoids compounds by high performance liquid chromatography coupled with diode array detector (HPLC-DAD)

Reverse phase chromatography analyses were carried out under gradient conditions using a Phenomenex C-18 column (4.6 mm x 150 mm) packed with 5 μm diameter particles. The mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and the composition gradient was: 13% of B until 10 min and changed to obtain 20%, 30%, 50%, 60%, 70%, 20% and 10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively (Ozturk et al., 2009; Boligon et al., 2012). *T. catigua* extract was analyzed

in the concentration of 5 mg/mL. The presence of phenolics and flavonoids compounds was investigated, namely, gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, ellagic acid, catechin, rutin, quercetin, and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.7 mL/min, injection volume 50 μL and the wavelength were 254 nm for gallic acid, 280 for catechin, 325 nm for chlorogenic, caffeic, rosmarinic and ellagic acids, and 365 nm for rutin,quercetin and kaempferol. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.020 - 0.200 mg/mL for catechin, quercetin, rutin and kaempferol; and 0.030 - 0.250 mg/mL for gallic, chlorogenic, caffeic, rosmarinic and ellagic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 500 nm). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by ICH (2005). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.4. Neurotoxic agents

Hydrogen peroxide (H_2O_2), sodium nitroprusside (SNP) and 3-nitropropionic acid (3-NPA) were used as neurotoxic agents in the study.

2.5. Animals

Male Wistar rats weighing 280–320 g and with age from 2.5 to 3.5 months from our own breeding colony (Animal House-holding, UFSM, Brazil) were kept in cages with free access to foods and water in a room with controlled temperature (22± 3°C) and in 12 h light/dark cycle. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil (23081.002435/2007-16).

2.6. Brain slices preparation and treatment

Animals were sacrificed by decapitation; the hippocampi were quickly dissected out and placed in cold artificial cerebrospinal fluid (aCSF) containing (in mM): 120 NaCl, 0.5 KCl, 35 $NaHCO_3$, 1.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 Na_2HPO4, 10 D-glucose (PH 7.4). Transverse sections of 400 μm were obtained using a McIlwain tissue chopper (Campden instruments). Hippocampal slices (3-5 per group in each plate) were pre-incubated in the presence or absence of *T. catigua* (10-100 μg/mL) for 30 min at 37°C, and then exposed to the neurotoxic agent (150-500 μM) for 1 h in an aCSF. The experiment with the extract (basal condition) or with the neurotoxic agent was performed separately using three slices per group in each plate for MTT reduction and DCFH oxidation assays, whereas 5 slices per group in each plate were used for the determination of lipid peroxidation levels.

2.7. MTT reduction assay (cellular viability)

MTT reduction was measured as an index of the mitochondrial dehydrogenase enzymes, which are involved in the cellular viability (Bernas and Dobrucki, 2002). After 1 h of hippocampal slices exposure to the neurotoxic agent, the media from treated and untreated slices were changed to a medium without plant extract. Then, 10 µL of MTT (final concentration of 50 µg/mL) was added and the plates were incubated for an additional 30 min at 37 °C. The purple formazan product formed was then dissolved in dimethyl sulfoxide (DMSO) (Mosmann, 1983). The optical density was measured using SpectraMax (Molecular Devices, USA) at 540 and 700 nm, and the net A540–A700 was taken as an index of cell viability. The results were corrected by the protein content and expressed as percent of control (untreated slices).

2.8. Determination of dichlorofluorescein (DCFH) oxidation in the incubation medium

After exposure of hippocampal slices to the neurotoxic agents, an aliquot of 900 µL from the media of treated and untreated slices were collected. Then, DCFH-DA (5 µM) was added to the incubation medium and the mixture was kept in the dark. Samples were read after 1 h by measuring the formation of the fluorescent product of DCFH oxidation (i.e., DCF) (Wang and Joseph, 1999; Halliwell and Gutteridge, 2007). The DCF fluorescence was measured using excitation and emission wavelengths of 488 and 525 nm, respectively, with slit widths of 1.5 nm (spectrofluorophotometer, Shimadzu RF-5301). The results were corrected by the protein content and expressed as percent of control (untreated slices).

2.9. Determination of thiobarbituric acid reactive substances (TBARS) in homogenates from hippocampal slices

At the end of the exposure to the neurotoxic agent, the slices from each sample (treated and untreated) were homogenized in 150 µL of aCSF, pH 7.4. Twenty microliters of 8.1% sodium docecyl sulfate (SDS), 100 µL of buffered acetic acid (pH 3.4) and 100 µL of 0.8% thiobarbituric acid (TBA) were then sequentially added to 80 µL of homogenates. The mixture was then incubated at 100°C for 1 h. After cooling, the reaction mixture was centrifuged at 2000xg for 10 min. The developed color was measured using SpectraMax (Molecular Devices, USA) at 532 nm. The results were calculated as nanomol (nmol) of MDA/mg of protein.

2.10. Protein Determination

The protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Software (version 5.0). Data were expressed as mean ± S.E.M (standard error of mean). Comparisons between experimental groups and respective controls were performed by paired t-test. The results were considered statistically significant for $p < 0.05$.

3. Results

3.1. Phenolics and Flavonoids profile of T. catigua barks extract by HPLC-DAD

The HPLC fingerprinting of *T. catigua* bark extract revealed the presence of phenolic compounds (gallic, chlorogenic, caffeic, rosmarinic and ellagic acids), flavonoids (quercetin, isoquercitrin, quercitrin, rutin and kaempferol) and tannins (catechin) (Fig. 1, Table 1). They were identified by comparing their retention time and UV spectra to authentic standards analyzed under identical analytical conditions. The quantification of these compounds by HPLC-DAD is presented in Table 1. It is worthy to note that similar results were obtained by Kamdem et al. (2012b). However, here we have done a more detailed characterization of the extract by using a different mobile phase and more standards.

3.2. Protective effect of T. catigua against H_2O_2, SNP and 3-NPA-induced cell death

T. catugua at different concentrations tested did not have any effect on cellular viability evaluated by MTT reduction (Fig. 2A). However, exposure of hippocampal slices to 150 µM of H_2O_2 (Fig. 2B), SNP (Fig. 2C) or 3-NPA (Fig. 2D) for 1 h, resulted in a significant decrease in MTT reduction (31.42%, 22.66% and 31.4% respectively) when compared to their respective controls (untreated slices) ($p < 0.05$, Fig. 2B-D). Pre-treatment for 30 min with *T. catigua* (10-100 µg/mL) blunted the neurotoxicity of H_2O_2 (Fig. 2B, $p < 0.05$), SNP (Fig. 2C, $p < 0.05$) and 3-NPA (Fig. 2D, $p < 0.05$) and restored the cellular viability to control values ($p > 0.05$) (Fig. 2B-D).

3.3. Effect of T. catigua extract on dichlorofluorescein (DCFH) oxidation levels in the incubation medium

Under basal conditions, only 40 µg/mL of *T. catigua* significantly decreased DCFH oxidation as compared to that found in the medium of untreated slices (control slices, Ctrl, $p < 0.05$; Fig. 3A). Exposure of slices to 150 µM H_2O_2 (Fig. 3B), SNP (Fig. 3C) or 3-NPA (Fig. 3D) for 1 h caused a significant increase in DCF fluorescence in the

incubation medium when compared to control medium ($p < 0.05$; Fig. 3B-D). The increase in DCFH oxidation was in the order H_2O_2 (66.83%, Fig. 3B) > SNP (35.43%, Fig. 3C) > 3-NPA (22.29%, Fig. 3D). Pre-treatment with *T. catigua* (40 µg/mL) for 30 min before exposure to H_2O_2 significantly reduced the DCFH oxidation when compared to H_2O_2 alone (Fig. 3B). Similarly, pre-treatment with 10 and 40 µg/mL *T. catigua* attenuated DCFH oxidation in the reaction medium as compared with SNP (Fig. 3C)- or 3-NPA (Fig. 3D)- treated slices ($p < 0.05$).

3.3. Effects of T. catigua on TBARS production induced by H_2O_2 (500 µM), SNP (150 µM) and 3-NPA (500 µM)

Incubation of hippocampal slices with 150 µM of SNP (Fig. 4C) or 500 µM of 3-NPA (Fig. 4D) for 1 h caused marked increase in TBARS production in slices homogenates as compared to their respective controls (Ctrl, $p < 0.05$). In contrast, H_2O_2 at 150 µM (data not shown) or at 500 µM did not induce TBARS formation (Fig. 4B, $p > 0.05$), but it tented to increase ($p = 0.162$).

SNP at 150 µM was a more potent inducer of TBARS formation than H_2O_2 and 3-NPA. Pre-treatment of slices with *T. catigua* extract (40-100 µg/mL) prevented LPO induced by the neurotoxic agents (Fig. 4B-D, $p < 0.05$). Additionally, *T. catigua* extract (10-40 µg/mL) significantly reduced TBARS formation in the homogenates of slices maintained under basal condition (Fig. 4A, $p < 0.05$). Paired t-test revealed a significant difference in TBARS formation between untreated slices (Ctrl) and those pre-treated with plant extract and exposed to the neurotoxic agent (Fig. 4B-D, $p < 0.05$).

4. Discussion

In traditional herbal medicine, numerous plants have been used to treat age related brain disorders and some of them have constituted a new direction for drug discovery (Adams et al., 2007; Gomes et al., 2009). In the present study, we examined the potential protective effect of *T. catigua* extract against H_2O_2-, SNP-, and 3-NPA-induced neurotoxicity in rat hippocampal slices. H_2O_2, SNP, and 3-NPA promote oxidative damage in a process likely involving reactive species generation, and lipid peroxidation (LPO). Whereas, pre-treatment of hippocampal slices with *T. catigua* extract (10-100 µg/mL) prior to the exposure to the neurotoxic agents protected hippocampal slices from H_2O_2, SNP, and 3-NPA deleterious effects.

During normal cellular metabolism, mitochondrial respiratory chain produces ROS and mitochondrial dysfunction has been associated with degenerative diseases. Consequently, it is important to identify compounds and/or plant extracts that could protect mitochondria from ROS-mediated toxicity (Lee et al., 2005; Gopi and Setty, 2010). Nitric oxide (NO) released from the decomposition of sodium nitroprusside (SNP, $[Na_2(Fe(CN)_5NO])$ has been reported to be one of the main component responsible for SNP-induced neurotoxicity. In particular, superoxide which is also generated under stress conditions can interact with NO to form peroxinitrite ($ONOO^-$) which in turn inhibits mitochondrial respiratory enzyme in an irreversible manner (Kirkinezos and Moraes, 2001; Zhang and Zhao, 2003). Similarly, 3-NPA is well known to impair mitochondrial function and energy production by inhibiting succinate dehydrogenase (SDH, mitochondrial complex II) irreversibly. The inhibition disrupts electron transfer chain and Krebs cycle (Alston et al., 1977; Browne et al., 1997; Wang et al., 2001), resulting in ATP depletion. The metabolic impairments caused by 3-NPA can culminate in excitotoxic cell death in the hippocampus (Beal et al., 1993; Greene

and Greenamyre, 1995). H_2O_2 has been reported to cause mitochondrial dysfunction by inactivation of Krebs cycle enzymes such as SDH, aconitase and alpha-ketoglutarate dehydrogenase (Sims et al., 2000; Nulton-Persson and Szweda, 2001). In the present study, the influence of ROS/RNS on mitochondrial redox potential was evaluated by measuring MTT reduction. Consistent with previous studies, our data demonstrated that exposure of hippocampal slices to H_2O_2, SNP and 3-NPA for 1 h resulted in a significant decrease in MTT reduction, which is consistent with mitochondrial dysfunction. Based on the fact that the three neurotoxic agents have different mechanisms of action, we suggest that the marked decrease in MTT reduction caused by H_2O_2 can be due to the formation of hydroxyl radical (OH^-). The significant decrease in MTT reduction, which gives an index of cell death, can be explained by the high vulnerability of the hippocampus to oxidative stress. Pre-treatment of slices with *T. catigua* (10-100 µg/mL) extract prior exposure to the neurotoxic agents significantly maintained cellular viability. This result suggests that the antioxidant mechanisms of *T. catigua* extract might be involved in the restoration of the brain SDH activity.

Lipid peroxidation (LPO) and its reactive products, such as malondialdehyde (MDA), can profoundly alter the structure and function of cell membrane and cellular metabolism, leading to cytotoxicity (Jia and Misra, 2007; Valko et al., 2007). In the current study, we found that SNP and 3-NPA triggered accumulation of MDA in hippocampal slices, which was inhibited by pre-treatment with *T. catigua* (10-100 µg/mL). These findings are in agreement with our previous report, which indicated a decrease in LPO products formation by *T. catigua* in rat brain homogenates (Kamdem et al., 2012a). In contrast, H_2O_2 did not induce LPO at 150 µM (data not shown) or at 500 µM (Fig. 4B). H_2O_2 cytotoxicity in the absence of LPO stimulation has also been observed in different cell types *in vitro* (Erba et al., 2003; Weidauer et al., 2004; Linden

et al., 2008). Those observations can be related to the lack of sensitivity of the TBARS method. Domínguez-Rebolledo et al. (2010) have recently compared the TBARS assay with BODIPYC_{11} probes for assessing LPO in red deer spermatozoa induced by H_2O_2. They demonstrated that the TBARS method offered comparatively limited sensitivity. Consequently, we can speculate that the TBARS assay was not sensitive enough to measure the LPO caused by H_20_2. SNP presented a more pronounced toxic effect by producing MDA followed by H_2O_2 and 3-NPA. Since the decomposition of SNP release cyanide, NO and free iron, it is possible that the effect of SNP in TBARS formation is a result of the sum of each of its pro-oxidant components. NO released from SNP in the incubation medium can undergo reaction with superoxide radicals forming peroxinitrite, a potent radical known to induce oxidative damage to several biomolecules, including membrane phospholipids. In addition, free iron released from SNP can induce TBARS formation in brain preparations (Pereira et al., 2009) via stimulation of Fenton reaction and its levels are increased in some degenerative diseases (Qian et al., 1997; Aisen et al., 1999; Bostanci and Bagirici, 2008). Another mechanism by which SNP might induce TBARS formation is via formation of iron complexes such as pentacyanoferrate complex (Arnold et al., 1984; Bates et al., 1990).

To clarify the protective mechanism of *T. catigua* extract against H_2O_2-, SNP- and 3-NPA-induced cell injury in hippocampal slices, we measured ROS/RNS generation released into the incubation medium by using DCFH-DA. We evaluated oxidative stress in the incubation medium because these results were expected to be more consistent since there was no manipulation of slices. Our results indicated a significant increase in DCF fluorescence (i. e. oxidized form of DCFH) in the medium obtained from slices exposed to H_2O_2, SNP and 3-NPA when compared to their respective control (Fig. 3B-D), suggesting that the plasma membrane was compromised. Interestingly, pre-

treatment of slices with *T. catigua* extract (10-40 µg/mL) prior exposure to the neurotoxic agents generally decreased DCFH oxidation to levels found in slices which were not exposed to pro-oxidant agents, an effect that could be attributed to its capacity to scavenge ROS/RNS. This result indicates that the neuroprotection conferred by the plant extract is due to its antioxidative effect of attenuating ROS/RNS generation and LPO. The brain is particularly sensitive to oxidative stress, owing to high oxygen consumption, relatively low concentration of antioxidants enzymes and its high content of polyunsaturated fatty acids. A number of studies have demonstrated the antioxidant properties of *T. catigua* extract, for instance, its ability to inhibit LPO in brain homogenates and to suppress liver mitochondrial ROS production (Brighente et al., 2007; Kamdem et al., 2012a).

It has been shown that a variety of phytochemicals in medicinal plants and dietary plants exert potent antioxidative properties (Park et al., 2011; Bornhoeft et al., 2012). *T. catigua* extract contains a variety of compounds (Fig. 1, Table 1) with pharmacological properties including antioxidant, anti-inflammatory, neuroprotective, etc (Crispo et al., 2010; Hunyadi et al., 2012; Sandhir and Mehrotra, 2013) that may protect CNS neurons from oxidative damage. Phytochemicals from *T. catigua*, particularly flavonoids and phenolics acids, have been reported to inhibit the propagation of free radical reactions and to protect the human body from diseases (Spencer, 2008; Rodrigo et al., 2011). They exert a multiplicity of neuroprotective action within the brain, including the potential to protect neurons against injury induced by neurotoxic agents, an ability to suppress neuroinflammation, and the potential to promote memory, learning and cognitive function (Spencer, 2008; Vauzour et al., 2008; Rodrigo et al., 2011; Shen et al., 2012; Vauzour, 2012).

5. Conclusion

The present work demonstrates that pre-treatment with *T. catigua* extract protected hippocampal slices from H_2O_2-, SNP- and 3-NPA-induced oxidative stress. The neuroprotection offered by *T. catigua* is at least in part, mediated through attenuation of cell death, reduction in ROS/RNS generation in the incubation medium and inhibition of LPO. These observations suggest that *T. catigua* may be useful in the prevention of diseases where cellular damage is a consequence of oxidative stress.

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Table caption

Table 1 Phenolics and flavonoids composition of *T. catigua* bark extract by HPLC-DAD

Compounds	***Trichilia catigua***		**LOD**	**LOQ**
	mg/g	Percent (%)	μg/mL	μg/mL
Gallic acid	14.06 ± 0.03	1.40	0.017	0.056
Catechin	6.03 ± 0.01	0.60	0.044	0.145
Chlorogenic acid	19.12 ± 0.05	1.91	0.036	0.119
Caffeic acid	5.27 ± 0.03	0.52	0.009	0.028
Rosmarinic acid	10.83 ± 0.01	1.08	0.011	0.036
Ellagic acid	2.96 ± 0.04	0.29	0.035	0.115
Rutin	4.75 ± 0.01	0.47	0.022	0.074
Isoquercitrin	7.39 ± 0.02	0.73	-	-
Quercitrin	4.83 ± 0.02	0.48	-	-
Quercetin	17.29 ± 0.03	1.72	0.028	0.092
Kaempferol	6.95 ± 0.04	0.69	0.031	0.103

Results are expressed as mean ± standard deviations (SD) of three determinations. LOD = Limit of detection, LOQ = Limit of quantification.

Figures captions

Fig.1. Representative high performance liquid chromatography (HPLC) profile of *Trichilia catigua.* Gallic acid (retention time, t_R = 11.92 min; peak 1), catechin (t_R = 19.58 min; peak 2), chlorogenic acid (t_R = 23.86 min; peak 3), caffeic acid (t_R = 26.09 min; peak 4), rosmarinic acid (t_R = 29.71 min; peak 5), ellagic acid (t_R = 31.84 min; peak 6), rutin (t_R = 40.25 min; peak 7), isoquercitrin (t_R = 44.97 min; peak 8), quercitrin (t_R = 47.73 min; peak 9), quercetin (t_R = 50.11 min; peak 10) and kaempferol (t_R = 60.49 min; peak 11).The chromatography peaks were confirmed by comparing its retention time (t_R) with those of reference standards (see Materials and methods). Calibration curve for gallic acid: Y = 12407x + 1359.8 (r = 0.9998); catechin Y = 11035x + 1358.4 (r = 0.9998); chlorogenic acid: Y = 12578x + 1295.7 (r = 0.9990); caffeic acid: Y = 14642x + 1581.3 (r = 0.9997); rosmarinic acid: Y = 11854x + 1497.9 (r = 0.9999); ellagic acid: Y = 13162x + 1074.3 (r = 0.9995); rutin: Y = 12492 + 1065.7 (r = 0.9999), quercetin: Y = 13195x + 1192.6 (r =0.9999) and kaempferol: Y = 11953x + 1376.4 (r = 0.9993).All chromatography operations were carried out at ambient temperature and in triplicate.

Fig. 2. Effect of *T. catigua* (A), H_2O_2 (B), SNP (C) and 3-NPA (D) on MTT reduction of hippocampal slices. Columns represent mean ± S.E.M. of four independent experiments. MTT reduction was significantly inhibited by the neurotoxic agents and pre-treatment with *T. catigua* prior to exposure markedly attenuated this effect. The results are expressed as percentage of control (untreated slices). * $p < 0.05$ versus Ctrl (control, untreated slices); # $p < 0.05$ versus H_2O_2/SNP/3-NPA-induced cellular injury. No significant differences were detected in MTT reduction when compared untreated slices (Ctrl) to those pre-treated with *T. catigua* extract (10-100 µg/mL) and exposed to

the neurotoxic agent (H_2O_2, Fig. 2B; SNP, Fig. 2C or 3-NPA, Fig. 2D) as analyzed by paired t-test ($p > 0.05$).

Fig. 3. Effect of *T. catigua* extract (A) on different neurotoxic agents (H_2O_2 (B), SNP (C) and 3-NPA (D))-induced DCFH oxidation in the incubation medium. 5 µMDCFH-DA was added to the incubation medium after 1 h exposure of slices (or not) to the neurotoxic agents and DCF fluorescence intensity was measured as a result of DCFH oxidation after 1 h of incubation in the dark. Columns represent mean ± S.E.M. resulting from four independent experiments and data are expressed as percentage of control (untreated slices). * $p < 0.05$ versus untreated slices (Ctrl), # $p < 0.05$ versus neurotoxic agent-treated slices. As it can be seen, all the neurotoxic agents caused a significant increase in DCFH oxidation, whereas *T. catigua* extract (10-40 µg/mL) caused a decrease in oxidative stress. Paired t-test indicated no significant difference in DCFH oxidation when compared the medium obtained from untreated slices with those obtained from slices pre-treated with *T. catigua* (10-100 µg/mL) and treated with the neurotoxic agent (H_2O_2, Fig. 3B; SNP, Fig. 3C or 3-NPA, Fig. 3D) ($p > 0.05$).

Fig. 4. Effect of *T. catigua* extract (A), H_2O_2 (B), SNP (C) and 3-NPA (D) on lipid peroxidation in rat hippocampal slices homogenates. TBARS is expressed as nanomol of malondialdehyde per mg of protein. After treatment with or without (basal) the neurotoxic agent for 1 h, slices were homogenates as described in materials and methods. Data show mean ± S.E.M. resulting from four independent experiments.* $p < 0.05$ as compared with untreated slices (Ctrl), # $p < 0.05$ as compared to the neurotoxic agent-treated slices.

Fig. 1.

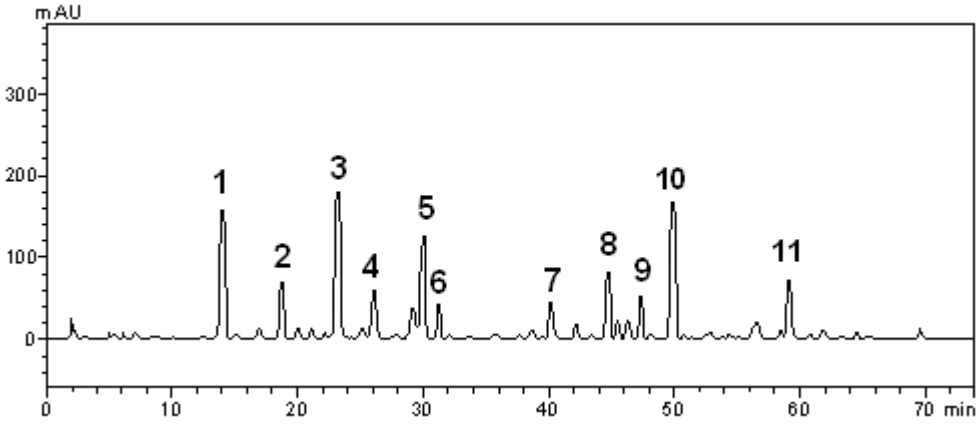
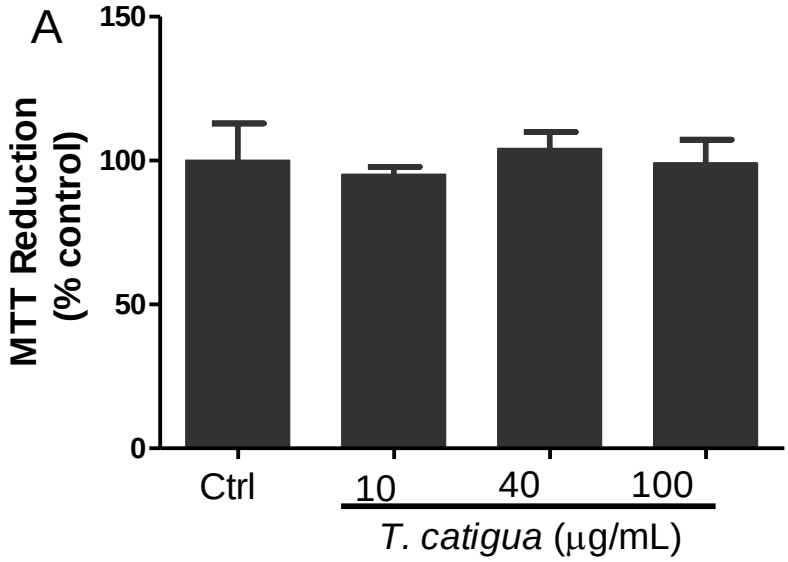


Fig. 2.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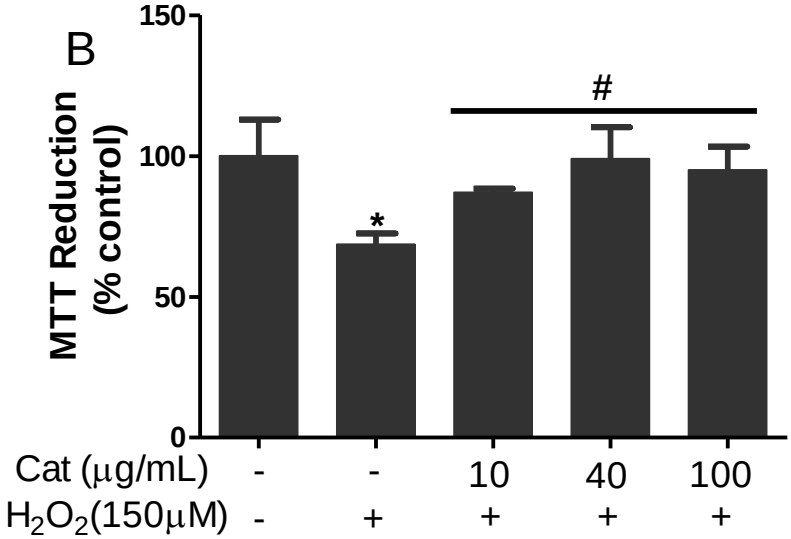
100
50
0
MTT Reduction
(% control)
Ctrl
10
40
100
T. catigua (μg/mL)


B
150
100
50
0
MTT Reduction
(% control)
*
#
Cat (μg/mL) - - 10 40 100
H2O2(150μM) - + + + +

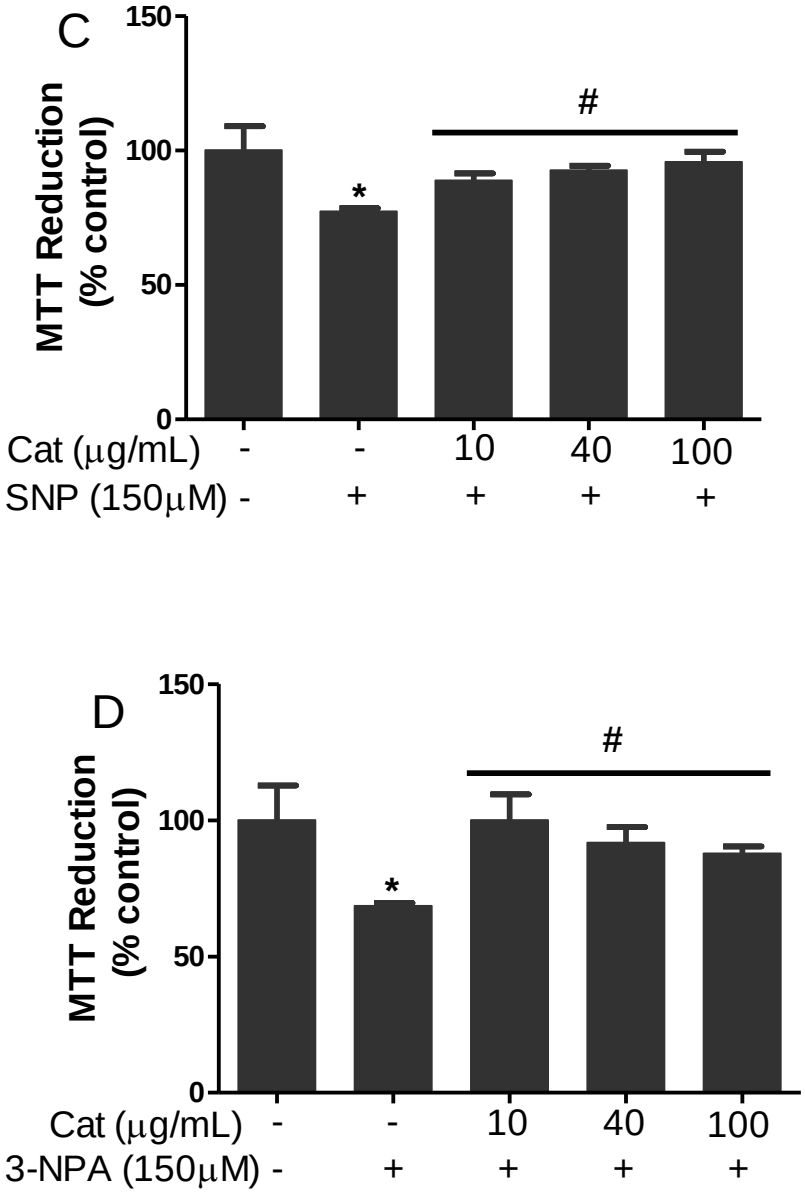
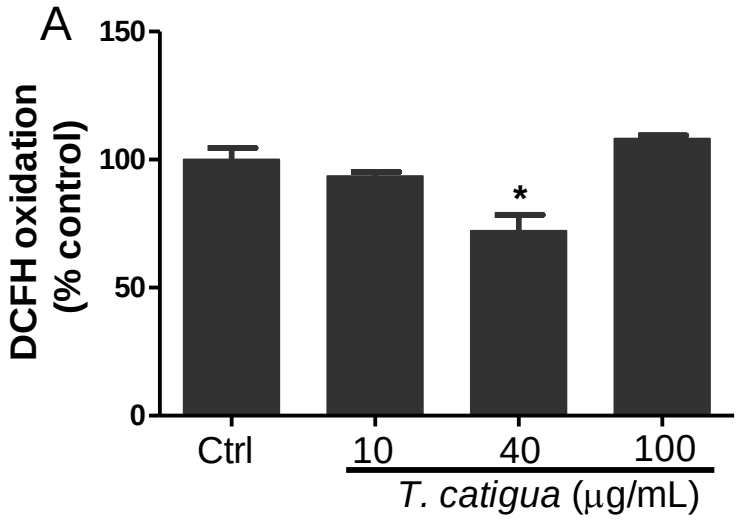
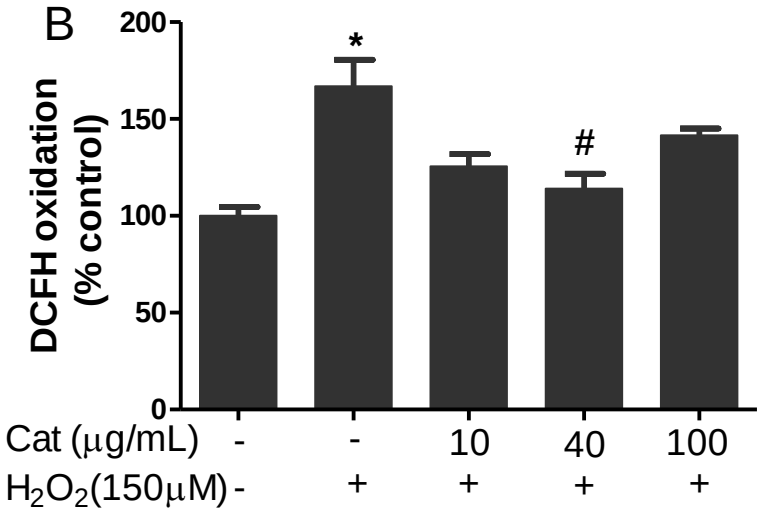

C
MTT Reduction (% control)
150
100
50
0
*
#
Cat (μg/mL) - - 10 40 100
SNP (150μM) - + + + +
D
MTT Reduction (% control)
150
100
50
0
*
#
Cat (μg/mL) - - 10 40 100
3-NPA (150μM) - + + + +

Fig. 3.


A
DCFH oxidation
(% control)
150
100
50
0
*
Ctrl
10
40
100
T. catigua (µg/mL)


B
DCFH oxidation
(% control)
200
150
100
50
0
*
#
Cat (µg/mL) - - 10 40 100
H2O2(150µM) - + + + +

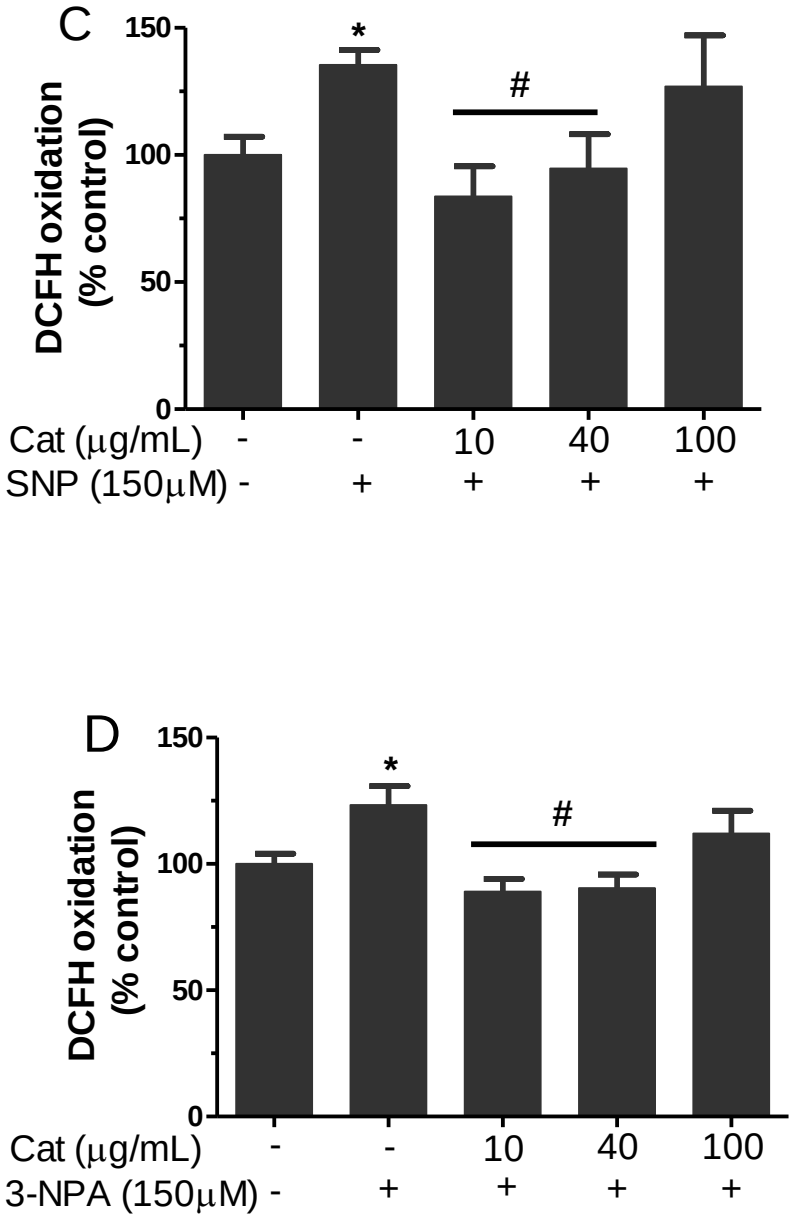
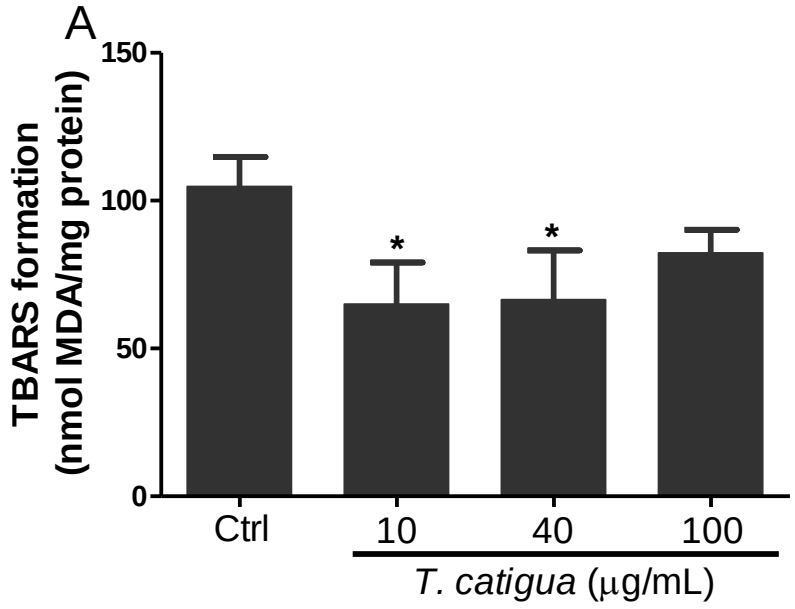
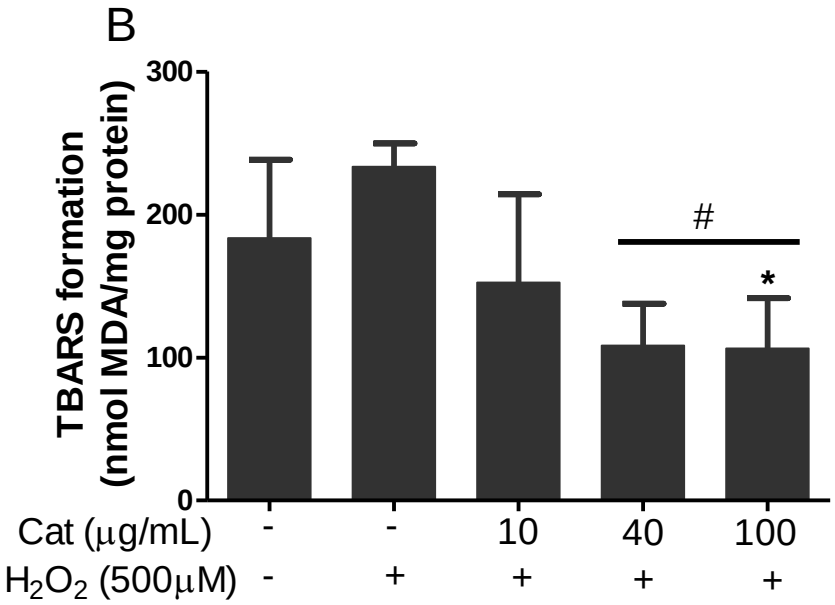
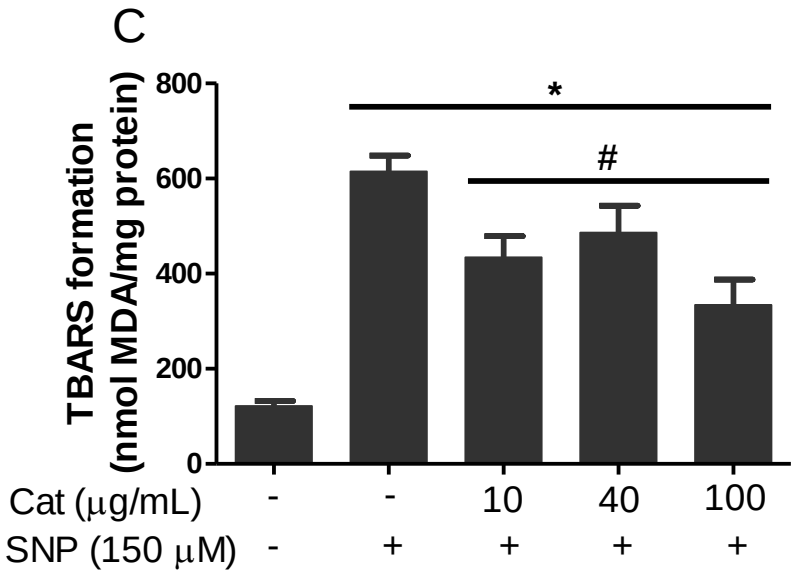
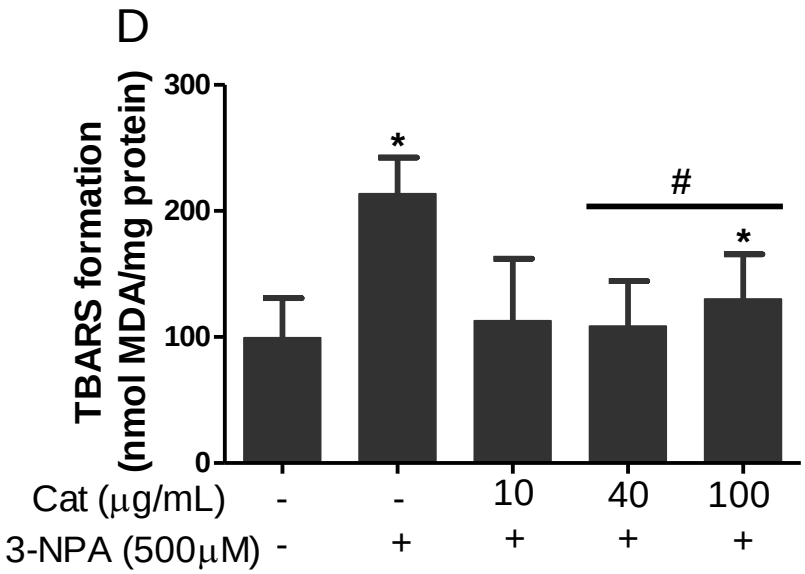

C
150
100
50
0
DCFH oxidation
(% control)
*
#
Cat (μg/mL) - - 10 40 100
SNP (150μM) - + + + +
D
150
100
50
0
DCFH oxidation
(% control)
*
#
Cat (μg/mL) - - 10 40 100
3-NPA (150μM) - + + + +

Fig. 4.


A
TBARS formation
(nmol MDA/mg protein)
150
100
50
0
*
*
Ctrl
10
40
100
T. catigua (μg/mL)


B
TBARS formation
(nmol MDA/mg protein)
300
200
100
0
#
*
Cat (μg/mL) - - 10 40 100
H2O2 (500μM) - + + + +


C
TBARS formation
(nmol MDA/mg protein)
800
600
400
200
0
*
#
Cat (μg/mL) - - 10 40 100
SNP (150 μM) - + + + +


D
TBARS formation
(nmol MDA/mg protein)
300
200
100
0
*
#
*
Cat (μg/mL) - - 10 40 100
3-NPA (500μM) - + + + +

PART III

Where Discussion, Conclusion, Perspectives and References are presented

4. DISCUSSION

Since the past decade, there is an increased global interest in the use of medicinal plants in the search for potential therapeutic agents, especially in the prevention and/or treatment of neurological diseases, including ischemic stroke (Simonyi et al., 2005; Adams et al., 2007; Gomes et al., 2009; Wu et al., 2010; Essa et al., 2012). In this context, the objective of this study was to evaluate the potential therapeutic effect of *Trichilia catigua* against ischemia-reperfusion (I/R) and different pro-oxidants mediated neurotoxicity in rat hippocampal slices. Based on the fact that the involvement of antioxidant ability of *T. catigua* in its pharmacological properties especially in the management of neurological-related diseases is scanty in the literature, the first step of the present study was to evaluate the potential antioxidant effects of *T. catigua* as well as the qualitative and quantitative analyses of selected chemical composition.

Considering the high susceptibility of the brain to free radicals attack, and the involvement of oxidative stress in neurodegenerative disorders, rat brain homogenates and hippocampal slices were used to evaluate the effects of *T. catigua* against oxidative stress induced by different pro-oxidant agents using the TBARS assay. The pro-oxidants used in this study were: Iron (Fe^{2+}), Hydrogen peroxide (H_2O_2), Sodium nitroprusside (SNP) and 3-Nitropropionic acid (3-NPA). They are known to induce oxidative stress through diverse mechanisms. The results obtained in this assay firstly demonstrated that all the solvent extracts (ethanolic, dichloromethane, ethyl acetate and n-butanol) as well as water extracts (cold and hot water) from *T. catigua* bark inhibited the lipid peroxidation (LPO) induced by Fe^{2+} (Kamdem et al., 2012a). But the ethanolic extract presented the strongest inhibition which was also observed in the DPPH radical scavenging activity. For these reasons, the ethanolic extract was used to continue our study.

Similar to that obtained with Fe^{2+} in brain homogenates, ethanolic extract of *T. catigua* significantly inhibited TBARS formation caused by H_2O_2, SNP and 3-NPA in slices homogenates (Kamdem et al., 2013). Lipid peroxidation is a complex process involving the interaction of oxygen-derived free radicals with polyunsaturated fatty acids. This phenomenon occurs through ongoing free radical chain reactions (Reed, 2011; Nowak, 2013). The ability of *T. catigua* to prevent LPO may be due to its high polyphenol content (Tang et al., 2007; Resende et al., 2011; Kamdem et al., 2012b; 2013). In

agreement, phenolics have been shown to form complexes with iron, probably related to the strong nucleophilic character of their aromatic rings (Moran et al., 1997), rendering them (i.e. iron) inactive or poorly active in the Fenton reaction. Furthermore, a plausible mechanism by which *T. catigua* is conferring protective action against H_2O_2-, SNP- and 3-NPA-induced TBARS production is that, it could not only interacting directly with Fe^{2+}, but may also assist in scavenging free radicals, thereby, preventing free radical chain reactions.

To further assess the antioxidant and anti-oxidative properties of *T. catigua*, we used the 2´,7´-dichlorofluorescin-diacetate (DCFH-DA), a useful indicator of ROS/RNS and oxidative stress. We measured the DCFH-oxidation both in isolated rat mitochondria (Kamdem et al., 2012a) and in the medium of hippocampal slices exposed either to oxygen-glucose deprivation (OGD) (Kamdem et al., 2012b) or to the pro-oxidants (Kamdem et al., 2013). Our results indicated that DCFH-oxidation stimulated by Ca^{2+} in isolated mitochondria was significantly reduced by *T. catigua* in a concentration dependent-manner (Kamdem et al., 2012a), reflecting an antioxidant property. Similarly, DCFH-oxidation was also attenuated in the incubation medium of slices exposed either to OGD or to the pro-oxidant agents, when *T. catigua* was present before OGD and during the reoxygenation periods (Kamdem et al., 2012b), and when the slices were pre-treated with *T. catigua* respectively (Kamdem et al., 2013). These results suggest that *T. catigua* could protect the DCFH from the oxidation by scavenging ROS/RNS, thus, resulting in decreased fluorescence intensity.

In addition to the aforementioned assays, we determined the effect of ethanolic extract of *T. catigua* on mitochondrial membrane potential (ΔΨm), since this assay can control ROS production. As a result, the extract at higher concentrations tested caused a decrease in mitochondrial ΔΨm, which seems to indicate that its toxicity does not overlap with its antioxidant activity (Kamdem et al., 2012a). Since *T. catigua* extract has been reported not to be toxic (Oliveira et al., 2005), and that it is generally accepted that pathophysiologic levels of ROS are produced at high ΔΨm values (Lu, 1999; Starkov and Fiskum, 2003; Liu, 2010; Suski et al., 2012; Sanderson et al., 2013); consequently, we can presume that the decrease in ΔΨm is associated with a reduction of ROS production and not with toxic effect of the plant.

The mitochondria have been reported to be the major source of ROS/RNS generation (Adam-Vizi, 2005). They play a central role in the maintenance of cell function by generating ATP indispensable for normal cellular homeostasis in the central nervous system (CNS) (Krieger and Duchen, 2002). In the present study, we evaluated the effects of *T. catigua*, OGD and pro-oxidants on mitochondrial activity or cellular viability. It was observed that *T. catigua* did not have any effect on cellular viability evaluated by MTT reduction. In contrast, exposure of slices to OGD or to the pro-oxidants resulted in a significant decrease in cellular viability (Kamdem et al., 2012b; Kamdem et al., 2013). Interestingly, this effect was blunted when *T. catigua* was present before ischemia and during the reperfusion periods (Kamdem et al., 2012b), and by *T. catigua* pre-treatment (Kamdem et al., 2013). Perturbations in the normal functions of mitochondria such as those induced by OGD or pro-oxidants can inevitably disturb cell function, resulting in the initiation of cell death (Krieger and Duchen, 2002). It should be stressed that mitochondria damage and lactate dehydrogenase (LDH) release are two associated phenomena, since the toxicity can start in the mitochondria and then can be "propagated" into the medium through damaged cell membrane. In line of this, the LDH leakage from hippocampal slices was measured in the incubation medium after I/R insult, as an index of membrane and cellular damage in oxidative stress (Freshney, 2000). As a result, the maximum leakage of LDH was obtained from the medium of slices exposed to OGD alone when compared the others groups, indicating an increase in membrane permeability due to oxidative stress (Kamdem et al., 2012b). Significant decrease in LDH leakage was found in the medium of slices when *T. catigua* was present before ischemia and during the reperfusion periods, when compared to OGD alone (without treatment).

Glutathione or non-protein thiol (NPSH), an important antioxidant molecule that controls endogenous free radical production was measured in slices homogenates after I/R insult. We observed that NPSH content was significantly reduced in slices exposed to OGD alone (without treatment) when compared to control slices (non-OGD, without treatment) (Kamdem et al., 2012b). However, *T. catigua* present before ischemia and during the reperfusion periods significantly prevented I/R-induced decline in NPSH content. Consequently, the possible mechanism underlying the neuroprotective effect of *T. catigua* extract might be the prevention of free radicals generation, due either to

direct interaction with free radicals generated during I/R or to an increase in NPSH content, which can, in turn, protect against oxidative bulk.

Phytochemically, *T. catigua* has been reported to possess polyphenols (flavonoids and phenolic acids) as their major component (Tang et al., 2007; Resende et al., 2011; Kamdem et al., 2012b; 2013). It contains flavonoids such as quercetin, rutin and other flavonoid glycosides (isoquercitrin, quercitrin), and phenolic acids such as chlorogenic, gallic, ellagic, caffeic and rosmarinic acids (Kamdem et al., 2012a,b) which are probably involved in the mechanism of free radical scavenging activity. They have been shown to possess a variety of potent biological action including free radical scavenging activity (Dajas, 2012; Schaffer et al., 2012; Quiñones et al., 2013).

In summary, the data of the present study shows the antioxidant action of *T. catigua* in *in vitro* models of neurototoxicity and suggest that further studies should be carried out on this plant, since it can be beneficial in the prevention of neurological disorders including ischemic stroke.

5. CONCLUSÕES

Com base nos resultados obtidos no presente estudo, em que avaliamos o potencial antioxidante e propriedades neuroprotetoras da *Trichilia catigua* (catuaba) *in vitro* contra a lesão de isquemia-reperfusão (I/R) e dos agentes pro-oxidantes em fatias de hipocampo de rato, pode concluir-se que:

Atividade antioxidante

- Todos os extratos (etanólico, diclorometano, acetato de etilo e n-butanol) foram capazes de sequestrar o radical DPPH, mas o extrato etanólico foi o mais potente quando comparado com os outros;
- Todos os extratos reduziram significativamente a peroxidação lipídica induzida pelo ferro;
- O extrato etanólico inibiu significativamente a geração das EROs/ERNs estimulada por Ca^{2+} e causou, em concentrações elevadas, uma redução no potencial de membrana mitocondrial ($\Delta\Psi m$).

Isquemia-reperfusão *in vitro*

- A *Trichilia catigua* presente no meio de incubação antes da privação de oxigênio e glicose (OGD) e durante a reoxigenação das fatias de hipocampo protegeu contra as lesões causadas pela isquemia e reperfusão (I/R);
- A *T. catigua* não protegeu as fatias de hipocampo *in vitro* contra a I/R quando adicionado ao meio após o insulto isquêmico (ou seja, quando usado como agente curativo).

Neurotoxicidade mediada pelos pro-oxidantes

- O pré-tratamento das fatias de hipocampo de ratos com *T. catigua* restaurou a atividade mitocondrial e diminiu a produção da EROs/ERNS no meio de incubação; impediu a formação de TBARS causada pelo H_2O_2, SNP e 3-NPA em fatias homogeneizadas;
- A análise fitoquímica do extrato da *T. catigua* por HPLC indicou a presença de compostos fenólicos e flavonóides com atividades antioxidantes reportadas, que podem estar envolvidos em seus efeitos neuroprotetores.

6. PERSPECTIVE

From the results presented in this study, the identification of phytochemical compound(s) directly associated with antioxidant and neuroprotective bioactivities of *Trichilia catigua* extract is a future research theme.

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