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# 2,2'-DISSELENETO DE DITIENILA, UM COMPOSTO ORGÂNICO DE SELÊNIO COM ATIVIDADE ANTIOXIDANTE E NEUROPROTETORA EM RATOS

**DISSERTAÇÃO DE MESTRADO**

**Cristiani Folharini Bortolatto**

**Santa Maria, RS, Brasil**
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**por**

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Dissertação apresentada ao Programa de Pós Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Mestre em Bioquímica Toxicológica**

**Orientador: Prof. Dr. Gilson Zeni**

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A Comissão Examinadora, abaixo assinada, aprova a Dissertação de Mestrado

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elaborada por

**Cristiani Folharini Bortolatto**

como requisito parcial para obtenção do grau de
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*Dedico este trabalho a minha mãe,*

*Alguém que sempre esteve e sempre estará ao meu lado em*

*todos os momentos!*

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"Enquanto estiver vivo, sinta-se vivo.

Se sentir saudades do que fazia, volte a fazê-lo.

Não viva de fotografias amareladas...

Continue, quando todos esperam que desistas.

Não deixe que enferruje o ferro que existe em você.

Faça com que em vez de pena, tenham respeito por você.

Quando não conseguir correr através dos anos, trote.

Quando não conseguir trotar, caminhe.

Quando não conseguir caminhar, use uma bengala.

Mas nunca se detenha."

(Madre Teresa de Calcutá)

# RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

## 2,2'-DISSELENETO DE DITIENILA, UM COMPOSTO ORGÂNICO DE SELÊNIO COM ATIVIDADE ANTIOXIDANTE E NEUROPROTETORA EM RATOS

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Data e Local da Defesa: Santa Maria, 2012

O estresse oxidativo tem sido implicado na patofisiologia de diversas doenças neurológicas uma vez que o cérebro é um órgão altamente susceptível ao dano oxidativo. A epilepsia do lobo temporal (ELT) tem sido amplamente estudada devido à alta taxa de prevalência e refratariedade ao tratamento medicamentoso. Além disso, a ELT pode estar associada com comorbidades psiquiátricas, tais como a depressão. Tendo em vista que muitos compostos orgânicos de selênio apresentam propriedades antioxidantes e neuroprotetoras, este trabalho investigou os efeitos do 2,2'-disseleneto de ditienila (DSDT) sobre as convulsões induzidas por ácido caínico (KA), um modelo experimental de ELT, bem como seu potencial antioxidante *in vitro* e atividade do tipo antidepressiva em ratos. Os resultados mostraram que o DSDT (100 mg/kg, via oral) reduziu as convulsões induzidas pela administração de KA (10 mg/kg, intraperitoneal), as quais foram demonstradas a partir de testes comportamentais e análise eletroencefalográfica. O aumento do conteúdo hipocampal de espécies reativas e de carbonilação de proteínas bem como a estimulação da atividade da $Na^+ K^+$ ATPase causados pelo KA foram reduzidos por DSDT (50 e 100 mg/kg). Além disso, o DSDT (100 mg/kg) protegeu contra a degeneração hipocampal resultante da exposição de ratos ao KA. O DSDT em baixas concentrações (na faixa de μM) reduziu o conteúdo de espécies reativas, carbonilação de proteínas e peroxidação lipídica em homogenato de cérebro de ratos *in vitro* e apresentou propriedades miméticas à deidroascorbato (DHA) redutase e à glutationa S-transferase (GST), enzimas importantes para a função antioxidante. Os resultados também revelaram que o DSDT foi efetivo em inibir a atividade da monoamino oxidase (MAO) A e B em homogenato de cérebro de ratos *in vitro* (25-100 μM) e em causar uma redução no tempo de imobilidade de ratos no teste do nado forçado (TNF) (50 e 100 mg/kg, via oral). Estes achados sugerem que o DSDT produziu uma ação anticonvulsivante no modelo do KA e atenuou o subseqüente dano oxidativo e a perda neuronal em hipocampo. Além disso, os dados mostraram que o DSDT teve efeito antioxidante e inibidor não-seletivo da MAO em cérebro de ratos *in vitro* bem como ação do tipo antidepressiva em ratos. Portanto, o DSDT pode ser útil como uma terapia para o tratamento da comorbidade ELT/depressão.

**Palavras-chave**: epilepsia do lobo temporal, neurotoxicidade, estresse oxidativo, depressão, selênio, ratos.

# ABSTRACT

Dissertation of Master's Degree
Federal University of Santa Maria, RS, Brazil

**2,2'-DITHIENYL DISELENIDE, AN ORGANOSELENIUM COMPOUND WITH ANTIOXIDANT AND NEUROPROTECTIVE ACTIVITIES IN RATS**

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CO-ADVISOR: CRISTINA WAYNE NOGUEIRA

Date and Place of the defense: Santa Maria, 2012

Oxidative stress has been implicated in the pathophysiology of several neurological diseases since the brain is an organ highly susceptible to oxidative damage. Temporal lobe epilepsy (TLE) has been widely studied due to the high prevalence rate and refractoriness to drug treatment. In addition, the ELT can be associated with psychiatric comorbidities, such as depression. Since many organoselenium compounds have antioxidant and neuroprotective properties, this study investigated the effects of 2,2'-dithienyl diselenide (DTDS) on seizures induced by kainic acid (KA), an experimental model of TLE, as well as its antioxidant potential *in vitro* and antidepressant-like activity in rats. The results showed that DTDS (100 mg/kg, per oral) reduced seizures induced by KA administration (10 mg/kg, intraperitoneal), which were demonstrated by behavioral tests and electroencephalographic analysis. The increase in the hippocampal content of reactive species and protein carbonylation as well as the stimulation of $Na^+ K^+$ ATPase activity caused by KA were reduced by DTDS (50 and 100 mg/kg). In addition, DTDS (100 mg/kg) protected against hippocampal degeneration resulting from exposure of rats to KA. DTDS, at low concentrations (μM range), reduced the content of reactive species, protein carbonylation and lipid peroxidation in rat brain homogenate *in vitro* and presented mimetic properties to dehydroascorbate (DHA) reductase and glutathione S-transferase (GST), important enzymes for antioxidant function. The results also revealed that DTDS was effective in inhibiting the activity of monoamine oxidase (MAO) A and B in rat brain homogenate *in vitro* (25-100 μM) and in causing a reduction on immobility time of rats in the forced swimming test (FST) (50 and 100 mg/kg, per oral). These findings suggest that DTDS produced an anticonvulsant action in the KA model and attenuated the subsequent oxidative damage and neuronal loss in hippocampus. Furthermore, the data showed that DTDS had antioxidant and MAO non-selective inhibitor effects in rat brain *in vitro* and antidepressant-like action in rats. Therefore, DTDS may be useful as a therapy for the treatment of comorbidity ELT/depression.

**Keywords:** temporal lobe epilepsy, neurotoxicity, oxidative stress, depression, selenium, rats.

# LISTA DE FIGURAS

## INTRODUÇÃO



## ANEXO - Resultados complementares

# LISTA DE ABREVIATURAS

**$(PhSe)_2$** - disseleneto de difenila

**4-HNE** - 4-hidroxinonenal

**ABTS -** 2,2'-Azino-bis(3-etilbenztiazolina-6-ácido sulfônico)

**AMPA** - α-amino-3-hidroxi-5-metil-4-isoxazol-7 ácido propiônico

**ANVISA** - Agência Nacional de Vigilância Sanitária

**ATP** - trifosfato de adenosina

**CAT** - catalase

**DHA** – dehidroascorbato

**DPPH –** 1,1-Difenil-2-picril-hidrazil

**DSDT** - 2,2'-disseleneto de ditienila

**EDTA -** ácido etilenodiamino tetra-acético

**EEG** - eletroencefalograma

**ELT-** epilepsia do lobo temporal

**GABA -** ácido gama-aminobutírico

**GPx** - glutationa peroxidase,

**GR** - glutationa redutase

**GSH** – glutationa reduzida

**GST** - glutationa S-transferase

**KA** - ácido caínico, cainato

**MAO** - monoamino oxidase

***m*-$CF_3(PhSe)_2$** - *m*-trifluormetil-difenil disseleneto

**MDA** - malondialdeído

**NMDA-** N-metil-D-aspartato

**NPS** - nitroprussiato de sódio

***p*-$Cl(PhSe)_2$** - *p*-cloro-difenil disseleneto

***p*-$OMe(PhSe)_2$** - *p*-metoxil-difenil disseleneto

**SNC** - sistema nervoso central

**SOD** - superóxido dismutase

**TBARS** - espécies reativas ao ácido tiobarbitúrico

**TCA**- teste do campo aberto

**TNF** - teste do nado forçado

# SUMÁRIO

# 1 INTRODUÇÃO

O elemento químico selênio (Se) foi descoberto pelo químico sueco Jöns Jakob Berzelius em 1817 e é pertencente ao grupo 16 da tabela periódica (calcogênios), apresentando-se em diversos estados de oxidação tais como selênio elementar ($Se^{o}$), selenito ($Se^{+4}$), selenato ($Se^{+6}$) e seleneto ($Se^{-2}$) (Porciúncula, 2003). O Se existe na natureza sob a forma orgânica (selenocisteína, selenocistina e selenometionina) e inorgânica (selenito e selenato) (Nakamuro et al., 2000). Devido à possibilidade de existir em diversos estados de oxidação, o Se pode levar à formação de diferentes compostos orgânicos e aminoácidos em substituição ao enxofre (S) (Tinggi, 2003). De fato, o Se compartilha diversas propriedades químicas e físicas com o S (Nogueira e Rocha, 2011) e esta similaridade permite a ocorrência de interações Se-S em sistemas biológicos. Cabe mencionar que o interesse inicial na atividade biológica do Se foi em razão de sua toxicidade (Moxon e Rhian, 1943; Nogueira e Rocha, 2011). Entretanto, atualmente o Se é também considerado um elemento traço essencial para a saúde (Papp et al., 2007).

O Se desempenha um papel crucial em diversas vias metabólicas, incluindo o metabolismo dos hormônios tireoidianos, a função imunológica, o crescimento celular e principalmente o sistema de defesa antioxidante (Stazi e Trinti, 2008). O Se está presente como resíduo de selenocisteína no sítio ativo das enzimas glutationa peroxidase (Flohé et al., 1973), tioredoxina redutase (Holmgren, 1985), 5'-deiodinase (Behne e Kyriakopoulos, 1990) e selenoproteina P (Ursini et al., 1990), sendo que a atividade redox do Se é essencial para o sítio catalítico dessas enzimas. Quantidades adequadas de Se são requeridas para a manutenção da saúde (Rayman, 2000) visto que concentrações insuficientes ou excessivas deste elemento podem ocasionar deficiência ou toxicidade, respectivamente. De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA), a ingestão diária recomendada para adultos deste micronutriente mineral é de 70μg. O Se é encontrado principalmente em alimentos como a castanha-do-pará, alho, cebola, brócolis, cogumelos, cereais, pescados, ovos e carnes (Dumont et al., 2006).

Baixos níveis de Se tem sido associados com a predisposição para o desenvolvimento de algumas doenças (Navarro-Alarcón e López-Martinez, 2000; Rayman, 2000) nas quais uma produção excessiva de espécies reativas (oxidantes) também tem sido reportada. Em níveis fisiológicos as espécies reativas têm importantes funções biológicas no organismo, entretanto quando a sua produção é aumentada pode ocorrer um desequilíbrio no estado redox celular. As espécies reativas de oxigênio compreendem o oxigênio *singlet* ($^1O_2$), o ânion

superóxido ($O_2^{\cdot-}$), o peróxido de hidrogênio ($H_2O_2$) e o radical hidroxil ($^{\cdot}OH$). As espécies reativas de nitrogênio como o óxido nítrico (NO) e o peroxinitrito ($ONOO^-$) também desempenham importante papel na patogênese de diversas doenças (Macásek et al., 2011). O organismo possui uma série de defesas antioxidantes, defesas estas que podem ser produzidas endogenamente ou adquiridas pela dieta, capazes de evitar o efeito deletério das espécies reativas geradas pelo metabolismo aeróbio. Estas estratégias de defesa podem atuar evitando a formação de espécies reativas, neutralizando-as ou ainda, promovendo a reparação de danos ocasionados por elas. Assim, o termo antioxidante pode ser considerado como qualquer substância que atrase, previna ou remova o dano oxidativo de uma molécula-alvo (Halliwell e Gutteridge, 2007). Entre as principais defesas antioxidantes não-enzimáticas estão as vitaminas C e E, carotenóides, flavonóides, pigmentos biliares, urato e o tripeptídeo glutationa (GSH) (Reischl et al., 2007). As defesas antioxidantes enzimáticas compreendem as enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), glutationa redutase (GR) (Halliwell e Gutteridge, 2007) e glutationa S-transferase (GST) (Sharma et al., 2004). Alterações relacionadas ao ataque de espécies reativas às biomoléculas podem ser causadas por sua excessiva formação e/ou ineficiência em sua interceptação pelas defesas antioxidantes, gerando o estresse oxidativo.

Devido ao importante papel do Se na defesa antioxidante do organismo, existe uma crescente busca pelo entendimento do seu papel biológico, em particular seu papel fisiológico na saúde e seu uso potencial como um agente terapêutico (Papp et al., 2007). Neste sentido, os efeitos neuroprotetores do Se têm sido amplamente estudados, uma vez que esse elemento traço desempenha importantes funções no cérebro. O Se é um potente agente protetor para os neurônios através da expressão de selenoproteínas que estão, na sua maioria, envolvidas na regulação do estado redox (Schweizer et al. 2004a). Além disso, modelos experimentais de doenças cerebrais como os de isquemia e demência resultante da doença de Alzheimer têm confirmado a eficácia farmacoterapêutica do Se (Yousuf et al., 2007; Ishrat et al., 2009).

Em uma dieta deficiente de Se, o cérebro exibe uma alta prioridade de conservar este elemento em relação aos outros órgãos, indicando a importância deste micronutriente para a função cerebral (Behne et al 1988; Buckman et al 1993; Whanger 2001;Schweizer et al., 2004a; Schweizer et al., 2004b). Sabe-se também que níveis insuficientes de Se no cérebro tem efeitos potencialmente negativos sobre o seu funcionamento podendo agravar a perda neuronal e disfunção subseqüentes aos estímulos endógenos ou exógenos, trauma e outras condições neurodegenerativas (Schweizer et al., 2004a) e até mesmo alterar a taxa de *turnover* de neurotransmissores (Castano et al., 1997). A deficiência moderada de Se tem sido

relacionada a diversas condições neurológicas, incluindo a Doença de Alzheimer e de Parkinson (Rayman, 2000). Além disso, uma associação entre a deficiência de Se e a epilepsia também tem sido reportada (Ashrafi et al., 2007a; Ashrafi et al., 2007b). Interessantemente, a suplementação oral com Se melhorou o estado clínico e o eletroencefalograma (EEG) de crianças com episódios epilépticos intratáveis e que apresentavam baixas concentrações sangüíneas de Se (Weber et al., 1991; Ramaeker et al., 1994).

Os compostos orgânicos de Se apresentam maior biodisponibilidade e geralmente são menos tóxicos que as formas inorgânicas deste elemento (Narajji et al., 2007). A bioquímica e a farmacologia de compostos orgânicos de selênio são temas de atual interesse científico, uma vez que muitos estudos têm sido concentrados no desenvolvimento de compostos de selênio estáveis que possuam potencial terapêutico para uma variedade de doenças humanas (Narajji et al., 2007). Os compostos orgânicos de Se, capazes de propagar o ciclo redox do Se, com a propriedade de mimetizar a química fisiológica redox de grupos selenóis/selenolatos, poderiam suplementar as defesas celulares naturais contra os agentes oxidantes (Arteel e Sies, 2001) e desta forma, ser úteis para a prevenção e/ou o tratamento de doenças relacionadas à excessiva produção de espécies reativas.

Nosso grupo de pesquisa tem extensivamente estudado o disseleneto de difenila [$(PhSe)_2$] e seus derivados. O cérebro parece ser um órgão-alvo dos disselenetos já que estes apresentam uma natureza lipofílica (Nogueira e Rocha 2010). Como demonstrado por Prigol et al. (2010), o $(PhSe)_2$ é capaz de atravessar a barreira cérebro-sangue de roedores. Corroborando com estes fatos, o $(PhSe)_2$ demonstrou atividades do tipo-antidepressiva (Acker et al., 2009; Savegnago et al., 2007, 2008; Wilhelm et al., 2010), ansiolítica (Savegnago et al., 2008) e melhorou a cognição de roedores (Stangherlin et al., 2008; Souza et al., 2010a; da Rocha et al., 2011). O $(PhSe)_2$ também apresentou efeitos protetores sobre o dano oxidativo cerebral induzido pela exposição de ratos jovens ao fumo passivo (Stangherlin et al., 2009) e pela exposição de camundongos ao cádmio (Luchese et al., 2007) ou ao acetaminofeno (da Silva et al., 2011). Em fatias hipocampais de ratos, este organoselênio protegeu contra o dano resultante da privação de glicose e oxigênio (Ghisleni et al., 2003) bem como contra o dano oxidativo induzido por $H_2O_2$ (Posser et al., 2008).

Recentes estudos revelam que os derivados substituídos no anel aromático do $(PhSe)_2$ também apresentam potencial farmacológico. O *p*-metoxil-difenil disseleneto [*p*-$OMe(PhSe)_2$] apresentou efeitos neuroprotetores em um modelo de demência esporádica decorrente da doença de Alzheimer em camundongos (Pinton et al., 2010; 2011). Adicionalmente, o *p*-cloro-difenil disseleneto [*p*-$Cl(PhSe)_2$] exerceu ação do tipo-

antidepressiva e melhorou a memória espacial de ratos velhos (Bortolatto et al., 2011). Por fim, o *m*-trifluormetil-difenil disseleneto [*m*-$CF_3(PhSe)_2$] induziu efeitos do tipo-antidepressivo e ansiolítico em camundongos (Brüning et al., 2009; 2011). Portanto, em adição à ação antioxidante, algumas propriedades neuroprotetoras também têm sido atribuídas aos disselenetos. Isto é bastante relevante do ponto de vista terapêutico, visto que o dano oxidativo está presente em muitas das doenças neurológicas (Ozcan et al., 2004; Mariani et al., 2005) já que o cérebro é especialmente sensível ao dano oxidativo (Mariani et al., 2005). A suscetibilidade do cérebro ao dano oxidativo deve-se ao fato de este órgão conter concentrações relativamente baixas de enzimas antioxidantes e grandes quantidades de ácidos graxos insaturados e catecolaminas, os quais são substratos suscetíveis ao ataque das espécies reativas (Reznick e Packer, 1993; Halliwell e Gutteridge, 2007). Outros fatores que contribuem para essa vulnerabilidade do cérebro a alterações do estado redox são: a presença de aminoácidos excitotóxicos; transporte de $Ca^{2+}$ através de membranas neuronais; uma alta taxa de consumo de oxigênio; presença de neurotransmissores auto-oxidáveis; geração de $H_2O_2$ pelo metabolismo cerebral através de reações enzimáticas e ainda a presença de células gliais (micróglia) que podem produzir $O_2^{\bullet-}$ e $H_2O_2$ quando ativadas (Halliwell e Gutteridge, 2007).

Uma doença neurológica que possui uma forte ligação com o dano oxidativo é a epilepsia. O termo epilepsia refere-se a uma ampla categoria de sintomas complexos em torno de funções cerebrais desordenadas, decorrentes de uma anormalidade e hipersincronia da atividade neuronal, podendo ser secundárias a uma variedade de processos patológicos (Engel e Pedley, 2008). A epilepsia está entre as doenças que mais freqüentemente acometem o sistema nervoso central (SNC) (Zielinski, 1988) e pode ser resultante de anormalidades congênitas no desenvolvimento cerebral, de alterações genéticas no metabolismo cerebral e na excitabilidade de neurônios e, ainda, a partir de insultos que lesam a anatomia e/ou a fisiologia do cérebro (Berg et al., 2010). Embora as epilepsias possam manifestar-se por várias vias diferentes, cada tipo de epilepsia tem em comum a importante característica de aumentar a excitabilidade neuronal, a qual se manifesta geralmente através da geração de convulsões (Mcnamara, 1999).

Anormalidades na neurotransmissão ocorrem através de um aumento na transmissão excitatória, diminuição da transmissão inibitória ou de ambos os eventos ocorrendo concomitantemente (Meldrum, 2000; Meldrum e Rogawski, 2007). Neurotransmissores excitatórios e inibitórios são mediadores da transmissão sináptica e controlam a estabilidade e eficiência das conexões sinápticas. O ácido gama-aminobutírico (GABA) é o mais importante

neurotransmissor inibitório e uma diminuição na neurotransmissão GABAérgica no SNC contribui para a hiperexcitabilidade, que é responsável pelo estado epiléptico (Onsen et al., 1999), uma condição aguda caracterizada por convulsões repetitivas ou prolongadas. O aminoácido glutamato é o mais abundante neurotransmissor excitatório no SNC e está envolvido na epileptogênese, tanto na iniciação quanto na propagação das convulsões e no dano neuronal resultante. O glutamato exerce suas ações através de receptores que funcionam como canais iônicos como receptores do tipo N-metil-D-aspartato (NMDA), α-amino-3-hidroxi-5-metil-4-isoxazol-7 ácido propiônico (AMPA) e KA permitindo o transporte de $Na^+$, $K^+$ e $Ca^{2+}$; e também através de cascatas sinalizadoras via receptores metabotrópicos (acoplados a proteínas G, que podem modular diferentes enzimas e canais iônicos) (Chapman, 2000; Bloss e Hunter, 2010). A ativação de receptores de aminoácidos excitatórios, tais como os de glutamato, pode ser um fator desencadeante à geração de espécies reativas (Engelborghs et al. 2000).

Estudos têm revelado os efeitos protetores de disselenetos, tais como o $(PhSe)_2$ e o *m*-$CF_3(PhSe)_2$, a partir da utilização de diferentes modelos experimentais de convulsão. O $(PhSe)_2$ foi efetivo em aumentar a latência para as convulsões químicas induzidas por 4-aminopiridina e prevenir a morte de camundongos expostos a esta neurotoxina (Brito et al., 2008). Ademais, o *m*-$CF_3(PhSe)_2$ atenuou as convulsões induzidas por pentilenotetrazol em camundongos provavelmente por inibir a captação de GABA (Prigol et al., 2009). Portanto, os efeitos protetores destes disselenetos frente às convulsões experimentais nos incentivaram a investigar a atividade anticonvulsivante de um composto quimicamente relacionado.

Segundo a Tabela de Classificação Internacional das Síndromes Epilépticas (Guerreiro et al., 2000), a epilepsia do lobo temporal (ELT) corresponde à aproximadamente 50% dos casos de epilepsia. A ELT foi definida pela *International League Against Epilepsy* (ILAE, 1989) como uma condição caracterizada por crises espontâneas recorrentes originadas da inibição e da ativação do lobo temporal lateral ou medial, sendo que em grande parte dos pacientes as crises evoluem para convulsões. A maioria das crises parciais complexas se origina no lobo temporal, ou seja, envolve estruturas do sistema límbico (Löscher, 1997). A estrutura mais danificada na ELT é a formação hipocampal que exibe uma perda neuronal seletiva resultando em esclerose (McNamara, 1994). Além disso, dentre as síndromes epilépticas refratárias a medicamentos, a ELT é a mais freqüente em que 20% dos pacientes mostram-se refratários aos anticonvulsivantes disponíveis (Yacubian, 1998). Por fim, a ELT é uma das mais novas doenças neurológicas em que o estresse oxidativo tem sido sugerido como um importante fator contribuinte (Waldbaum e Patel, 2010) e está também associada a

prejuízos na memória, psicoses, ansiedade e depressão (Sayin et al., 2004; Hermann et al., 2008).

A incidência de transtornos psiquiátricos, e de forma mais marcante de ansiedade e depressão, em pacientes com epilepsia é significativamente mais alta que na população geral. Depressão e epilepsia podem compartilhar mecanismos patogenéticos que facilitariam a ocorrência de um na presença do outro. Assim, o uso de fármacos antiepilépticos pode ter efeito direto no humor. Da mesma forma, suspeita-se que o uso de medicações antidepressivas possa exercer influência no limiar convulsivo (de Oliveira et al. 2007) de modo que a ativação noradrenérgica ou serotoninérgica parece contribuir para os efeitos anticonvulsivantes de antiepilépticos (Jobe et al., 1999; Jobe, 2003). Interessantemente, o selênio também exerce influência em patologias associadas aos transtornos de humor, uma vez que a carência de selênio parece levar a um estado de humor mais deprimido (Hawkes e Hornbostel, 1996) e o aumento no consumo deste elemento estabiliza o humor e diminui o estado depressivo e outros sintomas negativos do humor como ansiedade, confusão e hostilidade (Benton e Cook, 1991).

Os modelos experimentais são bastante úteis para a pesquisa de novas alternativas terapêuticas para o tratamento da ELT e da depressão. O KA, uma excitotoxina extraída de algas marinhas que quando administrada em animais mimetiza a ação do glutamato, é utilizado experimentalmente para induzir o estado epiléptico (Olney et al., 1974, Ben-Ari, 1985; Holopainen, 2008). Em situações de excessiva liberação de glutamato, tais como epilepsia e excitotoxicidade, o glutamato poder ser um fator chave de disfunção neuronal (Michaelis, 1998; Lees, 2000). Conforme reportado por Ferkany et al. (1982), a causa de neurotoxicidade induzida por KA, além de sua ação direta sobre receptores do tipo KA pós-sinápticos, é a ativação de receptores KA pré-sinápticos e a aumento da liberação de glutamato endógeno. O KA pode ainda ativar receptores do tipo AMPA (Farooqui et al., 2008). Portanto, O KA gera um aumento da excitabilidade cerebral causando atividade epileptiforme aguda e subaguda que pode durar por dias (Sperk, 1994). Em roedores, as convulsões agudas severas com subseqüente estado epiléptico gerado pelo KA induzem a uma série de eventos que simulam características comportamentais, eletrográficas e histopatológicas observadas na ELT humana (Ben-Ari, 1985). Por outro lado, a fim de se avaliar o potencial antidepressivo de novos compostos, um dos testes mais usados experimentalmente é o teste do nado forçado (TNF) (Porsolt et al., 1977), o qual apresenta um alto valor preditivo devido à resposta aos medicamentos antidepressivos utilizados na clínica.

O composto 2, 2'-disseleneto de ditienila (DSDT) é um disseleneto contendo tiofenos em sua estrutura. Este organoselênio tem sido empregado para a síntese de oligo(seleno-2,5-tienilenos) através de 2-tienilsselenilação eletrofílica de tiofeno (Tiecco et al., 2000), porém sua possível atividade biológica ainda não foi investigada. Além disso, estudos têm revelado o potencial antioxidante de diferentes compostos contendo tiofeno (Yanagimoto et al., 2002; Abu-Hashem et al., 2010). Portanto, a presença de tiofenos na molécula e a estrutura de disseleneto poderiam conferir ao DSDT uma possível ação antioxidante assim como outras ações neuroprotetoras.

Devido ao alto percentual de refratariedade aos tratamentos farmacológicos na ELT, a pesquisa por novas terapias tem grande relevância experimental, sendo o DSDT um possível candidato a agente terapêutico.

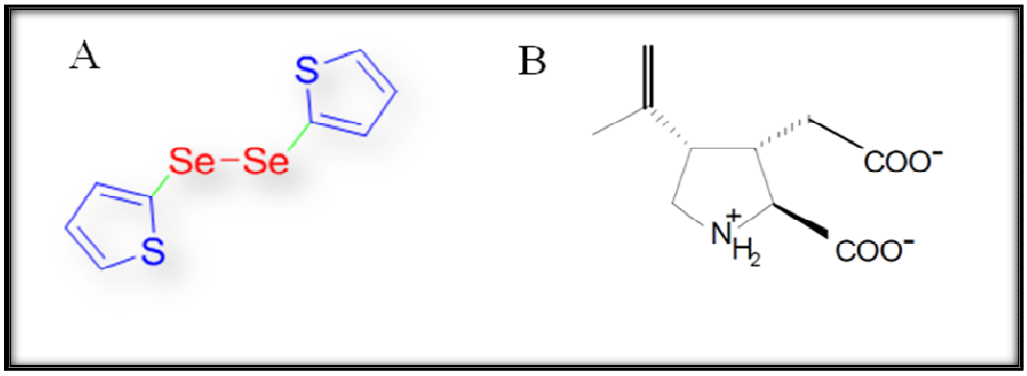



**Figura 1**. Estrutura química do DSDT (A) e do KA (B)

# 2 OBJETIVOS

## 2.1 Objetivo geral

Investigar as ações do DSDT sobre as convulsões induzidas por KA bem como seu potencial antioxidante e do tipo antidepressivo em ratos.

## 2.1 Objetivos específicos

Considerando os aspectos mencionados, os objetivos específicos deste estudo compreendem:

- Avaliar o possível efeito protetor do DSDT sobre as convulsões e alterações eletroencefalográficas induzidas por KA em ratos;
- Verificar o efeito do DSDT sobre o estresse oxidativo e a neurodegeneração induzidos por KA em ratos;
- Avaliar o potencial antioxidante do DSDT em homogenato de cérebro de rato bem como os mecanismos envolvidos nesta ação antioxidante *in vitro;*
- Avaliar o efeito do DSDT sobre a atividade das isoformas A e B da enzima monoamino oxidase (MAO) em cérebro de rato *in vitro;*
- Avaliar o potencial do tipo-antidepressivo do DSDT através do TNF em ratos.

# 3 ARTIGOS

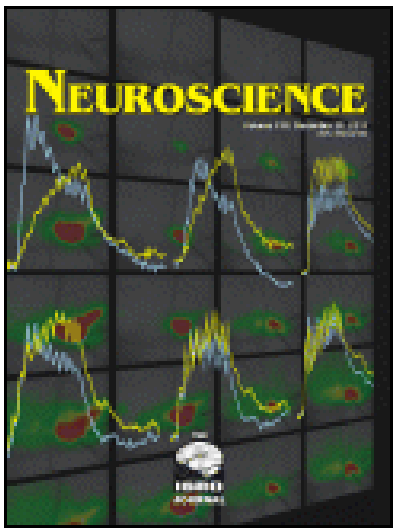
Os resultados que fazem parte dessa dissertação estão apresentados na forma de dois artigos científicos e de resultados complementares. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas dos artigos estão dispostos de acordo com a recomendação dos periódicos científicos aos quais foram enviados. Os resultados complementares estão divididos em Materiais e Métodos e Resultados e se encontram no anexo.

## 3.1 Artigo 1

**Efeito protetor do 2,2'-disseleneto de ditienila na neurotoxicidade induzida por ácido caínico em hipocampo de ratos**

**PROTECTIVE EFFECT OF 2,2'-DITHIENYL DISELENIDE ON KAINIC ACID-INDUCED NEUROTOXICITY IN RAT HIPPOCAMPUS**

Cristiani F. Bortolatto, Cristiano R. Jesse, Ethel A. Wilhelm, Leandro R. Ribeiro, Leonardo M. Rambo, Luiz Fernando F. Royes, Silvane S. Roman, Cristina W. Nogueira

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# PROTECTIVE EFFECT OF 2,2′-DITHIENYL DISELENIDE ON KAINIC ACID-INDUCED NEUROTOXICITY IN RAT HIPPOCAMPUS

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**Abstract—In this study, we investigated the effects of 2,2′-dithienyl diselenide (DTDS), an organoselenium compound, against seizures induced by kainic acid (KA) in rats. Rats were pretreated with DTDS (50 or 100 mg/kg) by oral route 1 h before KA injection (10 mg/kg, intraperitoneal). Our results showed that DTDS (100 mg/kg) was effective in increasing latency for the onset of the first clonic seizure episode induced by KA, as well as in decreasing the appearance of seizures and the Racine's score. DTDS also caused a decrease in the excitatory electroencephalographic (EEG) changes, resulting from KA exposure in hippocampus and cerebral cortex of rats. Besides, elevated reactive species (RS) and carbonyl protein levels and $Na^+$, $K^+$-ATPase activity in hippocampus of rats treated with KA were ameliorated by DTDS (50 and 100 mg/kg). Lastly, as evidenced by Cresyl-Violet stain, DTDS (100 mg/kg) elicited a protective effect against KA-induced neurodegeneration in rat hippocampus 7 days after KA injection. In conclusion, the present study showed that DTDS attenuated KA-induced status epilepticus in rats and the subsequent hippocampal damage.** 

**Key words: rats, kainic acid, seizures, hippocampus, oxidative stress, selenium.**

Epilepsies are among the most frequent CNS disorders affecting approximately 1.5% of the population worldwide (Majores et al., 2004). Temporal lobe epilepsy (TLE) is the most common form of adult epilepsy that involves the limbic structures (Hargus et al., 2011). Because of similar basic features, experimental models have allowed the determination of the basic molecular and cellular mechanisms of epileptogenesis and its relation to brain damage. This is particularly exemplified in the field of TLE (Ben-Ari and Cossart, 2000).

Systemic administration of kainic acid (KA) in rodents induces an epilepsy syndrome similar to human TLE, with mesial temporal sclerosis, spontaneous seizures, as well as significant deficits in learning and memory (Ben-Ari, 1985). Ionotropic glutamate receptor agonists, such as KA, typically increase cell death. Stimulation of glutamate receptors triggers sodium ($Na^+$) influx and neuronal depolarization, which lead to an opening of voltage-dependent calcium ($Ca^{2+}$) channels and stimulation of $Ca^{2+}$ influx (Park et al., 2004). Elevated $Ca^{2+}$ concentration generates reactive oxygen species (ROS), inducing injury and neuronal death (Allison and Pratt, 2003).

Oxidative stress is emerging as a key factor that not only results from seizures but may also contribute to epileptogenesis. In this sense, oxidative stress may be a brand new way for the development of new drugs that are neuroprotective. Synthetic antioxidants that protect mitochondrial targets and decrease neuronal death may be useful supplements for the clinical management of patients with status epilepticus (SE) or intractable epilepsy (Waldbaum and Patel, 2010). This is relevant because TLE is among the most frequent types of drug-resistant epilepsy (Engel, 2001).

The essential trace element selenium is of fundamental importance to human health. As a constituent of the small group of selenocysteine-containing selenoproteins, selenium elicits important structural and enzymatic functions (Papp et al., 2007). The pharmacotherapeutic efficacy of selenium has been confirmed in a number of experimental models of brain diseases (Yousuf et al., 2007; Ishrat et al., 2009). It is noteworthy that in a selenium-deficient diet, the brain exhibits a high priority to conserve this element, indicating the importance of this trace element to normal brain function (Schweizer et al., 2004a,b). With regard to epilepsy, an association between selenium deficiency and epilepsy has been reported (Ashrafi et al., 2007a,b). In fact, oral selenium supplementation has improved the clinical state and electroencephalogram of children with intractable epileptic seizures and low blood selenium concentrations (Weber et al., 1991; Ramaekers; et al., 1994). Reinforcing this idea, Savaskan et al. (2003) reported that selenium is effective in protecting neurons from primary damage in the course of excitotoxic lesions induced by KA in rats, suggesting that an adequate sele-

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*Abbreviations:* AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ANOVA, analysis of variance; DCHF, fluorescent dichlorofluorescein; DCHF-DA, 2′, 7′-dichlorofluorescein diacetate; DNPH, dinitrophenylhydrazine; DTDS, 2,2′-dithienyl diselenide; EEG, electroencephalographic; GABA, gamma-aminobutyric acid; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; KA, kainic acid; NMDA, N-methyl-D-aspartate; NPSH, nonprotein sulfhydryl; Pi, inorganic phosphate; p.o., orally; ROS, reactive oxygen species; RS, reactive species; SE, status epilepticus; S1, low-speed supernatant; TLE, temporal lobe epilepsy.

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nium supplementation is an important strategy to prevent the detrimental effects of excitotoxicity. Therefore, we believe that compounds containing selenium in their structure could also eventually reduce excitotoxic insults, such as seizures.

The interest in organoselenium chemistry and biochemistry has increased, mainly because these compounds show important biological activities, such as neuroprotection (Nogueira et al., 2004; Nogueira and Rocha, 2010). 2,2′-Dithienyl diselenide (DTDS), an organoselenium compound, has shown to be a promising antioxidant in rat brain homogenate *in vitro* (CF. Bortolatto, unpublished observations). Based on the above considerations, the objective of the present study was to investigate the protective effect of DTDS, an organoselenium compound, against seizures induced by KA in rats.

## EXPERIMENTAL PROCEDURES

### Animals

Adult male Wistar rats (200–300 g) were obtained from a local breeding colony. Animals were housed in cages with free access to food and water. They were kept in a separate animal room on a 12-h light/12-h dark cycle, with lights on at 7:00 AM, in an air-conditioned room (22±2 °C). Animal care and all experimental procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80–23, revised in 1996) and in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources from the Federal University of Santa Maria, Brazil. All efforts were made to minimize the number of animals used and their suffering.

### Chemicals

The compound DTDS (Fig. 1) was prepared according to the literature method (Tiecco et al., 2000). Analysis of hydrogen-1 nuclear magnetic resonance ($^{1}$H-NMR) and carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectra showed that the obtained compound presented spectroscopic data in full agreement with its assigned structure. The chemical purity of this compound (99.9%) was determined by GC-MS. KA, phenobarbital, chloral hydrate, 2', 7'-dichlorofluorescein diacetate (DCHF-DA), dinitrophenylhydrazine (DNPH), 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB), β-nicotinamide adenine dinucleotide phosphate reduced (NADPH) tetrasodium salt, ouabain, adenosine triphosphate (ATP), bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). All other chemicals were obtained from analytical grade and standard commercial suppliers. The DTDS chemical characteristics are given below:

Yield: 0.023 g (40%). $H^{1}$ NMR ($CDCl_3$, 200 MHz), δ (ppm): 7.50–7.47 (dd, *J*=5.3 Hz, 2H), 7.23–7.24 (dd, *J*=3.5 Hz, 2H), 7.00 (d, *J*=8.8 Hz, 1H), 7.00 (d, *J*=1.6 Hz, 2H). $C^{13}$ NMR ($CDCl_3$, 50 MHz), δ (ppm): 136.91, 132.91, 128.09, 125.518. MS (EI, 70 eV) m/z (relative intensity): 325 (34), 162 (100), 160 (53), 119 (24), 93 (4), 84 (27), 70 (32), 51 (5).

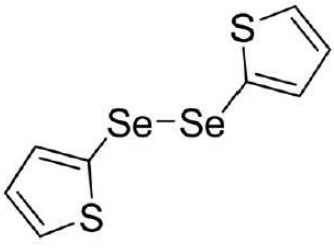


**Fig. 1.** Chemical structure of DTDS.

### Drug treatment and evaluation of behavioral changes

In a section of experiments, the animals were randomly assigned to six groups (*n*=8–10 per group). The rats were orally (p.o.) treated by gavage with DTDS (50 or 100 mg/kg dissolved in canola oil) or canola oil, and after 1 h the animals received i.p. KA (10 mg/kg dissolved in saline) or saline. The drugs were administered at a fixed volume of 1 ml/kg of body weight. The protocol used to elicit KA-induced seizures was based on previous studies (Morales-Garcia et al., 2009; Shin et al., 2009).

After injection of KA, the animals were put in individual cages, and they were attended for 4 h to evaluate the appearance of seizures, latency for the onset of the first seizure episode, as well as convulsive behavior according to the following rating scale described by Racine (1972): 0=no reaction; 1=stereotype mouthing, eye blinking and/or mild facial clonus; 2=head nodding and/or severe facial clonus; 3=myoclonic jerks in the forelimbs; 4=clonic convulsions in the forelimbs with rearing; and 5=generalized clonic convulsions associated with loss of balance, which were expressed as Racine's score. Latency for the onset of the first seizure episode was assumed as fourth stage of Racine's score. Because the dose of 100 mg/kg of DTDS showed the most effective protection against seizure induced by KA, this dose was chosen for subsequent electroencephalographic and histological analysis.

### Electroencephalographic (EEG) surgical procedures

A subset of animals (*n*=4 per group) was surgically implanted with electrodes under stereotaxic guidance. Animals were then anesthetized with Equitesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, two screw electrodes were placed bilaterally over the parietal cortex along with a ground lead positioned over the nasal sinus (coordinates from bregma: AP −4.5 mm; L 2.5 mm) (Paxinos and Watson, 1986). Bipolar nichrome wire Teflon-insulated depth electrodes (100 μm in diameter) were implanted 1 mm above the CA1 region of the dorsal hippocampus (coordinates relative to bregma: AP 4 mm, ML 3 mm, and V 2 mm) (Paxinos and Watson, 1986). The electrodes were connected to the multipin socket and were fixed to the skull with dental acrylic cement. EEG recordings were performed 5 days after the surgery.

### EEG recordings and analyses

Seizures were monitored in animals by EEG recordings. Rats were allowed to settle for habituation in a Plexiglas cage (25×25×60 $cm^3$) for at least 20 min, and then connected to the lead socket in a swivel inside a Faraday's cage. Routinely, a 10-min baseline recording was obtained to establish an adequate control period. The effect of oral administration of DTDS on seizure activity induced by KA was investigated by DTDS administration (100 mg/kg, p.o.) or its vehicle (canola oil), 1 h before the injection of KA (10 mg/kg, i.p.). The behavior of the animals was tracked during 4 h, and EEG was concomitantly recorded using a digital encephalographer. All EEG apparatus was obtained from Neuromap EQSA260, Neurotec Ltd., Itajubá, Minas Gerais, Brazil. EEG signals were amplified, filtered (0.1–70.0 Hz, bandpass), digitalized (sampling rate, 256 Hz), and stored in a PC for off-line analysis. Digitalized data from basal (10 min) DTDS administration (1 h) and post-KA (4 h) periods were divided in 30 s segments, and a 4 s sample from each segment was used to measure amplitude wave.

### Sample preparation for ex vivo analyses

After 4 h of KA administration, rats were decapitated. Brain was quickly removed, kept in ice, and dissected immediately on a cold plate for separation of hippocampus and cerebral cortex. The samples of hippocampus (hippocampal pool of two animals) and cortex were homogenized in 50 mM Tris–HCl, pH 7.4 (1/4, w/v), and centrifuged at 2400×g for 10 min to obtain the low-speed supernatant ($S_1$). $S_1$ was used for reactive species (RS), non-protein sulfhydryl (NPSH), glutathione peroxidase (GPx), and $Na^+$, $K^+$-ATPase assays, except for carbonyl protein levels.

RS, carbonyl protein, NPSH content, as well as GPx and $Na^+$, $K^+$-ATPase activities, were evaluated in cortex and hippocampus of rats exposed to KA, whereas brain regions are not equally injured after KA administration.

*Reactive species (RS) measurement.* The RS levels were determined in $S_1$ by a spectrofluorimetric method, using DCHF-DA assay (Loetchutinat et al., 2005). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCHF) is measured for the detection of intracellular RS. To estimate the level of RS, 10 μl of $S_1$ was added to 2.98 ml of 50 mM Tris–HCl (pH 7.4) and incubated with 10 μl of 1 mM DCHF-DA. The DCHF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 15 min after the addition of DCHF-DA to the medium. The RS levels were expressed as arbitrary units.

*Carbonyl protein levels.* Carbonyl protein content was assayed by a method based on the reaction of protein carbonyls with DNPH forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Hippocampal and cortex homogenates (1/4, w/v) were prepared in 50 mM Tris–HCl buffer, pH 7.4, without centrifugation. Homogenate was diluted with Tris–HCl buffer, pH 7.4, in a proportion of 1:8. Three tubes containing aliquots of 1 ml of dilution were incubated at 37ºC for 2 h. A volume of 200 μl of 10 mM DNPH dissolved in 2.0 M HCl was added to two of tubes (duplicate). In the third tube was added only 200 μl of 2.0 M HCl solution (blank). Tubes were incubated for 60 min at room temperature in the dark and vortexed every 15 min. After that, 0.5 ml of denaturizing buffer (sodium phosphate buffer, pH 6.8, containing 3% sodium dodecyl sulfate (SDS)), 1.5 ml of ethanol, and 1.5 ml of hexane were added. The mixture was vortexed for 40 s and centrifuged for 15 min at 2400×g. The pellet obtained was separated and washed two times with 1 ml of ethanol:ethyl acetate (1:1, v/v). The pellet was dissolved and mixed in 1 ml of denaturizing buffer solution. Absorbance was measured at 370 nm. Results were reported as carbonyl content (nmol carbonyl content/mg protein).

*Determination of non-protein thiols (NPSH) levels.* NPSH levels were determined by the method of Ellman (1959). To determine NPSH, $S_1$ was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded, and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer, pH 7.4, and 10 mM DTNB. The color reaction was measured at 412 nm. NPSH levels were expressed as μmol NPSH/g tissue.

*Glutathione peroxidase (GPx) activity.* GPx activity in $S_1$ was assayed spectrophotometrically through the glutathione/NADPH/glutathione reductase system by the dismutation of $H_2O_2$ at 340 nm (Wendel, 1981). In this assay, the enzyme activity is measured indirectly by means of NADPH decay. $H_2O_2$ is decomposed, generating oxidized glutathione (GSSG) from reduced glutathione (GSH). GSSG is regenerated back to GSH by the glutathione reductase present in the assay media, at the expense of NADPH. The enzymatic activity was expressed in nmol NADPH/min/mg protein.

*$Na^+$, $K^+$-ATPase activity.* The reaction mixture for $Na^+$, $K^+$-ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl, and 50 mM Tris–HCl, pH 7.4, in a final volume of 500 μl. The reaction was started by the addition of ATP to a final concentration of 3.0 mM. For obtaining the ouabain-sensitive activity, samples were carried out under the same conditions with the addition of 0.1 mM ouabain. The samples were incubated at 37 °C for 30 min; the incubation was stopped by adding trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. $Na^+$, $K^+$-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain). Released inorganic phosphate (Pi) was spectrofluorimetrically measured at 650 nm as described by Fiske and Subbarow (1925), and $Na^+$, $K^+$-ATPase activity was expressed as nmol Pi/mg protein/min.

*Protein quantification.* Protein concentration was measured by the method developed by Bradford (1976), using bovine serum albumin as the standard.

### Histology

Another subset of animals ($n$=3 per group) was used for histological analysis. DTDS was administered at the dose of 100 mg/kg, 1 h before the KA (10 mg/kg, i.p.) administration. Control rats were injected with canola oil (p.o.) plus saline (i.p.). After 7 days of KA administration (Liang et al., 2007), the animals were decapitated, and the hippocampus from individual rat was fixed in 10% formalin to histological evaluation. The hippocampus was embedded in paraffin, sectioned at 4 μm, and stained with Cresyl Violet for light microscopy examination. Cresyl Violet staining was performed to observe neuronal loss in the hippocampus induced by KA. Neuron morphology was observed in areas of the CA1, CA2, and CA3. Only neurons with a visible nucleus and with the entire outline of the cell apparent were considered normal.

### Statistical analysis

Seizure incidence was statistically analyzed by the $\chi^2$ method and Fisher's exact test. Data of Racine's score were analyzed by the nonparametric Kruskal–Wallis test. Statistical analysis of latency to the seizure onset was performed using one-way analysis of variance (ANOVA), followed by the Newman–Keuls test. *Ex vivo* analyses were performed using two-way ANOVA, followed by the Newman–Keuls test, when appropriate. EEG records were statis-

**Table 1.** Effect of pretreatment with DTDS on KA-induced seizures in rats

| Groups | Seizures (n/N)[a] | Latency (min)[b] | Racine's score[c] |
|---|---|---|---|
| Control | 0/8 | ns | 0 |
| DTDS 50 | 0/8 | ns | 0 |
| DTDS 100 | 0/8 | ns | 0 |
| KA | 10/10 | 62.51±12.31 | 4–5 (4.50±0.16) |
| DTDS 50+KA | 4/10 | 70.12±14.47 | 3–4 (3.25±0.25) |
| DTDS 100+KA | 2/10* | 91.21±5.65** | 1–2 (1.50±0.50)*** |

Rats were pretreated orally with DTDS (50 or 100 mg/kg, p.o.) or canola oil, and after 1 h they received KA (10 mg/kg i.p.).

[a] Number of animals that showed seizure episodes/number of animals per group.

[b] Time to the appearance for the first seizure episode (minutes).

[c] Interval of Racine's score according to scale of five points of severity (mean±SEM are presented between brackets).

* $P<0.05$ versus KA group ($\chi^2$ method and Fischer's exact probability test).

** $P<0.05$ versus KA group (one-way ANOVA).

*** $P<0.05$ versus KA group (Kruskal–Wallis test).

tically analyzed by two-way repeated-measures ANOVA. Data are expressed as means±SEM. Values of $P<0.05$ were considered statistically significant.

## RESULTS

### DTDS effects on KA-induced seizures

As shown in Table 1, there were no behavioral changes observed in animals treated with vehicle or DTDS (50 and 100 mg/kg, p.o.).

Behavioral analysis revealed that the systemic (i.p.) administration of an excitotoxic dose of KA (10 mg/kg) induced progressive motor alterations, similar to those previously described by Racine (1972). All animals pretreated with canola oil and then given KA showed behavioral seizures (10/10), and Racine's score reached 4.5± 0.16 points. In most of the animals that received canola oil plus KA, the seizure episodes were manifested by clonic seizures in the forelimbs with rearing and/or generalized clonic convulsions associated with loss of balance. The latency for the onset of the first clonic seizure episode induced by KA was 62.51±12.31 min (Table 1).

Pretreatment of rats with DTDS, at the dose of 100 mg/kg (p.o.), was statistically effective in decreasing the appearance of seizures (2/10) induced by KA, as well as in

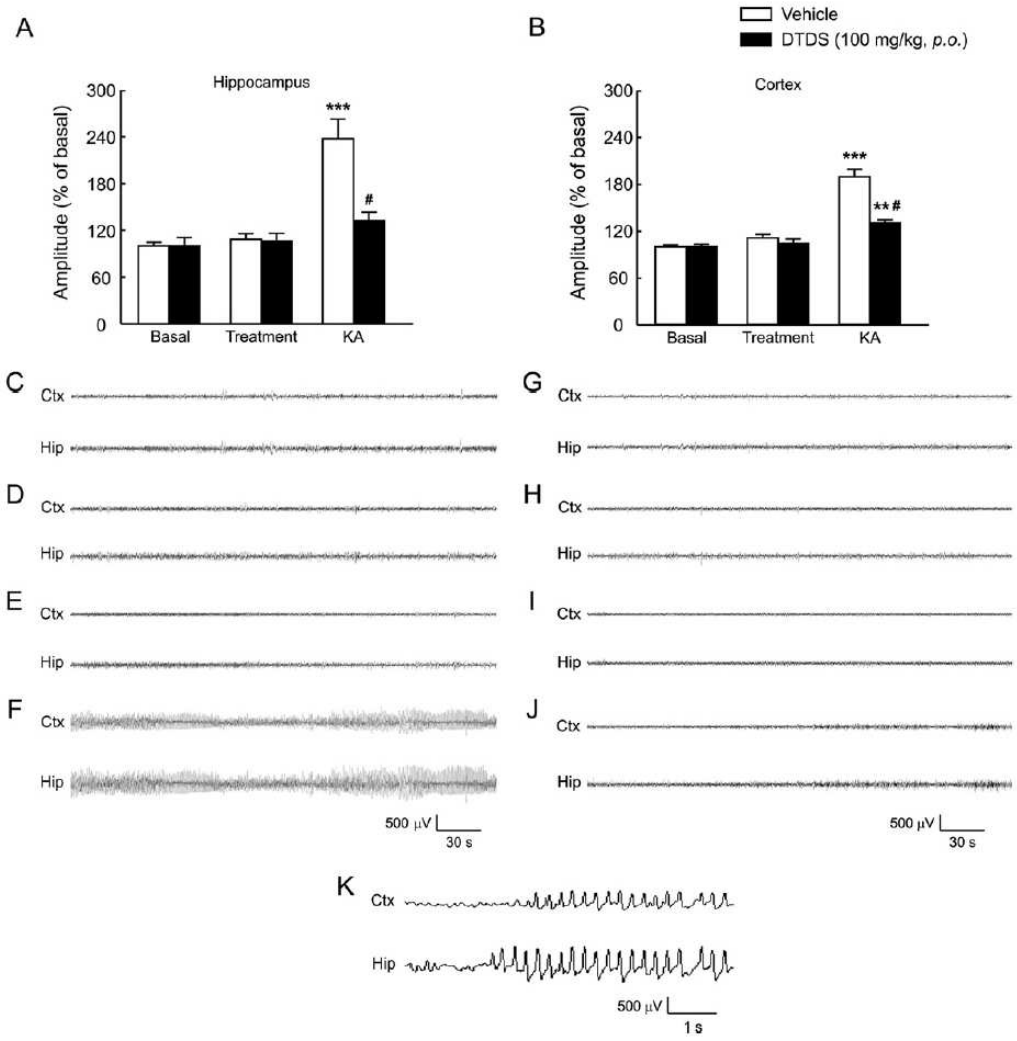


**Fig. 2.** Effect of pretreatment with DTDS on EEG alterations induced by KA exposure in rat cortex and hippocampus. Rats were pretreated orally with DTDS (100 mg/kg), and after 1 h they received KA (10 mg/kg, i.p.). The behavior of the animals was followedup during 4 h: (A) EEG amplitude in cortex; (B) EEG amplitude in hippocampus (*** $P<0.001$ vs. respective basal, ** $P<0.01$ vs. respective basal, # $P<0.001$ vs. KA, two-way repeated-measures ANOVA, $n=4$ rats/group). (C–J) Representative hippocampal EEG records of rats treated with vehicle (left panel, C–F) and DTDS (right panel, G–J), respectively, where (C) and (G) represent basal records; (D) and (H) represent posttreatment records; (E) and (I) represent records immediately after KA injection; and (F) and (J) represent records 2 h after KA injection; (K) represents the appearance of an epileptogenic focus in the hippocampus induced by KA, as can be evidenced by onset of discharges, with consequent spreading to the cerebral cortex.

**Table 2.** Effect of pretreatment with DTDS on RS, protein carbonyl and NPSH levels, and GPx activity in hippocampus of rats exposed to KA

| Groups | RS[a] | Protein carbonyl[b] | NPSH[c] | GPx[d] |
|---|---|---|---|---|
| Control | 8.69±0.24 | 1.53±0.04 | 4.70±0.04 | 29.48±1.50 |
| DTDS 50 | 5.33±0.22 | 1.50±0.06 | 4.05±0.22 | 28.00±1.11 |
| DTDS 100 | 6.10±1.00 | 1.81±0.32 | 4.54±0.10 | 25.80±2.39 |
| KA | 31.36±4.91* | 2.67±0.08* | 3.44±0.61 | 30.45±1.17 |
| DTDS 50+KA | 12.53±3.71** | 2.12±0.22** | 4.37±0.32 | 28.81±1.88 |
| DTDS 100+KA | 11.89±1.67** | 1.74±0.08** | 4.71±0.16 | 30.60±1.31 |

Rats were pretreated with DDT (50 or 100 mg/kg, p.o.) or canola oil, and after 1 h they received KA (10 mg/kg i.p.).
Data are reported as mean±SEM and expressed as follows: [a] arbitrary units,[b] nmol carbonyl content/mg protein,[c] μmol NPSH/g tissue,[d] nmol NADPH/min/mg protein (two-way ANOVA followed by Student–Newman–Keuls test); $n$=4–5 hippocampal pool/group (8–10 rats/group).
* $P<0.001$ versus control group.
** $P<0.05$ versus KA group.

increasing the latency for the onset of the first clonic seizure episode (91.21±5.65 min). A decreased Racine's score was observed in the rats that received a 100-mg/kg DTDS pretreatment (1.5±0.50; H(2)=10.59, $P<0.05$). When the animals were pretreated with DTDS plus KA, seizures were attenuated, and most of the animals expressed stereotype mouthing, eye blinking, and/or mild facial clonus, and/or head nodding, and/or severe facial clonus (Table 1).

On the other hand, the lowest dose of DTDS did not show a significant protection against behavioral alterations resulting from KA exposure, although a tendency in reducing KA-induced convulsive behavior was observed (Table 1).

### DTDS effects on KA-induced epileptiform EEG activity

As shown in Fig. 2, EEG recordings demonstrated that DTDS, at the dose of 100 mg/kg, did not alter baseline EEG amplitude in rat cortex (Fig. 2A) and hippocampus (Fig. 2B, see also Fig. 2G, H). KA administered at a convulsant dose (10 mg/kg, i.p.) induced EEG seizure activity at both brain structures (Fig. 2A, B). EEG recordings of hippocampus and cerebral cortex revealed that the systemic administration of KA induced the appearance of an epileptogenic focus in the hippocampus, which spread to the cerebral cortex (Fig. 2K). Fig. 2F illustrates hippocampal EEG alterations 2 h after KA administration.

Statistical analysis of quantitative cerebral cortex EEG revealed that DTDS pretreatment, at the dose of 100 mg/kg (p.o.), elicited a significant decrease on EEG alterations (but not back to basal levels) resulting from KA exposure ($F_{(2,12)}$=15.89; $P<0.001$) (Fig. 2A). However, at the dose of 100 mg/kg, DTDS pretreatment completely prevented the increase of EEG amplitude in hippocampus recording, reaching basal measurements, as shown in Fig. 2B ($F_{(2,12)}$=25.19; $P<0.001$) (compare also Fig. 2F, J).

### Oxidative stress parameters

*RS levels.* Two-way ANOVA of hippocampal RS levels revealed a significant DTDS × KA interaction ($F_{(2,21)}$=4.93; $P<0.0175$). Post hoc comparisons revealed that KA produced a significant increase in the hippocampal RS levels in comparison with the control. Preadministration of DTDS, at doses of 50 and 100 mg/kg, protected against the increase of hippocampal RS levels induced by KA. RS levels remained unaltered in the hippocampus of rats that received DTDS (50 and 100 mg/kg) when compared with the control group (Table 2). No alteration in RS levels was observed in cortex of rats exposed to KA and/or DTDS (Table 3).

*Carbonyl protein levels.* Two-way ANOVA of hippocampal carbonyl levels revealed a significant DTDS × KA interaction ($F_{(2,21)}$=6.58; $P<0.0060$). Post hoc comparisons showed that the exposure of animals to KA significantly increased the carbonyl protein levels in hippocampus when compared with the control group. DTDS, preadministered at the doses of 50 and 100 mg/kg, protected against the increase of the hippocampal carbonyl protein levels caused by KA exposure. DTDS, at both

**Table 3.** Effect of pretreatment with DTDS on RS, protein carbonyl and NPSH levels, and GPx activity in cortex of rats exposed to KA

| Groups | RS[a] | Protein carbonyl[b] | NPSH[c] | GPx[d] |
|---|---|---|---|---|
| Control | 7.60±0.45 | 1.19±0.05 | 4.58±0.06 | 35.01±0.80 |
| DTDS 50 | 6.49±0.46 | 1.02±0.09 | 4.18±0.07 | 31.07±1.40 |
| DTDS 100 | 7.06±0.61 | 1.45±0.10 | 4.52±0.07 | 37.77±1.14 |
| KA | 7.64±0.51 | 1.33±0.13 | 4.22±0.16 | 38.24±2.24 |
| DTDS 50+KA | 8.40±0.19 | 1.48±0.07 | 4.41±0.12 | 38.88±1.39 |
| DTDS 100+KA | 8.17±0.33 | 1.13±0.10 | 4.55±0.18 | 34.71±0.96 |

Rats were pretreated with DTDS (50 or 100 mg/kg, p.o.) or canola oil, and after 1 h they received KA (10 mg/kg, i.p.).
Data are reported as mean±SEM and expressed as follows: [a] arbitrary units, [b] nmol carbonyl content/mg protein, [c] μmol NPSH/g tissue, [d] nmol NADPH/min/mg protein (two-way ANOVA followed by Student–Newman–Keuls test); $n$=8–10 cortex/group.

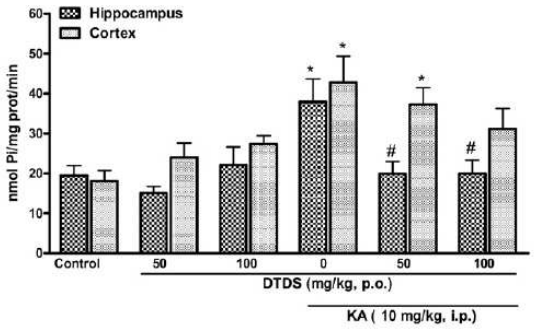


**Fig. 3.** Effect of pretreatment with DTDS on $Na^+$, $K^+$-ATPase activity in hippocampus and cortex of rats exposed to KA. Rats were pretreated orally with DTDS (50 or 100 mg/kg), and after 1 h they received KA (10 mg/kg, i.p.). The results are reported as mean±SEM and expressed as nmol Pi/mg prot/min. # $P<0.05$ versus respective control group * $P<0.05$ versus respective KA group by two-way ANOVA followed by Student–Newman–Keuls.

doses, did not alter hippocampal carbonyl levels when compared with the control group (Table 2). In addition, neither KA nor DTDS treatment altered carbonyl levels in cortex of rats (Table 3).

*NPSH levels and GPx activity.* There were no alterations on NPSH levels and GPx activity in the hippocampus (Table 2) and cortex (Table 3) of animals exposed to KA and/or DTDS.

*$Na^+$, $K^+$-ATPase activity.* Two-way ANOVA of hippocampal $Na^+$, $K^+$-ATPase activity demonstrated a significant DTDS × KA interaction ($F_{(2,21)}=3.61$; $P<0.0450$). Post hoc comparisons showed an increase in hippocampal $Na^+$, $K^+$-ATPase activity in the KA group in comparison with the control group. DTDS, at the doses of 50 and 100 mg/kg, was effective in protecting against $Na^+$, $K^+$-ATPase activity alterations resulting from KA exposure (Fig. 3).

Statistical analysis of cortical $Na^+$, $K^+$-ATPase activity showed a significant main effect of KA ($F_{(1,48)}=13.50$; $P<0.006$). Post hoc comparisons revealed that KA administration to rats increased $Na^+$, $K^+$-ATPase activity in cortex when compared with the control group. Pretreatment of animals with DTDS (50 and 100 mg/kg) was not effective in protecting against the increase of cortical $Na^+$, $K^+$-ATPase activity caused by KA exposure, although an attenuation of $Na^+$, $K^+$-ATPase activity in the animals treated with DTDS, at the highest dose, can be observed (Fig. 3).

As shown in Fig. 3, DTDS administration (50 and 100 mg/kg) did not modify $Na^+$, $K^+$-ATPase activity in hippocampus and cortex of rats when compared with the control group.

### DTDS effects on KA-induced histopathological changes

Histological analysis of hippocampus carried out under Cresyl Violet staining from control animals exhibited clearly visible neuronal layers. Seven days after KA injection, intense neuronal loss (cellular depopulation) was observed in CA1 (pyramidal), CA2, and CA3 regions from animals exposed to KA, although neuronal loss was more pronounced in the CA3 region. Hippocampi of KA-treated rats showed gliosis process, evidenced by an intense glial cellularity. Pretreatment with DTDS, at the dose of 100 mg/kg, 1 h before KA administration was effective in attenuating hippocampal neurodegeneration (Fig. 4).

## DISCUSSION

Behavior and EEG evidence found in this study demonstrated that DTDS relieved KA-induced seizures in rats. Besides, our data showed that DTDS was effective in preventing the hippocampal alterations of oxidative status and $Na^+$, $K^+$-ATPase activity. DTDS was also able in attenuating neuronal loss caused by KA exposure.

Organoselenium compounds have highly lipophilic nature (Nogueira and Rocha, 2010), leading us to infer the brain as a potential target for their action. In this context, diphenyl diselenide, an organoselenium compound, has been proven to quickly cross the blood–brain barrier of rodents (Prigol et al., 2010). In the present study, we showed that DTDS (100 mg/kg, p.o.) protected against KA-induced behavioral seizures in rats, providing evidence that DTDS can affect brain processes. Electrographically recorded seizures resulting from KA were characterized by the appearance of an epileptogenic focus in hippocampus that spread to the cerebral cortex. Our EEG findings are in accordance with the literature data, which have shown that the hippocampus is closely related to seizure onset (Liu et al., 2001). Besides, EEG revealed that previous DTDS administration significantly protected against KA-induced seizures in hippocampus, whereas in cerebral cortex, this reduction was partial.

The EEG findings seem to be related to those found for the $Na^+$, $K^+$-ATPase activity. DTDS (100 mg/kg) totally protected against the increase in the hippocampal $Na^+$, $K^+$-ATPase activity resulting from KA seizures, and there was a tendency of protection in cerebral cortex of rats. $Na^+$, $K^+$-ATPase is responsible for the maintenance of ionic gradient necessary for neuronal excitability (Freitas et al., 2003). Similar to investigators who found an increased activity of $Na^+$, $K^+$-ATPase as a result of the exposure of rodents to convulsants (Sztriha et al., 1987; Kinjo et al., 2007), we observed that KA stimulated the $Na^+$, $K^+$-ATPase activity in cortex and hippocampus. A plausible explanation for this fact is that activation of $Na^+$, $K^+$-ATPase could represent a compensatory mechanism in an attempt to control the brain excitability. It has been demonstrated that discharges produced by electrical stimulation are also followed by an increase in the $Na^+$, $K^+$-ATPase activity (Bignami et al., 1966; Harmony et al., 1968). Furthermore, the activation of glutamate receptors induces a $Ca^{2+}$-mediated activation of calcineurin, which dephosphorylates and activates the $Na^+$, $K^+$-ATPase (Monfort et al., 2002). In addition, nitric oxide, carbon monoxide, and metabotropic and N-methyl-D-aspartate (NMDA) glutamate receptors also modulate neuronal $Na^+$, $K^+$-ATPase activity by activating protein kinases C and G (Nathanson et al., 1995) in a complex phosphorylation cascade of regulatory

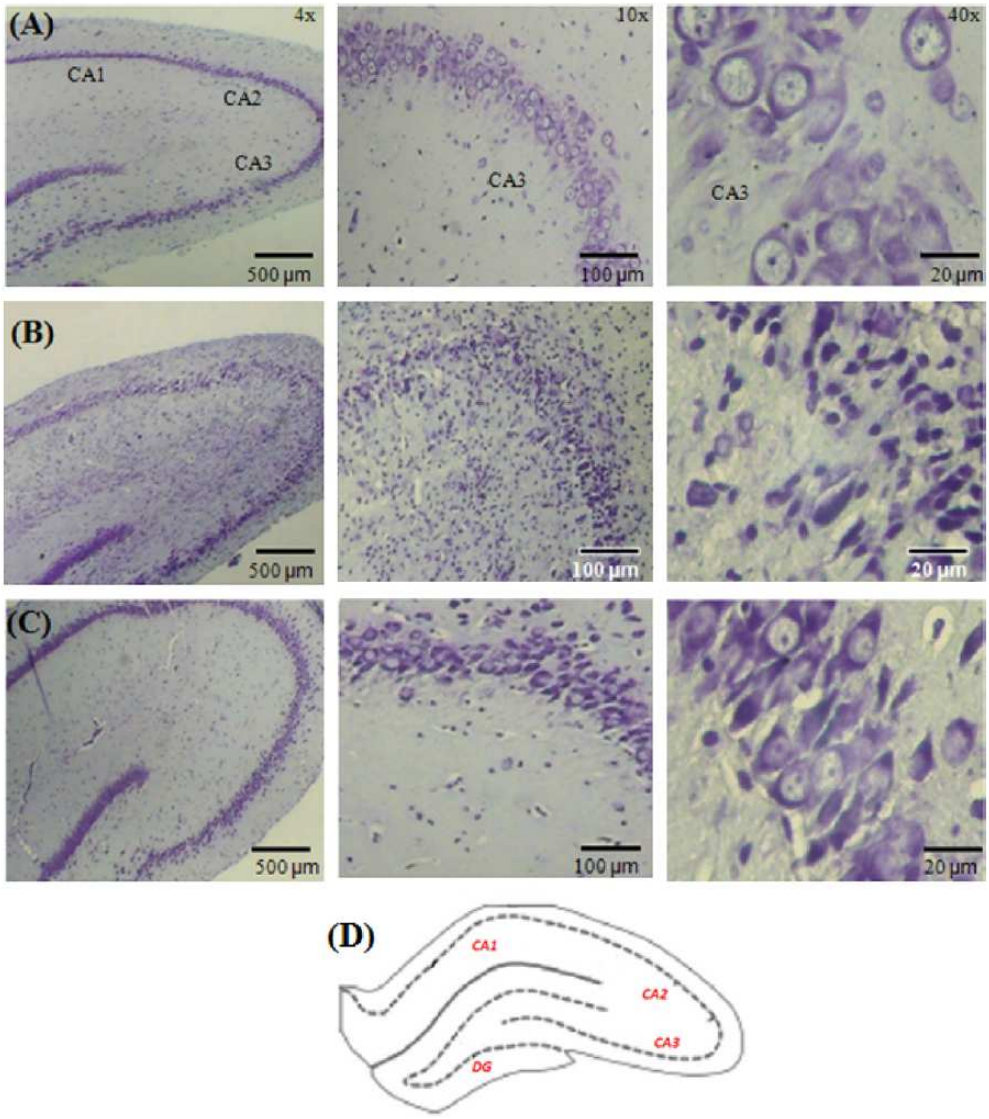


**Fig. 4.** Photomicrography of histopathological changes in rat hippocampus 7 d after KA exposure. (A) Control group; (B) KA; (C) DTDS, 100 mg/kg plus KA; (D) hippocampal map (CA1, CA2, CA3, dentate gyrus, DG). Wistar rats were pretreated with DTDS (100 mg/kg, p.o.) or canola oil, and the seizures were induced by KA (10 mg/kg i.p.) administration at 1 h after DTDS pretreatment. Cresyl Violet stain: 4, 10, and 40×, respectively. Calibration bars correspond to 500, 100, and 20 $\mu$m for augments of 4, 10, and 40×, respectively. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

proteins in which free radicals play a modulatory role (Therien et al., 2001).

Our results revealed that the KA administration induced oxidative damage in rat hippocampus, but not in cerebral cortex, evidenced by increased RS and protein carbonyl levels 4 h after its exposure. The present findings support the hypothesis that the pattern of oxidative injury induced by KA seems to be highly region specific, and the hippocampus is one of the most vulnerable areas to *in vivo* KA-mediated oxidative stress (Candelario-Jalil et al., 2001). Although many epilepsy studies have shown alterations in the antioxidant defenses by KA (Li et al., 2010; Kim et al., 2000; Yalcin et al., 2010), antioxidant defenses analyzed here were not modified. However, we cannot rule out the fact that another antioxidant defense, not evaluated, could be altered by KA exposure.

KA is thought to prosecute its action, at least in part, by increasing glutamate release through presynaptic receptors (Ferkany et al., 1982). In addition to KA receptors, KA also stimulates $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPA) receptors (Farooqui et al., 2008). It is generally accepted that the overactivation of

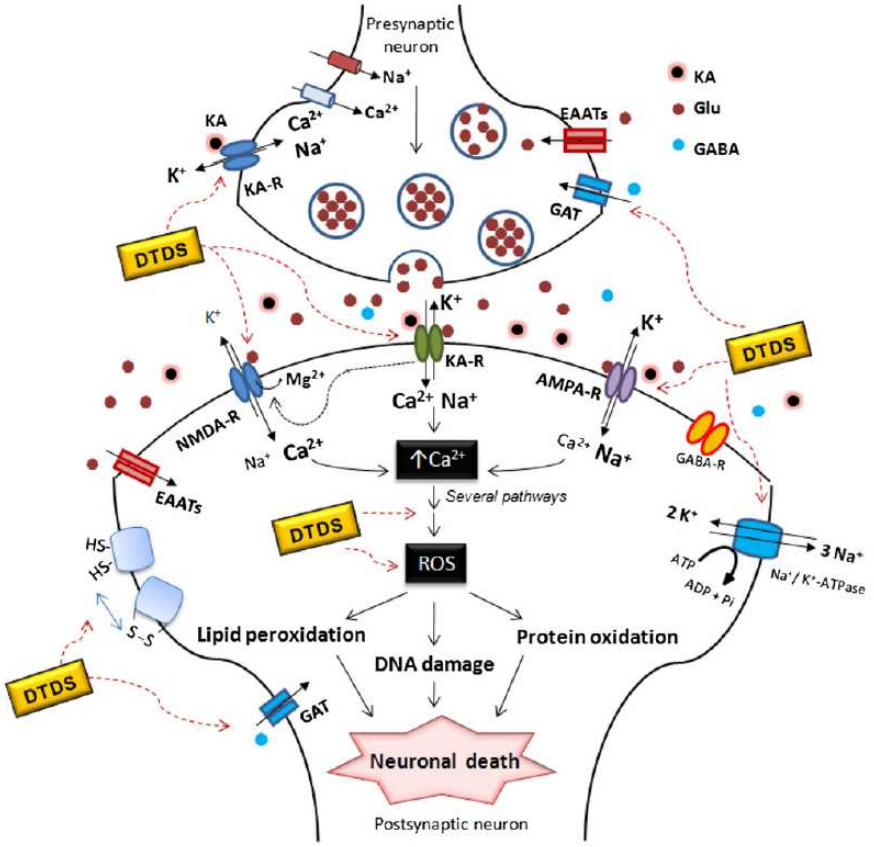


**Fig. 5.** Schematic presentation of signaling pathways involved in the excitatory amino acid-mediated excitotoxicity and possible targets, which could contribute to anticonvulsant and protective effects of DTDS. KA; Glu, glutamate; NMDA-R, NMDA receptor; KA-R, kainate receptor; AMPA-R, AMPA receptor; EEATs, excitatory amino acid transporters; GAT, GABA transporters.For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

excitatory amino acid receptors triggers a marked intracellular $Ca^{2+}$ rise, leading to the activation of $Ca^{2+}$-dependent proteases, kinases, and nucleases and the generation of ROS, with consequent neuronal damage (Allison and Pratt, 2003; Wang et al., 2005). Increased ROS levels may be critical in KA excitotoxic effects because ROS can damage lipids, proteins, and DNA and disrupt the membrane integrity, leading to cellular and mitochondrial dysfunctions that are associated with cell death (Lin and Beal, 2006) (Fig. 5).

We observed that DTDS pretreatment prevented the hippocampal oxidative damage resulting from KA injection. Although DTDS could act as an antioxidant in the present study, data of behavioral seizures and EEG seizure point to DTDS anticonvulsant action, thereby reducing the cascade of ROS formation. The results do not allow us to elucidate the exact mechanism by which DTDS caused the anticonvulsant action. However, it is interesting to note that some organochalcogenides are able to modulate neural circuits. It has been reported that acute exposure to ebselen inhibited $K^+$-stimulated [$^3$H]glutamate release by brain synaptosomes in rats (Nogueira et al., 2002). Prigol et al. (2009) have also demonstrated that *m*-trifluoromethyl-diphenyl diselenide attenuated pentylenetetrazole-induced seizures in mice by inhibiting gamma-aminobutyric acid (GABA) uptake in cerebral cortex slices. Moreover, some receptors and ion channels, such as NMDA receptor, voltage-gated $K^+$ channels, and GABA-A receptor, are known to be redox sensitive (Ruppersberg et al., 1991). In this context, Nogueira and Rocha (2010) suggest that redox modulation of specific high molecular weight thiol-containing molecules could contribute to the pharmacological effects of the organochalcogens. A schematic presentation of signaling pathways involved in the excitatory amino acid-mediated excitotoxicity and possible targets to DTDS is shown in Fig. 5.

Some authors have shown that systemic or intracerebral injection of KA causes epileptiform seizures in the CA3 region of the hippocampus. These seizures propagate to other limbic structures and are followed by a pattern of cell loss that is similar to those seen in patients suffering from TLE (Nadler, 1981; Ben-Ari, 1985). We demonstrated that DTDS was able to attenuate the neuronal loss in the hippocampal region observed 7 days after KA injection.

Although antioxidant mechanisms could be involved in the DTDS anticonvulsant action, we cannot rule out that the observed reduction of neuronal cell death is secondary to attenuated SE.

In conclusion, the present results showed that DTDS was effective in protecting against neurotoxicity induced by KA. However, the pharmacological mechanisms of anticonvulsant action of DTDS remain to be further elucidated.

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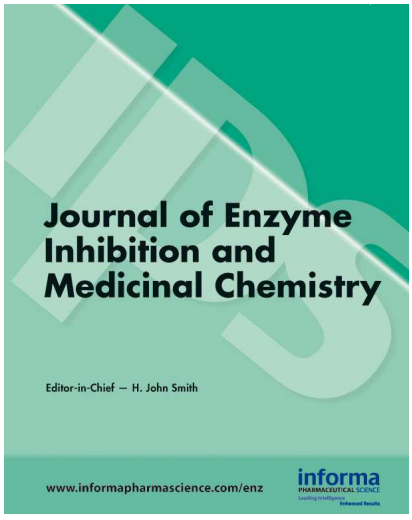
## 3.2 Artigo 2

**O 2,2'-disseleneto de ditienila, um composto orgânico de selênio, apresenta ação antioxidante e inibe a atividade da monoamino oxidase *in vitro***

**2, 2'-DITHIENYL DISELENIDE, AN ORGANOSELENIUM COMPOUND, ELICITS ANTIOXIDANT ACTION AND INHIBITS MONOAMINE OXIDASE ACTIVITY *IN VITRO***

Cristiani F. Bortolatto, Pietro M. Chagas, Ethel A. Wilhelm, Cristina W. Nogueira

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RESEARCH ARTICLE

# 2,2′-dithienyl diselenide, an organoselenium compound, elicits antioxidant action and inhibits monoamine oxidase activity *in vitro*

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**Abstract**

*Context:* Organoselenium compounds have been described as antioxidant and neuroprotective agents.

*Objective:* To evaluate the antioxidant action of 2,2′-dithienyl diselenide (DTDS) and its effects in brain monoamine oxidase (MAO) activity *in vitro*.

*Materials and methods:* Assays for reactive species (RS), lipid peroxidation, protein oxidation, MAO A and B activities in rat brain homogenate as well as mimetic dehydroascorbate reductase-like and glutathione S-transferase-like activities were performed using DTDS (μM range).

*Results:* DTDS was effective in decreasing the levels of RS as well as lipid peroxidation induced by malonate, sodium nitroprusside or $FeCl_2$/EDTA and protein carbonyl in the rat brain homogenate. DTDS elicited dehydroascorbate reductase-like and glutathione S-transferase-like activities. DTDS was effective in inhibiting both MAO-A and MAO-B activities.

*Discussion:* The results demonstrated that DTDS is an antioxidant agent with non-selective inhibitory effect on MAO activity.

*Conclusion:* DTDS is a promising molecule to be evaluated in experimental models of neurological diseases.

**Keywords:** Selenium, brain, rats

## Introduction

Oxidative stress, a perturbation of the redox homeostasis, has been implicated to the pathophysiology of several neurological disorders and plays a paramount role in the aging process[1,2]. In recent years, it has become increasingly clear that mitochondrial dysfunction and oxidative damage are major contributors to neuronal loss[3]. The brain is especially sensitive to oxidative stress because it utilizes high levels of oxygen, contains large amounts of lipids and exhibits a lower level of antioxidant defenses compared to other tissues[2]. Since natural or synthetic antioxidants can protect against oxidative stress, molecules with antioxidant action appear to be an attractive approach for prevention and/or adjuvant treatment of disorders linked to oxidative stress.

Chemicals such as sodium nitroprusside (SNP), malonate and $FeCl_2$/ethylenediamine tetraacetic acid (EDTA) are widely used to induce lipid peroxidation in order to screen novel antioxidant compounds *in vitro* since. These lipid peroxidation inductors can trigger reactive species (RS) generation by distinct ways[4–6]. The effects of novel compounds on the increase of RS induced by sodium azide, which causes mitochondrial dysfunction by inhibiting cytochrome oxidase activity[7], are also a valuable tool to screen antioxidant activity of compounds. Lastly, it is possible to apply assays for radical scavenging activity to characterize an antioxidant compound.

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Monoamine oxidase (MAO) is a flavoprotein located at the outer membranes of mitochondria in neuronal, glial and other cells. MAO is involved in the metabolic cerebral degradation of monoamines (serotonin, noradrenalin and dopamine) and appears to play important roles in many neurological and psychiatric disorders[8,9]. It is widely speculated that prolonged excessive activity of these enzymes may be conducive to mitochondrial damage and neurodegenerative disturbances[10]. The byproducts of MAO reactions include a number of potentially neurotoxic species, such as hydrogen peroxide and ammonia. In particular, hydrogen peroxide can trigger the production of reactive oxygen species (ROS) and induce mitochondrial damage and neuronal apoptosis[10]. In this sense, compounds having both antioxidant and MAO inhibitory properties could be interesting for treatment of some neurological diseases.

Selenium is an essential trace element and plays a crucial role in several major metabolic pathways, including antioxidant defense system[11]. Selenium exerts its antioxidant function mainly in the form of selenocysteine residues as an integral constituent of ROS-detoxifying selenoenzymes[12]. Besides, the preferential retention of selenium in the brain suggests that it plays important functions[13]. In this context, it has been highlighted the importance of the development of compounds containing selenium as preventive or therapeutic agents in neurological conditions.

Generally, organic forms of selenium are more bioavailable and less toxic than the inorganic forms[14]. The interest in the research of organic compounds containing selenium has increased considerably due to the fact that these compounds have been described to possess interesting pharmacological activities, such as neuroprotection[15,16]. In fact, the brain appears to be one of the target organs of organoselenides, since they have highly lipophilic nature[16]. In line with this, we have demonstrated that 2,2′-dithienyl diselenide (DTDS) plays anticonvulsant action in rats exposed to kainic acid[17].

Based on the pharmacological properties presented by synthetic organoselenium compounds, the aim of the present study was to evaluate the antioxidant action of DTDS *in vitro*. Moreover, since MAO activity is an important drug target for the treatment of neurological disorders, a second objective of this study was to investigate the *in vitro* effect of DTDS in cerebral MAO-A and MAO-B activities of rats.

## Material and methods

### Chemicals

DTDS (Figure 1) and ebselen were prepared according to the methods described by Tiecco *et al.*[18] and Engman[19], respectively. Analysis of $^{1}$HNMR and $^{13}$CNMR spectra showed that the compounds obtained presented spectroscopic data in full agreement with their assigned structures. The chemical purity of these compounds

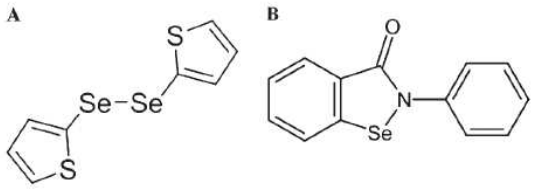


Figure 1. Chemical structures of (A) DTDS and (B) ebselen.

(99.9%) was determined by gas chromatography–mass spectrometry (GC/MS). Reduced glutathione (GSH), SNP and malonate were purchased from Sigma (St. Louis, MO, USA). 1-Chloro-2, 4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co (USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers. DTDS and ebselen were dissolved in dimethylsulfoxide (DMSO). The control group received the vehicle used for dissolving the compounds (DMSO). The DTDS chemical characteristics are given below:

Yield: 0.023 g (40%). $H^{1}$ NMR ($CDCl_3$, 200 MHz), $\delta$ (ppm): 7.47 (dd, $J$ = 1.2, 5.3 Hz, 2H), 7.23 (dq, $J$ = 1.2, 3.5 Hz, 2H), 7.00 (dd, $J$ = 3.5, 5.3 Hz, 2H). $C^{13}$ NMR ($CDCl_3$, 50 MHz) $\delta$ (ppm): 136.9 (2C), 132.9 (2C), 128.0 (2C), 125.5 (2C). MS (EI, 70 eV) $m/z$ (relative intensity): 325 (34), 162 (100), 160 (53), 119 (24), 93 (4), 84 (27), 70 (32), 51 (5).

### Animals

Male adult Wistar rats (200–300 g) were obtained from a local breeding colony. Animals were housed in cages with free access to food and water. Animals were kept in a separate animal room, on a 12-h light/12-h dark cycle, with lights on at 7:00 a.m., in an air-conditioned room (22 ± 2°C). The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

### Antioxidant assays

#### *Tissue preparation*

Rats were killed by decapitation and cerebral tissue (whole brain) was rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 50 mM Tris–HCl buffer, pH 7.4 (1/5, weight/volume). Homogenate freshly prepared was centrifuged at 2400×$g$ for 10 min to yield a pellet that was discarded and a low-speed supernatant ($S_1$). $S_1$ was used to determine the effect of different concentrations of DTDS on lipid peroxidation and reactive species levels. Protein carbonyl was assayed using the fresh brain homogenate (1/10, weight/volume) without centrifugation.

#### *RS measurement*

The RS levels were determined by a spectrofluorimetric method, using 2′, 7′-dichlorofluorescein diacetate (DCHF-DA) assay[20]. To estimate the levels of RS

production, 50 μL of $S_1$ was incubated with 2.9 mL of 10 mM Tris–HCl buffer, pH 7.4 and 10 μL of 1 mM DCHF-DA (prepared in ethanol and protected from light and warmth) in the presence or absence of a prooxidant (30 μL of 100 mM sodium azide) and 10 μL of DTDS at different concentrations (i.e., to achieve final concentrations of 1 to 25 μM) resulting in a final volume of 3 mL. The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCHF) is measured for the detection of RS levels. The DCHF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 15 min after the addition of DCHF-DA to the medium. During the 15 min of incubation at room temperature, tubes were maintained in dark. RS levels were expressed as arbitrary units (AU) of fluorescence.

### *Lipid peroxidation induced by malonate and SNP*

Malonate and SNP were used as inductors of lipid peroxidation. An aliquot of 100 μL of $S_1$ was added to the reaction mixture containing: 50 μL of 1.5 mM malonate or 0.3 mM SNP, 10 μL of DTDS at different concentrations (i.e., to achieve final concentrations of 1 to 25 μM) and 30 μL of 50 mM Tris–HCl, pH 7.4. Afterward the mixture was pre-incubated at 37°C for 1 h. The reaction product was determined using 500 μL thiobarbituric acid (TBA, 0.8%), 200 μL sodium dodecyl sulfate (SDS, 8.1%) and 500 μL acetic acid (pH 3.4) with subsequent incubation at 95 °C for 2 h. TBA reactive species (TBARS) were spectrophotometrically determined at 532 nm as described by Ohkawa *et al.*[21], using malondialdehyde (MDA, an end product of the peroxidation of lipids) as an external standard. Results were expressed as nmol MDA/g tissue.

### *Lipid peroxidation induced by $FeCl_2$/EDTA*

$FeCl_2$ plus EDTA were used as classical inductors of lipid peroxidation. An aliquot of 200 μL of $S_1$ was added to the reaction mixture containing: 30 μL of 500 μM EDTA solution (in water), 30 μL of 1.44 mM FeCl solution and 10 μL of DTDS at different concentrations (i.e., to achieve final concentrations of 1 to 25 μM) and water to complete a final volume of 300 μL. The FeCl solution was prepared in water, maintained in dark tube on the ice and immediately used. Afterward the mixture was pre-incubated at 37°C for 1 h. The reaction product was determined as described above Results were expressed as nmol MDA/g tissue.

### *Protein carbonyl determination*

Carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm[22]. Homogenate was diluted with Tris–HCl buffer, pH 7.4 in a proportion of 1:8 (homogenate:Tris–HCl). Aliquots of 940 μL of homogenate dilutions were incubated at 37°C for 2 h in the presence of 10 μL of DTDS at different concentrations (i.e., to achieve final concentrations of 1 to 25 μM) and 50 μL of 20 mM SNP. SNP was used to stimulate the protein carbonyl production and was prepared in water, maintained in dark tube on the ice and immediately used. In two tubes, it was added 200 μL of 10 mM DNPH in 2.0 M HCl. In the third tube, only 200 μL of 2.0 M HCl solution (blank) was added. All tubes were incubated for 1 h at room temperature, in dark and shaken using a vortex mixer every 15 min. After that, 0.5 mL of denaturizing buffer (sodium phosphate buffer, pH 6.8, containing 3% SDS), 1.5 mL of ethanol and 1.5 mL of hexane were added to all tubes. The tubes were shaken with a vortex mixer for 40 s and centrifuged for 15 min at 2400×*g*. The pellet obtained was separated, washed two times with 1 ml of ethanol: ethyl acetate (1:1, volume/volume), and dried at room temperature for 2 min. The pellet was immediately dissolved in 1 mL of denaturizing buffer solution with mixing. Absorbance was measured at 370 nm. Results were expressed as carbonyl content (nmol carbonyl content/mg protein).

### *Dehydroascorbate (DHA) reductase-like assay*

The DHA reductase-like activity of DTDS was assayed as described previously[23,24] with minor modifications. In brief, 10 μL of DTDS at different concentrations (i.e., to achieve final concentrations of 1 to 25 μM) were incubated (2 min) with 955 μL of 100 mM sodium phosphate buffer, pH 6.9, at 25°C in the presence of 10 μL of 100–300 mM GSH (final concentrations of 1–3 mM, diluted in water) in a final volume of 1 mL. The mixture was incubated at 25°C for 2 min. The DHA reductase assay was initiated by adding 25 μL of 20 mM DHA to a final volume of 1.0 mL. DHA solution was prepared on the day of experiments. For this end, ascorbic acid was added to a solution containing 10 mM sodium phosphate dibasic and 0.5 mM EDTA to achieve a final concentration of 20 mM ascorbic acid. The pH of mixture was adjusted to 5.5 with NaOH. After that, 10 μL of bromine to each 2 ml of ascorbic acid solution pH 5.5 were added and mixed at room temperature for 30 s. Afterwards, the solution was bubbled in argon for 10 min. The DHA solution obtained was stored protected from light in ice for up to 4 h. Ascorbic acid regeneration from DHA was recorded at 265 nm. A blank without DTDS was run, and the difference gave the DTDS DHA reductase activity in nmol/min using the molar extinction coefficient of ascorbic acid of 14,700 $cm^{-1}M^{-1}$. Ebselen (1–25 μM) was used as a positive control[25].

### *Glutathione S-transferase (GST)-like assay*

The reaction of GSH with CDNB is typically the preferred system used to measure the catalysis imparted by naturally occurring GSTs[26]. An aliquot of 10 μL of DTDS at different concentrations (i.e., to achieve final concentrations of 1 to 25 μM) was incubated with 20 μL of 50 mM GSH and 950 μL of 100 mM sodium phosphate buffer, pH 6.9 at 25°C for 3 min. The reaction was initiated by adding 20 μL of 25 mM CDNB to achieve a final volume of 1.0 mL and recorded for 3 min at 340 nm. CDNB was used as substrate. A blank without DTDS

was included and the difference was expressed as ΔAbs (delta absorbance)/min. Ebselen (1–25 μM) was used as a positive control[25].

#### *Scavenging activity of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical*

The determination of the $ABTS^{\bullet+}$ radical scavenging activity was performed according to the method described by Re *et al.*[27], with some modifications. Initially, the ABTS radical was generated by reacting 7 mM ABTS solution in water with 140 mM potassium persulfate in the dark for 12–16 h. In the day of the assay, the pre-formed ABTS radical solution was diluted in potassium phosphate buffer in a proportion of 1:88 (1 mL ABTS radical + 87 mL 10 mM potassium phosphate buffer, pH 7.0). Briefly, 1 mL of ABTS radical solution was added to tubes containing 10 μL of DTDS at different concentrations (i.e., to achieve final concentrations of 1 to 25 μM). The mixture was incubated at 25°C for 30 min in dark. The decrease in absorbance was measured at 734 nm. Ascorbic acid (1–25 μM) was used as a positive control. Results were expressed as percentage of the control.

#### *Scavenging activity of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical*

Radical-scavenging activity was determined by the reaction of the stable DPPH radical with the compound in accordance the method described by Choi *et al.*[28]. An aliquot of 10 μL of DTDS at different concentrations (i.e., to achieve final concentrations of 1 to 25 μM) was mixed with 1 mL of methanolic solution containing DPPH radical, resulting in a final concentration of 85 μM DPPH. The mixture was left to stand for 30 min at room temperature in dark and the absorbance was measured at 517 nm. Ascorbic acid (1–25 μM) was used as a positive control. Results are expressed as percentage of the control.

### MAO activity

#### *Preparation of cerebral mitochondria*

A preparation of cerebral mitochondria was carried out as described by Soto-Otero *et al.*[29]. Rat whole brain was removed and washed in ice-cold isolation medium (pH 7.4, $Na_2PO_4/KH_2PO_4$ isotonized with sucrose). Cerebral mitochondria were then obtained by differential centrifugation. Briefly, after removing blood vessels and pial membranes, brain were manually homogenized with four volumes (weight/volume) of the isolation medium. Then, the homogenate was centrifuged at 900×*g* at 4°C for 5 min. The supernatant was centrifuged at 12,500×*g* for 15 min. The mitochondria pellet was then washed once with isolation medium and centrifuged again under the same conditions. Finally, the mitochondrial pellet was reconstituted in a buffer solution ($Na_2PO_4/KH_2PO_4$ isotonized with KCl, pH 7.4). MAO activity was performed immediately after mitochondria isolation.

#### *Enzymatic assay*

MAO activity was determined as described by Krajl[30] with some modifications of Matsumoto *et al.*[31]. An aliquot of 100 μL of samples (100 μg of protein) was incubated at 37°C for 10 min in a medium containing buffer solution ($Na_2PO_4/KH_2PO_4$ isotonized with KCl, pH 7.4), specific inhibitors [selegiline (a MAO-B inhibitor, 250 nM) or clorgiline (a MAO-A inhibitor, 250 nM)] and DTDS (1–100 μM) at a final volume of 600 μL. Then 20 μL of kynuramine dihydrobromide was added to the reaction mixture (final concentration of 90 μM for MAO-A and 60 μM for MAO-B) as substrate. Samples were then incubated at 37 °C for 30 min. After incubation, the reaction was terminated by adding 300 μL of 10% trichloroacetic acid (TCA). After cooling and centrifugation at 3,000×*g* for 15 min, an aliquot of 1 mL of the supernatant was added to 1 mL of 1 M NaOH. The fluorescence intensity was detected spectrofluorimetrically with excitation at 315 nm and emission at 380 nm. The concentration of 4-hydroxyquinoline was estimated from a corresponding standard fluorescence curve of 4-hydroxyquinoline. MAO activity was expressed as nmol 4-OH quinoline/mg protein/min.

#### *Protein quantification*

Protein concentration was measured by the method of Bradford[32], using bovine serum albumin (1mg/mL) as the standard. For this, $S_1$ was diluted in 50 mM Tris–HCl buffer (pH 7.4) in a proportion of 1:10. Then, 50 μL of $S_1$ dilution was added to 2.5 mL of Coomassie (Bradford reagent) and mixed. After 10 min, the color product was measured at 595 nm.

### Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA), followed by the Newman-Keuls test when appropriate. The $IC_{50}$ values were determined by linear regression from individual experiments using "GraphPad Software" (GraphPad software, San Diego, CA, USA). The $IC_{50}$ values were calculated considering responses between 20 and 80% and reported as geometric means accompanied by their 95% confidence limits. The maximal inhibition (Imax) values were calculated at the most effective concentration used using "GraphPad Software".

## Results

### Effect of DTDS on RS levels induced by sodium azide

The increase in RS levels induced by sodium azide was protected by DTDS at concentrations equal or greater than 10 μM (Table 1). The values for $IC_{50}$ and $I_{max}$ were 23.89 μM (19.46–29.34) and 47 ± 30%, respectively (Table 2).

### Effect of DTDS on lipid peroxidation levels induced by malonate, SNP or $FeCl_2$/EDTA

In the present study, DTDS was effective against the increase in lipid peroxidation levels induced by malonate

at concentrations equal or greater than 1 μM (Table 1). The values for $IC_{50}$ and $I_{max}$ were 4.72 μM (2.15–10.33) and 82 ± 20%, respectively (Table 2).

In addition, DTDS showed antioxidant potential against lipid peroxidation induced by SNP at concentrations equal or greater than 10 μM (Table 1). The calculated $IC_{50}$ value was 5.94 μM (4.36–8.10) while the $I_{max}$ value was 92 ± 20% (Table 2).

The lipid peroxidation induced by $FeCl_2$/EDTA in rat brain was significantly decreased by DTDS at the concentration of 25 μM (Table 1). The values for $IC_{50}$ and $I_{max}$ were 15.33 μM (14.31–16.4) and 61 ± 12%, respectively (Table 2).

## Effect of DTDS on protein carbonyl formation induced by SNP

Statistical analysis demonstrated that DTDS, at concentrations equal or greater than 2.5 μM, was effective against protein oxidation induced by SNP (Table 1) in the rat brain homogenate. The calculated $IC_{50}$ value was 11.89 μM (6.31–22.40) while the $I_{max}$ value was 73 ± 60% (Table 2).

## DHA reductase-like activity

The data show that DTDS acted as a GSH-dependent DHA reductase, and the rate of reduction was closely proportional to the concentration of GSH and DTDS. DTDS, at concentrations equal or greater than 5 μM, was effective in reducing DHA to ascorbic acid (Figure 2A). Ebselen, the positive control, at concentrations of 10 μM and greater elicited DHA reductase-like activity, which was dependent on the GSH concentration (Figure 2B). The effect of DTDS was superior to that of ebselen DHA reductase-like activity.

## GST-like activity

In the presence of GSH, DTDS at concentrations equal or greater than 2.5 μM demonstrated GST-like activity.

Table 2. Calculated $IC_{50}$ (μM) and Imax (%) values for RS, lipid peroxidation (TBARS), protein carbonyl levels and MAO activity for DTDS.

| Assay | $IC_{50}$ (μM) | Imax (%) |
|---|---|---|
| RS | 23.89 (19.46–29.34) | 47 ± 30 |
| TBARS - Malonate | 4.72 (2.15–10.33) | 82 ± 20 |
| TBARS - SNP | 5.94 (4.36–8.10) | 92 ± 20 |
| TBARS - $FeCl_2$/EDTA | 15.33 (14.31–16.41) | 61 ± 12 |
| Protein Carbonyl | 11.89 (6.31–22.40) | 73 ± 60 |
| MAO-A | 44.56 (38.26–51.91) | 84 ± 60 |
| MAO-B | 33.28 (29.37–37.71) | 95 ± 20 |

The $IC_{50}$ values were reported as geometric means accompanied by their 95% confidence limits.

Table 1. *In vitro* action of DTDS in RS, TBARS and protein carbonyl levels in the rat brain homogenate.

| Concentration (μM) | RS [a] | TBARS Malonate[b] | TBARS SNP[b] | TBARS $FeCl_2$/EDTA [b] | Protein Carbonyl[c] |
|---|---|---|---|---|---|
| Control | 35.24 ± 0.71 | 53.34 ± 2.97 | 160.00 ± 28.20 | 299.60 ± 24.93 | 25.15 ± 0.82 |
| Induced | 69.63 ± 4.65* | 90.74 ± 12.10* | 697.00 ± 70.47* | 750.60 ± 11.92* | 50.25 ± 4.90* |
| 1 | 61.89 ± 3.44* | 61.94 ± 13.33# | 671.70 ± 66.16* | 702.50 ± 20.87* | 37.97 ± 1.66*# |
| 2.5 | 59.13 ± 3.44* | 49.94 ± 13.64# | 541.50 ± 46.09* | 663.00 ± 27.14* | 33.90 ± 2.92# |
| 5 | 54.64 ± 3.11*# | 39.31 ± 9.04# | 415.40 ± 62.78*# | 621.40 ± 18.67* | 28.03 ± 3.08# |
| 10 | 45.19 ± 4.31# | 25.29 ± 6.57# | 116.70 ± 25.37# | 568.90 ± 43.19*# | 22.93 ± 3.01# |
| 25 | 36.99 ± 2.07# | 16.68 ± 2.03# | 55.25 ± 11.11# | 293.90 ± 87.76# | 13.63 ± 3.25# |

Data are reported as the mean (s) ± S.E.M. of 3-4 independent experiments performed in duplicate and expressed as [a] arbitrary units of fluorescence, [b] nmol equivalents of MDA (malondialdehyde)/g tissue, [c] nmol carbonyl content/mg protein,. (*) denotes $p < 0.05$ as compared to the control, (#) denotes $p < 0.05$ as compared to induced (sample with inductor of oxidative damage) (One-way ANOVA/Newman-Keuls). The concentrations plotted in the table are related to DTDS. *Abbreviations:* RS - reactive species, TBARS - thiobarbituric acid reactive species, SNP - sodium nitroprusside.

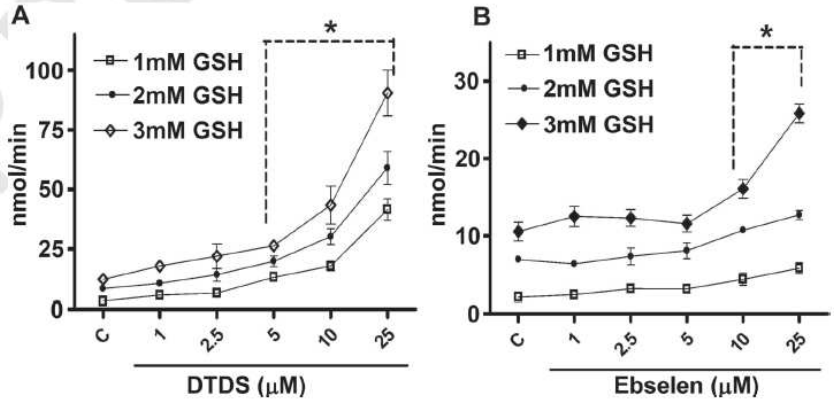


Figure 2. DHA reductase-like activity of (A) DTDS and (B) ebselen expressed as nmol ascorbic acid formed/min. Ebselen was used as a positive control. Values are the mean (s) ± S.E. (*) Denotes $p < 0.05$ as compared to the control (one-way ANOVA/Newman-Keuls).

Table 3. GST-like, ABTS and DPPH radical scavenging assays for DTDS and positive controls.

| | GST-like | | ABTS | | DPPH | |
|---|---|---|---|---|---|---|
| Concentration (μM) | DTDS | Ebselen | DTDS | Ascorbic acid | DTDS | Ascorbic acid |
| Control | 0.002 ± 0.001 | 0.006 ± 0.002 | 99.96 ± 0.23 | 99.54 ± 0.18 | 100.45 ± 0.75 | 99.87 ± 0.45 |
| 1 | 0.004 ± 0.003 | 0.006 ± 0.001 | 98.97 ± 0.34 | 94.86 ± 1.31 | 101.30 ± 2.95 | 96.39 ± 2.66 |
| 2.5 | 0.009 ± 0.001* | 0.008 ± 0.001 | 99.61 ± 2.36 | 89.09 ± 0.94 | 100.90 ± 2.34 | 87.54 ± 2.26* |
| 5 | 0.020 ± 0.003* | 0.013 ± 0.002 | 90.09 ± 4.76 | 78.95 ± 1.52* | 100.80 ± 3.73 | 74.78 ± 3.51* |
| 10 | 0.041 ± 0.004* | 0.023 ± 0.003* | 98.10 ± 5.37 | 58.30 ± 5.25* | 100.10 ± 3.66 | 42.42 ± 6.07* |
| 25 | 0.097 ± 0.010* | 0.035 ± 0.006* | 99.24 ± 4.97 | 15.64 ± 7.54* | 97.05 ± 6.08 | 5.32 ± 1.15* |

GST-like activity of DTDS and ebselen is expressed as Δ absorbance/min. Ebselen was used as a positive control. ABTS and DPPH radical scavenging activity were expressed as percentage (%) of blank. Ascorbic acid was used as a positive control. Data are reported as the mean (s) ± S.E. of 3-4 independent experiments. (*) Denotes $p < 0.05$ as compared to the control (one-way ANOVA/Newman-Keuls).

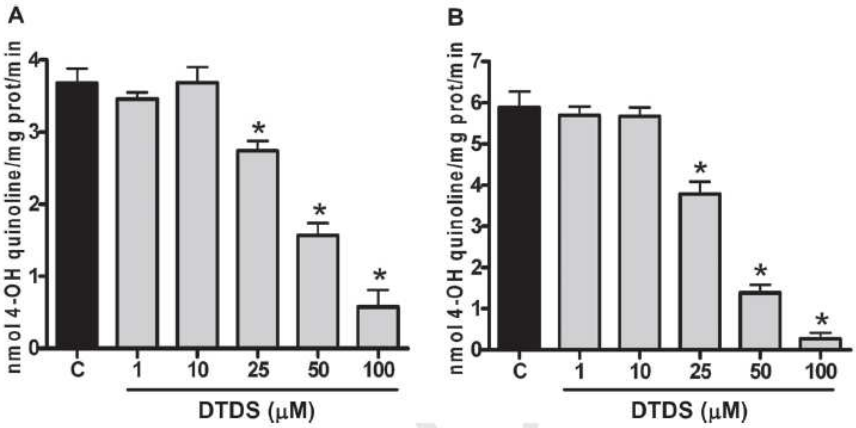


Figure 3. Effect of DTDS on (A) MAO-A and (B) MAO-B activities in the mitochondrial preparation of rat brain. Data are reported as the mean (s) ± S.E. of 3 independent experiments. (*) Denotes $p < 0.05$ as compared to the control (one-way ANOVA/Newman-Keuls).

Ebselen (positive control) at concentrations equal or greater than 10 μM also presented GST-like activity. The results demonstrated that GST-like activity of DTDS was more effective than that of ebselen, the positive control (Table 3).

### ABTS and DPPH radical-scavenging activity

DTDS, at all concentrations tested, had neither ABTS nor DPPH radical-scavenging activity ($p > 0.05$). Ascorbic acid (positive control) showed ABTS and DPPH radical-scavenging activity at concentrations equal or greater than 5 μM and 2.5 μM, respectively (Table 3).

### Effect of DTDS on MAO activity

Statistical analysis revealed that DTDS, at concentrations equal or greater than 25 μM, significantly inhibited both MAO-A (Figure 3A) and MAO-B (Figure 3B) activities when compared to the control tube. The $IC_{50}$ values were 44.56 μM (38.26–51.91) for MAO-A and 33.28 μM (29.37–37.71) for MAO-B. The $I_{max}$ values for MAO-A and MAO-B by DTDS were 84 ± 60% and 95 ± 20%, respectively (Table 2).

## Discussion

Results of this study demonstrate that DTDS exhibited antioxidant action in vitro by a mechanism that involves DHA reductase- and GST-like activities. In addition, DTDS revealed to be a non-selective MAO inhibitor since it was effective in inhibiting MAO-A and MAO-B activities in vitro.

High RS levels can be critical for cells since RS can attack various biomolecules including lipids and proteins. Emerging data from a number of neurological diseases suggest that there may be common features of toxicity that are related to oxidative damage[3]. On the other hand, selenium has been described as a potent protective agent for neurons through redox regulation[13]. Here, DTDS, an organic compound containing selenium in its structure, showed antioxidant action by decreasing the augment of RS levels induced by sodium azide. Although selenium is probably involved in the antioxidant effect, it is interesting to consider the contribution of tiophene portion of molecule in this effect since it is known that several thiophene compounds have antioxidant property[33].

In the central nervous system, lipids are among the main targets of RS because their membranes are rich in polyunsaturated fatty acids that are highly susceptible to lipid peroxidation[2]. The results clearly indicate that the compound DTDS protected against brain lipid peroxidation induced by SNP, malonate and $FeCl_2$/EDTA exerting its antioxidant effect irrespective of the chemical. These results suggest that DTDS is a promising

antioxidant agent and is effective in protecting lipid biomolecules by preventing the generation of toxic products resulting from several chemical inductors of lipoperoxidation.

The protein carbonyl formation can occur as a result of oxidative stress and has been shown to play an important role in a number of human diseases[34]. ROS are known to convert amino groups of proteins and thereby alter protein structure or function[34]. Interestingly, the protein carbonyl levels were reduced by DTDS in the rat brain homogenate. This finding substantiates the antioxidant action of DTDS and demonstrates that it is effective in protecting different biomolecules against the RS action.

The present data show that DTDS, at low concentrations (5 μM and greater), acted as a GSH-dependent DHA reductase, an enzyme that catalyzes the reduction of DHA to ascorbic acid[24]. Moreover, DHA reductase-like activity of DTDS was superior to that of ebselen, a well-recognized antioxidant[25]. Ascorbic acid is able to protecting against lipoperoxidation by acting as a scavenger of ROS and by one-electron reduction of lipid hydroperoxyl radicals via the vitamin E redox cycle[35]. Thus, the antioxidant activity of DTDS can be attributed, at least in part, to its DHA reductase-like activity, since this recycling leads to the accumulation of ascorbic acid in tissue increasing their antioxidant capacity.

GST-like activity can also contribute to the antioxidant effect of DTDS, since this compound showed GST-like activity starting at the concentration of 2.5 μM. It is known that GSTs play an important role in cellular protection against oxidative stress[36] besides its detoxification function of xenobiotics[37]. GSTs can reduce lipid hydroperoxides through their selenium-independent glutathione peroxidase activity and also detoxify lipid peroxidation end products, such as 4-hydroxynonenal[36]. The findings presented here demonstrated that the GST-like activity of DTDS was more effective than that of ebselen, the positive control used in this test.

It was demonstrated in this study that DTDS had neither ABTS nor DPPH radical-scavenging activity, radicals widely used as antioxidant activity screening assays[38]. Therefore, the antioxidant activity of DTDS probably is not related to its ability in stabilizing non-natural radicals.

In addition to the antioxidant action, the findings demonstrated that DTDS acted as nonselective MAO inhibitor, since this compound was effective in inhibiting the activity of the isoforms -A and -B of MAO in the mitochondrial preparation of rat brain. Thus, there is no evidence that MAO inhibitors need to be selective for therapeutic effect since each MAO isoform can assume the function of the other when one is inhibited[39]. This suggests that nonselective MAO inhibitors might have broader therapeutic effects than selective MAO-A or MAO-B inhibitors[40].

Oxidative deamination of monoamines by MAO is accompanied by the reduction of molecular oxygen to hydrogen peroxide[41], a major contributor to oxidative stress. As there are suggestions that oxidative stress plays a role in neurological diseases, we proposed that DTDS, by eliciting both antioxidant and nonselective MAO inhibitory properties, could represent an interesting neuroprotective strategy. These effects confer to DTDS a potential to be tested in experimental models of brain diseases.

## Conclusions

The present study revealed that DTDS, at low concentrations (μM range), exhibited antioxidant action *in vitro* evidenced by the reduction of RS, lipid peroxidation and protein oxidation levels in the rat brain homogenate. The ability to mimic DHA-reductase and GST enzymes may significantly contribute to the antioxidant potential of this organoselenium compound. In addition, the results showed that DTDS is effective in inhibiting MAO-A and MAO-B activities *in vitro*, characterizing it as a nonselective MAO inhibitor. Therefore, DTDS might be a good candidate for future drug development for prevention or treatment of neurological diseases linked to oxidative stress.

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## Declaration of interest

The authors declare that there are no conflicts of interest.

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# 4 DISCUSSÃO

Os disselenetos são compostos orgânicos de selênio amplamente estudados em virtude de suas propriedades sintéticas, farmacológicas e toxicológicas. Este trabalho revelou o potencial farmacológico de um disseleneto contendo tiofenos em sua estrutura, o DSDT, o qual é utilizado como um intermediário reacional na síntese orgânica, mas que ainda não possuía atividades biológicas descritas. Nós demonstramos pela primeira vez que o DSDT apresentou ação anticonvulsivante em um modelo experimental de ELT em ratos. Além disso, os resultados aqui reportados demonstraram que o DSDT é um antioxidante sintético e que possui a capacidade de inibir a atividade da MAO A e B *in vitro* além de apresentar ação do tipo-antidepressiva em ratos no TNF.

Diversos trabalhos têm documentado a participação de espécies reativas na patofisiologia de várias doenças neurológicas tais como a epilepsia (Reznick e Packer, 1993; Przedborski *et al*., 1996; Ben-Menachem *et al*., 2000, Herken et al., 2007). Recentes evidências sugerem que o estresse oxidativo afeta processos metabólicos e pode assim contribuir para a excitabilidade neuronal e o desenvolvimento de crises epilépticas (Santos et al., 2008; Freitas, 2009; Freitas e Tomé, 2010, Waldbaum e Patel, 2010 a,b). De acordo com isso, um estudo realizado por López et al. (2007) revelou um aumento em marcadores de dano oxidativo bem como alterações na atividade de enzimas antioxidantes em pacientes com ELT.

Tendo em vista que os compostos orgânicos de Se possuem ações farmacológicas *in vitro* e *in vivo* que podem ser atribuídas, em parte, às suas propriedades antioxidantes (PATAI 2012), o possível efeito protetor do DSDT frente a convulsões, dano oxidativo e neuronal induzidos por KA foi investigado. Como demonstrado no **artigo 1**, o DSDT, pré-administrado pela via oral em ratos, atenuou as convulsões bem como o subseqüente dano hipocampal resultante da administração sistêmica de KA.

Racine (1972) atribuiu escores (1 a 5) às convulsões límbicas induzidas pelo KA, os quais nos permitem avaliar o grau de severidade da atividade convulsiva. De acordo com os nossos resultados, a administração intraperitoneal de KA causou convulsões severas em ratos (escores 4 e 5 da escala de Racine) e a intensidade destas pôde ser também detectada através do EEG. O EEG revelou que as crises convulsivas induzidas por KA tiveram um foco inicial no hipocampo e que se propagaram para o córtex cerebral. Nossos achados estão de acordo com o fato de que os receptores do tipo KA possuem uma distribuição heterogênea no SNC (Candelario-Jalil et al., 2010). Estudos mostram que a administração sistêmica ou

intracerebroventricular de KA causa convulsões epileptiformes na região CA3 do hipocampo que se propaga para outras regiões (Nadler, 1981; Ben-Ari, 1985), mostrando que o hipocampo está relacionado ao foco inicial das convulsões (Liu et al., 2001). Como observado nos resultados, o DSDT, na dose de 100 mg/kg, reduziu significantemente a incidência de convulsões clônicas e o escore de Racine bem como aumentou a latência para o primeiro episódio convulsivo naqueles animais que apresentaram convulsões clônicas. O DSDT, na dose de 50 mg/kg, atenuou as alterações comportamentais resultantes da exposição de ratos ao KA, embora seu efeito protetor não tenha atingido significância estatística. Além disso, o DSDT (100 mg/kg) reverteu completa e parcialmente o aumento na amplitude eletroencefalográfica induzido pelo KA no hipocampo e córtex de ratos, respectivamente. Nós concluímos a partir destes resultados que o DSDT demonstrou ação anticonvulsivante em ratos no modelo de ELT induzida por KA.

Visto que a $Na^+$ $K^+$-ATPase possui diversas funções neurais, modulando direta ou indiretamente a sinalização, liberação e captação de neurotransmissores bem como a neurogênese (Choi 1988; Xie e Askari 2002; Deisseroth et al. 2004), a atividade desta enzima foi mensurada em córtex e hipocampo de ratos expostos ao KA e/ou DSDT. A $Na^+$ $K^+$-ATPase é uma proteína integral de membrana responsável pelo co-transporte de três íons $Na^+$ para o meio extracelular e dois íons $K^+$ para o meio intracelular, para cada molécula de trifosfato de adenosina (ATP) hidrolisada. Ela consome cerca de 40-60% do ATP cerebral para manter o gradiente eletroquímico necessário à excitabilidade neuronal, regulação do volume celular, balanço osmótico e para o transporte de moléculas ligadas ao co-transporte de $Na^+$; como glicose, aminoácidos e neurotransmissores (Erecińska e Silver, 1994; Kaplan, 2002; Jorgensen et al., 2003).

Os resultados deste estudo revelaram que o KA estimulou da atividade da $Na^+$ $K^+$-ATPase em córtex e hipocampo de ratos. Uma ativação da atividade desta enzima também tem sido reportada em outros protocolos experimentais de convulsão (Sztriha et al., 1987; Kinjo et al., 2007). É possível que as alterações sobre a atividade da $Na^+$ $K^+$-ATPase estejam diretamente relacionadas às propriedades excitotóxicas do KA, ou seja, esta enzima poderia estar atuando de forma compensatória a fim de controlar o aumento na excitabilidade gerada pelo KA, bloqueando assim as descargas epileptiformes e restaurando o potencial de membrana. Corroborando com isso, Bignami et al. (1966) e Harmony et al. (1968) demonstraram que descargas produzidas por estimulação elétrica também são capazes de gerar uma estimulação da atividade da $Na^+$ $K^+$-ATPase. E não menos importante, a ativação de receptores glutamatérgicos do tipo NMDA induz a uma estimulação da atividade da $Na^+$

$K^+$-ATPase (Monfort et al., 2002). Isso ocorre porque a ativação de receptores NMDA causa a entrada de íons $Na^+$ e $Ca^{2+}$ para o interior do neurônio pós-sináptico. O $Ca^{2+}$ liga-se à calmodulina e ativa a proteína fosfatase calcineurina, que desfosforila a $Na^+$ $K^+$-ATPase. Essa desfosforilação aumenta a atividade enzimática com um aumento no consumo de ATP, permitindo a remoção do excesso de $Na^+$ (Marcaida et al., 1996). Conforme mostrado no **artigo 1**, DSDT, na dose de 50 e 100 mg/kg, restaurou a atividade da $Na^+$ $K^+$-ATPase no hipocampo de ratos expostos ao KA. No córtex cerebral, a dose de 100 mg/kg apresentou uma tendência em reduzir a atividade desta enzima quando comparada ao grupo KA, o que parece relacionar-se aos resultados encontrados no EEG. É possível que o DSDT interaja diretamente com a enzima para modular sua atividade ou ainda que atue sobre outros alvos moleculares evitando a excitotoxicidade, de forma que não seja necessária a super estimulação da atividade da $Na^+$ $K^+$-ATPase para neutralizar os efeitos resultantes da administração de KA.

Outro achado bastante interessante no **artigo 1** é que o KA levou à degeneração hipocampal em ratos. De fato, as convulsões geradas pela administração de KA são seguidas por um padrão de perda celular que é bastante similar àquele encontrado em pacientes que sofrem de ELT (Ben-Ari, 1985; Nadler, 1981) e que é clinicamente chamado de esclerose mesial temporal. A extensiva perda celular ocorre nas células localizadas nas regiões piramidais CA1 e CA3 do hipocampo. Os efeitos epileptogênicos do KA na região CA3 são causados pela ativação de receptores de alta afinidade do tipo KA que se expressam preferencialmente na região das fibras musgosas. Já os receptores glutamatérgicos do tipo NMDA são mais concentrados na região CA1 (Geddes et al., 1986). Assim a região CA3 é o marca-passo para a geração de atividades sincronizadas que subseqüentemente propagam-se para a CA1 e outras regiões (Ben-Ari e Cossart, 2000). Além disso, uma hiperatividade da $Na^+$ $K^+$-ATPase resultante da administração de KA poderia também resultar em déficit energético, contribuindo para a morte celular.

Alterações no sistema glutamatérgico desempenham um significante papel no desenvolvimento da epileptogênese. Receptores glutamatérgicos quando ativados, permitem um influxo de $Ca^{2+}$ que em altas concentrações pode levar à morte celular (Li et al., 2010). A ativação da fosfolipase $A_2$, fosfolipase C, proteína quinase C, endonucleases, óxido nítrico sintase e proteases dependentes de $Ca^{2+}$ podem contribuir para os danos celulares resultantes do aumento da concentração intracelular de $Ca^{2+}$ (Siesjo, 1992; Cheng e Sun, 1994; Tymianski e Tator, 1996). Como muitas destas reações enzimáticas podem gerar espécies reativas (Orrenius et al., 1992), a formação destas espécies oxidantes pode ser crítica para os efeitos excitotóxicos do KA (Carriedo et al., 1998; Sun et al., 1992; Cheng e Sun, 1994).

Neste contexto, as espécies reativas causam dano oxidativo às biomoléculas e disfunção mitocondrial, processos que podem resultar na morte celular. Com base nisso, alguns parâmetros para a avaliação do estado redox foram mensurados em córtex e hipocampo de ratos expostos ao KA.

Como mostrado no **artigo 1**, o KA induziu dano oxidativo com características região-específicas, ou seja, um aumento nos níveis de espécies reativas e na oxidação de proteínas foi encontrado em hipocampo de animais expostos ao KA, mas não em córtex. Isto está de acordo com um estudo publicado por Candelario-Jalil et al. (2001), no qual foi demonstrada a diferença de vulnerabilidade de estruturas cerebrais frente ao dano oxidativo induzido por KA em ratos, sugerindo que as regiões cerebrais não estão sujeitas a mesma intensidade de injúria oxidativa. Estes autores demonstraram que o hipocampo está entre as áreas mais vulneráveis ao dano oxidativo por KA, o que se relaciona com os achados histopatológicos em pacientes e em modelos animais de ELT. Dentre os mecanismos que podem contribuir para este diferente padrão de dano oxidativo está a distribuição heterogênea dos receptores do tipo cainato (KA) no SNC (Chittajallu et al., 1999), uma vez que as áreas que mais expressam estes receptores são as que possuem maior suscetibilidade à injúria oxidativa mediada pelo KA (Candelario-Jalil et al., 2001). Portanto, o dano oxidativo no hipocampo de animais que receberam o KA parece contribuir de forma significativa para a neurodegeneração encontrada nesta estrutura.

Corroborando com a hipótese do papel das espécies reativas na neurodegeneração hipocampal causada pelo KA, Chung e Han (2003) demonstraram que a perda neuronal seletiva no hipocampo pode também envolver a ativação da micróglia, já que o KA pode ativar células microgliais e aumentar a produção de espécies reativas, exacerbando o dano neuronal. De fato, um aumento no número de células gliais, processo conhecido como gliose, foi encontrado em hipocampo de animais expostos ao KA. A contribuição de espécies reativas na morte neuronal induzida pelas convulsões é embasada, em parte, na observação de que as convulsões prolongadas resultam em um aumento da oxidação de macromoléculas celulares (Bruce e Baudry, 1995), enquanto que diversos compostos com propriedade antioxidantes previnem a excitotoxicidade *in vitro* (Monyer et al., 1990; Lafon-Cazal et al., 1993; Puttfarcken et al., 1993; Patel, 1996) e *in vivo* (Schulz et al., 1995; MacGregor et al., 1996). Neste sentido, pesquisadores encontraram uma correlação entre os produtos de oxidação avançada de proteínas e o tempo de evolução da doença (López et al., 2007). De acordo com estes achados, o presente trabalho revelou que os níveis de carbonilação de proteínas estavam aumentados no hipocampo de ratos injetados com o KA. Adicionalmente, um déficit do sistema da glutationa em pacientes com epilepsia tem sido também descrito (Mueller et al.,

2008). Entretanto nós não observamos alterações nas defesas antioxidantes enzimática (atividade da GPX) e não-enzimática (níveis de tióis não-protéicos) em hipocampo de ratos expostos ao KA. Isto pode ser explicado pelo fato dessas defesas não terem exibido uma rápida resposta frente à formação aumentada de espécies reativas. Cabe ainda considerar que existem outras defesas antioxidantes que não foram avaliadas neste estudo e que poderiam estar alteradas em resposta à excitotoxicidade causada pelo KA.

O DSDT (50 e 100 mg/kg) preveniu o aumento dos níveis de espécies reativas e de carbonilação de proteínas gerados pelo KA. A propriedade anticonvulsivante do DSDT parece contribuir de maneira efetiva para os seus efeitos sobre a injúria oxidativa induzida pelo KA. Assim, a redução da excitotoxicidade pelo DSDT com a conseqüente redução na geração de espécies reativas e no dano oxidativo às proteínas poderia estar relacionada à prevenção das alterações histopatológicas encontradas no hipocampo de ratos dias após a administração de KA. De fato, o DSDT (100 mg/kg) protegeu contra a neurodegeneração hipocampal e também reduziu a gliose em animais expostos ao KA. Assim, a neuroproteção exercida pelo DSDT parece estar relacionada principalmente à sua propriedade anticonvulsivante, reduzindo o estado epiléptico induzido pelo KA. Isto é apoiado pelo fato de o DSDT bloquear as convulsões eletroencefalográficas induzidas pelo KA no hipocampo de animais expostos ao KA.

Os resultados aqui apresentados nos remetem a idéia de que o DSDT module de alguma forma o balanço entre a excitação e a inibição neuronal, impedindo que o KA desencadeie os seus efeitos deletérios incluindo a excessiva formação de espécies reativas e degeneração neuronal. Embora não possamos elucidar o mecanismo anticonvulsivante do DSDT a partir dos resultados expostos no **artigo 1**, sabe-se que alguns organocalcogênios, como por exemplo, ebselen, $(PhSe)_2$ e *m*-$CF_3(PhSe)_2$ modulam a captação e/ou liberação de glutamato (Nogueira et al., 2002; Souza et al., 2010b) ou a captação de GABA (Prigol et al, 2009), principais neurotransmissores excitatório e inibitório do SNC, respectivamente. Além disso, alterações do tipo redox são determinantes na função de canais iônicos (DiChiara e Reinhart, 1997) e alguns receptores como o receptor NMDA e $GABA_A$ são conhecidos por ser sensíveis à modulação redox (Ruppersberg et al., 1991). Neste sentido, Nogueira e Rocha (2010) consideram que a oxidação de enzimas, receptores e canais iônicos contendo grupos -SH possa estar envolvida em algumas propriedades farmacológicas dos organocalcogênios. Assim, as ações do DSDT sobre sistemas envolvidos no controle da excitabilidade neuronal são relevantes para delinear os mecanismos de ação deste composto, entretanto permanecem a ser investigados.

Como as alterações no estado redox são um fator chave nos mecanismos de neurotoxicidade induzida pelo KA, os efeitos neuroprotetores do DSDT poderiam indicar, em parte, a sua habilidade de agir como um antioxidante além de sua ação anticonvulsivante. Neste sentido, as propriedades antioxidantes do DSDT poderiam também contribuir para a manutenção do equilíbrio redox celular e preservação da morfologia nas áreas hipocampais CA1, CA2 e CA3 do hipocampo. Por isso o potencial antioxidante do DSDT foi investigado em cérebro de ratos *in vitro*.

Nós demonstramos no **artigo 2** que o DSDT apresentou efeito antioxidante em homogenato de cérebro de rato *in vitro* em concentrações na faixa de μM. Este efeito antioxidante foi evidenciado pela proteção contra o aumento de espécies reativas induzido por azida de sódio, substância que gera disfunção mitocondrial através da inibição da atividade da enzima citocromo oxidase (Chen et al., 2003). Níveis elevados de espécies reativas podem resultar em oxidação de proteínas, levando à produção de grupos carbonila. Neste sentido, nós demonstramos que o DSDT reduziu a carbonilação de proteínas induzida por nitroprussiato de sódio (NPS), um doador de NO e/ou cianeto (Bates et al., 1991; Dawson et al., 1991; Rauhala et al., 1998). Portanto, além de evitar a formação de espécies reativas e/ou neutralizá-las, o DSDT também protegeu as proteínas contra a oxidação.

O cérebro é um órgão bastante suscetível a lipoperoxidação e um dos produtos gerados pela oxidação de lipídios é o malondialdeído (MDA) que pode ser determinado através da medida quantitativa de espécies reativas ao ácido tiobarbitúrico (TBARS) (Reznick e Packer, 1994). Como demonstrado no **artigo 2**, DSDT reduziu os níveis de peroxidação lipídica induzida por diferentes agentes químicos como malonato, NPS e $Fe^{2+}$/ ácido etilenodiamino tetra-acético (EDTA). O malonato é um inibidor da enzima succinato desidrogenase que induz disfunção mitocondrial e produção do radical $O_2^{\cdot-}$. Como já descrito, o NPS é um doador de NO, que juntamente ao $O_2^{\cdot-}$ produz o $ONOO^-$, levando à peroxidação lipídica e produção de mais espécies reativas (Beckman et al., 1990). A formação de espécies reativas está também relacionada ao estado redox dos metais de transição, tais como o ferro. O $H_2O_2$ é convertido a radicais $^{\cdot}OH$ através da reação de Fenton, que requer ferro reduzido ($Fe^{2+}$) (Halliwell e Gutteridge, 1990). O $Fe^{2+}$ também se liga ao $O_2$ e forma o íon perferril ($Fe^{2+}$-$O_2$) (Miller e Aust, 1989). As espécies $^{\cdot}OH$ e $Fe^{2+}$-$O_2$ são altamente reativas e agem como espécies geradoras da peroxidação lipídica (Fridovich, 1989). Portanto, DSDT reduziu os níveis de peroxidação lipídica independentemente do agente indutor de lipoperoxidação utilizado. A partir dos resultados obtidos no **artigo 2**, nós verificamos que o DSDT protegeu diferentes

biomoléculas (proteínas e lipídios) contra a oxidação, revelando-se um promissor agente antioxidante.

A fim de investigar os mecanismos envolvidos na ação antioxidante do DSDT *in vitro*, nós realizamos os ensaios de captura dos radicais 2,2'-azino-bis(3-etilbenztiazolina-6-ácido sulfônico) (ABTS) e 1,1-difenil-2-picril-hidrazil (DPPH) e de atividade mimética às enzimas GST e dehidroascorbato (DHA) redutase. O ácido ascórbico é um antioxidante que reduz as espécies reativas a moléculas estáveis (Bendich et al., 1986; Meister, 1994; Winkler et al., 1994) e auxilia na proteção de biomembranas contra a peroxidação lipídica através do ciclo redox da vitamina E (Halliwell e Gutteridge, 1999), além de servir como co-fator na biossíntese enzimática de colágeno, carnitina, catecolamina e neuropeptídeos (Burns, 1957; Levine, 1986; Wilson, 2002). As células dos mamíferos eficientemente transportam e reduzem DHA a ácido ascórbico e, portanto, essa reciclagem leva ao acúmulo de ácido ascórbico nos tecidos (Welch et al., 1995; Wilson, 2002) aumentando a sua capacidade antioxidante. A redução do DHA a ácido ascórbico é catalisada pela enzima DHA redutase com o consumo de GSH (Wells et al., 1995; Xu et al., 1996). O **artigo 2** mostra que o DSDT reduziu quimicamente o DHA a ácido ascórbico na presença de GSH de maneira mais eficiente que o controle positivo, ebselen, agindo de maneira análoga à enzima DHA redutase. Este mecanismo pode servir para explicar, em parte, as propriedades antioxidantes e outros efeitos farmacológicos do composto.

O DSDT também apresentou propriedades miméticas à enzima GST que foram superiores ao ebselen (controle positivo). Além de sua função na detoxificação de xenobióticos, a GST serve também como defesa antioxidante (Mosialou e Morgenstern, 1989; Hayes e Pulford, 1995; Fiander e Schneider, 1999). A GST desempenha um papel crucial nos mecanismos de defesa contra a peroxidação lipídica através da redução de hidroperóxidos lipídicos e fosfolipídicos, espécies capazes de propagar as reações de lipoperoxidação. Além disso, a GST é capaz de proteger as células por detoxificar produtos tóxicos formados pela peroxidação lipídica, como o 4-hidroxinonenal (4-HNE) e a acroleína, uma vez que estas espécies possuem um centro eletrofílico que pode ser conjugado ao GSH (Sharma et al., 2004). Portanto, como mostrado no **artigo 2**, a atividade antioxidante do DSDT pode também estar relacionada a sua capacidade de mimetizar a GST. Entretanto, nós demonstramos que o DSDT, em contraste ao ácido ascórbico, não estabilizou os radicais sintéticos ABTS e DPPH.

Além disso, nossos resultados revelaram que o DSDT inibiu as isoformas A e B da MAO em homogenato de cérebro de rato *in vitro*. A MAO é uma enzima responsável pela degradação de monoaminas como serotonina, noradrenalina e dopamina e a desaminação

oxidativa destas monoaminas é acompanhada da formação de $H_2O_2$ (Rigby et al., 2005). Visto que a MAO parece desempenhar um importante papel em muitas doenças neurológicas e psiquiátricas (Shih e Thompson, 1999; Shih et al., 1999) e que inibidores desta enzima apresentam potencial terapêutico para o tratamento das Doenças de Alzheimer e Parkinson (Chen et al., 2007) bem como da depressão (Pacher e Kecskeméti, 2004), o fato de o DSDT apresentar atividade antioxidante e inibitória da MAO pode conferir efeitos neurofarmacológicos adicionais a este composto.

As desordens de humor são as mais freqüentes comorbidades psiquiátricas em pacientes com epilepsia, com uma prevalência estimada entre 11 e 60% em pacientes com convulsões recorrentes (Mendez et al., 1986). A depressão tem um impacto negativo sobre a qualidade de vida dos pacientes com ELT (Harden, 2002; Jones et al., 2005; Kanner, 2005; Schmitz et al, 2005) e pode estar relacionada com a esclerose hipocampal (Quiske et al., 2000). A existência de uma relação bidirecional entre as desordens depressivas e a epilepsia tem emergido (Kanner, 2008) e implica em uma complexa interação entre mecanismos neurobiológicos (incluindo genéticos), iatrogênicos e psicossociais (Gonçalves e Cendes, 2011). Neste sentido, o uso de um antiepiléptico com propriedades estabilizadoras do humor tem um grande apelo clínico (Katherine et al., 2011). Portanto, a possível ação do tipo antidepressiva do DSDT foi investigada através do TNF em ratos.

Como mostrado no **anexo (resultados complementares)**, O DSDT, administrado pela via oral nas doses de 50 e 100 mg/kg, reduziu o tempo de imobilidade de ratos no TNF bem como a atividade locomotora e exploratória no teste do campo aberto (TCA). Isto mostra que o DSDT apresentou atividade do tipo antidepressiva em ratos. Embora o DSDT tenha causado alterações no TCA, nós descartamos a possibilidade de um resultado falso-positivo já que os efeitos observados no TCA não foram de natureza psicoestimulante. Assim, a ação do tipo antidepressiva do DSDT poderia contribuir para o tratamento da depressão resultante da ELT. Entretanto, estudos adicionais da atividade farmacológica bem como estudos toxicológicos são relevantes para a avaliação do DSDT como uma futura terapia para o tratamento da comorbidade ELT/depressão.

# 5 CONCLUSÃO

Os resultados apresentados nesta dissertação nos permitem concluir que:

- ✓O DSDT apresenta ação anticonvulsivante no modelo agudo do KA em ratos evidenciada por uma redução das alterações comportamentais e eletroencefalográficas induzidas pelo KA;

- ✓O dano oxidativo bem como a neurodegeneração em hipocampo de ratos expostos ao KA foram atenuados pelo DSDT;

- ✓Este organoselênio apresentou atividade antioxidante *in vitro*, que pode ser parcialmente explicada por sua atividade mimética às enzimas DHA redutase e GST;

- ✓O DSDT agiu como um inibidor não-específico da MAO cerebral *in vitro;*

- ✓O DSDT apresenta ação do tipo antidepressiva visto que sua administração oral reduziu o tempo de imobilidade de ratos no TNF.

# 6 PERSPECTIVAS

Tendo em vista os resultados obtidos neste trabalho, as perspectivas para trabalhos posteriores são:

- Estudar possíveis mecanismos envolvidos nos efeitos farmacológicos do DSDT em ratos;

- Visto que alguns antidepressivos podem induzir supressão do apetite, temos como perspectiva avaliar a possível ação supressora do apetite por DSDT e outros disselenetos em ratos e as vias envolvidas nesta ação.

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# 8. ANEXO

## 8.1 Resultados complementares

### 8.1.1 Materiais e Métodos

8.1.1.1 Drogas

O composto DSDT foi preparado e caracterizado em nosso laboratório pelo método previamente descrito por Tiecco et al. (2000). O composto foi diluído em óleo de canola e administrado pela via oral em volume fixo de 1 mL/kg de peso corporal.

8.1.1.2 Animais

Foram utilizados ratos Wistar adultos machos, pesando entre 220-280g, provenientes do Biotério Central da Universidade Federal de Santa Maria (UFSM). Os animais foram acondicionados sob condições de temperatura de 22 ± 2ºC e mantidos em um ciclo de 12h luz/12h escuro. A dieta foi constituída de ração comercial (GUABI, RS, Brasil) e água fresca *ad libitum*. Os animais foram divididos em três grupos (n=10/grupo).

8.1.1.3 Tratamento

O DSDT foi administrado aos ratos nas doses de 50 ou 100 mg/kg, pela via oral, através de sonda intragástrica. Os animais controle receberam apenas o veículo do DSDT (óleo de canola). Após 1 hora do tratamento, os animais foram submetidos aos testes comportamentais para a avaliação da atividade locomotora e exploratória e do tipo-antidepressiva. As doses, a via de administração e o tempo de pré-tratamento do DSDT foram baseados no **artigo 1**.

8.1.1.4 TCA

Resultados falso-positivos podem ser obtidos no TNF quando as drogas em teste apresentam um efeito psicoestimulante (Borsini e Meli, 1988). Para excluir um possível resultado falso-positivo é importante a investigação de alterações sobre a atividade locomotora e exploratória dos animais. Neste sentido, os efeitos do DSDT sobre a atividade locomotora e exploratória dos animais foram avaliados através do TCA.

A arena para o TCA é constituída de madeira nas dimensões de 30 cm de altura, 45 cm de comprimento e 45 cm de largura e dividida em 9 quadrantes iguais (3 x 3). Cada animal foi colocado individualmente no centro do aparato e observado por 4 min para avaliação da

atividade locomotora (número de segmentos cruzados com as quatro patas) e exploratória (número de elevações com as patas dianteiras) (Walsh e Cummins, 1976).

8.1.1.5 TNF

O TNF, originalmente descrito por Porsolt et al. (1977), é um modelo farmacológico bastante utilizado para predizer o efeito antidepressivo de novas drogas em roedores por apresentar alto valor preditivo devido à resposta aos medicamentos antidepressivos utilizados na clínica. Neste teste os roedores são expostos a uma situação aversiva, nadar em um tanque cilíndrico com água (20 cm de diâmetro e 60 cm de altura, contendo 40 cm de água a 24±1ºC), onde eles não podem tocar o fundo do cilindro ou fugir (escape). Com o tempo os animais percebem que não tem como sair do local e desenvolvem uma postura de imobilidade. Os animais são considerados imóveis quando flutuam imóveis ou fazem apenas os movimentos necessários para manter sua cabeça acima da água. Uma redução no tempo de imobilidade serve como indicativo de efeito do tipo antidepressivo. Neste trabalho o TNF foi conduzido ao longo de dois dias, isto é, um dia para uma sessão de treino da natação (15') e o teste propriamente dito (6'), realizado 24h após o treino.

8.1.1.6 Análise Estatística

Todos os resultados foram expressos como média ± erro padrão (EP). Os dados foram analisados pelo teste de normalidade de D'Agostino seguido pela comparação dos grupos experimentais usando o teste ANOVA de uma via. Os valores de $p < 0,05$ foram considerados significativos.

**8.1.2 Resultados**

8.1.2 1 Efeito do DSDT no TCA e no TNF em ratos

O tratamento dos animais com DSDT (50 e 100 mg/kg), administrado pela via oral, reduziu o número de cruzamentos e de elevações com as patas dianteiras no TCA quando comparado com os animais do grupo controle (Figura 1a e 1b).

A administração de DSDT, nas doses de 50 e 100 mg/kg pela via oral, resultou em uma diminuição significante no tempo de imobilidade dos animais no TNF em comparação ao grupo controle (Figura 2).

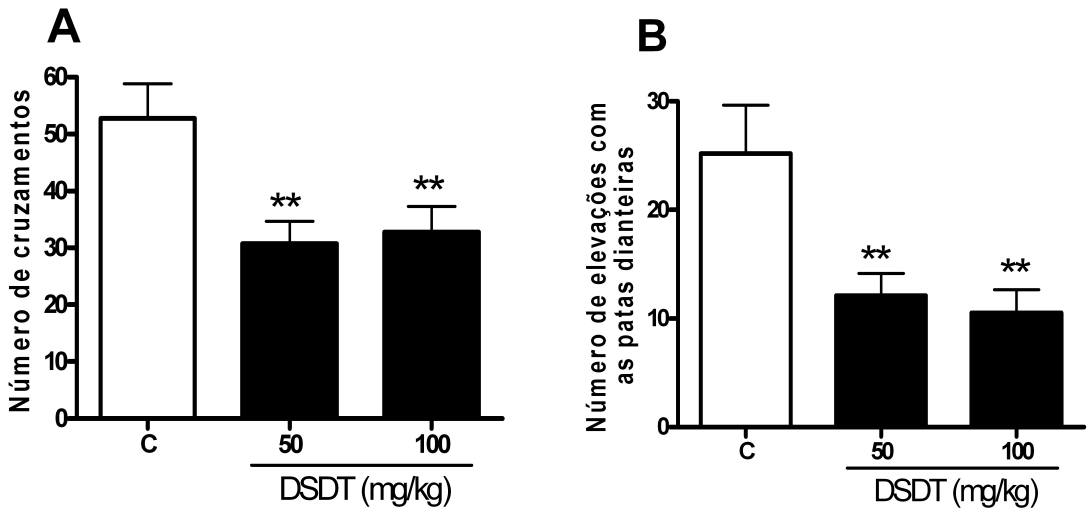


**Figura 2.** Efeito do DSDT sobre a atividade locomotora (número de cruzamentos, A) e exploratória (número de elevações com as patas dianteiras, B) de ratos no TCA. O composto DSDT foi administrado nas doses de 50 ou 100 mg/kg, pela via oral, 1h antes do teste. Os animais do grupo controle receberam apenas o veículo. Os resultados foram expressos como média ± EP (n = 10 animais/grupo). Os asteriscos denotam o nível de significância em comparação ao grupo controle ** $P < 0.01$ (ANOVA de uma via).

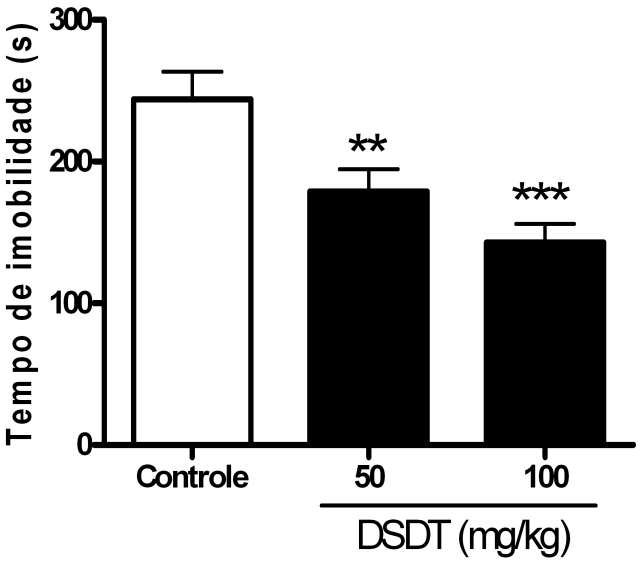


**Figura 3.** Efeito do DSDT sobre o tempo de imobilidade (s) de ratos no TNF. O composto DSDT foi administrado nas doses de 50 ou 100 mg/kg, pela via oral, 1h antes do teste. Os animais do grupo controle receberam apenas o veículo. Os resultados foram expressos como média ± EP (n = 10 animais/grupo). Os asteriscos denotam o nível de significância em comparação ao grupo controle *** $P < 0.001$, ** $P < 0.01$ (ANOVA de uma via).