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CARACTERIZAÇÃO FITOQUÍMICA E EFEITOS ANTINOCICEPTIVOS E ANTI-INFLAMATÓRIOS DE *Sida tuberculata* R.E. Fries "GUANXUMA"

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Uruguaiana,
Agosto de 2017

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica *Stricto sensu* da Universidade Federal do Pampa, como requisito parcial para obtenção do grau de Doutor em Bioquímica.

Orientador: Prof. Dr. Vanderlei Folmer

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RESUMO

O presente estudo avaliou os efeitos antinociceptivos e anti-inflamatórios dos extratos obtidos de *Sida tuberculata* (ST), popularmente conhecida como "guanxuma", em modelos agudo e crônico em camundongos. Além disso, foram feitas caracterizações fitoquímicas e análises do potencial antioxidante e citotóxico *in vitro*. De acordo com os dados encontrados, os extratos das folhas e raízes apresentaram um significativo efeito antioxidante ($p<0.05$) a partir das doses de 0.015 e 0.03 $mg.mL^{-1}$ respectivamente, porém o extrato das folhas teve uma ação mais pronunciada comparado ao extrato das raízes ($<IC_{50}$) sendo selecionado para os testes *in vivo*. Através das análises químicas, foi desenvolvido um método analítico por UHPLC para determinação do marcador fitoquímico, 20-hydroxyecdysone (20-HE), e também foram identificados oito compostos, sendo 20HE o majoritário juntamente com um derivado de Canferol. Nos testes de citotoxicidade *in vitro*, os extratos metanólicos das folhas (*Sida tuberculata leaf extract* - STLE) e raízes (*Sida tuberculata root extract* - STRE) demonstraram uma ação antiproliferativa contra as linhas celulares tumorais HepG2 e MCF-7 (IC_{50} entre 543.6 - 593.4 $\mu g.mL^{-1}$ para STLE, e 397.1 - 493.9 $\mu g.mL^{-1}$ para STRE). Também, STLE diminuiu a viabilidade em leucócitos humanos a partir da dose de 10 $\mu g.mL^{-1}$. Já os resultados dos ensaios de dor e inflamação *in vivo* demonstraram que o STLE (10-300 $mg.kg^{-1}$) administrado pela via oral (v.o.), 1h antes do teste, inibiu significativamente a nocicepção das fases neurogênica e inflamatória para o modelo de formalina (31,3 e 40,1% respectivamente). Do mesmo modo, STLE diminui significativamente as contorções induzidas por ácido acético, chegando a 71,8% de inibição na dose de 100 $mg.kg^{-1}$. Os experimentos mostraram que, pelo menos em parte, o mecanismo de ação de STLE envolve os sistemas opióides e adenosinérgicos uma vez que seu efeito foi revertido pela Naloxona (inibidor não seletivo dos receptores opióides) e pelo DPCPX (inibidor seletivo do receptor de adenosina A1) no modelo de ácido acético. A avaliação por *Docking* computacional demonstrou que os compostos Canferol e 20HE, presentes no extrato, podem interagir principalmente com o receptor opióide μ. O pré-tratamento com STLE (100 $mg.kg^{-1}$) também reduziu a migração celular total, o número de neutrófilos, a atividade da MPO, e o níveis das citocinas IL-1β, IL-6 e TNF-α no fluído peritoneal de animais com peritonite induzida por carragenina. No mesmo modelo foi verificado uma redução dos níveis de TBARS e um

aumento nos níveis de NPSH. Além disso, o tratamento com STLE a 100 mg.kg^{-1} uma vez ao dia também apresentou efeito antinociceptivo no modelo de dor inflamatória crônica induzida por CFA (i.pl.), como ainda diminuiu o edema de pata após o quinto dia de tratamento. As doses administradas diariamente (100 mg.kg $^{-1}$ durante 15 dias) não apresentaram alteração macroscópica e nem sobre o peso absoluto e o peso relativo dos órgãos vitais (coração, pulmão, fígado, baço e rins). Portanto, o conjunto de dados apresentados sugerem que ST possui uma significativa atividade antinociceptiva e anti-inflamatória frente a modelos agudo e crônico em camundongos, com mecanismo de ação envolvendo, parcialmente, sua ação antioxidante e a interação sobre o sistema opióide e adenosinérgico, e também ação redutora de mediadores pró-inflamatórios. Desta forma, a espécie ST apresenta uma atividade potencialmente terapêutica que corrobora com seu uso popular. Porém mais estudos são necessários a fim de elucidar mais precisamente seu mecanismo de ação e se o mesmo é atribuído a um ou mais compostos do extrato.

Palavras chave – *Sida tuberculata*, fitoquímica, 20-hydroxyecdysone, antioxidante, citotoxicidade, nocicepção e inflamação.

ABSTRACT

The present study evaluated the antinociceptive and anti-inflammatory effects of extracts obtained from *Sida tuberculata* (ST), popularly known as "guanxuma", on acute and chronic models in mice. In addition, were performed a phytochemical characterization and antioxidant and cytotoxic analysis *in vitro*. According to the data, leaf and root extracts presented a significant antioxidant effect ($p < 0.05$) from the 0.015 and 0.03 $mg.mL^{-1}$ doses respectively, however the leaves extract had a more pronounced action compared to the leaves root extract (<IC50) being selected for *in vivo* tests. Through the chemical analyzes, an analytical method was developed by UHPLC to determine the phytochemical marker, 20-hydroxyecdysone (20-HE), and eight compounds were identified, being 20HE the major compounds together with a Kaempferol derivative. In cytotoxicity tests, *S. tuberculata leaf extract* (STLE) and *S. tuberculata root extract* (STRE) demonstrated antiproliferative action against HepG2 and MCF-7 tumor cell lines (IC50 between 543.6 - 593.4 $\mu g.mL^{-1}$ for STLE, and 397.1 - 493.9 $mg.mL^{-1}$ for STRE). Besides, STLE decreased human leukocytes viability from dose of 10 $\mu g.mL^{-1}$. *In vivo* assays demonstrated that STLE (10-300 $mg.kg^{-1}$) given orally (p.o.) 1 h before the test, significantly inhibited the neurogenic and inflammatory phases in the formalin model (31.3 and 40.1% respectively). Likewise, STLE significantly decreased the contortions induced by acetic acid, reaching 71.8% inhibition at the dose of 100 $mg.kg^{-1}$. The experiments showed, at least in part, the STLE mechanism of action involves the opioid and adenosinergic systems since its effect was reversed by Naloxone (non-selective opioid receptor inhibitor) and DPCPX (selective A1 adenosine receptor inhibitor) in acetic acid model. The evaluation by computational docking demonstrated that Kaempferol and 20HE compounds might interact primarily with the μ opioid receptor. Pretreatment with STLE (100 $mg.kg^{-1}$) also reduced total cell migration, neutrophil number, MPO activity, and levels of IL-1β, IL-6 e TNF-α cytokines in the peritoneal fluid of animals submitted to carrageenan-induced peritonitis. In the same model, there was a reduction of TBARS levels and an increase in NPSH levels. In addition, treatment with STLE at 100 $mg.kg^{-1}$ once daily also presented antinociceptive effect in chronic inflammatory pain model induced by CFA (i.pl.), and paw edema decreased after the fifth day of treatment. The doses administered daily (100 $mg.kg^{-1}$ for 15 days) did not present macroscopic alteration and

did not change the absolute weight and organs relative weight (heart, lung, liver, spleen and kidneys). Therefore, the data presented suggest that ST has a significant antinociceptive and anti-inflammatory activity against acute and chronic models in mice, with a mechanism of action involving, partially, its antioxidant action and the interaction on the opioid and adenosinergic system, as well as reducing action of pro-inflammatory mediators. Thus, the ST species presents a potential therapeutic action that corroborates with its popular use. However, more studies are needed in order to elucidate more precisely its mechanism of action and whether it is attributed to one or more compounds in the extract.

Key words - *Sida tuberculata*, phytochemistry, 20-hydroxyecdysone, antioxidant, cytotoxicity, antinociception and inflammation.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AINEs – anti-inflamatórios não esteroidais;
ATP – adenosina trifosfato;
cAMP – monofosfato de adenosina cíclico;
CHA - ciclohexil adenosine, agonista de receptores adenosinérgicos A1;
DPCPX - antagonista seletivo de receptores adenosinérgicos A1;
EROs – Espécies Reativas de Oxigênio;
GPx – Glutationa peroxidade;
GSH – Glutationa reduzida;
H_2O_2 – Peróxido de hidrogênio;
MDA – Malondialdeído;
mgEAG – miligramas por equivalente de Ácido gálico;
mgER – miligramas por equivalente de Rutina;
CFA - adjuvante completo de Freund;
Cg – carragenina;
Dexa – Dexametasona;
ERNs - espécies reativas de nitrogênio;
EROs -espécies reativas de oxigênio;
20HE – 20-hydroxyecdysone;
5-HT - serotonina;
IC50 – Concentração inibitória de 50%;
i.p. - intraperitoneal;
i.pl. – intra-plantar;
IL-1β - interleucina 1- beta;
IL-4 - interleucina 4;
IL-6 - interleucina 6;
IL-8 – interleucina 8;
IL-10 – interleucina 10;
K^+ATP – canal de potássio sensível a ATP;
MPO – Mieloperoxidase;
nNOS - óxido nítrico sintase neuronal;
NO - óxido nítrico;
NPSH – tióis não proteicos;
PKG – quinase de proteína G;
SNC – Sistema nervoso central;
SOD – superóxido dismutase;
ST – *Sida tuberculata*;
STLE – extrato das folhas de *Sida tuberculata*;
STRE – extrato das raízes de *Sida tuberculata*;
TNF-α – fator de necrose tumoral alfa;
v.o. – via oral.

SUMÁRIO

APRESENTAÇÃO

No presente trabalho, dentre os tópicos destacados no Sumário, ressalta-se que o título **Resultados** está organizado sob a forma de um **Artigo científico (publicado) e Manuscritos I, II e III,** os quais contemplam os objetivos propostos e as normas do Programa de Pós-Graduação em Bioquímica da UNIPAMPA, Campus Uruguaiana. Dessa forma, cada um desses manuscritos contém sua *Metodologia*, *Resultados*, *Discussão* e *Referências* inseridos no corpo do texto e que representam a íntegra do estudo.

Ao final dos Resultados encontra-se o tópico **Discussão geral** que apresenta interpretações e comentários gerais sobre todos os resultados alcançados. Essa seção discorre sucintamente sobre os resultados correlatos na literatura científica e sugere proposições que sustentam a Tese gerada.

Por fim, salienta-se que as **Referências Bibliográficas** representam citações a parte dos manuscritos, pois contemplam a literatura mencionada na **Introdução**, **Fundamentação teórica** e **Discussão geral.**

1 INTRODUÇÃO

A biodiversidade da flora brasileira, estimada em 20-22% da total existente no planeta, tem dado sua contribuição à descoberta de novas estruturas químicas biologicamente ativas (DUTRA et al., 2016; VALLI et a., 2013; BOLZANI et al., 2012). Dados apontam que entre os anos 2011 e 2013 pesquisadores brasileiros publicaram mais de 10 mil artigos científicos relacionados a plantas medicinais e seus compostos. Além disso, o mercado brasileiro de componentes fitoterápicos movimentou aproximadamente 260 milhões de dólares americanos em 2014. Isto ainda é considerado modesto em comparação ao mercado mundial de fitoterápicos, que movimentou cerca de 27 bilhões de dólares no mesmo período (DUTRA et al., 2016).

A população brasileira tem uma longa tradição no uso de plantas medicinais para o tratamento de diferentes doenças agudas e crônicas. Entretanto, a despeito da biodiversidade e do interesse da população na medicina tradicional, é sempre um desafio selecionar uma planta de interesse científico e pouco explorada em pesquisas farmacológicas. Assim, baseado no conhecimento etnofarmacológico e empírico, o presente estudo investiga a espécie botânica *Sida tuberculata* (ST) em diferentes "*approaches*" dentro das áreas fitoquímica, bioquímica e farmacológica.

ST (Malvaceae) está entre as 95 espécies do gênero presentes no território brasileiro (DINDA et al., 2015). Trata-se de uma planta com porte herbáceo/subarbustivo, caule lenhoso, bastante resistente e tamanho variando entre 40 cm até mais de 1m em alguns indivíduos. Seu uso tradicional está associado, principalmente, a sua possível ação hipoglicemiante, antimicrobiana e anti-inflamatória.

Recentemente foi demonstrada uma ação antifúngica para os extratos das folhas e raízes de ST frente a linhagens de *Candida krusei* (ROSA et al., 2015). Além disso, identificou-se as principais classes de compostos, com destaque para os ecdisteróides (majoritários), flavonoides e alcaloides. Outros dados da literatura apontam essa classe como comum em espécies de *Sida* (ARCINIEGAS et al., 2017; CHAVES et al., 2017; WANG et al., 2008; DINAM et al., 2001a). Levando em consideração, que os fitoconstituintes majoritários apresentam núcleo esteroidal (fitoecdisteróides), gerou-se a hipótese de que extratos obtidos de ST pudessem apresentar uma possível ação analgésica e anti-inflamatória.

Trabalhos realizados com outras espécies de Sida, tais como *S. rhombifolia* (MAH et al., 2017; GANGU et al., 2011; FRANZOTTI et al., 2000) *S. cordifolia* (NARENDHIRAKANNAN & LIMMY, 2012; SUTRADHAR et al., 2006 e 2007) e *S. acuta* (KONATÉ et al., 2012), já demonstraram um efeito antinociceptivo e anti-inflamatório para o gênero, corroborando com nossa perspectiva.

No tratamento da dor, os anti-inflamatórios não esteróides (AINEs, em inglês *NSAIDs*) estão entre os fármacos mais amplamente utilizados, uma vez que bloqueiam os estímulos de dor no tecido sensorial (FERNANDEZ-DUENAS et al., 2008). O agravante é que cada vez mais se descobrem efeitos adversos provocado pelos AINEs, tais como gastrointestinais, renais, cardíacos e vasculares (VARGA et al., 2017; AL-SAEED, 2011; HAAG et al., 2008; ANTMAN et al., 2005; OATES et al., 1998).

Assim, embora a etiologia da dor seja um fenômeno complexo, o estudo com plantas e seus subprodutos tem ajudado no entendimento de mecanismos biológicos relacionados a dor. Portanto, o presente trabalho se propôs a caracterizar quimicamente extratos obtidos de ST e determinar seu potencial antioxidante e citotóxico *in vitro* e investigar seu efeito antinociceptivo e anti-inflamatório *in vivo*.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 *SIDA TUBERCULATA*

Sida tuberculata R.E. FRIES (ST), comumente conhecida como “guanxuma, vassourinha e/ou malva-branca” é uma espécie pertencente à família Malvaceae amplamente distribuída na região do Pampa, sul do Brasil (BOVINI et al., 2001). ST apresenta um porte herbáceo quando jovem e subarbustivo em indivíduos maduros, (Figura 1).

Figura 1. Foto de espécimes de *Sida tuberculata* (Malvaceae)
Fonte: Autor (2017).

De maneira geral, seus espécimes mostram flores com um tom amarelo-creme, as quais ficam abertas por um período de 2 a 3 horas aproximadamente entre as 9 horas da manhã e as 14 horas da tarde dobrando as pétalas após este período (SILVA-PERIRA et al. 2003). Embora considerada como uma espécie infestante de plantações e monoculturas, a população local utiliza suas folhas e raízes para tratar algumas

enfermidades. Dentre as suas aplicações na medicina popular, destacam-se o uso das infusões de suas folhas e raízes com ação antimicrobiana, anti-inflamatória, cicatrizante e hipoglicêmica (ROSA et al., 2015, 2016).

Algumas espécies de *Sida* são amplamente usadas e estudadas em vários países. Espécies como *Sida acuta*, *Sida cordifolia*, *Sida rhombifolia*, *Sida spinosa* e *Sida veronicaefolia* possuem um diversificado uso na medicina tradicional da Índia (Ayurveda), China e lugares da América e África (ARCINIEGAS et al., 2017; SIDDIQUI et al., 2016; DINDA et al., 2015; IBIRONKE et al., 2014; MOMIN et al., 2014; PAWA et al., 2011).

Mais de 140 compostos têm sido identificados em espécies do gênero *Sida*, sendo fitoecdisteróides, flavonoides e alcaloides os grupos predominantes (ARCINIEGAS et al., 2017; CHAVES et al., 2017; DINDA et al., 2015; SUTRADHAR et al., 2007; JADHAV et al., 2007). Alguns destes trabalhos apontam os fitoecdisteróides como a principal classe detectada no gênero (WANG et al., 2008; JADHAV et al., 2007; DARWISH & REINECKE, 2003; DINAN et al., 2001a). Resultados prévios do nosso grupo corroboram com esses dados, além do que, o ecdisteróide 20-hydroxyecdysone (20HE) foi proposto como majoritário nas folhas e raízes de ST (ROSA et al., 2015).

Esta classe conhecida primeiramente como ecdisteróides foi inicialmente descoberta em insetos, onde exercem funções hormonais responsáveis pelo crescimento, metamorfose, embriogênese, diapausa e reprodução destes invertebrados (DINAN et al., 2001b). Na década de 60 foi identificada uma classe análoga em plantas, que mais tarde foi chamada de “fitoecdisteróides” para diferenciar dos compostos análogos presentes nos animais (DINAN et al., 2001b). Atualmente diversos efeitos farmacológicos têm sido atribuídos aos fitoecdisteróides, entre eles antidepressivo (ISHOLA et al., 2014), benefícios contra atrofia muscular (HIRUNSAI et al., 2016); protetor neuronal em Parkinson (LIU et al., 2016; KHURANA & GAJBHIYE, 2013). Além disso, alguns estudos têm demonstrado que os fitoecdisteróides, especialmente 20HE, possui a capacidade de aumentar a síntese proteica (ANTHONY et al., 2015; PARR et al., 2015, 2014; GORELICK-FELDMAN et al., 2010), assim, 20HE tem sido adicionado a suplementos dietéticos nos Estados Unidos (WANG et al., 2008).

Outras classes de metabólitos secundários dentro do gênero *Sida*, como alcaloides e flavonoides, também apontaram importantes atividades farmacológicas.

Chaves et al. (2017; 2013) demonstrou efeito vaso relaxante de alcaloides isolados de *S. rhombifolia.* Trabalhos realizados por Ahmed et al. (2011) e Jang et al. (2003) demonstraram um possível efeito citotóxico para o alcaloide Criptolepina isolada de *S. acuta*. Além disso, derivados de tocoferol isolados de *S. acuta* também mostram um significativo efeito antioxidante (CHEN et al., 2007). Sutradhar et al. (2006; 2008) estudando flavonoides isolados de *S. cordifolia* encontrou um significativo efeito anti-inflamatório e analgésico em modelos de contorções induzidas por ácido acético e edema de pata por carragenina. Outros estudos também determinaram uma ação anti-inflamatória e analgésica para *Sida* sp. (MAH et al., 2017; KONATÉ et al., 2012, 2010; BONJARDIM et al., 2011; FRANZOTTI et al., 2000).

Embora estes trabalhos tenham demonstrado a relevância das espécies de *Sida* spp. em determinadas atividades biológicas, cabe destacar que pesquisas com espécies nativas do bioma Pampa e adaptadas às características singulares dessa região podem revelar componentes e efeitos exclusivos em espécimes existentes nessa área. Além do que, estudos bioquímicos e farmacológicos com a espécie ST até o presente momento, além do grupo proponente dessa proposta, praticamente inexistem na literatura.

2.2 DOR E NOCICEPÇÃO

A dor é um mecanismo de alerta sobre possíveis ameaças ao bem estar e a integridade geral do organismo, capturando assim, a atenção do indivíduo para a identificação da sua causa e consequente resolução do problema. (CHAPMAN & GAVRIN, 1999; WALL, 1999). Dessa forma, ela se torna um sinal clínico fundamental para a detecção e determinação de uma gama ampla de enfermidades, bem como, para gerar um comportamento preventivo ou de recuperação e, consequentemente, limitação de danos (WOLF, 2000; ALMEIDA et al., 2004).

De acordo com a Associação Internacional para o Estudo da Dor (IASP) a definição de dor é "*uma desagradável experiência sensorial e emocional, deflagrada por uma ameaça real, potencial ou que é percebida desta forma"*. Segundo Oaklander (2011) a dor é tão privilegiada que está acima de outros pensamentos e atividades, e ainda ajuda a formar memórias permanentes. Por exemplo, um dedo que se queimou evitará para sempre o fogo.

Portanto, devido a influência do estado emocional, psicológico e cultural, o estudo da dor em seres humanos é complexo. Cada ser humano percebe e encara a dor de uma forma singular. A partir desta definição, sabemos que há um componente fisiológico e outro psicológico/emocional, e que a união de ambos os humanos entendem por dor. Em modelos animais estes fatores são minimizados, muito embora ainda seja um fenômeno complexo, avalia-se a dor de forma indireta, propiciando uma análise mais eficiente dos processos analgésicos (RUSSO & BROSE, 1998; TJØLSEN & HOLE, 1997). Nestes modelos se toma em consideração apenas o aspecto fisiológico da dor, chamado de **nocicepção**. Assim, enquanto a dor trata-se de um fenômeno mais subjetivo, a nocicepção responde somente aos aspectos fisiológicos sensoriais. O sinal de alerta da dor advém da ativação específica de fibras sensoriais específicas, sensibilizadas quando o estímulo é potencialmente perigoso, ou seja, excedem uma determinada faixa considerada como inócuo (MILLAN, 1999).

De acordo com estudos de Sherrington (1906) já se acreditava na ocorrência de um caminho neural para a percepção da dor, distinto das fibras nervosas sensoriais, que mais tarde foi identificado como um neurônio sensorial primário, ativado por um estímulo capaz de causar lesão tecidual, chamado de **nociceptor** (JULIUS & BASBAUM, 2001; PERL, 2007). Atualmente, os nociceptores são referenciados como neurônios aferentes (sensoriais) que respondem a estímulos perigosos (potencialmente prejudiciais) mecânicos, térmicos ou químicos, gerando e transmitindo potenciais de ação em direção ao sistema nervoso central (SNC) (OAKLANDER, 2011; BASBAUM et al, 2009; BASBAUM & JESSELL, 2000).

Os **neurônios nociceptivos** são distintos de outras fibras nervosas sensoriais, porque eles separam um estímulo nocivo de um inócuo, característica inexistente em neurônios aferentes não nociceptivos. No geral, as fibras sensoriais primarias são compostas tanto um ramo axonal periférico, inervando tecidos periféricos, quanto um ramo axonal central, fazendo a ligação com a medula espinal, podendo ser classificadas em três grupos baseados em critérios anatômicos e funcionais resumidas na Figura 2 (BASBAUM et al., 2009).

As fibras nociceptivas transmitem o impulso nervoso em diferentes velocidades. Existem as responsáveis pela resposta rápida a dor que são mielinizadas (A-delta, Aδ) e a fibras nociceptoras de condução lenta, sem mielinização (fibras C). Embora, o conhecimento médico que se tenha hoje, as funções dessas fibras ainda não são

completamente esclarecidas, porém o papel de ambas se sobrepõe na resposta a dor (OAKLANDER, 2011). A estimulação dessas fibras nociceptoras periféricos faz com que a informação nociceptiva seja levada por meio das fibras aferentes até o SNC (SANN & PIERAU, 1998). Também existem as fibras nociceptivas silenciosas ("*silent*" ou "*sleeping*"), são fibras aferentes, que normalmente não são responsivos a estímulos comuns, somente sob influência de mediadores inflamatórios (JULIUS & BASBAUM, 2001).

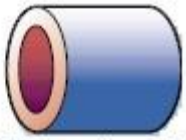
Tipo de Fibra	 Fibras Aα e Aβ	 Fibra Aδ	 Fibra C
Mielinazação	muita	pouca	ausente
Diâmtro	10µm	2 – 6µm	0.4 – 1.2µm
Velocidade de condução	30-100m/s	1.2-30m/s	0.5-2m/s
Temperatura	não reconhece	Tipo I > 53°C Tipo II > 43°C	> 43°C
Tipo de sinal	propriocepção (toque leve)	nocicepção (térmica, mecânica e química)	nocicepção (térmica, mecânica e química)

Figura 2. Tipos de fibras sensoriais primárias com diferentes graus de mielinização e velocidade de resposta**.**
Fonte: Autor (2017).

A ativação dos nociceptores ocorrida, por exemplo, em casos de mudança de estímulo térmico nocivo, estímulo mecânico nocivo, e estímulo nocivo químico, leva a liberação local de mediadores químicos tais como bradicinina, prótons, óxido nítrico (NO), serotonina (5-HT), histamina, metabólitos do ácido araquidônico, ATP, adenosina, citocinas, aminoácidos excitatórios, Substância P, opióides e acetilcolina, entre outros. Estes metabólitos atuam sobre receptores específicos da fibra nociceptiva,

levando a uma propagação do sinal nociceptivo até o SNC (Figura 3) (JULIUS & BASBAUM, 2001). Estes potenciais de ação chegando até o cérebro são interpretados como dolorosos (desagradáveis) e desencadeiam ações protetoras, memórias e emoções.

Vale ressaltar que essa ativação por mediadores químicos, pode ser iniciada por células não neuronais como células teciduais lesadas, células inflamatórias, células endoteliais, plaquetas, fibroblastos e células de Schwann (BESSON, 1997).

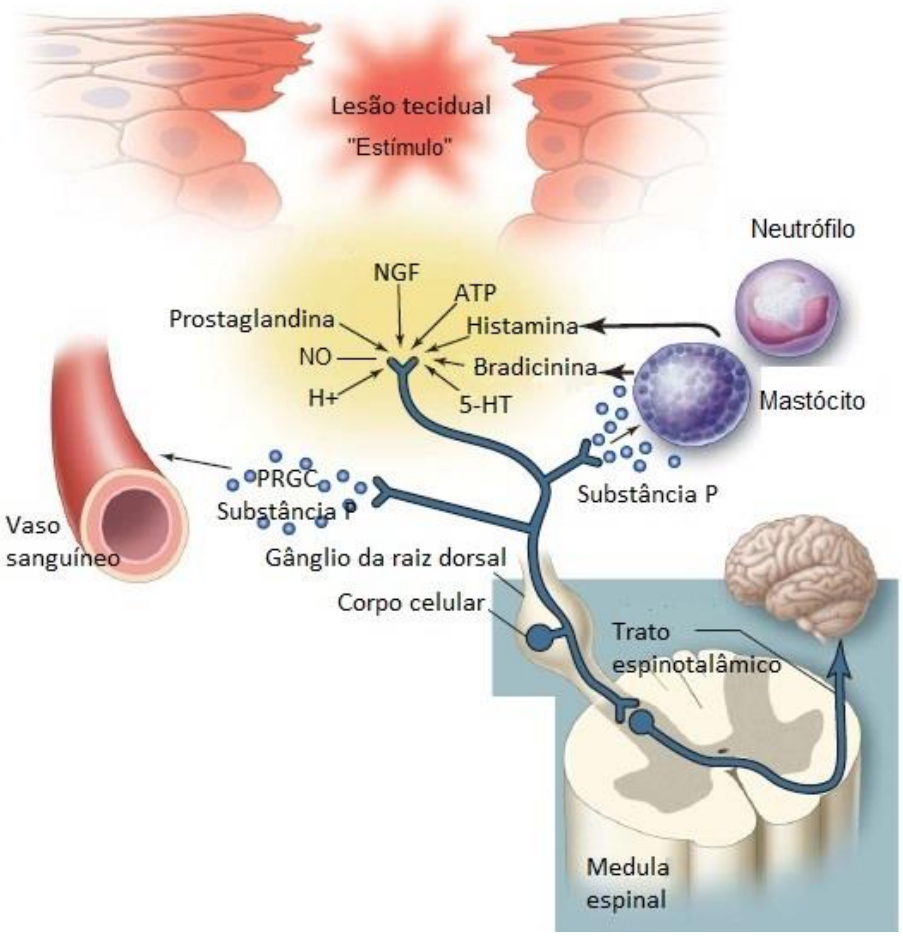


Figura 3. Ativação de nociceptores sob influência de mediadores no sítio da lesão. ATP = trifosfato de adenosina; NGF = fator de crescimento de nervo (*nerve growth fator*); NO = óxido nítrico; PRGC = peptídeo relacionado ao gene da calcitonina**.**
Fonte: Oaklander (2011).
Acesso: https://www.medicinanet.com.br/m/conteudos/acp-medicine/

De acordo com Garland (2012) e Oaklander (2011), o sinal nociceptivo é transmitido a várias estruturas do SNC que exercem controle sobre a sinalização da dor

por meio de projeções descendentes, estas inibem ou amplificam a excitabilidade das vias nociceptivas ascendentes. As fibras sensoriais primárias, que conduzem os estímulos dolorosos, possuem seus corpos celulares localizados no gânglio espinal da raiz dorsal e suas terminações centrais entrando na medula espinal pelo corno posterior (dorsal). Neste local novos potenciais de ação são gerados com neurônios de segunda ordem no mesmo lado da medula (ipsilateral) e ascendem para o encéfalo via tálamo (chamado trato espinotalâmico). Quando chegam ao encéfalo, estes neurônios de segunda ordem fazem sinapse com neurônios de terceira ordem no tálamo (diencéfalo). Por fim, os neurônios de terceira ordem chegam e se projetam no interior do córtex somatossensorial. E também se difundem para regiões responsáveis pelas respostas emocionais e a dor (Figura 4) (GARLAND, 2012; OAKLANDER, 2011). O SNC possui mecanismos próprios para o controle da dor por meio da via descendente que envolve uma série de estruturas encefálicas, bem como sistemas de neurotransmissores, tais como os sistemas opióide, serotoninérgico, noradrenérgico, gabaérgico, adenosinérgico, além de canabinóides entre outras substâncias (MILLAN, 2002).

Nocicepção é o centro de muitos estados dolorosos, mas a dor pode ocorrer sem nocicepção (periférica) e vice-versa, sob influência de fibras lesadas ou do estado emocional (LOESER & TREEDE, 2008). Assim, ainda hoje pesquisadores buscam continuamente tratamentos que visam o alívio da dor, seja pela descoberta de novas drogas, uso mais eficiente de drogas já disponíveis ou mesmo por tratamentos não farmacológicos que aliviam a dor (DUTRA et al., 2016).

Referente a duração, a dor pode ser transitória, aguda ou crônica. Na dor transitória, a ativação de nociceptores ocorre na ausência de dano tecidual. Já, na dor aguda geralmente existe lesão e ativação de nociceptores no sítio lesionado. Por sua vez, a dor crônica, geralmente, é ocasionada por lesão ou enfermidade, podendo durar por longo tempo devido a fatores que não os causadores (LOESER & MELZACK, 1999).

Quanto à sua origem, a dor pode ser classificada em: nociceptiva, neurogênica, neuropática, psicogênica e inflamatória (SILVA, 2013). A dor nociceptiva deve-se à estimulação excessiva dos nociceptores localizados na pele, vísceras e outros tecidos. A dor neurogênica reflete dano de tecido neuronal na periferia ou no SNC. Quando há disfunção ou dano de um nervo ou grupo de nervos, resultando em quadro álgico, denomina-se dor neuropática. No entanto, quando a dor não é proveniente de fonte somática identificável e pode refletir fatores psicológicos, chama-se dor psicogênica

(MILLAN, 1999). Por fim, na dor inflamatória, ocorre significativo dano tecidual, com dor geralmente mais persistente e acompanhada de inflamação. Nestas circunstâncias geralmente ocorre quadro de hipersensibilidade causado pela ativação e sensibilização dos nociceptores periféricos por mediadores químicos, produzidos pela lesão tecidual e pela inflamação (DRAY, 1997).

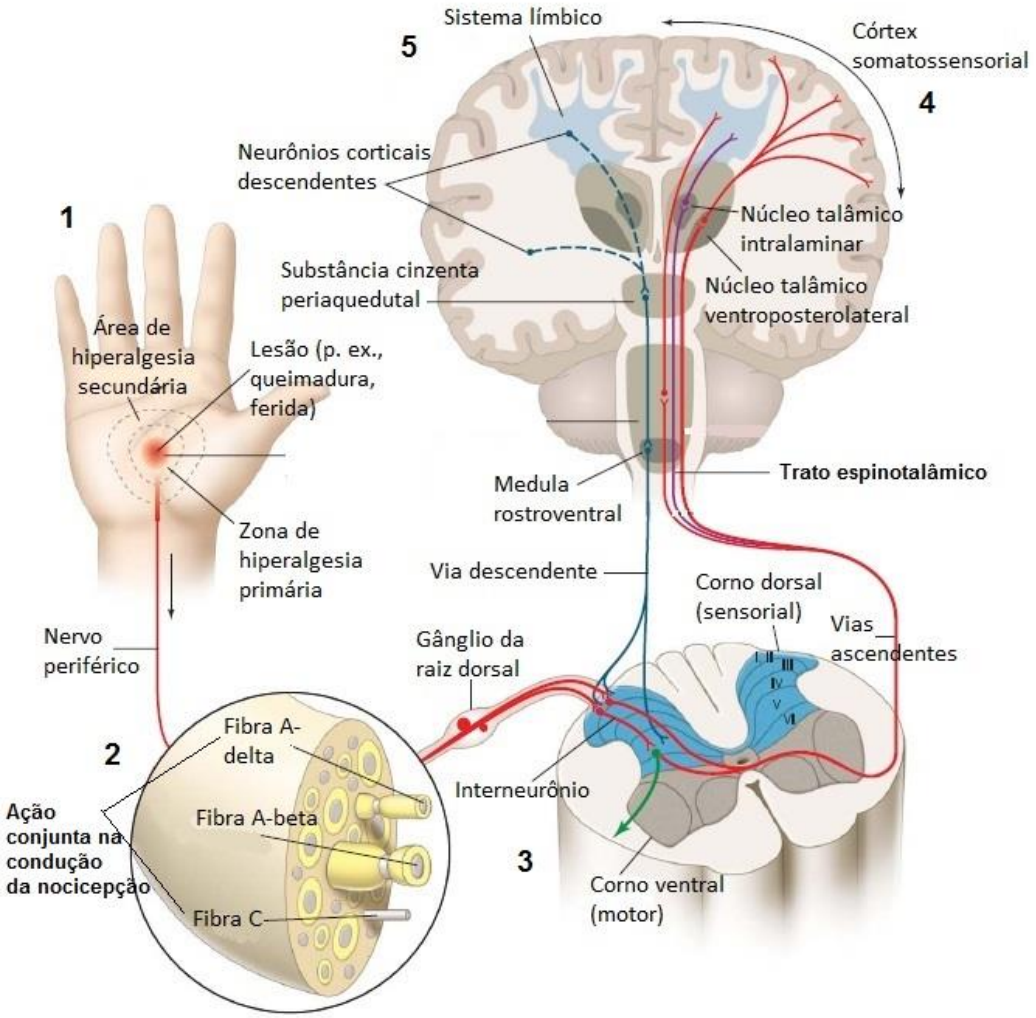


Figura 4. O processamento sensorial da nocicepção em múltiplos níveis (periférico e central). (1) Ativação das fibras Aδ e C pela lesão (dor); (2) Nociceptores conduzem impulsos aferentes na até a medula espinal. (3) O corno dorsal os sinais nociceptores que chegam são modulados e ascendem. (4) Ativação de áreas localizadas no tálamo e no córtex cerebral, as projeções secundárias ao nível do trato espinotalâmico, trato da coluna dorsal e outras vias levam à percepção da dor.
Fonte: Oaklander (2011).
Acesso: https://www.medicinanet.com.br/m/conteudos/acp-medicine/

2.3 PROCESSO INFLAMATÓRIO

Na existência de uma lesão tecidual em nosso organismo, são acionados mecanismos imunes de controle com o propósito de limitar os danos e auxiliar a regeneração. Estes mecanismos fazem parte da *resposta inflamatória*, caracterizada por quatro sinais cardinais: dor, rubor, calor, edema e, em alguns casos, culminando com perda total ou parcial da função (SCHMID-SCHÖNBEIN, 2006).

A inflamação pode ser dividida em dois estágios, o agudo e o crônico, mas apresentam manifestações comuns no início, ao longo e no final do processo. O estágio agudo é um processo inicial, mediado principalmente pela ativação das células do sistema imunitário, tais como os leucócitos polimorfonucleares (neutrófilos), e o sistema complemento. Este estágio da inflamação persiste por um curto período de tempo e geralmente traz resultados benéficos para a reparação do dano (NATHAN, 2012). Se o processo inflamatório persiste por um período maior, semanas ou meses, então o estágio considerado crônico está instalado, ocorrendo intensa atividade leucocitária (macrófagos e linfócitos) na tentativa do reparo tecidual (RICKLIN & LAMBRIS, 2013).

Os primeiros mediadores químicos liberados na inflamação são a histamina e a serotonina (5-HT) produzidos por mastócitos, basófilos e plaquetas sanguíneas (FRANCISCHETTI, 2010). A histamina provoca vasodilatação e aumento da permeabilidade vascular (eritema e edema) e estimula as terminações nervosas sensoriais. A serotonina por sua vez, no sistema imune, ativa monócitos humanos e impede a sua apoptose, modula a produção de citocinas e quimiocinas em monócitos (SOGA et al, 2007; DÜRK et al., 2005).

Na fase aguda, a inflamação promove alterações fisiológicas que envolvem uma sequência coordenada de eventos, como a vasodilatação, possibilitando o aumento do fluxo sanguíneo no local e da permeabilidade vascular, a migração celular de leucócitos juntamente com aumento na expressão de moléculas de adesão, prejuízo tecidual por atividade de proteases e espécies reativas de oxigênio (EROs), necrose e apoptose, além da liberação de inúmeros mediadores pró-inflamatórios como as citocinas fator de necrose tumoral do tipo alfa (TNF-α) e interleucina 1 beta (IL-1β) (HUERRE & GOUNON, 1996; VIVIER & MALISSEN, 2005). Vários destes fatores agem sobre células residentes no local (tais como fibroblastos, células endoteliais, macrófagos

teciduais e mastócitos) e também das células inflamatórias recém recrutadas (monócitos, linfócitos, neutrófilos e eosinófilos) (FEGHALI & WRIGHT, 1997). A Figura 5 ilustra a migração celular na fase aguda da inflamação.

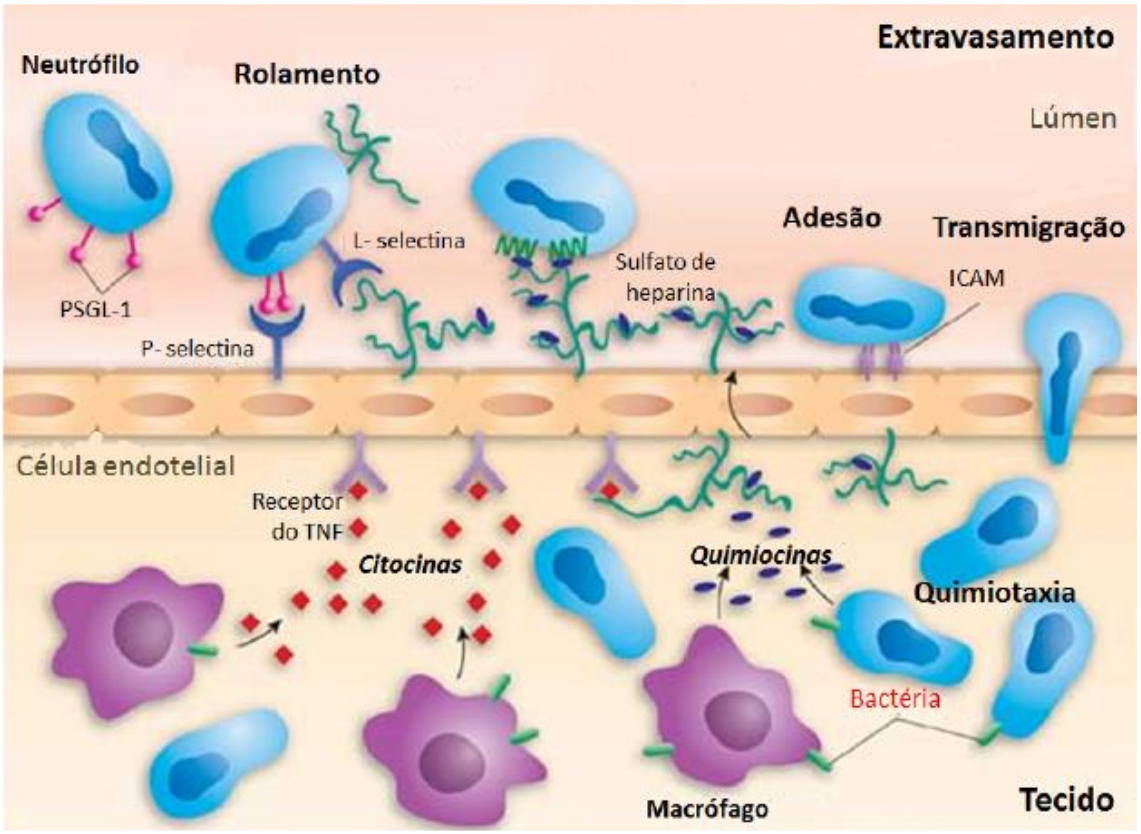


Figura 5. Migração celular no processo inflamatório agudo.
Fonte: Silva (2003).

A dor irá ocorrer devido aos efeitos diretos de mediadores liberados a partir do dano inicial como da resposta inflamatória em si, e ainda pela compressão dos nervos sensoriais ocasionada pelo edema local

Entre os principais sistemas envolvidos na inflamação estão o sistema do complemento, de coagulação e a cascata do ácido araquidônico. Esta última por ação de enzimas específicas irá originar prostaglandinas (PGs) pela ação das ciclooxigenases (COX 1 e 2), leucotrienos e tromboxanos (pela ação de lipooxigenases), todos mediadores envolvidos no processo inflamatório (RANG & DALE, 2004).

Dentre as células envolvidas podem ser citadas as células do sistema imune, como neutrófilos, linfócitos e mastócitos e os macrófagos, que estão envolvidos na

liberação de uma gama de mediadores e radicais livres que contribuirão para o processo inflamatório (HAVSTEEN, 2002). Entre estes mediadores liberados pelos macrófagos merecem destaque as citocinas, que entre outras ações induzem a liberação de enzimas envolvidas no processo inflamatório, tais como as COX (SCHMID-SCHÖBEIN, 2006).

As citocinas credita-se um papel crítico na resposta inflamatória tanto aguda como na crônica. Algumas são chamadas de pró-inflamatórias pois promovem e estimulam a inflamação, no entanto outras chamadas de anti-inflamatórias auxiliam a supressão da inflamação. As citocinas envolvidas na resposta aguda são IL-1, TNF-α, IL-6, IL-11, IL-8 e algumas quimiocinas. Destaca-se que IL-1 (α e β) e TNF-α são mediadores inflamatórios extremamente potentes, e juntamente com IL-6 e IL-8, são as principais citocinas na inflamação aguda, desempenhando uma ação crítica como mediadores endógenos de moléculas de adesão (FRANCISCHETTI et al, 2010; FEGHALI & WRIGHT, 1997). Soma-se ainda, que NO, IL-1β e TNF-α são indutores potentes da COX-2 nas células envolvidas com a resposta imunológica (COLEMAN, 2001).

Outras moléculas geradas em células inflamatórias, são as EROs, as quais decorrem principalmente por ação catalítica das enzimas NADPH oxidase (formação do radical ânion superóxido (O*), e da enzima Mieloperoxidase (MPO), que catalisa a formação do ácido hipocloroso a partir do peróxido de hidrogênio e cloreto. Ressalta-se que a MPO é a enzima mais abundante em neutrófilos e seu poder de catálise lhe confere a versatilidade de reagir com uma série de substratos que não somente o cloreto (SILVA, 2003). Dentre estes substratos estão polifenóis, catecolaminas, aminoácidos, superóxido, nitrito, ácido ascórbico (vitamina C) e o ácido úrico. A oxidação destes substratos gera intermediários como radicais livres, peróxidos orgânicos, quinonas, entre outros produtos capazes de oxidar estruturas celulares do hospedeiro e alterar respostas celulares e, dependendo a extensão da oxidação, induzir dano tecidual (SILVA, 2003).

A grande preocupação dos mecanismos que participam da inflamação é o seu envolvimento em doenças crônicas, incluindo câncer, diabetes, doenças neurodegenerativas, cardiovasculares e reumatológicas (GARCIA, 2005). Além disso, os efeitos adversos de vários anti-inflamatórios não esteroidais (AINEs) utilizados na clínica, fazem da pesquisa por novos compostos com ação moduladora das condições dolorosa e processos inflamatórios uma área sempre em atualização.

Assim, o desenvolvimento de novos fármacos para o tratamento de processos inflamatórios, incluindo o alívio da dor, recebe considerável atenção tanto por parte da comunidade científica, quanto pelas indústrias farmacêuticas. Levando em consideração que esses fármacos possam advir de plantas medicinais, os mesmos possuem grande aceitação e uso terapêutico pela população em geral. Porém, até chegar a medicamento, existe a necessidade de pesquisa básica, pré-clínica e de cunho acadêmico. Esses trabalhos precisam ser mais detalhados e bem embasados para encontrar, verificar e apontar à indústria, compostos de origem vegetal com propriedades farmacológicas confiáveis, dignos de investimento científico.

2.4 MODELOS ANIMAIS DE NOCICEPÇÃO E INFLAMAÇÃO

Nesse contexto, vários modelos experimentais são ferramentas importantes e tornam possível o estudo da nocicepção e inflamação. Dentro dos modelos clássicos de nocicepção aguda utilizados, rotineiramente se usa um estímulo álgico, que pode ser térmico, mecânico, elétrico ou químico. Boa parte dos protocolos descrevem sua aplicação em uma parte do corpo do animal, geralmente nas patas traseiras, na cauda ou no abdômen, levando a um comportamento que denote dor, ou ainda à retirada defensiva do membro afetado em resposta à dor, de maneira que essas respostas possam ser mensuradas de uma forma relativamente fácil (BARROT, 2012; MOGIL, 2009). Neste trabalho os modelos de nocicepção aguda ou crônica foram baseados em modelos de dor induzidos por agentes químicos.

Entre os modelos químicos agudos amplamente empregados em experimentos *in vivo*, estão o teste de contorções induzidos por ácido acético e a nocicepção pelo teste da formalina (37% formaldeído). (BARROT, 2012; MOGIL, 2009). O modelo de nocicepção visceral por indução de ácido acético, envolve a injeção intraperitoneal (i.p.) do mesmo em ratos ou camundongos, levando o animal a uma série de espasmos abdominais características, que podem ser acompanhadas pela extensão de um ou dos dois membros traseiros, além de torções laterais, todos contabilizados num determinado período de tempo. (WHITTLE, 1964). Este ensaio descrito como um modelo comum de nocicepção inflamatória visceral, uma vez que libera mediadores pró-inflamatórios, permite aferir a atividade antinociceptiva de substâncias que atuam tanto em nível central quanto periférico (KOSTER et al, 1959; TJØLSEN & HOLE, 1997).

O outro teste descrito para avaliação da nocicepção é a formalina (solução de 37% de formaldeído (DUBUISSON & DENNIS, 1977; HUNSKAAR et al, 1985). A dor gerada nesse teste sobre o animal permite a avaliação de dois tipos característicos de nocicepção, além de ser considerado um modelo inflamatório de curto prazo. Neste ensaio, observa-se uma primeira fase ou fase neurogênica, qualificada pela ativação dos nociceptores aferentes primários e liberação de neurotransmissores excitatórios no corno dorsal da medula espinal (PUIG & SORKIN, 1996; SKILLING et al, 1988), Essa fase é seguida uma fase quiescente caracterizada pela ativação de vias inibitórias descendentes, o que gera uma redução do comportamento nociceptivo do animal. A outra fase que pode ser avaliada trata-se da fase secundária ou fase inflamatória mediada tanto pela atividade de aferentes primários como pelo aumento da sensibilidade espinal aos mediadores excitatórios liberados, dentre estes cininas e citocinas inflamatórias (SWEITZER et al, 2004; PUIG & SORKIN, 1996; TAYLOR et al, 1995; CODERRE et al, 1990; SKILLING et al, 1988; DICKENSON & SULLIVAN, 1987).

Juntamente com avaliações de dor, os agentes químicos álgicos podem ser utilizados para avaliar parâmetros inflamatórios. Pois, posteriormente aos danos teciduais causados pela exposição a agentes irritantes, o sistema imunológico libera mediadores inflamatórios que irão ativar e sensibilizar o sistema nociceptivo (MARCHAND et al, 2005). Portanto, a grande parte dos modelos de dor inflamatória ocorre por administração de substâncias que desencadeiam um processo inflamatório pelo sistema imune (BARROT, 2012; NEGUS et al, 2006). Entre estes, ressaltamos o modelo inflamatório induzido por injeção (i.p.) de carragenina (Cg), polissacarídeo linear sulfatado obtido a partir de extratos de algas marinhas vermelhas, o qual induz uma reação inflamatória local que ativa proenzimas e cininas, gerando edema, migração celular (principalmente neutrófilos) e atingindo uma resposta máxima em aproximadamente 3 a 4 horas após a administração. (MOREAU et al, 2005; PAGANO et al, 2002). Além desse, o modelo inflamatório que emprega o adjuvante completo de Freund (CFA, *Mycobacterium tuberculosis*, 1 mg/mL) proposto em 1937, é considerado um importante modelo crônico, uma vez que, desencadeia uma resposta imune inflamatória prolongada, elevada e duradoura. O CFA é um dos mais potentes adjuvantes descritos para diversos antígenos, sendo usado extensivamente em procedimentos experimentais que pretendem avaliar cronicamente a evolução do processo inflamatório (STILLS & BAILEY, 1991).

Por conseguinte, esses modelos amplamente utilizados e bem descritos na literatura científica compõe a ideia central desse trabalho, que objetivou avaliar o potencial anti-inflamatório e antinociceptivo, dos extratos obtidos de ST, frente a modelos de dor aguda e crônica.

2.5 IMPORTÂNCIA DOS FITOQUÍMICOS COMO ANTIOXIDANTES

Há uma estimativa de que 2 a 5% do oxigênio metabolizado nas células aeróbicas resulte em espécies reativas de oxigênio (EROs), tais como superóxido (O_2^-), e consequentemente gerando os radicais hidroxila (OH^-) e peróxido de hidrogênio (H_2O_2) (Figura 6). Por sua vez, existe um mecanismo de defesa antioxidante, primariamente endógeno, que está continuamente neutralizando as EROs em outros compostos relativamente estáveis. Esse sistema de detoxificação funciona através de um mecanismo enzimático envolvendo principalmente as enzimas superóxido dimsutase (SOD), glutationa peroxidase (GPX), glutationa redutase (GSR), catalase (CAT) (HALLIWELL & GUTTERIDGE, 2007; MOSKOVITZ et al., 2002).

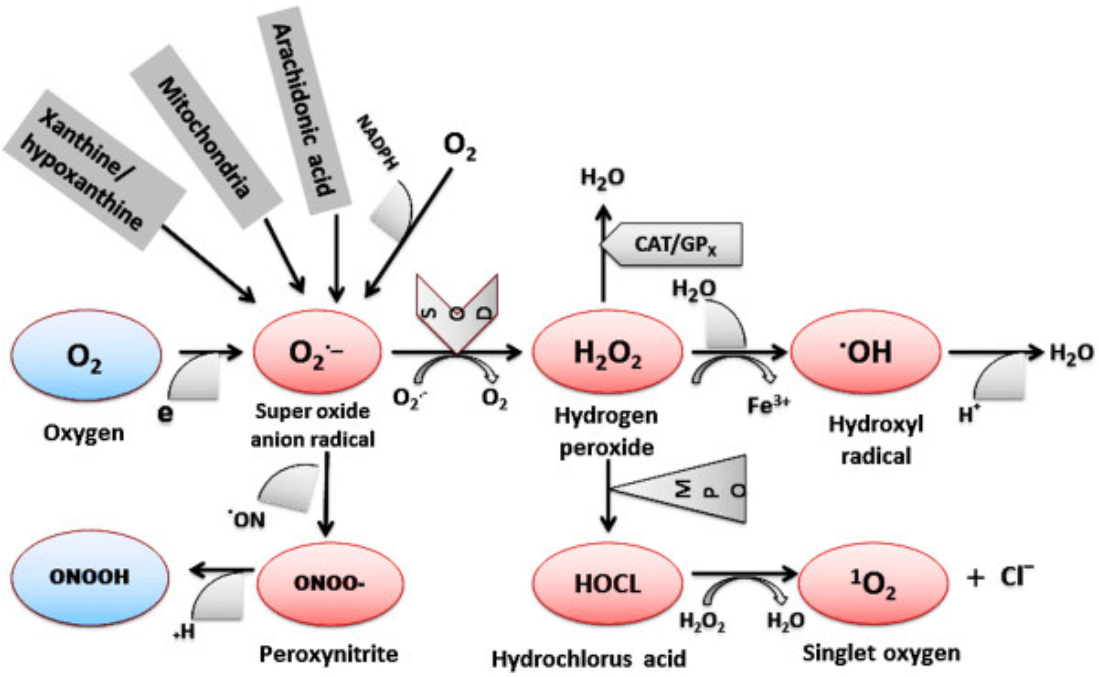


Figura 6. Principais fontes de radicais livres e seu metabolismo
Fonte: Shah et al. (2014).

Em baixos níveis e dentro das condições aceitáveis para o organismo, as EROs possuem papéis importantes na homeostase sistêmica (principalmente sinalização celular e produção óxido nítrico (NO) e monofosfato cíclico de guanosina, GMPc) (BELLÓ, 2002; DRÖGE, 2002). Entretanto, quando ocorre um aumento na produção de EROs, ou ainda, uma relativa diminuição nas defesas antioxidantes, as EROs podem causar danos a moléculas biológicas tais como proteínas, lipídeos e o DNA, gerando o estresse oxidativo. Assim, essas estruturas denominadas radicais livres (RL) formam um importante fator na etiologia de várias condições patológicas tais como a doença de Alzheimer, Parkinson, artrite, reumatismo, infarto do miocárdio, distúrbios do sistema imune, neoplasias, acidente vascular cerebral, diabetes e outras doenças degenerativas, e ainda, no envelhecimento (SHAH et al., 2014; AL-SHOBAILI et al., 2011; HALLIWELL & GUTTERIDGE, 2007).

Dentro dessa condição de desequilíbrio no estado redox da célula, chamado estresse oxidativo, entra o papel de antioxidantes não enzimáticos, minimizando os efeitos deletérios dos RL. De acordo com Halliwell & Gutteridge (2007), considera-se um antioxidante uma substância que, quando presente em baixas concentrações, comparadas a de um substrato oxidável, retarda ou inibe significativamente a oxidação deste substrato. Assim, agentes de baixo peso molecular com ação *scavenger* (substâncias que neutralizam ou minimizam a ação de um RL ou espécie reativa), tais como carotenoides, bioflavonóides, indóis, catecóis, glutationa, α-tocoferol (vitamina E), betacaroteno (vitamina A), bilirrubina, ácido ascórbico (vitamina C) e ácido úrico, são compostos que auxiliam a manter a homeostase celular.

Nesse contexto, evidências apontam para um importante papel de componentes fitoquímicos como antioxidantes, auxiliando na manutenção dos radicais livres e consequentemente, na resistência a doenças (LOBO et al., 2010; CUI et al., 2004). Estudos recentes têm mostrado a eficácia de plantas medicinais e seus extratos em modelos *in vitro*, ex vivo e *in vivo* (SALGUEIRO et al., 2016a; COLPO et al., 2016; LIMA et al., 2017; PORTELA et al., 2016).

A presente espécie deste trabalho, ST, tem demonstrado um significativo efeito antioxidante *in vitro* tanto para extratos das folhas como das raízes (ROSA et al., 2016). Outras espécies de Sida, já demonstraram efeitos *free radical scavenger in vitro* e *in vivo*, tais como *S. acuta*, *S. alba*. *S. cordifolia* e *S. rhombifolia* (ARCINIEGAS et al.,

2017; PREETHIDAN et al., 2013; KONATE & SOUZA 2010; THOUNAOJAM et al., 2011, 2012).

Compostos vegetais, com destaque para os compostos fenólicos, já se revelaram como sendo eficazes neutralizadores de radicais livres e inibidores de peroxidação lipídica (efeito danoso a membranas celulares) (RICE-EVANS et al., 1997). Existe um interesse considerável em produtos nutracêuticos e medicina preventiva no desenvolvimento de antioxidantes naturais de origem vegetal, devido à sua capacidade de proteção contra o dano oxidativo e também o baixo risco de efeitos colaterais (SOUSA et al., 2015; BENAYAD et al., 2014; KCHAOU et al., 2013). Segundo Pisoschi & Pop (2015) acredita-se que a suplementação com antioxidantes exógenos pode incrementar a capacidade do organismo em controlar o estresse oxidativo quando as defesas endógenas estão diminuídas. Assim, o possível uso de suplementos antioxidantes num nível aquedado e profissionalmente supervisionado, auxiliaria na manutenção contra o excesso de EROs em células humanas, e seria uma medida preventiva contra situações patológicas. (SEIFRIED et al., 2007). Cabe ressaltar, que os compostos antioxidantes se encontram em diferentes classes de compostos, os quais podem interferir nos ciclos oxidativos para inibir ou retardar o dano oxidativo as biomoléculas (TUBEROSO et al., 2013).

É interessante notar, que as substâncias consideradas como princípios ativos vegetais advém, na sua maioria, do metabolismo secundário das plantas, ou seja, são componentes intimamente envolvidos na relação planta-ambiente. Dessa forma, na sua grande parte, possuem aspectos ecológicos e biológicos relevantes. Destaca-se que tais moléculas podem agir inicialmente como defesa química (contra insetos, micro-organismos), funções gustativas, odoríferas e visuais (coloração) com seletividade para determinada classe de insetos, e ainda, podem inferir sobre processos simbiônticos intra e interespécies (MAZID et al., 2011; VERPOORTE & MEMELINK, 2002).

Consequentemente, a busca por compostos vegetais farmacologicamente úteis ainda representa um nicho da ciência com bastante relevância em vista daquilo que já proporcionou a medicina e daquilo que ainda pode proporcionar.

2.6 FERRAMENTAS COMPUTACIONAIS NA PESQUISA DE COMPOSTOS NATURAIS

Na pesquisa e desenvolvimento (P&D) de um novo fármaco, existe uma demanda de tempo bastante significativa, o que torna todo o processo dispendioso e moroso. Otimizar esse procedimento torna-se um ganho para todos os envolvidos. A estimativa aponta que de cada 30 mil moléculas estudadas apenas nove chegam ao mercado após mais de uma década de estudos além da aprovação dos órgãos regulatórios. Portanto, uma abordagem planejada pode incrementar a eficiência e trazer resultados satisfatórios num período menor de tempo (CALIXTO & SIQUEIRA, 2008).

A P&D pode ser simplificadamente dividido em: (i) descoberta, fase pré-clínica onde se conhece e escolhe o alvo terapêutico, e também se identifica a molécula bioativa; (ii) otimização, melhoramento estrutural do composto juntamente com estudos de absorção, distribuição, metabolismo, excreção e toxicidade – ADMET; (iii) desenvolvimento, ocorre o aprimoramento da formulação aliado a estudos clínicos (GUIDA et al., 2010; BARREIRO & FRAGA, 2008)

Em cada etapa da P&D de fármacos, diferentes abordagens tecnológicas estão sendo aplicadas a fim de reduzir custos e riscos envolvidos nesse processo. Assim, nos últimos anos, segmentos da indústria farmacêutica tem aperfeiçoado metodologias computacionais que auxiliam na seleção e triagem dos compostos de interesse terapêutico (HUALIANG, 2006). Aliado a esse fator, questões éticas têm levado a um controle mais rigoroso em relação ao uso de animais em estudo pré-clínicos Assim, há a necessidade de se desenvolver e padronizar testes que poupem ou minimizem a utilização de animais superiores, e que da mesma forma sejam eficientes em detectar, entre outros, toxicidade e possíveis ações farmacológicas.

Entre as mais recentes metodologias utilizadas estão os ensaios *in silico* (via computacional), como modelagem molecular, *Docking* molecular, estudos de relação estrutura-atividade (*structure-activity relationship* e *quantitative structure-activity relationship* – SAR e QSAR), avalições toxicológicas por comparação de fragmentos moleculares entre outros. Tais abordagens permitem uma triagem mais direta e eficiente de protótipos de fármacos e análogos, sem que ocorra experimentação aleatória e desnecessária (ARROIO et al., 2010).

Nesse contexto, programas computacionais de química juntamente com os bancos de dados em rede se mostram indispensáveis para o surgimento desses novos agentes terapêuticos. Pois uma rápida análise da atividade biológica *versus* propriedades físico-químicas de uma série de moléculas de interesse se torna possível por meio

dessas ferramentas (CARVALHO 2003). Assim, executando essas avaliações *in silico*, a atividade biológica do composto de interesse pode ser aperfeiçoada e/ou ter a sua toxicidade reduzida.

Dentre as vantagens relacionadas aos modelos computacionais estão a redução da síntese de compostos análogos e redução de testes *in vitro* e *in vivo* para aferição da atividade biológica. Além disso, esses ensaios podem ser empregados nas fases iniciais de estudo, usando apenas a estrutura "virtual" dos compostos investigados. Juntamente com isso, permitem uma avaliação prévia e em grande escala de grupamentos chamados farmacóforos (região da molécula de maior interação com seu receptor) por meio de bibliotecas contendo até milhões de compostos já conhecidos (HUALIANG et al., 2006).

Nesse cenário, os constituintes ativos de plantas podem ser selecionados e teoricamente dirigidos, objetivando seu melhor aproveitamento na pesquisa básica. Uma vez que estes compostos são uma importante fonte de agentes farmacêuticos novos, sua relevância precisa ser considerada. De acordo com Pan et al. (2013) aproximadamente 80% das drogas antimicrobianas, cardiovasculares, imunopressoras e anticancerígenas disponíveis no mercado, são derivadas ou desenvolvidas a partir de produtos naturais, geralmente vegetais. Hoje, centenas de alvos farmacológicos importantes na medicina mostram interações com produtos naturais, os quais, muitas vezes, possuem uma ação pleiotrópica (sobre múltiplos alvos) (GOEL et al., 2011). Tais informações são úteis na descoberta de fármacos baseado em plantas medicinais.

Soma-se a isso o fato dos extratos vegetais serem misturas complexas de diferentes metabólitos, que após uma elucidação estrutural podem ser estudados de maneira independente. Consequentemente, essas avaliações prévias podem predizer possíveis ações terapêuticas e/ou tóxicas destes fitoquímicos (ARVIDSON et al., 2008).

Por fim, estudos com compostos de plantas utilizando ferramentas computacionais tem mostrado a viabilidade e eficiência de tal metodologia (SALGUEIRO et al., 2016b; KADIR et al., 2013; BARLOW et al., 2012; ARVIDSON et al., 2008). Esse contexto sugere que um "*computer-based approach*" pode ser extremamente útil na avaliação de fitoconstituintes biologicamente ativos.

3 JUSTIFICATIVA

Embasado pelo conhecimento etnofarmacológico, a pesquisa com plantas medicinais torna-se uma possível fonte de compostos biologicamente ativos de interesse farmacêutico. Assim, tona-se necessário estudos que elucidem princípios ativos obtidos de plantas, avaliando de maneira multidisciplinar questões levantadas quanto a sua possível ação terapêutica e/ou toxicológica.

Essa proposta apresenta a espécie vegetal *S. tuberculata*, pouco estudada e nativa do Bioma Pampa. Em estudos realizados previamente pelo nosso grupo na Universidade Federal do Pampa, os extratos de *S. tuberculata* demonstraram atividades antimicrobianas significativas contra cepas de *Candida krusei*. Além disso, numa abordagem fitoquímica inicial, revelou-se a presença de fitoconstituintes de diferentes classes, tais como fitoecdisteróides (majoritários), flavonoides e alcaloides.

Portanto, baseado no seu perfil fitoquímico, que entre outros apresenta compostos esteroidais, surge a possibilidade de uma possível ação analgésica e anti-inflamatória dos extratos. Aliados a ensaios que já demonstraram a capacidade antioxidante, incrementam a possibilidade de uma ação farmacológica da planta. Portanto, nossa proposta buscou elucidar mais detalhadamente a composição química da espécie *S. tuberculata*, e também fornecer bases quanto a qualidade do material vegetal e apontar compostos que permitam uma abordagem mais eficiente da dor aguda e crônica.

Por fim, além de fomentar a pesquisa na região do pampa, de onde advém a matéria prima, estudos bioquímicos e farmacológicos com a espécie *S. tuberculata* são raros na literatura científica. Gerando assim, perspectivas para estudos futuros complementares em áreas correlatas e transversais.

4 OBJETIVOS

4.1 GERAL

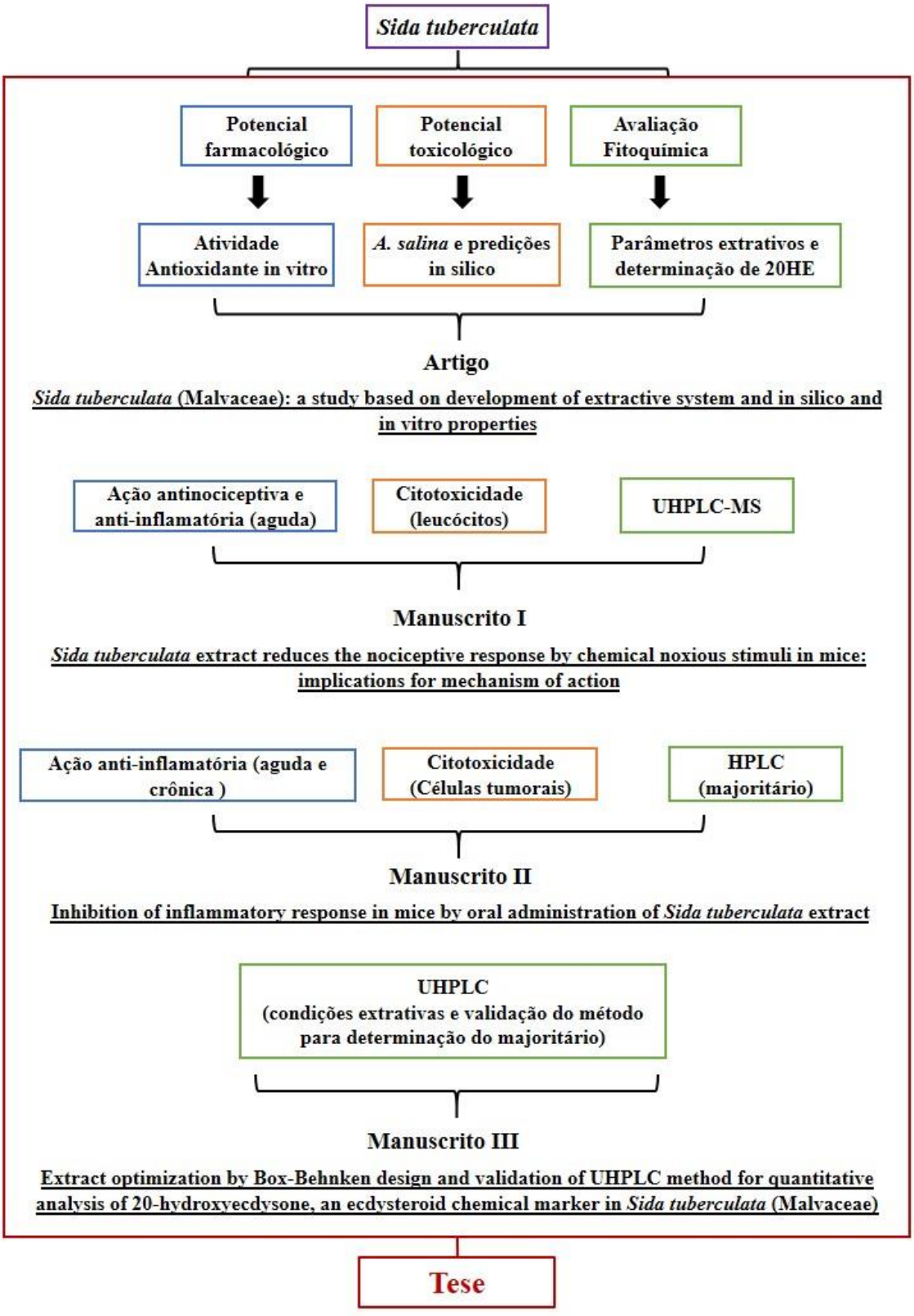
Caracterizar a composição fitoquímica e avaliar os efeitos antinociceptivos e anti-inflamatórios de extratos obtidos de *Sida tuberculata*

4.2 ESPECÍFICOS

- Identificar os principais fitoconstituintes presentes em diferentes extratos de ST*;*
- Desenvolver e validar um método cromatográfico para a determinação de um marcador fitoquímico em ST;
- Avaliar o efeito antioxidante dos extratos de ST por meio de ensaios *in vitro*;
- Investigar o potencial citotóxico dos extratos de ST *in vitro*;
- Determinar potencial antinociceptivo e anti-inflamatório *in vivo* dos extratos de ST em modelos de dor aguda e crônica;
- Investigar possíveis mecanismos de ação do extratos de ST.

5 RESULTADOS

Os resultados aqui expostos, em forma de um artigo e três manuscritos científicos, abrangem os objetivos propostos e o regulamento do Programa de Pós-Graduação em Bioquímica da UNIPAMPA, Campus Uruguaiana. Veja o mapa conceitual abaixo.



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Sida tuberculata (Malvaceae): a study based on development of extractive system and *in silico* and *in vitro* properties

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Abstract

Sida tuberculata (Malvaceae) is a medicinal plant traditionally used in Brazil as an antimicrobial and anti-inflammatory agent. Here, we aimed to investigate the different extractive techniques on phytochemical parameters, as well as to evaluate the toxicity and antioxidant capacity of *S. tuberculata* extracts using *in silico* and *in vitro* models. Therefore, in order to determine the dry residue content and the main compound 20-hydroxyecdysone (20E) concentration, extracts from leaves and roots were prepared testing ethanol and water in different proportions. Extracts were then assessed by *Artemia salina* lethality test, and toxicity prediction of 20E was estimated. Antioxidant activity was performed by DPPH and ABTS radical scavenger assays, ferric reducing power assay, nitrogen derivative scavenger, deoxyribose degradation, and TBARS assays. HPLC evaluation detected 20E as main compound in leaves and roots. Percolation method showed the highest concentrations of 20E (0.134 and 0.096 mg/mL of extract for leaves and roots, respectively). All crude extracts presented low toxic potential on *A. salina* (LD_{50} >1000 μg/mL). The computational evaluation of 20E showed a low toxicity prediction. For *in vitro* antioxidant tests, hydroethanolic extracts of leaves were most effective compared to roots. In addition, hydroethanolic extracts presented a higher IC_{50} antioxidant than aqueous extracts. TBARS formation was prevented by leaves hydroethanolic extract from 0.015 and 0.03 mg/mL and for roots from 0.03 and 0.3 mg/mL on egg yolk and rat tissue, respectively ($P<0.05$). These findings suggest that *S. tuberculata* extracts are a considerable source of ecdysteroids and possesses a significant antioxidant property with low toxic potential.

Key words: *Sida tuberculata*; 20-hydroxyecdysone; Toxicity prediction; Antioxidant

Introduction

Sida species are widespread around the world, occurring predominantly in the tropics, particularly in South America. Some species of this genus has been employed in traditional medicine for a long time, such as *S. rhombifolia*, *S. acuta* and *S. cordifolia* (1,2). In Brazil, *Sida* species are used in folk medicine for treatment of stomatitis, blenorrhea, asthmatic bronchitis and other inflammatory processes (3,4). Among the several species of this genus is *S. tuberculata* (Malvaceae), a medicinal plant widely distributed in South Brazil. Traditionally, leaves and roots of this species have been used as anti-inflammatory, hypoglycemic and antimicrobial agents.

Previous studies with different extracts and isolated compounds of this genus have described important biologic effects. Aqueous extracts from *S. cordifolia* reduced the damage caused by rotenone and presented a therapeutic action in Parkinson's disease (5). *S. acuta* revealed a significant hepatoprotective effect against liver damage induced by paracetamol overdose (6). Leaf extracts of *S. rhomboidea* demonstrated a significant cardiovascular protective effect (7). The anti-inflammatory activity also was investigated for *S. tiagii* extracts, which presented similar results to the standard drugs tested (8). Our group previously found a significant antimicrobial effect of *S. tuberculata* extracts against *C. krusei* strain (9).

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Chemical investigations of *Sida* spp. have indicated the presence of a wide variety of compounds. Among the main classes of chemicals detected, ecdysteroids (10), alkaloids (11) and flavonoids (12) are predominant. Within the ecdysteroids class, polyhydroxylated ketosteroids and its derivatives are the most frequent (13,14). Ecdysteroids are produced primarily in arthropods and plants, but are also present in fungi, and even in marine sponges (15). Interesting observations on the potential importance of ecdysteroids have justified studies on function and biological properties of this class.

Recently, our research group identified, among others, 20-hydroxyecdysone (20E), a major ecdysteroid in *S. tuberculata*, as well as flavonoids and alkaloids (9). Thus, considering our interest about *S. tuberculata* biological properties, in the current work, different extracts were evaluated based on phytochemical parameters and assessed for their toxicological and antioxidant potential.

Material and Methods

Plant material

The whole plant was collected in Uruguaiana (Rio Grande do Sul, Brazil), a city located at the western border with Argentina. A specimen was identified and a voucher (*Sida tuberculata* R.E. Fries; ICN 167493) was deposited at ICN Herbarium (Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil).

Extract obtainment

Initially, the plant was separated into leaves and roots. Each material was submitted to drying at 40°C, reduced to a powder and submitted to extraction by maceration, reflux, and percolation techniques. Aiming to evaluate the most adequate system, three ethanol concentrations were tested on extraction: 20, 30 and 40% (v/v) for leaves and 50, 70 and 90% (v/v) for roots. This choice was based on the plant's tissue rigidity. In all cases, the plant:solvent proportion was standardized at 1:10 (w/v). Aqueous infusions (tea) were also prepared for toxicity and antioxidant assays according to methodologies described below.

Dry residue determination

Dry residue assay was performed according to Brazilian Pharmacopoeia (16). Briefly, 2.0 mL of each extract were submitted to drying at 105°C until a residual mass correspondent to the concentration of dried extractives was obtained. All developed extracts were assayed in triplicate.

Chromatographic analysis

Extract samples were evaluated by a high performance liquid chromatograph coupled with diode array detection (HPLC-DAD) (9). Chromatography analysis was performed with a Prominence liquid chromatograph (Shimadzu, Japan), equipped with a binary pump LC-20AD with SIL-10AF auto sampler and SPDM10A PDA detector. Mobile phase consisted of (A) 0.05% phosphoric acid in water and (B) acetonitrile, prepared daily, filtered through a 0.45-mm membrane filter (Millipore, Germany) and sonicated before use. The separation was accomplished using a Phenomenex Luna C-18(2) column (250 × 4.6 mm, 5 μm) with a gradient elution protocol of 0.01–23 min, 10–40% solvent B; 23.01–40 min, 10% solvent B, at a flow rate of 0.8 mL/min. Injection volume was 20 μL and DAD detector was operated at 250 nm.

20-hydroxyecdysone monitoring. Aiming to determine the concentration of the major compound 20E in extract, five concentrations (10, 50, 100, 200 and 500 μg/mL) of standard solution (20E, Sigma Aldrich, USA, 93% purity) were prepared in methanol. Chromatographic injections were made in triplicate. All solutions were freshly prepared and filtered through a 0.45-mm membrane filter (Millipore), prior to analysis.

Computational prediction

Aiming to identify possible nutraceuticals, we evaluated the predictive toxicity of 20E. For this, we analyzed the risks of damages, such as genotoxic damage, endocrine disruption, irritation and hERG (the human ether-à-go-go-related gene) inhibition. Data were generated on-line using ADME-Tox web server software, Advanced Chemistry Development, Inc. (ACD/Labs, ACD/Percepta Platform, version 12.01, Canada, www.acdlabs.com, 2013) (17), which predicts the fragments that could lead to possible toxic effects.

In addition, the 20E was subjected to the drug-likeness evaluation and drug-score profiles using the Osiris Property Explorer program available on the web [http://www.organic-chemistry.org/prog/peo] (18), comparing it with a reference substance.

Artemia salina assay

Based in dry residue and HPLC analysis, we selected extracts (ethanol 70 and 40% for roots and leaves, respectively) to be used in this test. Extracts were concentrated and ethanol was evaporated.

The test was performed according to Meyer et al. (19) with minor modifications. Briefly, *A. salina* eggs were incubated in seawater with 3% NaCl at room temperature for 24 h. After, *A. saline* larvae (10 approximately) were transferred to ELISA plate wells containing different extract concentrations (100–1000 μg/mL) prepared by diluting the extract in 10 mL of the artificial saline solution. For control, larvae were incubated with seawater only. Plates were maintained at 28 ± 1°C for 24 h and the survival rate (%) was counted for lethal dose 50% (LD_{50}) determination. Three independent experiments were performed.

Determination of antioxidant capacity

For antioxidant activity protocols, solutions obtained by percolation with ethanol 70 and 40% for roots and leaves, respectively, were selected. The aqueous extracts were also

evaluated with these protocols. For all assays described below, the samples were diluted to obtain a concentration range of 0.003–0.3 mg/mL. It is important to emphasize that the color controls were used for all extracts, avoiding probable interference of extracts color in results. Moreover, except for thiobarbituric acid reactive substances (TBARS) protocols in animal tissue, results are expressed in half maximal inhibitory concentration (IC_{50}).

DPPH• assay. Antiradical activity of *S. tuberculata* extracts was determined using the DPPH• method (20). Different concentrations of extracts were added to DPPH• solution. After 30 min of incubation at room temperature, the reduction in the number of free radicals was measured by reading the absorbance at 517 nm. Values are reported in IC_{50} based on percentage of inhibition of DPPH• absorbance in relation to the control values without extracts.

ABTS•+ scavenger activity. $ABTS^{\bullet+}$ radical cation (21) was obtained by the reaction between the ABTS solution with the $K_2S_2O_8$ (140 mM) solution for 12–16 h in the dark at room temperature. Antioxidant assay was performed by incubation of extract samples and $ABTS^{\bullet+}$ (final volume of 1.5 mL) during 6 min in the dark. The absorbance was measured at 734 nm. Ethyl alcohol was used as blank to calibrate the spectrophotometer.

Ferric reducing potential assay (FRAP). The ferric reducing power of *S. tuberculata* extracts was determined using a modified version of the FRAP assay (based on the chemical reduction of Fe^{3+} to Fe^{2+}) (22). Briefly, aliquots of the extract were added to freshly prepared and pre-warmed (37°C) FRAP reagent and incubated at 37°C for 30 min. Reduction was monitored by measuring the change of absorbance at 593 nm.

Nitrogen derivative species scavenging activity. According to Marcocci et al. (23), the assay is based on the reaction of nitric oxide radical (NO•) produced by sodium nitroprusside in aqueous solution at physiological pH 7.2. Under aerobic conditions, NO• reacts with oxygen to produce nitrogen derivative products (i.e., nitrate and nitrite) which can be determined using Griess reagent. Values report the percentage of nitrite reaction inhibition with Griess reagent depicted by the *S. tuberculata* extracts as an index of the NO• scavenging activity.

Deoxyribose assay. This assay was performed in accordance with modifications proposed by Puntel et al. (24). Here, hydroxyl radicals were generated by Fenton reaction. Antioxidant capacity was evaluated by the extract's ability to neutralize hydroxyl radicals. Results are reported in IC_{50} based on percentage of inhibition.

Lipid peroxidation assay. Using egg-yolk homogenates, a modified TBARS protocol was employed to measure the formed lipid peroxide (25). Briefly, egg yolk was homogenized and mixed with *S. tuberculata* extracts and $FeSO_4$. This mixture was incubated at 37°C for 60 min, and used in the TBARS assay. Values are reported in equivalents of malondialdehyde (MDA) generated by lipid peroxidation and corrected by mg of tissue.

TBARS in brain and liver of rats. A total of 4 adult male rats (Wistar) were maintained and used in accordance with guidelines of the Committee on Care and Use of Experimental Animal Resources (Protocol approved #001/2012, UNIPAMPA). The animals were sacrificed by decapitation and the brain and liver were removed, quickly homogenized in NaCl (150 mM) and kept on ice. TBARS content was determined as described by Ohkawa et al. (26), using a standard curve of MDA. Briefly, after homogenization, samples were centrifuged at 4000 *g* at 4°C for 10 min to yield a low speed supernatant fraction (S1). The obtained S1 was used for basal and/or pro-oxidants ($FeSO_4$) induced lipid peroxidation. This mixture was incubated at 37°C for 60 min, and after used in the TBARS assay. Values are reported in nmol of MDA generated by lipid peroxidation and corrected by protein content.

Statistical analysis

Data are reported as means ± SD for at least three independent determinations for each experimental step. Statistical differences between groups were determined by two-way ANOVA with the Tukey's post tests. Values of $P \leqslant 0.05$ were considered the limit for significance.

Results

Dry residue

Results obtained from dry residue assay are described in Table 1. The higher yield of dry content occurred when the extracts were prepared by percolation. In terms of alcoholic concentration, the most efficient solvents were hydroethanolic solutions at 40% for leaves and at 70% for roots, when the dry residue parameter was considered alone.

Table 1. Results obtained from dry residue assay for hydroethanolic extracts of *S. tuberculata* prepared using different extraction techniques.

Solvent	Dry residue (% w/w)		
	Extraction technique		
	Maceration	Percolation	Reflux
Leaves			
Ethanol 20%	1.42 (1.5%)	1.84 (0.49%)	1.92 (2.0%)
Ethanol 40%	1.44 (4.4%)	2.88 (0.32%)	2.17 (1.0%)
Ethanol 70%	1.20 (2.9%)	1.75 (4.73%)	1.79 (2.4%)
Roots			
Ethanol 50%	1.21 (1.4%)	2.00 (1.5%)	1.24 (4.9%)
Ethanol 70%	1.34 (0.6%)	2.93 (4.3%)	1.26 (2.1%)
Ethanol 90%	1.03 (0.1%)	0.91 (3.0%)	1.26 (3.9%)

Data are reported as the average of three analyses and relative standard deviation (RSD, % w/w).

Chromatographic analysis

HPLC analyses were performed evaluating *S. tuberculata* extracts obtained from maceration, percolation and reflux. In leaves, two major peaks were identified, with retention times (Rt) of approximately 17.2 (peak 1) and 18.0 min (peak 2). The most representative ecdysteroid (20E) present in the leaves (peak 1) was also accompanied by significant amounts of another phenolic compound, a kaempferol derivative (peak 2). In roots, 20E was detected as the major compound.

In order to evaluate extraction efficiencies, the peak areas of 20E were considered. Data showed that percolation was the most effective technique followed by reflux and maceration (Figure 1). In addition, results revealed that 20E concentration was greater in leaves than roots extracts, 0.134 and 0.096 mg/mL, respectively (Table 2).

***In silico* predictions**

The 20E compound was submitted to computational prediction of toxic effects using ACD/Labs. This effect was

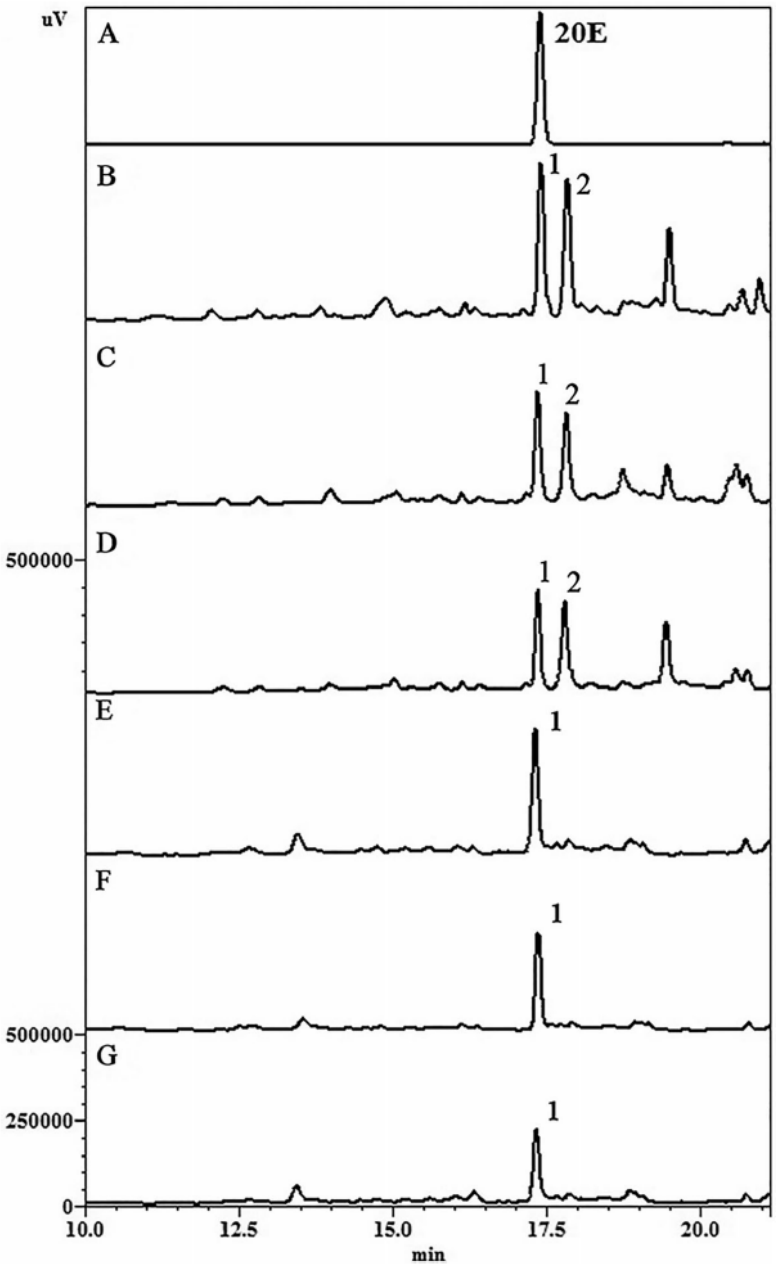

Figure 1. Comparative chromatogram of *S. tuberculata* extracts, using 40% ethanol for leaves and 70% ethanol for roots. *A*, reference substance of 100 μg/mL 20-hydroxyecdysone (20E). *B*, *C*, and *D*, leaf hydroethanolic extract obtained by percolation, reflux and maceration, respectively. *E*, *F*, and *G*, root hydroethanolic extract obtained by percolation, reflux and maceration, respectively. Peak 1: 20E, Peak 2: Kaempferol derivative, acquired at 250 nm.

evaluated by the probability of producing a positive Ames Test outcome (Ames test against *Salmonella typhimurium* TA97a, TA98, TA100, TA102, TA104, TA1535, TA1537 and TA1538), a positive endocrine disruptor test outcome, hERG inhibition and irritant effects. 20E showed a low probability to cause toxic effects in all the parameters evaluated (Table 3). Thus, these data suggest that 20E may be non-genotoxic, non-reproductive-system toxic, non-cardiotoxic and non-irritant. The software also predicted that 20E does not present hazardous fragments, which are interconnected with mutagenic effects.

The Osiris Property Explorer calculated the drug-likeness and drug-score characteristics based in the list of all available fragments from 3300 traded drugs as well as 15,000 commercially available chemicals (Fluka, Germany). In this work, Osiris results showed that 20E had a positive drug-likeness (0.62) and drug-score (0.2) values (Table 4). The 20E values were greater than those of α-tocopherol (-6.2 drug-likeness; 0.11 drug-score, respectively), and greater than drug-likeness of ascorbic acid (0.02).

Table 2. 20-hydroxyecdysone concentration in *S. tuberculata* hydroethanolic extracts using different extraction techniques.

Solvent	20-hydroxyecdysone (mg/mL of extract)		
	Extraction technique		
	Maceration	Percolation	Reflux
Leaves			
Ethanol 20%	0.073 (3.1%)	0.087 (1.7%)	0.089 (1.8%)
Ethanol 40%	0.074 (4.8%)	0.134 (3.0%)	0.101 (1.7%)
Ethanol 70%	0.062 (3.8%)	0.081 (2.7%)	0.083 (2.0%)
Roots			
Ethanol 50%	0.045 (3.1%)	0.065 (2.3%)	0.059 (3.3%)
Ethanol 70%	0.050 (4.4%)	0.096 (3.4%)	0.060 (3.5%)
Ethanol 90%	0.038 (2.2%)	0.030 (2.9%)	0.060 (2.7%)

Data are reported as the average of three analyses and relative standard deviation (RSD, %).

Table 3. *In-silico* screening using toxicity predictions for 20-hydroxyecdysone compound present in *S. tuberculata* extracts.

Toxicity modules	Probability*	Toxicity risk
Genotoxic (Ames test)	0.10	(+)
Estrogen receptor alpha binding	0.01	(+)
Irritant	0.00	ND
hERG inhibitor	0.03	(+)

*Probability of causing toxic effects. The scale of toxicity risk ranges from low (+), medium (++), to high (+++) and not detected (ND) calculated by using ACD/Labs program.

Artemia salina toxicity

Results of *A. salina* toxicity are shown in Table 5. *S. tuberculata* presented low toxicity to *A. salina* larvae (LD_{50} >1000 μg/mL) for both leaves and roots extracts.

Antioxidant assays

In the DPPH• assay, the hydroethanolic extracts from leaves showed the most effective result with IC_{50} activity at 0.116 mg/mL followed by hydroethanolic root with IC_{50} of 0.142 mg/mL (Table 6). Aqueous extracts showed the lowest antioxidant capacity.

In the $ABTS^{\cdot+}$ assay, IC_{50} values ranged from 0.014 (hydroethanolic leaf) to 0.545 mg/mL (aqueous root). Compared to DPPH• assay, hydroethanolic leaves had the highest potential scavenger, approximately 10-fold. In addition, hydroethanolic leaf extract possessed the highest $ABTS^{\bullet+}$ inhibition followed by root extract.

In the FRAP protocol, the oxidative form of iron (Fe^{+3}) is converted to ferrous (Fe^{+2}) by antioxidant compounds. Extracts of *S. tuberculata* expressed great reducing activity. As shown in Table 6, hydroethanolic leaf extract had the most pronounced effect of all assessed protocols (IC_{50}=0.006 mg/mL). Similarly, aqueous extracts of leaves were also potent in FRAP activity (IC_{50}=0.011 mg/mL).

The nitrogen reactive species scavenger test illustrates percentage inhibition of nitrogen reactive species by extracts from leaves and roots of *S. tuberculata*. The IC_{50} value of hydroethanolic and aqueous extracts ranged from 0.118 and 0.996 mg/mL (Table 6). Both extracts, leaves and roots, showed a significant ($P<0.05$) scavenging

Table 4. Values of drug-likeness and drug-score for 20-hydroxyecdysone (20E) and reference antioxidant compounds (α-tocopherol and ascorbic acid) by the Osiris Property Explorer program.

	20E	α-tocopherol	Ascorbic acid
Drug-likeness	0.62	–6.27	0.02
Drug-score	0.20	0.11	0.74

Osiris Property Explorer program <http://www.organicchemistry.org/prog/peo/>

Table 5. Values of 50% lethal dose (LD_{50}) obtained from *Artemia salina* assay to *S. tuberculata* extracts.

Plant extract	LD_{50} (μg/mL)	
	Hydroethanolic	Aqueous
Leaves	>1000 (3.1%)	>1000 (2.8%)
Roots	>1000 (4.0%)	>1000 (4.9%)

Data are reported as the average of three analyses and relative standard deviation (RSD).

Table 6. Maximal inhibitory concentration (IC_{50}) values of antioxidant assays from different extracts of *S. tuberculata*.

Assays	IC_{50} (mg/mL)			
	Leaves extract		Roots extract	
	Hydroethanolic	Aqueous	Hydroethanolic	Aqueous
DPPH	0.116 ± 0.88*	0.470 ± 0.68	0.142 ± 1.02*	0.401 ± 0.98#
ABTS	0.014 ± 0.02*	0.115 ± 0.01#	0.058 ± 0.01*	0.545 ± 0.04
FRAP	0.006 ± 0.003#	0.011 ± 0.07#	0.109 ± 0.014	0.129 ± 0.028
NO scavenger	0.118 ± 2.06*#	0.248 ± 2.77#	0.272 ± 1.04*	0.996 ± 2.61
Deoxyribose assay	0.012 ± 1.61*	0.029 ± 0.85#	0.013 ± 0.81*	0.259 ± 1.36

Data are reported as mean ± SD for n=3. *$P < 0.05$ between the hydroethanolic and aqueous solvents in the same extract. #$P < 0.05$ differences within the same solvent between leaves and roots (ANOVA with Tukey's post-test).

activity. However, extracts of leaves had more scavenger property than root extracts.

In deoxyribose degradation assays, extracts presented a potent scavenger activity of hydroxyl radical, one of the most aggressive oxidants formed from Fenton reactions. In this regard, *S. tuberculata* extracts significantly inhibited the oxidation of deoxyribose in low concentrations (Table 6). Overall, hydroethanolic extracts showed stronger inhibition activity than aqueous extracts.

Analyses of lipid peroxidation from egg yolks (Figure 2A and B) showed that both extracts of *S. tuberculata* inhibited lipid peroxidation. Hydroethanolic extracts exhibited significant inhibition from 0.015 and 0.03 mg/mL for leaves and roots, respectively ($P \leqslant 0.05$). Aqueous extracts showed significance from 0.15 mg/mL for both parts of plants. Comparing leaves and roots, the leaves had greater antioxidant activity than roots.

Figure 2C and D shows the effects of *S. tuberculata* extracts on lipid peroxidation caused by Fe^{+2} in rat brain homogenates. The iron concentration tested (0.01 mM) induced a significant oxidative damage ($P \leqslant 0.05$). We observed that hydroethanolic extracts from leaves and roots presented a significant decrease on TBARS formation ($P \leqslant 0.05$) from 0.015 and 0.03 mg/mL concentrations, respectively. Aqueous extracts showed a significant reducing effect from 0.03 and 0.3 mg/mL concentrations for leaves and roots, respectively.

In liver tissue, all extracts of *S. tuberculata* inhibited TBARS production (Figure 2E and F). However, the hydroethanolic extracts exerted a more pronounced effect. At concentrations of 0.03 and 0.3 mg/mL, hydroethanolic extracts of leaves and roots inhibited lipid peroxidation to almost baseline levels.

Discussion

The present study describes an investigation about the medicinal plant *S. tuberculata* from Brazilian Pampa biome. We evaluated the dry residue and the concentration of the major compound (20E) with different extraction techniques applied on *S. tuberculata*. The data were used to select the extracts to be applied in toxicity and antioxidant assays. This method has a central role in obtaining products with constant composition and reproducible biological properties.

Considering the parameters evaluated for leaves and roots, percolation was the most effective technique. Results showed that percolation method improved the dry residue and 20E concentration. This finding may be related to the technique's exhaustive extraction and solvent renewal. Differently from maceration and reflux, in percolation the solvent remains 1 h in contact with the sample and then elutes through the column more than once with fresh solvent. Moreover, percolation methodology does not involve heating, an important aspect taking into account the thermolability of some phytoconstituents.

Our chromatographic analysis confirmed 20E as the main metabolite in leaves and roots. Moreover, a kaempferol derivative was detected only in leaves. This finding is in accordance with a previous phytochemical study by our group (9). In addition, we observed the presence of more metabolites in leaves than in roots. This result may be partially explained by the sunlight influence on biosynthesis of some compounds such as flavonoids (27).

The major identified compound (20E) belongs to the ecdysteroids class. It has a steroidal nucleus and a polyhydroxylated chain (Figure 3). Ecdysteroids or "phytoecdysteroids" are the plant analogues of insect growth hormones. Their function in plants is unclear; however, they may be involved in the deterrence of invertebrate predators by acting as antifeed-systems, or yet by interfering in the ingestion of phytophagous insects (16). In mammals, 20E has demonstrated therapeutic properties including memory improvement, reduction of lipid storage and anabolic effects (28–30).

The predictive Ames test used here is performed worldwide as an initial screening to determine genotoxic

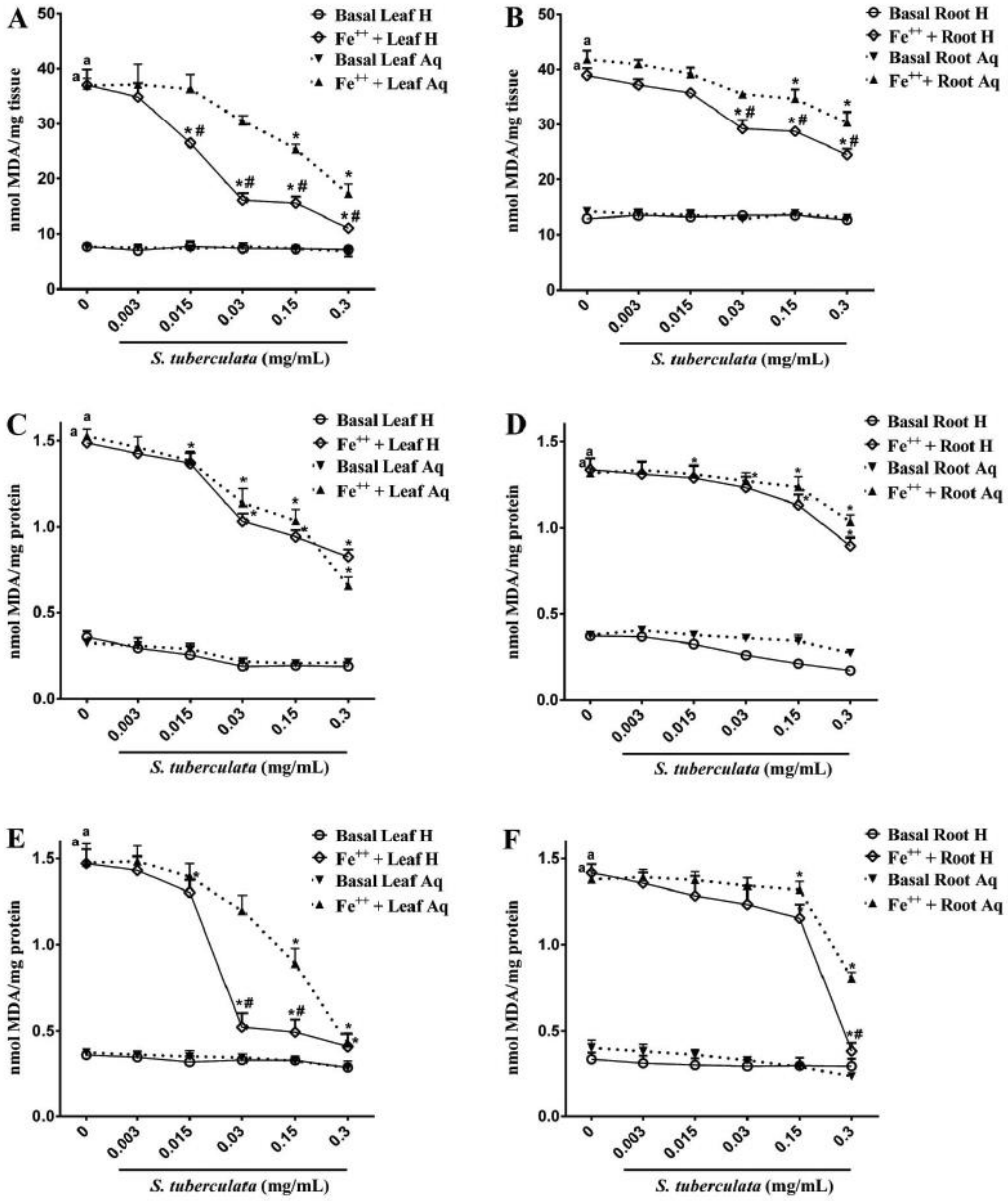

Figure 2. Effects of *S. tuberculata* extracts on TBARS production in egg yolk lipids (*A*, *B*), brain (*C*, *D*) and liver (*E*, *F*) of rats. Hydroethanolic leaf extract (Leaf H), aqueous leaf extract (Leaf Aq), hydroethanolic root extract (Root H), and aqueous root extract (Root Aq) were evaluated. Results are reported as nmol of MDA per mg of tissue (*A*, *B*) or mg of protein (*C*–*F*) for (n=3). $^{a}P \leqslant 0.05$ compared to basal not induced (i.e., zero concentration); $^{*}P \leqslant 0.05$ compared to basal in the same group (i.e., zero concentration); $\#P \leqslant 0.05$ compared to the same concentration of the other extracts evaluated (two-way ANOVA with the Tukey's post tests).

properties of new chemical entities to be used by the pharmaceutical industry. It is a quick test, based on bacterial reverse mutation performed on various bacterial strains. The genotoxicity predicted by the Ames Test is based on an iterative model built using structural toxic fragments from a database as descriptors. 20E showed a low probability for genotoxicity (10%).

The endocrine disruptor test is associated with the binding of compounds to alpha estrogen receptor, which may be linked to reproductive toxicity and cancers (31). The 20E was classified as binder/non-binder, due to their relative binding affinities (RBA) compared to a reference ligand in the ACD/Labs database (17). Two cut-offs were used: LogRBA $\geqslant 3$ ("general binding"), and LogRBA > 0 ("strong binding") and 20E presented a 1% probability to be a LogRBA $\geqslant 3$. This measurement is important since the 20E has a steroidal structure and may interact with *in vivo* hormonal receptors.

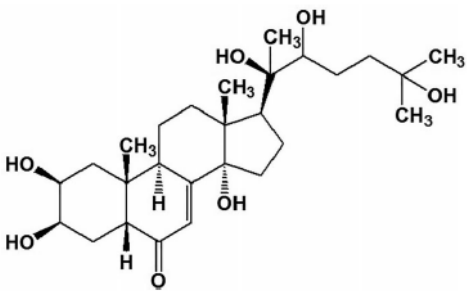

Figure 3. Representation of 20-hydroxyecdysone molecular structure ($C_{27}H_{44}O_7$). PubChem CID: 5459840.

Another assessed toxic effect was the hERG inhibition, which is an ion channel that, when inhibited, is related to cardiovascular damage (32). It is essential to investigate any chemical entity for this potential cardiotoxic effect. A large number of drugs have been withdrawn from clinical trials or from the market due to the fatalities associated to hERG inhibition. The hERG predictive inhibition for 20E was 3%. Similarly, irritant properties related to skin and eye tissues were evaluated, and non-toxic results were found. The low toxic probability of 20E, may be in part due to the absence of hazardous fragments (predictive data), which are known toxic agents.

The *A. salina* toxicity assay is practical, inexpensive, simple, reliable and an important tool in routine plant toxicity screening. Our data showed very low toxicity of hydroethanolic and aqueous extracts from leaves and roots. As the test implies the presence of cytotoxic constituents, as initial screening, our results indicate that aqueous extracts used as folk medicine have a low risk of acute toxicity. However, further studies are necessary to establish the toxicological endpoints at systemic level.

Since this work proposed to investigate the antioxidant potential of *S. tuberculata* extracts, we evaluated the overall potential of its major compound to be qualified as a nutraceuticals product and, therefore, be available in the market. Thus, the compound 20E was subjected to an *in silico* screening to evaluate its theoretical drug-likeness and drug-score in comparison with antioxidants references, ascorbic acid and α-tocopherol. The positive values of drug-likeness indicated that 20E contains fragments as good as the references. These results increase the possibility of establishing the therapeutic actions of these fragments, known as pharmacophores, and of this compound becoming a possible nutraceuticals product.

The assessment of antioxidant activity was applied for 40% (leaves) and 70% (roots) hydroethanolic extracts, obtained by percolation technique. It is important to emphasize that the best extractive system was defined observing the results of all evaluated parameters, and trying to maintain the stability of metabolites. Aqueous infusions were assayed, with the purpose of evaluating the method usually employed by the population in preparing home remedies.

The antioxidant capacity of an extract or compound can be analyzed by several assays with different mechanisms (33). Generally, the chemical reaction involved in antioxidant *in vitro* assays fall into two categories: hydrogen atom transfer (HAT) assays, which use a competitive reaction between an antioxidant and a substrate, where both compete for peroxyl radicals thermally generated, and single electron transfer (ET) reaction assays, that measure the potential of an antioxidant to reduce an oxidant, which changes color when reduced. All these elements, advantages and limitations, need to be considered when evaluating and selecting a potential antioxidant.

In view of the above comments, we used six different methods to evaluate the antioxidant capacity of *S. tuberculata* extracts: DPPH$^{\bullet}$, ABTS$^{\bullet+}$, NO and FRAP based in ET assays, and deoxyribose and TBARS, which are based in HAT. Results indicated that both leaves and roots extracts, could act by ET and HAT mechanisms. Moreover, data showed that hydroethanolic extracts present a better antioxidant potential than aqueous extracts. This fact may be related with a greater extraction ability of ethanol than water alone. Yea et al. (34), evaluating the effect of different solvents on phenolic content, found a higher extraction capacity in aqueous alcohols than water.

We also observed that leaves presented higher scavenger properties than roots. This finding probably occurred due to the diversity of phytoconstituents present in leaves. In fact, our analysis identified a kaempferol derivative detected only in the leaves. In this context, phenolic compounds are known for its antioxidant properties, such as free radical scavenging and chelation of metal ions. Therefore, the presence of phenolic compounds may explain the notable antioxidant activity in leaves. Moreover, concomitant occurrence of phenols and ecdysteroids may improve antioxidant potential by synergistic effects (35). However, it is not possible to know precisely if ecdysteroid class compounds exert antioxidant effects.

Our results for DPPH$^{\bullet}$, ABTS$^{\bullet+}$, FRAP and NO assays are agreement with Pawar et al. (36) and Shah et al. (37), who reported a great antioxidant activity for *S. cordifolia* and *S. cordata*. It should be noted that the effect of *S. tuberculata* detected with Griess reaction may be due to scavenger activity of extracts for nitrogen derivative species, i.e., NO_2, N_2O_3, N_2O_4, peroxynitrite ($ONOO^-$) or even for different redox forms, such as nitrosonium (NO^+) and nitroxyl anion (NO^-) generated or interconverted from nitric oxide under physiological conditions. Therefore, the antioxidant activity is an important property, since it prevents the formation of deleterious oxidants that can react with biological molecules, particularly oxidizing iron/sulfur centers, zinc fingers, and protein thiols, which plays a relevant role in cardiovascular and neurological diseases (23,38).

In this context, it is known that iron plays a significant role in noxious oxygen species production. Iron initiates a chain of reactions leading to lipid peroxidation and consequent cellular damage. Our data showed a protective effect of all extracts of *S. tuberculata* against oxidative damage by deoxyribose and TBARS assays.

One possible protection mechanism against lipid peroxidation damage may be related to Fe^{2+} chelating activity. In this case, the extract binds to metal preventing it to interact with H_2O_2 avoiding hydroxyl radical ($OH^{\bullet}$) generation and consequently the damage. In other words, chelating compounds may decrease metal bioavailability inhibiting its participation in $OH^{\bullet}$ generation by the Fenton reaction (39,40). *S. tuberculata* extracts presented a significant Fe^{2+} chelating activity (data not shown), which may support the observed decrease in lipid peroxidation. Another possibility would be $OH^{\bullet}$ neutralization by atom transfer. Thus, considering the *S. tuberculata* scavenger effects on $DPPH^{\bullet}$ and $ABTS^{\bullet}$ radicals, we may suggest that the $OH^{\bullet}$ radical scavenging potential of extracts interfered in the oxidation process.

In conclusion, *S. tuberculata* presented different classes of metabolites, predominantly phytoecdysteroids, which, together with polyphenols, may be involved with antioxidant activity. In addition, 20E showed an *in silico* low risk, and crude extracts had very low cytotoxicity against *A. salina* larvae. Thus, due the medicinal potential revealed by *S. tuberculata*, it is important to conduct further *in vivo* studies, as well as to consider 20E as a promising molecule for further investigations on oxidative stress from a nutraceutical source.

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Manuscript I

Sida tuberculata leaves extract reduces the nociceptive response by chemical noxious stimuli in mice: implications for mechanism of action

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Abstract

Ethnopharmacological relevance: *Sida tuberculata* R.E.Fries (Malvaceae) commonly known as "guanxuma" in Southern Brazil, has been traditionally employed as tea for diabetes, hypercholesterolemia, inflammatory process and insect bite relief.

Aim of the study: To investigate the antinociceptive potential from *S. tuberculata* extract and explore the possible action mechanisms involved. In addition, to analyze the phytochemical profile and cytotoxicity *in vitro*.

Materials and methods: Initially the *S. tuberculata* methanolic extracts obtained from leaves (STLE) and roots (STRE) was submitted a chemical characterization by UHPLC-MS analyses and cytotoxic evaluation using human leukocytes (non-malignant cell line). The *in vivo* antinociceptive property of STLE (10-300mg.kg^{-1}) was assessed in mice subjected the formalin model and acetic acid-induced abdominal writhes. The involvement of opioid and adenosinergic systems was tested by agonist/antagonist use. After, it was performed a computational docking to elucidate which extracts compounds are able to interact with opioid receptors.

Results: The main class detected in methanolic extract from *S. tuberculata* were the Ecdysteroids, and 20-Hrydoxyecdysone (20HE) confirmed as the major phytoconstituent. STLE displayed a significant decrease in cell viability, however neither STLE nor STRE demonstrated genotoxicity. The pre-treatment with STLE (10 and 30 mg.kg^{-1}) produced a significant inhibition on both, first and second phase in formalin-induced licking response. In acetic acid-induced writhes model, STLE (from 10 mg.kg^{-1}) reduced significant the abdominal contortions. The results suggest that action mechanisms of STLE effect may be an interaction with opioid and adenosine systems. Molecular docking suggested that μ opioid-receptor (MOR) may interacting with extract compounds, specially Kaempferol derivative and 20HE.

Conclusion: *S. tuberculata* extracts treatments significantly reduced antinociceptive action in mice, which may be related to modulation of opioid and adenosine receptors with ecdysteroids and flavonoids.

Key words*:* *Sida tuberculata*, antinociception, opioid, adenosine, phytochemical, docking molecular

1. Introduction

Pain is an uncomfortable condition considered as a global health problem. Although it is an organism defense action, the affected individuals can suffer different pain stages as acute, chronic, intermittent, or a combination of them (Reardon et al., 2015; Goldberg & McGee, 2011). Thus, if not treated, some painful processes may induce to neurological and emotional problems, including inability to work, disrupted social relationships, depression and consequently, decreased life quality (Dutra et al., 2016; Bond, 2011).

Physiology and etiology of pain is a complex phenomenon and in most cases, it involves the activation of nociceptive fibers. Some of these high-threshold neurons are considered the primary afferent nociceptors. They response to varied mechanical, thermal or chemical stimuli, detected by many types of ionotropic channels and metabotropic receptors in nociceptor surface (Rodrigues et al., 2012; Tominaga, 2007).

Binding site structure of a target receptor and its interaction with different ligands are a common parameter for the development of new pain therapies (Chaplan et al., 2010). In this context, some computational molecular docking tools have been developed to use information related to the arrangement at the receptor binding sites and to calculate the energies of interactions and repulsions with different linkers (Nunes-Alves & Arantes, 2014). Thus, the use of molecular docking in virtual screening and discovery of new chemical binders is a viable alternative to the costs reduction with reliable results of biological activity process.

Several alternative approaches have been tested to reduce nociceptive, neurogenic, neuropathic and inflammatory pain (Simões et al., 2017; Lima Cavendish et al., 2015; Silva et al., 2011; Yi et al., 2010). Plants derivatives are, in turn, potential sources for new pain therapies. Notably, the main drugs currently used as analgesics were derived from plants or were synthesized based on natural products (Atanasov et al., 2015). Thus, based on popular knowledge, our group have been investigate the composition and biological effects of *S. tuberculata* (ST) extracts.

ST (Malvaceae family) is an herbaceous/sub-shrub plant, widely found in Southern Brazil, which has its leaves and roots used in popular medicine for inflammatory processes, diabetes and vascular disorders. Recently, previous studies identified the main phytochemical classes in ST extracts, such as phytoecdysteroids,

flavonoids and alkaloids (ROSA et al., 2015 and 2016) Moreover, a significant antifungal and antioxidant properties were detected. The plants of *Sida* genus has been used for a long time in folk medicine and it revealed important pharmacological properties (Dinda et al., 2015; Rejitha et al., 2015; Momin et al., 2014; Khurana & Gajbhiye, 2013).

In nociception context, Bonjardim et al. (2011) demonstrate that *S. cordifolia* has a pronounced antinociceptive activity on orofacial nociception in mice. Other study, the polyphenol-rich fractions obtained from *S. urens* produced significant analgesic effects in acetic acid-induced writhing method (Konaté et al., 2013). *S. cordifolia* also presented analgesic and anti-inflammatory activity in nociception models induced by acetic acid and carrageenan (Sutradhar et al., 2008).

Given the therapeutic potential of *Sida* sp, the present study proposed to investigate antinociceptive effects of extract obtained from leaves of ST and explored mechanism of action. Moreover, we analyzed the chemical composition and cytotoxicity *in vitro* from leaves and roots extracts.

2 Material and methods

2.1 Plant collection and extract preparation

S. tuberculata whole plant was collected in south Brazil (between September and February months) in a biome Pampa area under geographical coordinates 29º50'06.3"S and 57 º05'50.5"W, Uruguaiana city, RS, Brazil. In sequence, botanical department identified the species and one exemplar was deposited to ICN Herbarium as *S. tuberculata* R.E. Fries; ICN 167493 voucher specimen, Federal University of Rio Grande do Sul.

The plant was initially separated into leaves and roots, which were submitted to drying at 35 ºC and ground to powder. The extractive technique employed methanol on a heating plate at 40 ° C for 5h (ratio of 1:10, w/v). Every hour the material was sonicated in an ultrasonic bath for 30 seconds. Next, the crude material was filtered and subjected to a degreaser process by liquid-to-liquid partition. Thus, using a separatory funnel with methanolic crude extract/hexane p.a. in ratio 1:1 (v/v) the separation process was performed. After, methanolic fraction was collected, concentrated in a rotary evaporator and stored for further assays.

2.2 UHPLC-MS analysis

The instrumentation employed was an Ultra Performance Liquid Chromatograph (UHPLC-Shimadzu, Japan), equipped with a binary pump LC-20AD with CBM-20A controller and SPD-20A PDA detector. Chromatographic analyses were performed using a reverse-phase system with the following conditions: Nano Separation Technologies (NST) C_{18} column (250 x 4.6 mm, 5 mm); mobile phase of phosphoric acid 0.1 % in water (A) and phosphoric acid 0.1 % in methanol (B) with a flow rate of 0.7 mL/min; DAD detection at 250-340 nm; injection volume of 10 μL. The elution gradient was 0.01–1.0 min, 5% solvent B; 1.01– 4.0 min, 5-90% solvent B; 4.0-6.0 min, 10% solvent B; 6.01 – 7.0 min, 90-5%; 7.01-12 remained at 5% solvent B.

The MS analyses were conducting on a micrOTOF-QII (Bruker® Scientific; Billerica, MA, USA) with an electrospray ionization interface (ESI). The mass spectrometry equipment was operated in positive-ion mode, according to the following conditions: capillary voltage 4000V; collision energy 8.0 eV; drying gas flow rate 9L/min.; dry temperature 200 °C; sample cone 40V; N2 was used as the nebulizing. Mass spectra were recorded by using full scan mode ranging from 200 to 800 m/z.

2.3 Cytotoxicity in vitro

2.3.1 Cell culture preparation

The leukocytes cultures were prepared using 0.5 ml of venous blood (collected by voluntary venipuncture) and immediately transferred to a culture medium containing 10 ml of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin, as described by Dos Santos-Montagner et al. (2010). The Research Ethics Committee of the Federal University of Pampa (n°. 27045614.0.0000.5323) approved the experiment. STLE and STRE were added with the medium to obtain the concentrations 1, 10 and 100 µg.mL^{-1}. The cells were placed in a greenhouse at 37°C in a 5% CO_2 environment for 72 hours. Hydrogen peroxide at 10 µM was used as positive and PBS 7.4 buffer as negative control.

2.3.2 Cell viability test

After 72h, cell viability was determined by the loss of membrane integrity using trypan blue method (Burow et al., 1998). The viable cells were counting in a Neubauer

chamber and the results were expressed as the percentage of cell viability in relation to negative control group by means of three independent experiments.

2.3.3 Micronuclei frequency assay

Micronucleus presence in leukocyte samples were determinate by panoptic method according Schmid (1975) with minor modifications. Cells were centrifuged twice in 1000 rpm for 5min and about 1 mL containing the cell pellet was drained and spread over a glass slide in triplicate. After dry (at room temperature) the slides were stained and analyzed by optical microscope. Results were result expressed a 1000 cell-count.

2.3.4 Comet DNA assay

The comet DNA assay was performed as previously described Dos Santos-Montagner et al. (2010) and Tice et al. (2000). The slides containing approximately one hundred leukocyte per slide were analyzed in triplicate under blind conditions. Cell viability limit was of 50%. The cells length tail was used to calculate DNA damage index (DI) based on different comet levels ranging from 0 (no damage) to 4 (maximum damage).

2.4 Behavioral tests in vivo

24.1 Animals

Male Swiss mice (25–40 g) obtained from the Central Biotery of the Federal University of Santa Catarina (Florianópolis, Brazil) were housed under standardized conditions at constant temperature (21±2 °C), with free access to food/water and 12-h light/dark cycle. Animals were habituated to laboratory conditions for at least 1 h before testing and the experiments were conducted during daylight, in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). The Ethics Committee approved the tests (protocol number PP00745 of Federal University of Santa Catarina) and each group contained between 5-10 animals. We emphasize that based on previous results of our group (Rosa et al., 2016) STLE was chosen to *in vivo* tests due its greater antioxidant capacity and large amounts of ecdysteroids compounds than STRE.

2.4.2 Formalin test

Experiment was conducted as described by Santos et al. (1998). Mice received oral doses (p.o.) of STLE (10, 30, 100 and 300 $mg.kg^{-1}$) or vehicle (saline solution) 1 hour before noxious stimuli. The right hind paw was injected with 20 µl (2.5% formalin solution at 0.92% formaldehyde in saline) in ventral surface and the animals were immediately placed in an acrylic chamber. Time spent licking and biting the injected paw was recorded between 0-5 min (neurogenic phase) and 15-30 min (inflammatory phase). The values were considered as nociception response.

2.4.3 Acid acetic test

Abdominal writhing model was induced by intraperitoneal (i.p) acetic acid injection (0.6 %, 0.45 ml/mouse) in mice according Santos et al. (1998). Each animal were pretreated orally (p.o.) with STLE (10, 30, 100 and 300 $mg.kg^{-1}$) or vehicle (saline solution) 1 hour before initiating the noxious stimulation. After, mice were placed in an acrylic chamber, and the intensity of nociceptive behavior (abdominal writhes) was quantified over a period of 20 minutes, starting 5min after min after stimulus injection.

2.4.4 Open field test

Locomotor and anxiety behavior were assessed by open field test according Bond and Giusto, (1977) with modifications. Briefly, mice were pretreated with vehicle (saline solution 0.9%, p.o.) or STLE (10 -300 $mg.kg^{-1}$, p.o.), and one hour later they were enclosure a white circular apparatus (100 cm diameter, with 12 equally spaced square) in a silent room. The animals behavior response was recorded by a video camera during 6 min period and the parameters evaluated were: (1) number of crossing with all paws, (2) rearing, times standing on hind legs, and (3) self-grooming frequency (Whimbey and Denenberg, 1967).

2.4.5 Opioid system involvement

To verify the possible opioid system involvement in antinocicpetive ST action on acid acetic model, it was followed the methodology describe by Lapa et al. (2009). The mice were pre-treated with naloxone, a non-selective opioid receptors, ($1mg.kg^{-1}$, i.p.) 30 min before administration of STLE ($100\ mg.kg^{-1}$, p.o.), vehicle (saline solution) or morphine ($2.5\ mg.kg^{-1}$, subcutaneous). After the irritant treatment, acetic acid

intraperitoneal injection, the nociceptive response was evaluated by counting the abdominal constrictions cumulatively over a period of 20 min.

2.4.6 Adenosine system involvement

The procedure used was similar to that described previously. The animals were pre-treated with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective A1 receptor antagonist, (1 $mg.kg^{-1}$, i.p.) 20 min before the test compounds, STLE (100 $mg.kg^{-1}$, p.o.), vehicle (saline solution) or positive control ciclohexil adenosine, CHA, (selective agonist A1 – 0.1 $mg.kg^{-1}$, i.p.). The nociceptive response to an intraperitoneal injection of 0.6% acetic acid was evaluated 30 minutes after the CHA injection or 60 minutes STLE or vehicle administration.

2.5 Molecular modeling and computational docking study

Docking studies were conducted with the two major compounds in *S. tuberculata* leaves extract [20-Hydroxyecdysone (20HE) and Kaempferol-3-(6”-p-coumaroyl)-glucopyranoside (KCG)] in order to predicting possible mechanisms involved in the biological activity studied. DFT B3LYP/6-311G* basis in gas phase methodology, evaluable in Spartan’08 for Windows software (Wavefunction Inc., Irvine, USA) was used to geometry optimization and conformational analyisis. The geometry of compounds was optimized followed by submitting to systematic conformational analysis with torsion angle increment set of 30^o in the range $0\text{-}360^o$. The lowest energy conformer for chemical structure was saving in mol2 file before to use in docking studies.

The structure of μ opioid receptor (MOR) from *Mus musculus*, encoded PDB ID: 4DKL (Manglik et al. 2012) was downloaded from Protein Data Bank (PDB), before to perform the docking studies, and this 3D structure was prepared by remove the water molecules and adding polar hydrogens using Autodock Tools 1.5.6 (Morris et al. 2009). Computational investigations were executed using iGemdock 2.1 software (Yang and Chen, 2004) in which the individual binding poses 20HE and KCG were assessed and submitted to dock in the active site of the 4DKL, entitled BFO. Docking calculations were performed using by bounded ligand option, binding center of 3 to 8 Angstroms considering the original ligand morphinan. The software was also used at drug screening Docking Accuracy Setting with GA parameters set for population size,

generation and number of solutions as 200, 70 and 3, respectively, and Gemdock score function of hydrophobic and electrostatic (1:1 preference). iGemdock software was used to infers the pharmacological interactions between biological receptor and the compound studied. Results obtained allow ranking the screening compounds by combining the pharmacological interactions and energy-based scoring function of iGemdock.

2.5 Statistical analyses

Data are presented as the mean ± standard error of mean (SEM). *In vivo* analyses represent the mean of 5–10 mice per group. The results were statistically analysed using one-way or two-way ANOVA followed by post-hoc Bonferroni or Dunnet. In all cases, differences were considered significant when $p < 0.05$.

3. Results

3.1 Phytochemical composition

UHPLC-MS analyses performed in positive mode and by DAD achievement on ST extracts from leaves and roots revealing a complex matrix of compounds (ecdysteroids, alkaloids and flavonoids derivatives) (Table 1).

Table 1. Compounds identified in *S. tuberculata* with equivalent absorbance in Uv-vis maximum, [M+H]+ ions, key fragments in positive mode by UHPLC/ESI-MS.

nº	UV vis (abs. max.)	$[M+H]^+$ *m/z*	Molecular Formula	MS fragments m/z	Compound suggested
1	248	643	$C_{33}H_{54}O_{12}$	625 $[M+H-H_2O]^+$	20-Hydroxyecdysone-3-glucopyranoside
2	248	481	$C_{27}H_{44}O_7$	463 $[M+H-H_2O]^+$, 445 $[M+H-2H_2O]^+$, 427 $[M+H-3H_2O]^+$	20-Hydroxyecdysone
3	250	497	$C_{27}H_{44}O_8$	479 $[M+H-H_2O]^+$,463 $[M+H-2H_2O]^+$, 445 $[M+H-3H_2O]^+$,427 $[M+H-4H_2O]^+$	5,20-dihydroxyecdysone*
4	248	465	$C_{27}H_{44}O_6$	447$[M+H-H_2O]^+$,429$[M+H-2H_2O]^+$,	Ecdysone *
5	248	613	$C_{32}H_{52}O_{11}$	481$[M+H\text{-hexose}]^+$, 463$[M+H\text{-hexose-}H_2O]^+$, 445 $[M+H\text{-hexose-}2H_2O]^+$, 427 $[M+H\text{-hexose-}3H_2O]^+$	20-Hydroxyecdysone-3-deoxyhexose
6	312	595	$C_{30}H_{26}O_{13}$	449 $[M+H-C_9H_6O_2]^+$, 287$[M+H- C_9H_6O_2\text{-glu}]^+$	Kaempferol-3-(6-p-Coumaroyl) glucopyranoside

7	317	314	$C_{18}H_{19}O_4N$	282 $[M+H-CH_3O]^+$	N-Feruloyltyramine
8	280	189	$C_{11}H_{10}N_2O$	171 $[M+H-H_2O]^+$	Vasicine *

The chemical profile confirmed the six phytoconstituents previously detected by our group (see Rosa et al., 2015). Here, a detailed assessment of the mass fragments allowed the suggestion of three unpublished compounds for this species, 5,20-dihydroxyecdysterone $[M+H]^+$ at 497 m/z, alpha-Ecdysone $[M+H]^+$ at 465 m/z and Vasicine $[M+H]^+$ at 189 m/z (Table 1). In addition, the signals intensity analysis displayed that 20HE is major ecdysteroid in leaves and roots extract. The proposed chemical structures and their MS fragmentations are illustrated in Fig.1. As polyhydroxylated group, phytoecdysteroids showed successive water losses, $[M–H–mH2O]^+$ and these characteristic ions were used to propose the known and unknown components in the ST extracts.

3.2 Cytotoxicity and genotoxicity testing

Using human leukocyte cultures, we evaluated cytotoxicity and genotoxicity in non-malignant cells. Different STLE and STRE concentrations, ranging from 1-100 µg/ml, were used to verify the *in vitro* potential of cell damage. According to the results presented Fig. 2 (A), STLE displayed a significant reduction in cell viability in all doses tested. Percentage of amount cell reduced significantly ($p<0.001$) at the higher STLE concentration tested ($100\mu g.mL^{-1}$) similarly to positive control H_2O_2 (10µMol). However, no significant reductions were observed for STRE at any of the concentrations evaluated. Moreover, data from comet and micronucleus assays demonstrated no significant genotoxic changes to all doses tested either leaf or root extracts (Fig.2 B and C).

3.3 Behavioral tests

Crude extract obtained from leaves of *S. tuberculata* (STLE) was chosen to assess the ability to reduce pain behavior. Based on previous results of our group (Rosa et al., 2016; 2015) that point out the leaves extracts present more antioxidant action and

phytochemicals than roots extracts, behavior assays were tested on *in vivo* nociceptive models induced by noxious chemicals.

Figure 1

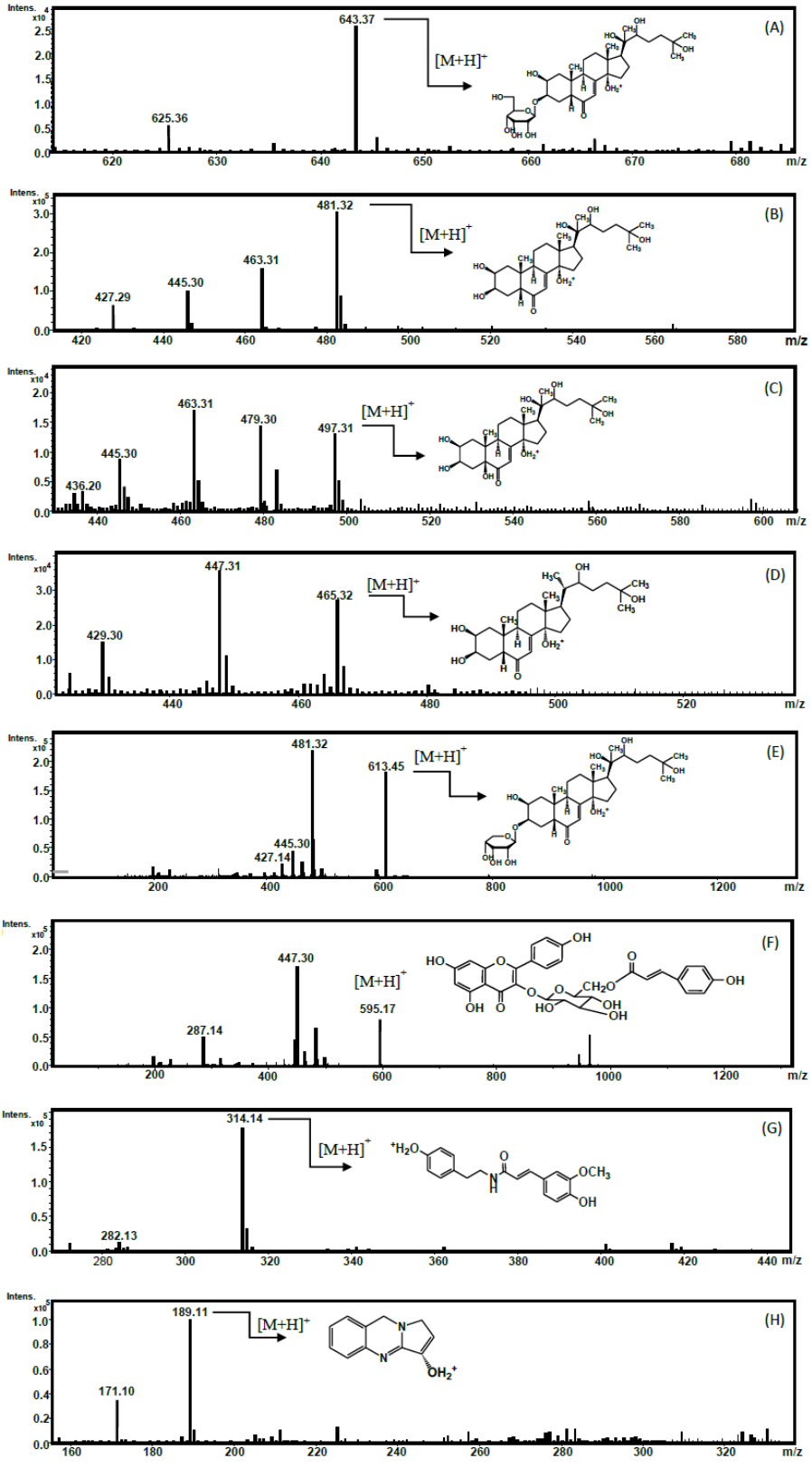
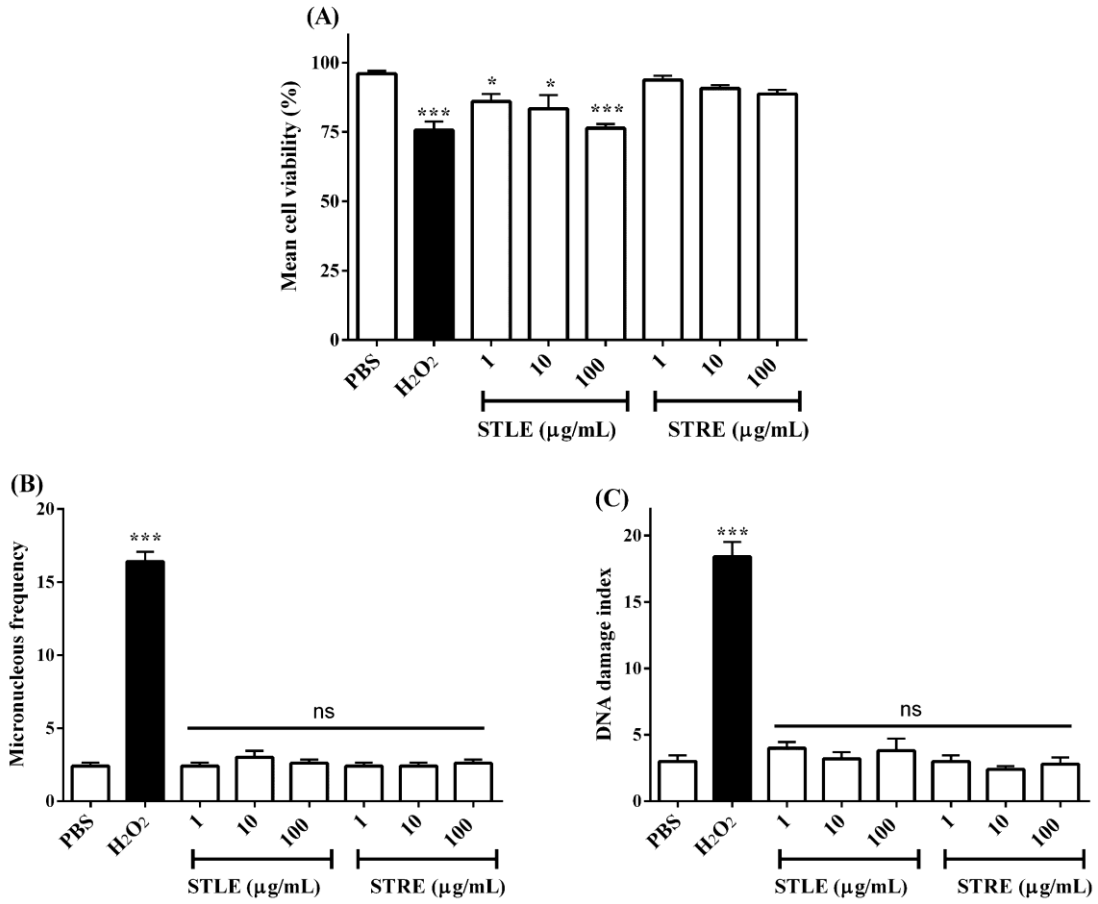


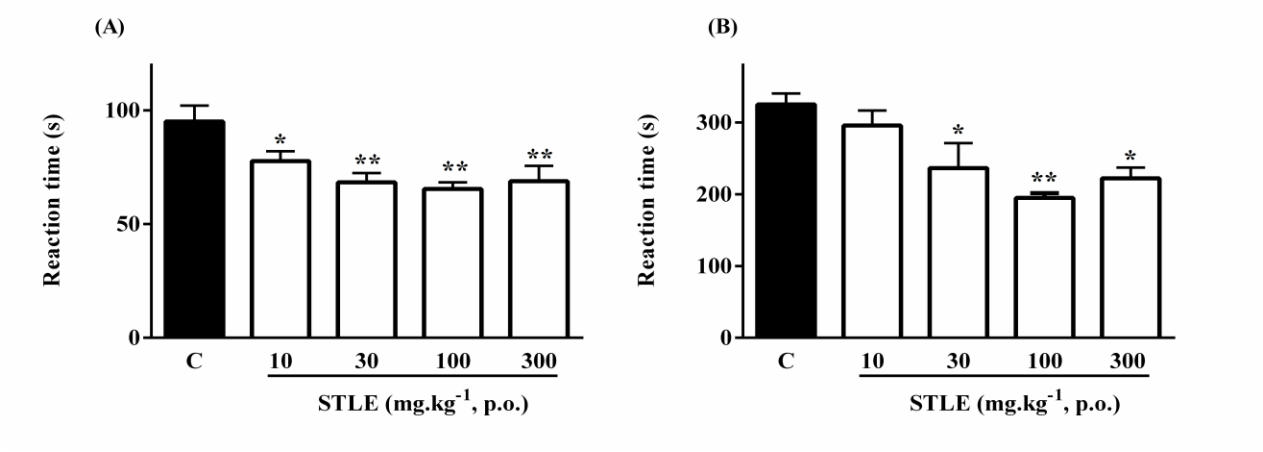
Figure 2



3.3.1 Formalin

Firstly, the investigations were established on the formalin test, widely studied as persistent pain model. STLE concentrations ranging from 10 – 300 mg.kg^{-1} were tested. In effect, as can be seen in Fig. 3, formalin intra-plantar injection produced nociceptive response in both, the first and second phases. STLE produced a significantly ($p<0.05$) inhibition in licking and biting behavior in both phases. The percentage of inhibition ranged from 18 to 31% in the neurogenic phase (Fig.3A), and 24 to 40% in second phase (Fig. 3B). The most pronounce antinociceptive response in both phases was observed at 100 mg.kg^{-1} dose.

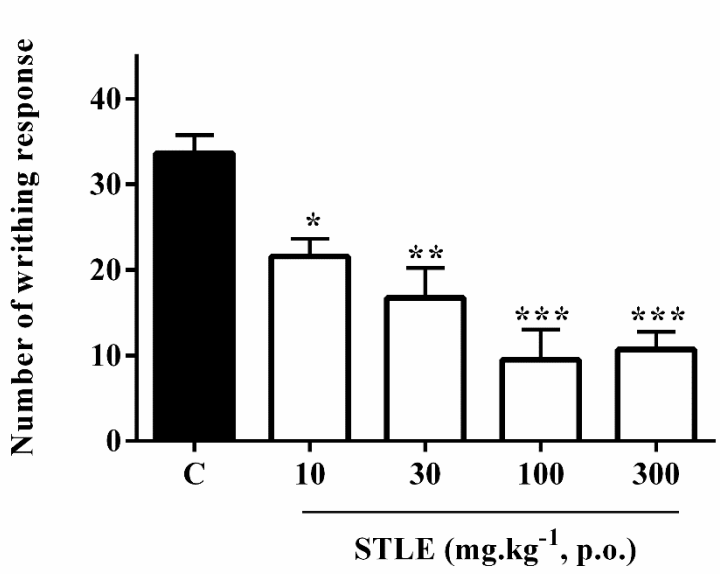
Figure 3



3.3.2 Acetic acid test

Results from acetic acid-induced pain model showed a cumulative amount of abdominal writhings in mice treated with noxious stimuli only (Fig.4). Differently, a single STLE dose was able to reduce significantly abdominal constrictions from lowest dose tested (10 mg.kg-1). Additionally, the pre-treatment with 100 mg.kg^{-1} inhibited the writing numbers in approximately 71.8 %.

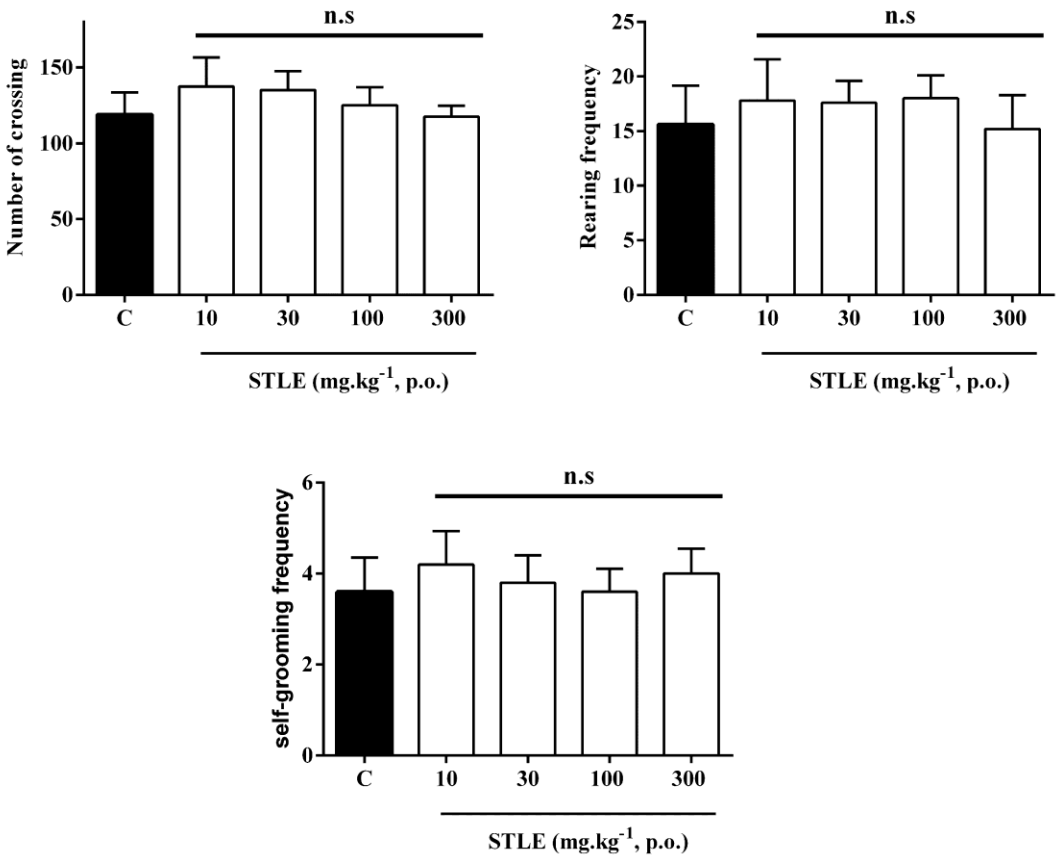
Figure 4



3.3.3 Locomotor and anxiety measurement by open field test

STLE pre-treatment with the same doses (10-300 mg.kg^{-1}, p.o.) tested in acute nociception and inflammation models no affect the spontaneous locomotor and emotional response (Fig.5). The open field assay showed no significant difference in the number of crossing, rearing and grooming frequency between STLE concentrations tested and control group.

Figure 5



3.4 Systems involved in action mechanisms

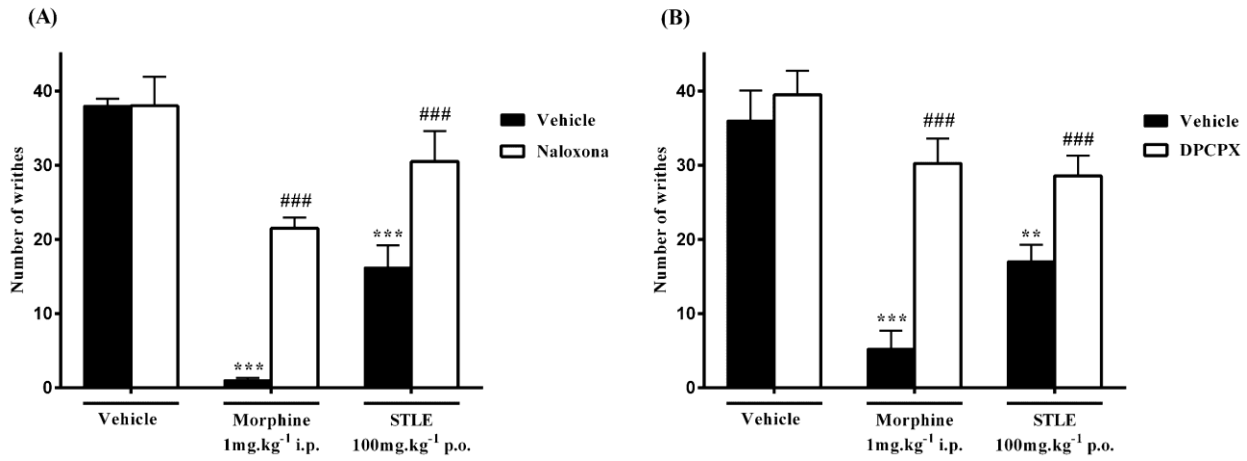
Opioid and Adenosine system tests

Based on acetic acid test results, we investigate the opioid and adenosine systems involvement through the agonist/antagonist usage. Thus, purposing to evaluate the most significant STLE dose in acetic acid model and probable action mechanism, we pre-treat the animals with opioid receptor antagonist naloxone or saline prior to

morphine (1 mg.kg^{-1}) or STLE (100 mg.kg^{-1}). Data demonstrated that morphine group pre-treated with saline fully prevented the abdominal constrictions, whereas the morphine group pre-treated with naloxone inhibited the morphine action (Fig. 7A). A similar effect was observed in the STLE groups. Those animals pre-treated with saline present a significantly reduction in nociceptive response. In other hand, those pre-treated with naloxone, the STLE effect was disabled, suggesting that STLE has its mechanism, at least in part, linked to opioid system (Fig. 6A).

In relation to the adenosinergic system, as shown in Fig. 6 (B), the anti-nociceptive effects of CHA (0.03 mg.kg^{-1}, adenosinergic agonist) were reversed by pre-treatment of animals with DPCPX (3 mg.kg^{-1}, A1 antagonist). Similarly, the antagonist DPCPX was able to inhibit the STLE (100 mg.kg^{-1}) on the acetic acid-induced nociception model. Thus, it may be that anti-nociceptive effect demonstrated by STLE, also involves receptor A1 from adenosine system.

Figure 6



3.5 Molecular docking

In order to investigate which of two major molecules (20HE and KCG, illustrated in Fig. 1 B and E) detected in STLE extract would present better interaction with opioid receptors, we performed a molecular docking simulation. In these studies, the compounds 20HE and KCG were geometrically placed to interact with specific amino acids residue in opioid receptor site and results from H-bond, Van der Waals

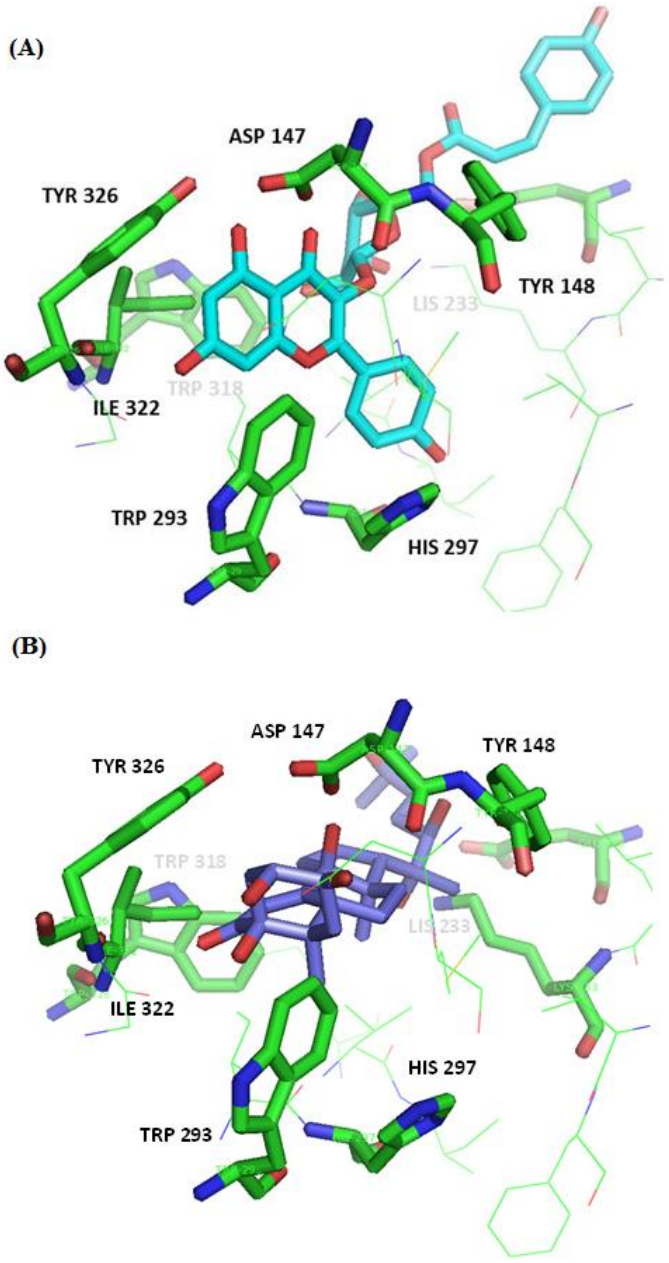
(VDW) and electrostatic interactions energy are showed in Tables 2. A detailed interaction between compounds and main amino acid residues from MOR (active site BFO) are illustrated in Fig. 7 (A and B).

Table 2. Central pharmacological interactions (Van der Walls, H-bonding, and electrostatic) of *S. tuberculata* compounds and residues involved in the binding site of μ opioid receptor (active site BFO) applying the Residues Consensus Analysis.

Compounds	**Interactions**	**ASP 147** (Kcal.mol^{-1})	**TYR 326** (Kcal.mol^{-1})
Kaempferol-3-(6"-p-coumaroyl)-glucopyranoside	Van der Walls	-4.9	-8.4
	H-Bonding	-6.3	-2.5
	Electrostatic	0.0	0.0
20-Hydroxyecdysone	Van der Walls	-0.4	-9.3
	H-Bonding	0.0	0.0
	Electrostatic	0.0	0.0

The empirical scoring function of iGemDOCK is the estimated as total sum of VDW, H-bonding and electrostatic energy. Here, the ligands 20HE and KCG demonstrated affinity energy of -82.10 and -117.30 Kcal mol-1 respectively. Specifically the VDW and H-bond values for KCG molecule were -97.11 and -20.20 respectively, and for 20HE values were -53.65 and -28.45 Kcal mol-1. No electrostatic interactions of both compounds and amino acids residues from MOR was observed.

Figure 7



4. Discussion

This work explore the antinocieptive action in mice pre-treated with *S. tuberculata* methanolic extract. Besides that, presents an evaluation on its chemical composition and *in vitro* cytotoxicity. Previous phytochemical analyses with *S. tuberculata*, ethanolic and aqueous extract, were performed by our group and allowed identification the main classes of secondary metabolites, of which phytoecdysteroids

were the most abundant (Rosa et al., 2015 and 2016). Here, we used other extraction technique and instrumentation analyses, nevertheless we confirm these results and propose three additional compounds. Thus, through our UHPLC-MS, also based on maximum Uv absorption, standard fragmentation and literature data, we identified 05 ecdysteroids molecules, being 20HE the major compound in STLE/STRE extracts, and KCG the second compound more abundant in STLE agreeing with our previous results (Rosa et al., 2016). Moreover, the analyses allow us suggest the presence of unpublished compounds, 5,20-dyhydroxyecdysone (5,20HE), Ecdysone and Vasicine for this species (Table 1). The compound 5,20HE, which was substituted with hydroxyl groups on the core at C-5, has been detected from *S. rhombifolia* and here the fragmentation detected is in agreement with Wang et al (2008) suggesting a 20HE derivative. Considering the main ion $[M+H]^+$ observed at m/z 465 and the fragments 447 and 429 from compound 4, we believe corresponding two water molecules losses ($M+H - 3H_2O$), which is characteristic from ecdysteroids, so, together with its Uv absorption profile (250 nm), this substance may be describe as Ecdysone compound, also named alpha-Ecdysone. This metabolite and its derivatives has already been described for other *Sida* sp (Jadhav et al., 2007). In addition, the fragments observed in our data are in accordance with the same profile described by Wang et al. (2008) studying samples of *S. rhombifolia*. Relative the compound 8, the high intensity ion peak at 189 m/z, with Uv maximum at 280 nm and the abundant product ion *m/z* 171, also probably due loss of water, may be described as the alkaloid Vasicine. Singh et al. (2015) and Madhukar et al. (2014) observed the same pattern of breakage studying the alkaloid Vasicine in *Adhatoda vasica*. In addition, this compound has already been identified in *Sida* sp (Silveira et al., 2003; Ghosal et al., 1975).

Human leukocytes is considered a suitable model for cytotoxicity and genotoxicity evaluation on non-malignant cells. Fig. 2 shown a treating cultured human leukocytes with different concentrations of leaves extract from *S. tuberculata*. According to results, STLE significantly altered the cell viability in all doses tested. Although previous studies by our group have reported low toxic effect to aqueous extract of *S. tuberculata* (Rosa et al., 2016), it is important emphasize here we employed a methanolic extract, which may be extracting more and/or other phytochemicals with potential cytotoxicity. The quinazoline alkaloid Vasicine present in this extract is one example of structure with potential cytotoxic effect. Some studies

point to an antimicrobial and cytotoxic action by vasicine and vasiciene acetylated (Duraipandiyan et al., 2015; Ignacimuthu & Shanmugam, 2010). This results open perspectives in cytotoxic assays with cancer cells. In other hand, in comet and micronucleus assays the extract from both, leaves and roots, did not presented genotoxicity. Micronuclei frequency and comet assay are significant indicative of DNA mutagenesis and damage. Therefore, the data indicated weak genotoxic potential from STLE and STRE.

Pain is often associated with various types of diseases such as low back pain, fibromyalgia, rheumatoid arthritis, migraine, among others. Thus, currently due to life style and ageing population this condition probable will be frequently present in next generations. This is one of the reasons for continuous search of new molecules to treat painful conditions. The first model used in this study to evaluate the antinociceptive potential of STLE was formalin, which causes an immediate response in mice (licking and/or bite the injected paw). This test allows evaluating two pain phases, neurogenic (nociceptors activation) and inflammatory (pro-inflammatory mediators release). STLE produced a significant inhibition on both phases assessed, being 100 $mg.kg^{-1}$ dose more pronounced. According Lima Cavendish et al. (2015) and highlight by Malmberg & Yaksh (1992) substances with central action, on formalin test, may block both phases. On the contrary, peripherally acting substances, such as NSAIDs (non-steroidal anti-inflammatory drug), only affect the second phase.

To estimate the possible central and peripheral STLE action, we performed abdominal writhing model induced by intraperitoneal acetic acid injection. This model, although not specific, presents prominent sensitivity and it has been used to evaluate visceral pain originating from chemical mediators (Moreira et al., 2016). Studies have been indicated that acetic acid injection increase the endogenous mediators such as bradykinin, prostaglandins, histamine, cytokines (TNF-α, IL-1β) and consequently reduce the nociceptive threshold by excitation of primary afferent nociceptive fibers (Ikeda et al, 2001; Ribeiro et al., 2000; Duarte et al.,1988; Martinez et al.,1999). The STLE, in all doses tested, significantly reducing the nociceptive behavior caused by acetic acid. Results demonstrated that from 10 to 100 $mg.kg^{-1}$, inhibition occurred in a dose-dependent manner. This fact suggest, at least in part, that ST extract may decrease inflammatory response through interference on synthesis, release, and/or metabolism of endogenous mediators involved in visceral pain. To exclude the possibility that the

antinociceptive response evidenced by STLE in acetic acid and formalin models could occur through adverse effects on the motor and emotional states of animals, open field test was performed with same STLE doses assessed. Thus, results demonstrated that STLE was not able to induce significant alterations in the parameters evaluated, discarding this hypothesis.

To propose the mechanism of STLE act in visceral pain were used *in vivo* antagonist experiments. Several endogenous molecules and their receptors are involved in analgesia, among these, opioid peptides and their receptors have an important role in visceral pain maintenance (Yin et al., 2016; Jinsmaa et al., 2005; Reichert et al., 2001). Besides, adenosine receptors are widely involved in pain since they are present in the spinal cord and brain areas linked nociceptive transmission, as well as, peripheral sensory afferents or adjacent fibers (Wang et al., 2015; Curros-Criado & Herrero, 2005).

Thus, we examined the effects of both, naloxone, a non-selective opioid receptor antagonist, and DPCPX, a selective A1 adenosine receptor antagonist, on the properties of STLE in acetic acid model. The results indicated that the antinociceptive action of ST extract on writhing response was partially prevented by naloxone and DPCPX. Therefore, we hypothesized that STLE may be interacting through opioid receptor-mediated and A1 adenosine receptor, suggesting a concomitant involvement of the opioid and adenosine receptor systems in STLE antinociceptive effect in the acetic acid test. It is important emphasize, the evidence indicates that the pathway activated by A1 receptors may be similar to the action of endogenous opioids. In part, activation of these receptors (A1 and opioids) is connected to the NO/cGMP/PKG/ K^+ ATP (nitric oxide, guanosine cyclic monophosphate, protein kinase G and potassium-sensitive ATP channel) pathway (Lima et al., 2010; Toda et al., 2009; Pol, 2007; Sachs et al., 2004). This pathway is sensitized by NO generated through nNOS (neuronal nitric oxide synthase) by directly activating cGMP or indirectly PKG, which leads to the opening of the ATP-sensitive K^+ ion channels, which in turn lead to a hyperpolarization of the cell, and consequent decrease in neuronal excitability (Sachs et al., 2004). Therefore, we suggested that STLE contains compounds with at least partial ability to interact to these receptors and modulate the analgesic response.

Computational docking study was performed to provide the information on interaction between STLE compounds and specific receptors involved in pain pathway signalization. To suggest a possible interaction between the main molecules in extract

and opioid receptors, we performed a computational study. A previous and initial evaluation with opioids receptors: μ (Mi – PDB ID: 4DKL), δ (Delta – PDB ID: 4EJ4), κ (Kappa – PDB ID: 4DJH) downloaded from protein data bank (Data not showed), allow us a selection of the MOR. This receptor and cavity BFO were chosen according the positive activity characteristic for compounds assessed compared with others receptors. Moreover, the MOR has been demonstrated interaction capacity with flavone structures, like kaempferol (Ruiu et al. 2015). 20HE present a steroidal nucleus, which some studies have been point out interact with opioid receptors (Capasso & Loizzo, 2008).

According docking data experiments, the principal interactions of both compounds with residues ASP 147, TYR 148, MET 151, GLU 229, LYS 233, TRP 293, ILE 296, HIS 297, VAL 300, TRP 318, ILE 322, GLY 325, and TYR 326. After this step it was applied the post-screening analysis and the residue TYR 236 was determined by Consensus Analysis for two compounds, with Z-score 2.15 and WPharma 1.00. To KCG derivative the ASP 147 was also detected as the residue evolved in this ligand-receptor binding, with Z-score 2.07 and WPharma 1.00. Fig. 6A showed the interactions of KCG and main amino acids residues from MOR, which the flavone structure nucleus present interactions with ASP 147, TRP 293, ILE 322, HIS 297and TYR 326 residues. The residues interacting are the same observed in active site determined in docking study of MOR flavones performed by Ruiu et al. (2015), which suggest the similar results for flavonoid Kaempferol structure.

The data generated suggest the KCG may be better fitted in MOR (active site BFO) in comparison with 20HE, main in consequence of low energy of interaction and interaction with ASP 147 and TYR 326, since the 20HE has a tendency to interact less with ASP 147 residue. However, more *in vivo* studies with these isolated compounds and specific receptors need to elucidate better the action mechanisms. Besides, we do not rule out the possibility of a synergistic effect between all molecules of extract with the receptor. In other hand, the presence of these compounds in plant extract may be also an indicative to opioid activity.

5. Conclusions

In summary, our results displayed a significant antinociceptive action of STLE in experimental *in vivo* behavior models, including reduction in formalin-induced liking and acetic acid writhing response. The investigations showed a probable involvement of opioid and adenosinergic systems on STLE effect, which, at least in part, may be related to the interaction among MOR and A1-AR receptors with major compounds in extract (Kaempferol derivative and 20HE). Phytochemical analyses confirm the phytoecdysteroids as the main class in STLE and STRE. In addition, *in vitro* tests open perspective in studies with cancer cells, due moderate cytotoxicity presented by STLE without cause mutagenotoxicity. Thus, future studies should be considered to evaluate the isolated chemical compounds from *S. tuberculata,* to prove its efficacy and safety as important plant in management the acute pain.

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Figure captions

Fig. 1. Main fragments ions (m/z) in the mass spectra obtained from *S. tuberculata* extracts by UHPLC/ESI-MS in positive mode. The illustrative compounds corresponding to metabolites suggested in Table 1. (A) 20-Hydroxyecdysone-3-glucopyranoside; (B) 20-Hydroxyecdysone; (C) 5,20-dihydroxyecdysone; (D) Ecdysone; (E) 20-Hydroxyecdysone-3-deoxyhexose; (F) Kaempferol-3-(6-p-Coumaroyl) glucopyranoside; (G) *N*-Feruloyltyramine; (H) Vasicine.

Fig. 2. Leukocyte viability assay (A); micronuclei frequency test (B); comet assay (C), after treatment (72h) with different concentrations of methanolic *S. tuberculata* leaves extract (STLE) and roots extract (STRE). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns = no significant statistical differences compared to negative control (PBS, phosphate buffer saline pH 7.2). As positive control was used H_2O_2 (hydrogen peroxide at 10µM).

Fig. 3. Effects of *S. tuberculata* methanolic extract from leaves (STLE 10–300 $mg.kg^{-1}$, p.o.) on Formalin-induced nociception in mice: (A) first phase (neurogenic pain) and (B) second phase (inflammatory pain). In the first phase 0-5min- (panel B) and second – 15-30 min - (panel C). Each bar represents the mean ± S.E.M. of 5–10 animals. Significance levels are indicated by * $p<0.05$ and ** $p<0.01$ when compared to the control group (ANOVA followed by the Dunnet test).

Fig. 4. Effects of *S. tuberculata* methanolic extract from leaves (STLE 10–300 $mg.kg^{-1}$, p.o.) on acetic acid-induced nociception in mice. Results expressed as number of writhes. Each bar represents the mean ± S.E.M. of 5–10 animals. Significance levels are indicated by * $p<0.05$ and ** $p<0.01$ when compared to the control group (ANOVA followed by the Dunnet test).

Fig. 5. Effects of *S. tuberculata* methanolic extract from leaves (STLE 10–300 $mg.kg^{-1}$, p.o.) on the motor and emotional response in mice by open field test. Each bar represents the mean ± S.E.M. of 5 animals. Statistical comparison was performed using ANOVA followed by the Bonferroni's test.

Fig. 6. Opioid and adenosinergic systems involvement on the antinociceptive action of *S. tuberculata* leaves extract (STLE). Pre-treatment with opioid antagonists naloxone (1 $mg.kg^{-1}$, i.p., panel A) or adenosine antagonists DPCPX (1 $mg.kg^{-1}$, i.p., panel B) on the inhibitory action of STLE (100 $mg.kg^{-1}$ p.o.), Morphine (1 $mg.kg^{-1}$ i.p.) or CHA (0.03 mg $.kg^{-1}$, i.p.) in nociception induced by acetic acid in mice. Each column represents the mean ± standard error of the mean of 6 to 10 animals. Significance levels are indicated by ** $p < 0.01$; *** $p < 0.001$ when compared with the saline control group a; ### $p < 0.001$ significant compared with morphine or CHA agonists (two-way ANOVA/ Dunnet test).

Fig. 7. Binding of Kaempferol-3-(6-p-Coumaroyl)-glucopyranoside and 20-Hydroxyecdysone in the active site of BFO (PDB: 4YAY) from μ opoiod-receptor. Graphic visualization obtained using PyMOL (v.0.99) **(A)** Kaempferol in receptor site; **(B)** 20-Hydrxyoecdysone in receptor site.

Manuscript II

Antitumor activity *in vitro* and inhibition of inflammatory response *in vivo* by *Sida tuberculata* extracts

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Abstract

Considering the traditional use of *Sida tuberculata* (Malvaceae), the current study aimed to assess the *in vitro* cytotoxicity and *in vivo* anti-nociceptive and anti-inflammatory effects of *S. tuberculata* (ST) methanolic extracts in acute and chronic models. The antiproliferative capacity of ST was evaluated against HepG-2, MCF-7 and 3T3 cells line. The anti-inflammatory properties of ST leaves extract (STLE) were investigated on two experimental models: carrageenan-induced peritonitis and complete Freund's adjuvant (CFA) induced mechanical nociception in paw of mice. According *in vitro* IC_{50} results, the ST extracts from leaves (ranging from 543 to 800 $\mu g.mL^{-1}$) and roots (ranging from 378 to 852 $\mu g.mL^{-1}$) presented significant antiproliferative activity. For *in vivo* tests, STLE (10-100 $mg.kg^{-1}$, p.o. 60 min early), inhibited the inflammation parameters (TBARS, NPSH, MPO activity, cell influx and cytokines release) in peritonitis model. In addition, STLE (100 $mg.kg^{-1}$, p.o.) administered once a day, significantly reversed tong-term the mechanical hypernociception and paw oedema caused by CFA in mice. Therefore, our findings suggest ST extracts minimize acute and chronic inflammation process supporting traditionally use.

Key words: cytotoxicity, pain, inflammation, Sida tuberculata.

Introduction

Inflammatory process are a sequence of coordinated events in response to noxious stimuli, such as infections and tissue damage, in order to restore homeostasis. Thus, this response generates an increase in vascular permeability and consequent release of inflammatory mediators, causing edema and cell migration to the inflammation site [1]. Several can be the causes these inflammatory reactions, including contact with microbial fragments or toxins (lipopolysaccharide (LPS), complete Freund's adjuvant (CFA) from bacteria), irritant chemicals (such as carrageenan) and autoimmune reactions.

Among different cells involved in inflammation process, mast cell, neutrophils and macrophages play important role in acute and chronic inflammation [2]. The chemostatic neutrophils action at the site of inflammation may lead to production of numerous pro-inflammatory mediators such as cytokines (IL1β, IL6, TNF-α), reactive oxygen and nitrogen species, specific enzymes (metalloproteases, myeloperoxidase) which may further intensify the inflammatory response and injure [3, 4]. These events promotes the classical inflammatory signals pain, heat, redness, swelling and loss of function [5, 6].

The inflammatory pain is a direct effect from mediators released, activation of resident cells, compression of the sensory nerves, resulting from both the initial damage as well as the inflammatory response itself [5]. In this sense, these alterations in affected tissue promote a threshold reduction and augment the response of nociceptors that innervate the tissue injured [7, 8]. All these inflammatory parameters can be assess for several *in vivo* models, among then, CFA and carrageenan-induced inflammation [9-12] as chronic and acute models respectively.

Due the principally side effects of anti-inflammatory and analgesics available currently, the investigation and development of safe new therapies are needed. In this context, plants constitute an important source of molecules with therapeutic potential. The main drugs currently used to treat pain and some inflammation process are derived from plants metabolites or were remodeled based on natural products [13].

Sida tuberculata R.E.Fries (Malvaceae) has been investigate by our group as potential source of biological activities. *S. tuberculata* is common plant found in Southern Brazil, traditionally has its roots and leaves employed for treat inflammation process, vascular complications and diabetes. Recently studies showed alkaloids, phenolic compounds and phytoecdysteroids are the most significant classes present in *S. tuberculata* extract. A significant antioxidant and antimicrobial capacity were detected for leaves extract from *S. tuberculata* [14, 15].

Therefore, in this work, we aimed to investigate the anti-inflammatory and analgesics properties of leaves extracts obtained from *S. tuberculata*. In addition, an *in vitro* antitumor assay was performed to assess the possible cytotoxic action from extract.

Materials and Methods

Plant material

S. tuberculata whole plant was collected from the Uruguaiana city, Pampa biome region, Southern Brazil, under geographical coordinates 29º50'06.3"S and 57 º05'50.5"W. The samples collection occurred at temperature of 22 ± 2 ºC after rained day to facilitate plant removal from the soil. A voucher specimen (ICN 167493) was deposited at the Herbarium of Federal University of Rio Grande do Sul (UFRGS),

Department of Botany. M. Grings and O. Bueno gently contribute to plant identification.

Extraction obtainment

Initially, the plant was separated into leaves and roots, dried at 40 ºC for 5 days in oven. After ground to powder, the samples were extracted using methanol under magnetic stirring at 40ºC for 5 hours (1:10 w/v of plant:solvent). Periodically every hour, the material was sonicated for 30 seconds. The crude material resultant was filtered and subjected to a liquid-to-liquid partition with hexane (1:1 v/v of extract/solvent). In sequence, methanolic fraction was collected and concentrated in rotary evaporator resulting in *S. tuberculata* leaves and roots extracts (STLE and STRE respectively), which were employed for further analyses.

HPLC-PDA analysis

The chromatrographic analyses were performed using a Prominence Liquid Chromatograph (Shimadzu, Kyoto, Japan) equipped with an DGU-20As degasser, LC-20AT pump, SIL-20A auto sampler, SPD-M20A PDA detector, CTO-20A oven and CBM-20A controller. The separation procedure was conducted using a Phenomenex Luna C-18(2) column (250 x 4.6 mm, 5 mm) and mobile phase consisted of 0.05% phosphoric acid in water (A) and acetonitrile (B) at a flow rate of 0.8 $mL.min^{-1}$. The gradient system used was: 0.1–23 min, 10–40% of solvent B in A; 23.01–40 min, 10% solvent B and 90% solvent A. The detection was achieved on DAD detector set at 250 nm. The extracts samples and mobile phase were filtered through a 0.45 mm membrane filter (Millipore) before use. The standard solution (20HE major compound) was diluted in methanol and injected in triplicate .

Antitumor activity

The tumor cell lines HepG2 (human hepatocyte carcinoma) and MCF-7 (human breast cancer) were used as *in vitro* models to study the antitumor activity of the STLE and STRE. Moreover, the non-tumor cell line 3T3 (murine Swiss albino fibroblasts) was used as negative control of the antitumor activity. All cells were grown in DMEM medium (4.5 g/l glucose), supplemented by 10% (v/v) FBS, at 37ºC with 5% CO_2. They were routinely cultured in 75 cm^2 culture flasks and harvested using trypsin-EDTA when the cells reached approximately 80% confluence.

HepG2 (8.5 x 104 cells/ml), MCF-7 (1 x 105 cells/ml) and 3T3 (1 x 105 cells/ml) cell lines were seeded into the 60 central wells of 96-well cell culture plates in 100 µl of complete culture medium. Cells were incubated for 24 h under 5% CO_2 at 37ºC and the medium was then replaced with 100 µl of fresh medium, supplemented by 5% (v/v) FBS, containing the extracts at the concentration range 7.8-1000 µg/ml. Untreated control cells were exposed to medium with 5% (v/v) FBS only. The cell lines were exposed for 24 h to each treatment, and their viability was assessed by the MTT assay.

The MTT endpoint is based on the protocol first described by Mossmann [16], which is a measurement of cell metabolic activity. After complete the cell treatment time, the medium was removed, and 100 µl of MTT in PBS (5 mg/ml) diluted 1:10 in medium without FBS was then added to each well. The microplates were further incubated for 3 h under 5% CO2 at 37ºC, after which the medium was removed. Thereafter, 100 µl of DMSO was added to each well to dissolve the purple formazan product. Plates were then placed on a microtiter-plate shaker for 10 min at room temperature, and the absorbance of the resulting solutions was measured at 550 nm

using a Multiskan FC (Thermo Scientific, San Jose, CA, USA) microplate reader. Cell viability was calculated as the percentage of tetrazolium salt reduced by viable cells in each sample.

The cytotoxicity of each extract in each cell line was expressed as percentage of viability with regard to untreated control cells (the mean optical density of untreated cells was set at 100% viability). The IC_{50} doses (concentration causing 50% death of the cell population) were calculated for each cell line by curve fitting of percent cell survival against concentrations of the extract.

In vivo assays

Based on results from antioxidant capacity of ST extracts, we select STLE for *in vivo* experiments due its higher scavenger activity compared to STRE [15].

Animals

Male Swiss mice with approximately 40 days old and 30–40 g body weight were obtained from the animal house of the Federal University of Santa Maria (UFSM). The mice were randomly distributed into experimental groups (4 animals per cage) and kept at 25±2 °C in 12 h light–dark cycles (lights on at 07:00 h) with water and food *ad libitum*. The animals remain in this condition at least 7 days prior to assays. Experiments were carried out in accordance with the ethical guidelines for investigation of experimental pain in conscious animals [17] and were performed after the approval and under the consent and surveillance of the Ethics Committee for Animal Research (CEUA: 009/2016), Federal University of Pampa. The number of animals and the intensity of the noxious stimuli were the minimum necessary to demonstrate the consistent effects of drug treatments.

Carrageenan-induced Peritonitis

Peritonitis was induced by intraperitoneal (i.p.) injection of 0.5 mL of carrageenan (0.75 mg per cavity) diluted in sterile saline according to procedures described previously by Pagano et al. [18]. Six groups (n = 6 – 8) were randomly selected for experiments accomplishment. Control group received saline solution injection (0.9%, i.p.). Test groups were pre-treated with Dexamethasone (0.5 $mg.kg^{-1}$, i.p.) or STLE (10, 30 and100 $mg.kg^{-1}$, orally) 30 and 60 min before carrageen injection respectively. Four hours after carrageenan (Cg) or saline injection, the animals were sacrificed by CO_2 inhalation and the peritoneal cavity was washed with 1.5 mL of phosphate buffer saline (PBS). Samples of exudate and tissue peritoneal were collected for subsequent analyses as describe bellow. The measurement of protein levels in the tissue samples was performed according Bradford [19].

Inflammatory parameters evaluated

Total leukocyte and cell differential counting

Total leukocyte count was performed in the Neubauer chamber, after diluting the peritoneal fluid with Türk's solution (1:20) [18, 22].

Differential counting was performed with peritoneal cells smear stained with fast panoptic dye kit. The counting was accomplished with aid of microscope. The results are presented as neutrophils % per ml of peritoneal exudation.

Myeloperoxidase test

Myeloperoxidase (MPO) activity in the peritoneal liquid was determined as describe by Bradley et al. [23] with modifications. Briefly, peritoneal exudate samples

(100 µl) were homogenized in 0.5% hexadecyltrimethyl ammonium bromide buffer (pH 5.4) and centrifuged (10.000 x g for 20 min at 4 °C). An supernatant aliquot was used to react with a solution of 18.4 mM Tetramethylbenzidine in Dimethylformamide, phosphate buffer sodium 80 and 22 mM (pH 5.4), and hydrogen peroxide 0.017% in 96-well plate. The plate was incubated at 37 ° C for 6 min, and after the reaction was stoped by the addition of sodium acetate (1.46 M, pH 3.0). MPO activity was estimated by colorimetric measurements using the plate reader (BMG Labtec, Germany), at 650 nm. The activity was expressed as OD/mL [24].

Cytokines measurement

The collected peritoneal fluid was used to evaluate cytokine levels by enzyme immunoassay kit (ELISA- R & D Systems, Minneapolis, U.S.A.). ELISA kits were performed according to the manufacturer's instructions. Aliquots (100 µL) were used to assessed IL-1β, IL-6, IL-10 and TNF-α cytokine levels. The absorbance for cytokines studied were achieved at 450 nm and 550 nm absorbance in microplate reader (Berthold Technologies - Apollo 8 - LB 912, KG, Germany). Results were expressed in pg/mL [25].

Oxidative parameters assessed

TBARS assay

The thiobarbituric acid reactive species (TBARS) was determined according to the method proposed by Ohkawa et al. [20] with adaptations. TBARS levels were measured in the peritoneum tissue of animals. Briefly, tissue samples were homogenized and centrifuged at 4000 g at 4°C for 10 min to yield a supernatant fraction (SF1), which was used in TBARS test. The readings were obtained

spectrophotometrically (at 532 nm) using a malonaldehyde standard curve (MDA) to correct in protein content.

Non-protein SH (NPSH) assay

NPSH assay was executed according Ellman [21] with modifications. Concisely, peritoneum tissue samples from SF1 (100 µL) were added to the system containing phosphate buffer Ellman reagent (DTNB100 mM) and (potassium phosphate buffer 250 mM pH 7.4). The NPSH levels were determined by spectrophotometer reading at 412 nm and the results were calculated in relation to glutathione (GSH) standard curve. The results were expressed as nanomoles SH per milligram of protein.

CFA-induced persistent inflammatory pain

The chronic inflammatory model was performed according to the methodology described by Ferreira et al. [26] with minor modifications. Thus, the animals receiving an intra-plantar injection of 20 µL CFA (1 $mg.ml^{-1}$ of heat killed *Mycobacterium tuberculosis* in paraffin oil 85% and mannide monoleate 15%) in the ventral surface of the right hind paw. Control animals received 20 µL of saline solution (0.9%) in the hind paw. To assess the effects of the STLE treatment against CFA-induced chronic inflammatory pain, animals received the STLE (100 $mg.kg^{-1}$, intragastrically, v.o., dose selected based in previous results provided by acetic acid model, data not show) 24 h following CFA intra-plantar injection. Mechanical hypersensitivity was evaluated at 0, 0.5, 1, 2, 4 hours after treatment to verify the time-course of STLE dose effect. To evaluate the long-term effects by STLE treatment, a dose of 100 $mg.kg^{-1}$ was administered (p.o.) once a day (every 24h) during 11 days. This repeated treatment was interrupted for 3 days, on eleventh day, and was re-initiated, for more two days, to

investigate the tolerance response. The total time of hypersensitivity evaluation were 15 consecutive days.

Hypersensitivity and paw oedema measurement

CFA experiment was assessed as previously described by Palmer et al. [27] with changes. After an adaptation period of 60 min (in small-enclosed testing arenas on top of a wire mesh floor) the hypersensitivity was evaluated as response for paw withdrawal threshold by application of different von Frey monofilaments (VFH, Stoelting, Chicago, USA). The filaments with forces ranging from 0.02 to 4.0g were applied perpendicularly to the plantar surface of injected paw for 5 seconds, causing a slight curvature in the filament. The median of the paw withdrawal threshold was determined using an Up-Down Dixon [28]. Once the baseline values have been determined, those animals that received CFA and STLE (100 $mg.kg^{-1}$) were evaluated daily as describe above.

The paw oedema progression was verified by electronic caliper (Starrett, model 797B-8, with LCD display), and was expressed in milliliters as the difference between the CFA injected paw and contralateral paw (left paw). The paw measurements were assessed daily for 15 days.

Organ to body weight ratio

At the end of the fifteenth day of CFA experiment, the animals were euthanized by cervical dislocation and the vital organs (heart, lungs, liver, spleen and kidney) were quickly removed, cleaned with saline and weighted. Each organ to body weight proportion was calculated as (weight of organ/body weight of rat on day of sacrifice) ×100% [29].

Statistical analysis

Results are reported as means ± standard error of mean (SEM). *In vitro* assays represent three independent determinations for each experimental step. Statistical differences between groups were determined by two-way ANOVA with the Tukey's post tests. Values of $P<0.05$ were considered the limit for significance.

Results

HPLC analysis

Chromatographic evaluations were performed examining *S. tuberculata* extracts obtained from methanolic solution under magnetic stirring. Previous studies by our group have been elucidated main compounds in *S. tuberculata* extracts [14, 15]. Here, we intend to detect and quantify the major compound, 20-Hydroxyecdysone (20HE), obtained by methanolic solvent using other extraction technique. In STLE and STRE, the compound 20HE was detected in the retention time (Rt) at 17.4 min (Fig. 1). 20HE corresponding the major peak at 250 nm (abs) in both extracts, with concentration of 0.27 and 0.25% g of dry extract, respectively.

Antitumor test

The *in vitro* antitumor activity of STLE and STRE was assessed using two different tumor cell lines, HepG2 and MCF-7. In addition, a non-tumor 3T3 cell line was used to compare the results. These *in vitro* models provide rapid and effective results on cytotoxicity potential. Initially, STLE and STRE were evaluated against HepG2 cell line, and both showed significant results (Fig. 2A). STLE presented significant reduction in HepG2 viability from 15.6 $\mu g.mL^{-1}$ dose and STRE from lowest

dose tested (7.8 µg.mL^{-1}). The IC50 values for STLE and STRE were 543.6±13.4 and 378.9±37.8, respectively. In MCF-7 assay, both extracts displayed significant results from 7.8 µg.mL^{-1} dose and the decreased in cell viability reaching 72.2 and 70.4 % for STRE and STLE at 1.000 µg.mL^{-1} dose respectively (Fig. 2B). IC50 values in this tested was found at 593.4±49.1 for STLE and 493.91±27.7 for STRE. Likewise, 3T3 non-tumor cells test presented significant reduction in viability (Fig. 2C), but less sensitive as shown by IC50 values (800.6±15.6 and 852.7±18.9 for STLE and STRE respectively).

Carrageenan-induced peritonitis

The anti-inflammatory property of the crude extract acquired from *S. tuberculata* leaves was evaluated by carrageenan-induced peritonitis model. First, MPO activity was investigated. As can be seen in Fig. 3 (A), the animals that received only carrageenan presented a significant increase ($P < 0.05$) in the MPO activity 4 h after the induction. On the other hand, pretreatment with STLE reduced MPO activity significant from lowest ($P < 0.001$) concentration tested , 10 mg.kg^{-1}, similar results was found to Dexa group. There was no difference among all STLE dose tested.

Moreover, TBARS and NPSH assays were used as oxidative marker on groups evaluated. TBARS levels, also were incremented in the carrageenan group compared to saline group ($p < 0.05$) (Fig. 3 B). However, Dexa pretreatment was able to prevent oxidative damage ($p < 0.05$). In addition, the groups STLE, 30 and 100 mg.kg^{-1}, presented significantly decreased in TBARS generation ($p < 0.05$). Fig. 3 C presents the NPSH levels in carrageenan-induced peritonitis model. As can be seen, NPSH levels were significantly decreased in carrageenan group ($p < 0.05$). STLE treatment at 100 mg.kg^{-1} prevent this reduction restoring the NPSH to normal levels.

The inflammatory cytokines levels (IL-1β, IL-6 and TNF-α) in the peritoneal fluid of animals that received Cg, increased when compared to saline group (Figure 5 A). In addition, as can be seen, the IL-10 levels, an anti-inflammatory cytokine, decreased in the peritoneal exudate of Cg group compared to saline animals (Figure 5 B). STLE pre-treatment reduced significant ($p<0.01$) IL-1β, IL-6 and TNF-α levels from lowest dose tested (10 $mg.kg^{-1}$), but was not able to prevent the IL-10 depletion.

Carrageenan injection resulted in a significant ($P<0.05$) increase in leukocytes in the peritoneal exudate 4 hours after its administration. There was an increment, principally, in leukocytes cells in peritoneal cavity compared to the saline group (Fig. 5). In addition, the mononuclear cells number was decreased in carrageen group in relation the saline group (Fig. 5B). However, STLE pretreatment promotes a significant inhibition in polymorphonuclear leukocytes migration from the lowest dose assessed (10 $mg.kg^{-1}$) and prevented the diminution in mononuclear cells (Fig. 5 A and B)

CFA-induced inflammation

Considering the antinociceptive and anti-inflammatory effect of STLE in acute models, we evaluated its action on a chronic model, in this case an inflammatory model induced by CFA injection (i.pl.). Von Frey filaments were used to assessed mechanical hypersensitivity. The evaluation started 24 hours after the CFA injection and extending up to the 15th day post-injection. As shown in Figure (Fig. 6 A), CFA injection induced a decrease in the paw withdrawal threshold relative to the control group treated with vehicle. In the other hand, STLE (100 $mg.kg^{-1}$, p.o.) was not able to reverse the hypersensitivity acute until 4 hours after orally administration (Fig. 6A). However, the hypersensitivity was reduced significantly by STLE only from 6^{th} day treatment. This significant effect remained and until 11^{th} where STLE presented its most prominent

action (Fig. 6B). When the treatment was interrupted for three days, the hypersensitivity was observed again. Conversely, when treatment was restored on day 14, STLE again was able to increase the paw withdrawal threshold, with a response similar to that observed on the 11th day. Moreover, Fig. 6 C shown that the CFA i.pl. injection of lead an significant increase in the paw volume. In other hand, the post-treatment once a day (24 h after CFA injection) of animals with STLE (100 mg.kg^{-1}, p.o.) reduce the CFA-induced paw oedema significantly after 72h of treatment. This result remained until the last of experiment.

The animals euthanized showed no macroscopic alteration, and there was no significant variation in heart, lungs, liver, spleen and kidney weight from both, treated and un-treated mice (Table 1). The organ-to-body weight ratio of the all groups assessed also remained unchanged.

Discussion

In the present study, *S. tuberculata* extract was evaluated for its analgesic and anti-inflammatory potential in acute and chronic inflammatory models. Moreover, a preliminary anti-tumor activity was assessed for its extracts.

Previous studies by our group have been characterized chemically extracts from leaves and roots of *S. tuberculata* [14, 15]. Therefore, based on the diversity structural of its compounds, principally steroidal metabolites, we hypothesized the analgesic and anti-inflammatory capacity for STLE. *S. tuberculata*, as describe previously by our group, is categorized mainly for phytoecdysteroids, alkaloids and phenolic compounds. Among these, 20HE was identified as main compound in extracts from leaves and roots. This compound has been detected in other Sida spp., and are the most abundant phytoecdysteroid found in plant families [30-33]. Although the ecdysteroids role in

plants is not known for sure, probably these metabolites are involved in plant defense against insects and nematodes [30; 34].

The cytotoxicity studies of *S. tuberculata* extracts were performed using tumor (HepG2, MCF-7) cell lines and 3T3 (non-tumor cell). This evaluation was the first in literature for *S. tuberculata* and it was performed based in cytotoxic results on human leucocytes (Data not shown). However, according IC_{50} values detected in this study, although STLE and STRE have showed cytotoxic effects against all cell lines tested, this effect may be considered low, since it displayed, in general, IC_{50} values > 500 $mg.mL^{-1}$ of cell viability, exception STRE on HepG2 (IC_{50} of 378.97 ± 37.8) . Studies have been demonstrated cytotoxic activity for *Sida* species, as *S. acuta,* assessed against HepG2 cells showed a weak activity to methanolic extract with IC_{50} of 475.33 µg/mL [35]. Moreover, *S. cordifolia* and *S. rhombifolia* methanolic extract presented lower antiproliferative activity for HepG2 cell compared to *S. acuta* [36]. However, when compared to non-tumor cell test, STLE and STRE presented a pronounced and specific anti-tumor activity. This is important, because suggest that other mechanism may be involved in cytotoxicity activity against tumor cells. Some authors attribute, at least in part, the cytotoxic potential another frequent compound class in *Sida* spp., alkaloids [37, 38]. Previous results by our group, detected representative of this class in *S. tuberculata* [14]. However, it is inconclusive to affirm that the antiproliferative capacity in *S. tuberculata* is due to alkaloids. Thus, more studies are necessary to elucidate this hypothesis.

Chemical constituents obtained from medicinal plants and other natural products have been significantly contributed in the study of many inflammatory diseases [13]. In the present work, we demonstrated that STLE decrease inflammatory parameters in peritonitis model. Our results show STLE reduced peritoneal oxidative marker

(TBARS) stress in mice. TBARS test is a recognized marker widely employed to detect lipid damage on cells and tissues, and its augmented levels are an indirect indicative of malondialdehyde and oxidative stress by excessive reactive oxygen species (ROS) production. Thus, TBARS diminution may be related to antioxidant capacity present in *S. tuberculata* extract [15]. Similarly, an increase in NPSH levels, may be a suggestive antioxidant protection by STLE against depletion in glutathione (GSH) levels. GSH is a non-protein thiol present in aerobic species, its intracellular antioxidant role includes the detoxification of xenobiotics and ROS, being responsible for the maintenance of the thiol redox balance [39].

Acute inflammation involves, among other factors, increase in vascular permeability, inflammatory exudate, including increased plasma proteins, cell migration (principally neutrophils), and proliferation of endogenous chemical mediators at the injury [40]. Our data showed that pre-treatment with STLE (100 $mg.kg^{-1}$ p.o.) before carrageenan injection, produced a decreased in MPO activity and in the cell influx, manly neutrophils. Neutrophils are the major cellular subtype involved in the defense of the organism, and their migration from blood vessels to tissues is a crucial process during host response against infections by microorganisms and / or other harmful agents. Perhaps, the extract may act prevent signaling between cells and mediators. Results found by Rosa et al. [15] displayed ethanolic extract from *S. tuberculata* can exert scavenger activity against nitric oxide derivative species detected by Gries reaction [41]. Nitric oxide (NO) plays important role in inflammation steps, including, leukocyte adhesion and transmigration [42], thus, the STLE could be acting as NO captor, diminishing cell migration. But this suggestion needed to be investigate in more detail.

In addition, the polymorphonuclear leukocytes recruited in the inflammation induced by carrageenan synthesize and release a large number of mediators, including the proinflammatory cytokines TNF-α, IL-1β and IL-6 responsible for leukocytes chemotaxis [40]. According our results, STLE, similarly to dexamethasone, significantly reduced TNF-α, IL-1β and IL-6 concentrations. Thus, the inhibition of leukocyte migration could be related to previously treatment with STLE, which are able to decrease cytokines production. However, on the contrary of Dexamethasone group, STLE was not able to affect the IL-10 levels, which was decreased by carrageenan injection. The IL-10 cytokine is produced by macrophages and it is recognized by its anti-inflammatory action, which plays an important role in the inflammation control [43].

In other hand, may be possible that phytoecdysteroid compounds present in the STLE [14], are acting in the modulation of the inflammatory process. Studies have been demonstrated the phytoecdysteroids potential in to act on inflammation process. Ochieng et al. [11] evaluating seven phytoecdysteroids from stem bark of *Vitex doniana* found a significant inhibition in paw oedema development due to carrageenan-induced inflammation in rat. Other work showed that Muristerone A, can to interact with the NF-kB system with similar effects to dexamethasone and retinoic acid, leading an apoptotic signaling in murine lymphoma WEHI-231 cells [44]. In addition, Peschel et al [45] studying *Leuzea carthamoides* extract in immune response displayed that 20HE, although not alone, may exert effects in NF-κB inhibition. Sun and Yasukawa [46] demonstrated that Ergostane-type ecdysteroid isolated from *Polyporus umbellatus* exhibited potent anti-inflammatory capacity on TPA-induced inflammation model in mice. Thus, further studies with isolated phytoecdysteroids and other compounds from

S. tuberculata are needed to investigate the possible involvement of these structures in inflammation process.

Aiming assessment the effects of STLE in chronic models of pain, we investigated the nociceptive response of mice treated with STLE on inflammatory pain induced by CFA intra-plantar injection. A characteristic of this nociception model is the hypernociceptive responses to mechanical stimuli applied to the site of the injury [47]. Here, we evaluated mechanical response by application of Von Frey filaments. The long-term pain caused by CFA intra-plantar injection involves central sensitization due to the release of several inflammatory and pain mediators (among then, cytokines, glutamate, NO, prostaglandins, histamine, 5-hydroxytryptamine and immunological agents) resulting in increase in the sensitivity of both peripheral sensory afferents fibers at the damage site [48-50]. The present study demonstrated that prolonged mice treatment with STLE reduced mechanical hypersensitivity and paw oedema in chronic inflammatory pain. However, the antinociception effect was only observed from 6^{th} day and paw oedema reduction from 5^{th} day of treatment, showing a possible extract involvement in long-term inflammatory response. After detected, this result of STLE was not susceptible to tolerance, since repeated STLE administration did not cause a decrease on effect. Besides that, the treatment discontinuation caused hypersensitization of the animals again, being inhibited when the STLE treatment was restored. In other hand, STLE was not able to reduce the hypersensitivity in an acute treatment. Therefore, STLE may works at as slow acting drug, inhibiting inflammatory markers release. Its important emphasize that STLE administrated once a day per 15 days.

Finally, although detected a significant cytotoxicity capacity, STLE at 100 $mg.kg^{-1}$ showed no alterations in absolute and relative organs weight (Table 4), revealing, at least in part, a low systemic toxicological potential. These data are a

sensitive indicator to assess the toxicological effect of an experimental compound. Absolute organ weight is a reliable tool because significant differences in organ weight between treated and untreated animals may occur in the absence of any morphological changes. Other important and additional toxicity parameter is organ weight to body weight ratio, to explain for differences in body weight. Evaluation of organ weight changes in the presence of body weight differences has resulted in the use of additional tools such as relative organ weight [51]. Thus, this toxicological parameter suggest a low damaging potential at systemic level from STLE in the conditions evaluated in this study. However, others specifics toxicological methodologies need to be investigated in future studies.

Conclusion

In summary, the present *in vitro* results indicates a significant and specific cytotoxicity to STLE and STRE against HepG2 and MCF-7 tumor cells line. *In vivo* assays, demonstrated that pretreatment with STLE suppress inflammation parameters by carrageenan-induced peritonitis in mice. In addition, this work provides evidence that STLE exerts a long-term significant anti-hypersensitivity in mechanical nociceptive and paw oedema response caused by CFA. The exactly STLE mechanisms of action are not completely known, however its ability to inhibit oxidative parameters, cell influx and cytokines release may be responsible, at least in part, for its antinociceptive and anti-inflammatory action.

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Figures captions

Fig. 1 – HPLC-DAD of *S. tubercualta* methanolic extract and its major compound at 250 nm. (A) 20-Hydroxyecdysone (20HE) standard at 100µg.mL^{-1}; (B) Extract from leaves (STLE); (C). Extract from roots (STRE).

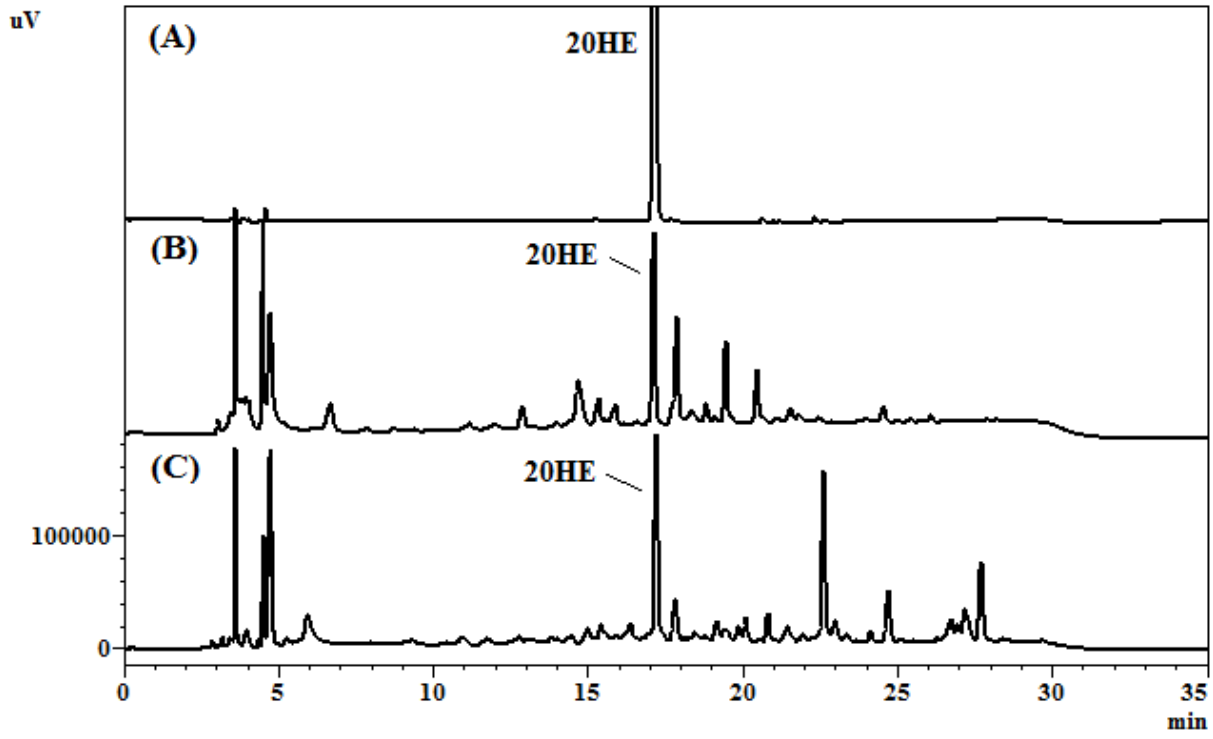


Fig. 2 – Antiproliferative effect of *S. tuberculata* extracts against cell lines (STLE – leaves extract; STRE – roots extract). (A) HepG2 - human hepatocyte carcinoma; (B) MCF-7 – human breast cancer; (C) 3T3 - murine Swiss albino fibroblasts. Results are expressed as percentage of cell viability (%) calculated in relation to the negative control (100% of viability). * $p < 0.05$, significant compared to negative control. # $p < 0.05$, significant compared the same dose of the other extract.

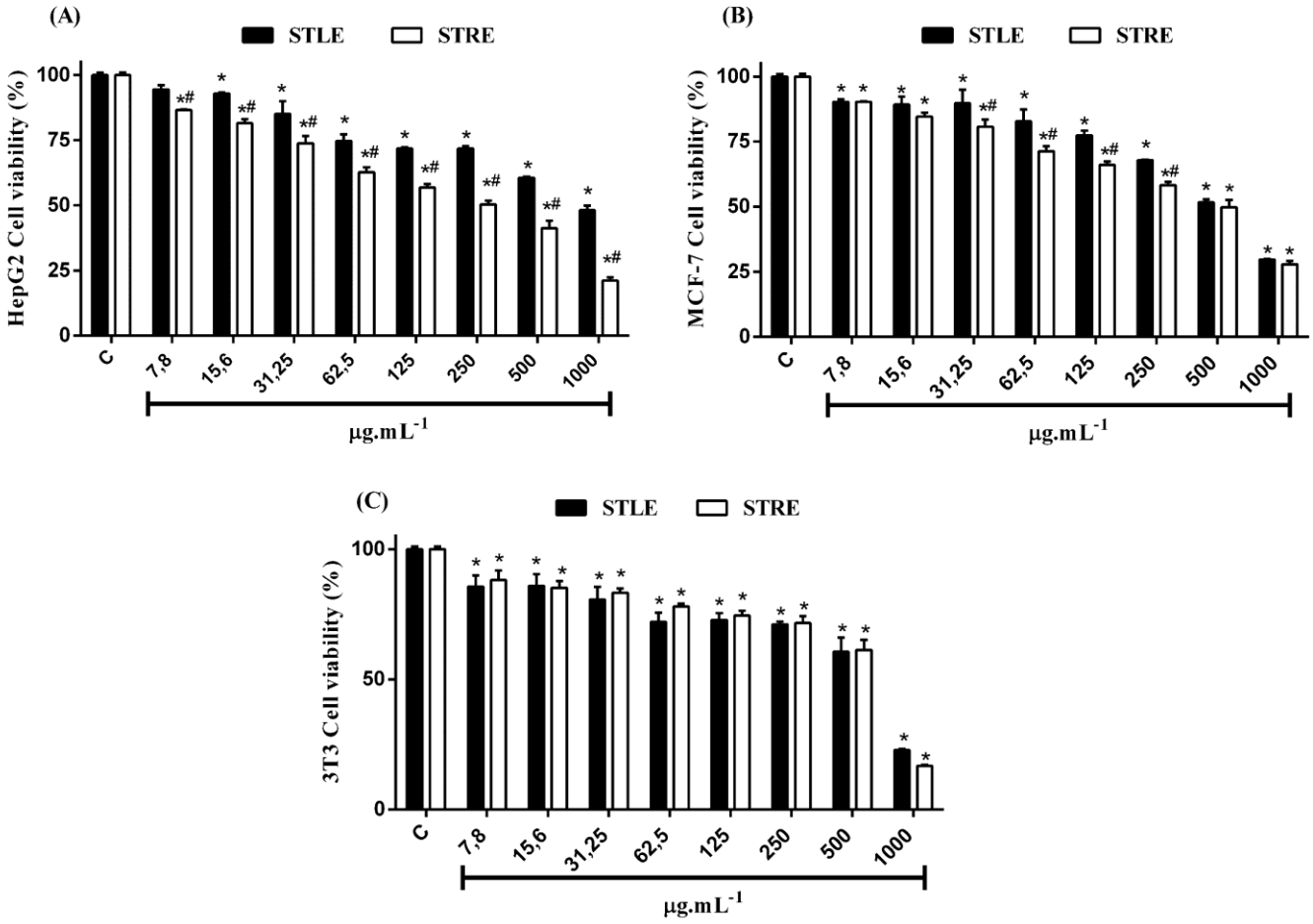


Fig. 3 – TBARS **(A)** and Non-protein –SH levels (NPSH) **(B)** on peritoneum inflammation induced by carrageenan in mice pre-treated with *S. tuberculata* leaves extract (STLE 10-100 mg.kg^{-1}, p.o.). Abbreviations, V = Vehicle group (NaCl, 0,9%, p.o.), Dexa = Dexamethasone, Cg = Carrageenan (750 μg/mice cavity). Symbols # ($p<0.05$), ### ($p<0.001$) indicates significant in comparison to vehicle group. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), significant compared to Carrageenan group.

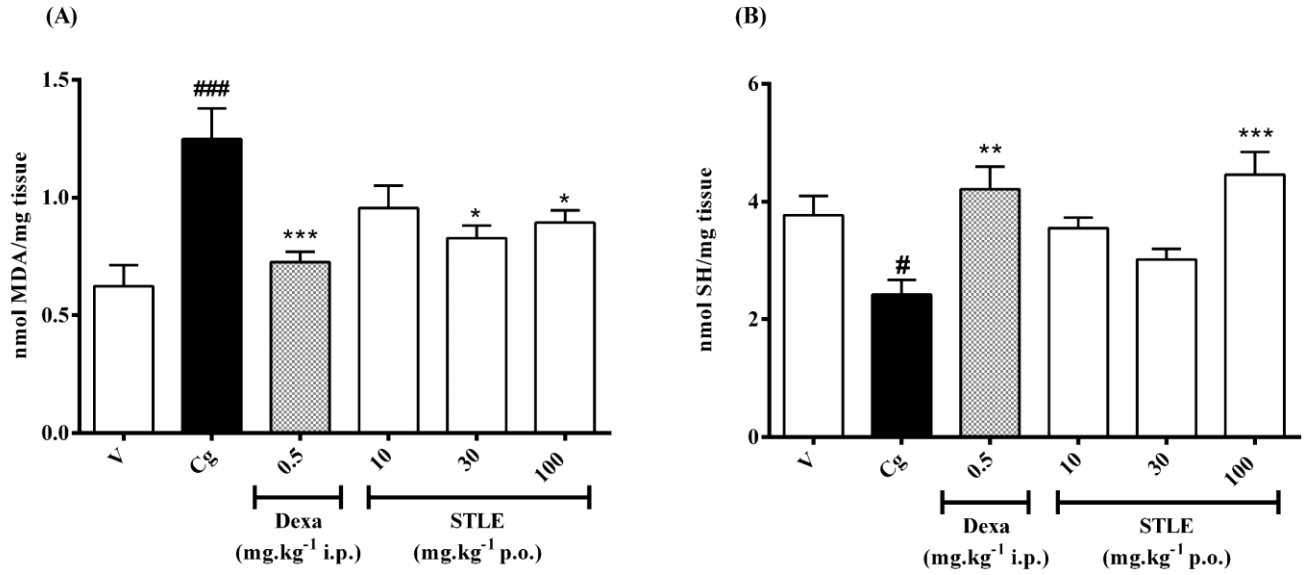


Fig. 4 – Cell migration and myeloperoxidase activity in peritoneal fluid of carrageenan-induced peritonitis in mice pre-treated with *S. tuberculata* leaves extract (STLE 10-100 mg.kg^{-1}, p.o.). **(A)** Total leukocytes, **(B)** Mononuclears cells number; **(C)** Neutrophils number; **(D)** Myeloperoxidase activity (MPO). Abbreviations, V = Vehicle group (NaCl, 0,9%, p.o.), Dexa = Dexamethasone, Cg = Carrageenan (750 μg/mice cavity). Symbols # ($p<0.05$), ## ($p<0.01$), ### ($p<0.001$) indicates significant in comparison to vehicle group. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), significant compared to Cg group.

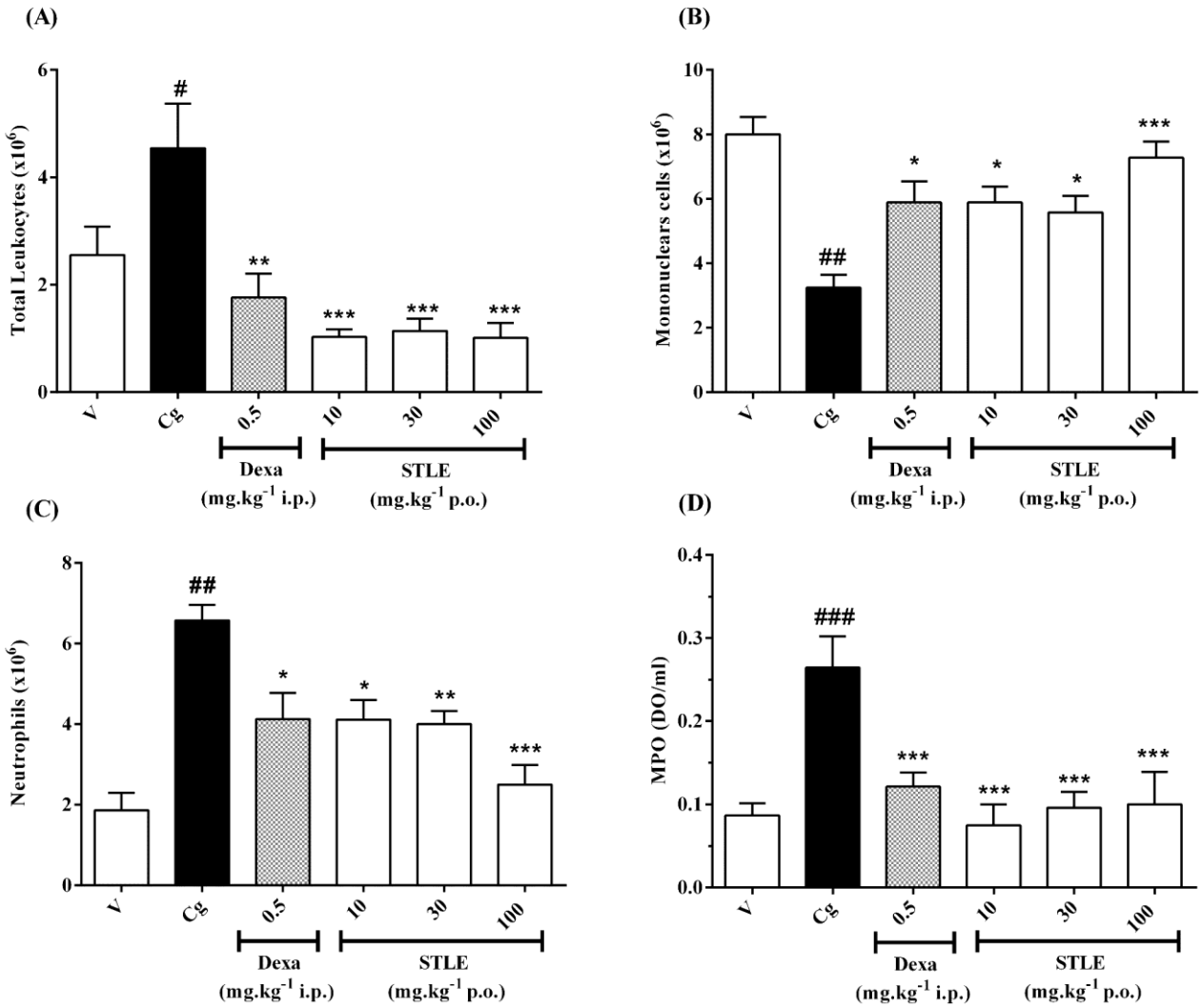


Fig. 5 – Cytokines levels in peritoneal fluid of carrageenan-induced peritonitis in mice pre-treated with *S. tuberculata* leaves extract (STLE 10-100 mg.kg^{-1}, p.o.). **(A)** IL-1β levels, **(B)** TNF-α levels, **(C)** IL-6 and **(D)** IL-10 levels. Abbreviations, V = Vehicle group (NaCl, 0,9%, p.o.), Dexa = Dexamethasone, Cg = Carrageenan (750 μg/mice cavity). Symbols # ($p<0.05$), ### ($p<0.001$) indicates significant in comparison to vehicle group. ** ($p<0.01$), *** ($p<0.001$), significant compared to Cg group.

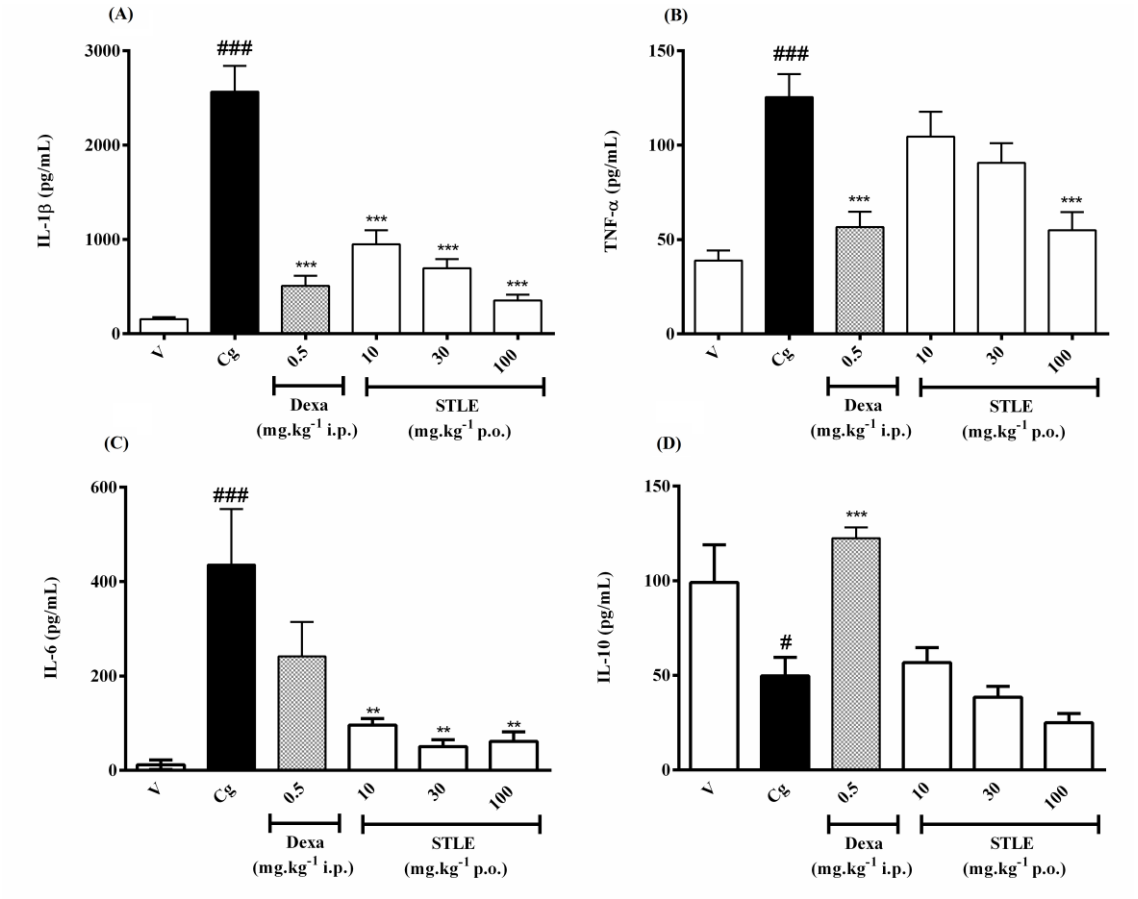
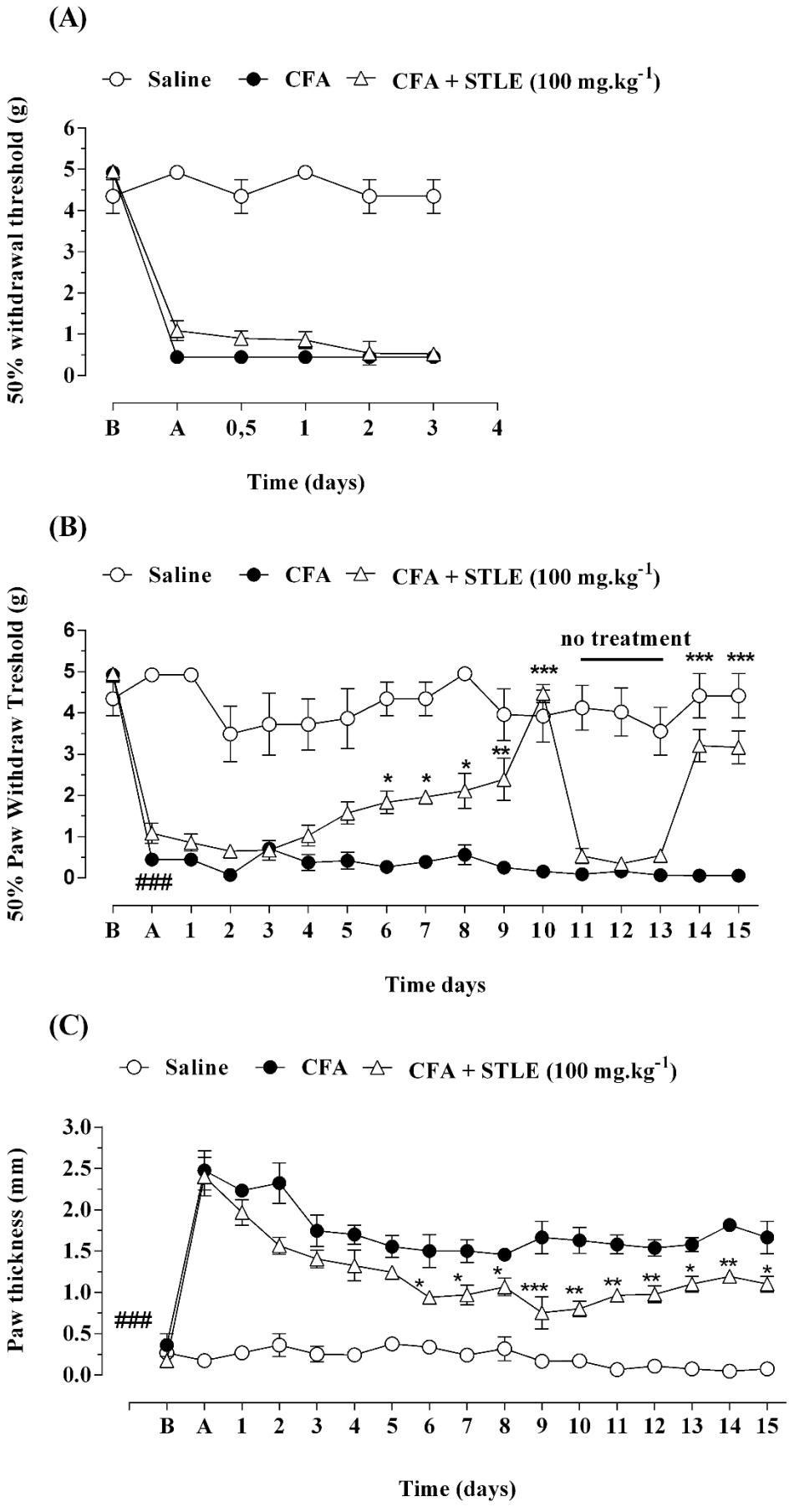


Fig. 6 – Hypersensitivity and paw oedema induced by CFA in mice treated once a day with *S. tuberculata* leaves extract (STLE at 100 $mg.kg^{-1}$, p.o.). **(A)** Mechanical hypersensitivity assessed by von Frey filaments in the first 4 hours after STLE treatment (60 min before), **(B)** Mechanical hypersensitivity assessed by von Frey filaments during 15 days, **(C)** Paw oedema evaluated by electronic caliper. Each group represent the mean± standard error of mean (SEM) from 4-7 animals per group. Abbreviations, CFA = complete Freund adjuvant (20 μL i. pl., 1 $mg.ml^{-1}$ of heat killed *Mycobacterium tuberculosis* in paraffin oil 85% and mannide monoleate 15%), Saline group (NaCl, 0,9%, p.o.). Symbols ### ($p<0.001$) indicates significant in comparison to saline group. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), significant compared to CFA group.


(A)
Saline
CFA
CFA + STLE (100 mg.kg-1)
50% withdrawal threshold (g)
B
A
0,5
1
2
3
4
Time (days)
(B)
Saline
CFA
CFA + STLE (100 mg.kg-1)
50% Paw Withdraw Treshold (g)
no treatment
###
Time days
(C)
Saline
CFA
CFA + STLE (100 mg.kg-1)
Paw thickness (mm)
###
Time (days)

Tables

Table 1 - Relative organ weights of mice orally treated with STLE (100 $mg.kg^{-1}$) for 15 days during CFA model.

Groups	**Organs evaluated**				
	Heart	**Lungs**	**Liver**	**Spleen**	**Kidneys**
Saline	4.15 ± 0.11	4.29 ± 0.20	13.69 ± 0.22	3.14 ± 0.19	7.15 ± 0.08
CFA	3.89 ± 0.14	4.44 ± 0.09	13.26 ± 0.27	3.09 ± 0.16	7.03 ± 0.17
CFA + STLE	4.09 ± 0.09	5.27 ± 0.48	13.83 ± 0.55	3.74 ± 0.22	7.02 ± 0.16

Values are expressed as mean ± standard error of the mean (n = 4-6 for each group).

Manuscript III

Extract optimization by Box-Behnken design and validation of UHPLC method for quantitative analysis of 20-hydroxyecdysone, an ecdysteroid chemical marker in *Sida tuberculata* (Malvaceae)

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Abstract

Sida tuberculada (ST) is a Malvaceae species widely distributed in southern Brazil. In traditional medicine, ST has been employed as hypoglycemic, hypocholesterolemic, anti-inflammatory and antimicrobial. Previous studies revealed its chemical composition contains mainly flavonoids, alkaloids and phytoecdysteroids. The present work aimed to optimize the extractive technique and to validate an UHPLC method for the determination of 20-hydroxyecdsone (20HE). Using ST leaves, the method optimization was performed in two steps (extractive method selection and optimization by Box-Behnken Design (BBD) using the statistical software MiniTab 17®. The extractive methods tested were: static and dynamic maceration, ultrasound, ultra-turrax and reflux. In the Box-Behnken three parameters were evaluated in three levels (-1, 0, +1), particle size, time and plant:solvent ratio. In validation method, the parameters of selectivity, specificity, linearity, limits of detection and quantification (LOD, LOQ), precision, accuracy and robustness were evaluated. The results indicate static maceration as better technique to obtain 20HE peak area in ST extract. The optimal extraction from surface response methodology was achieved with the parameters granulometry of 710nm, 9 days of maceration and plant:solvent ratio 1:54 (w/v). The UHPLC-PDA analytical method provided a selective, linear, precise, accurate and robust method for 20HE detection in ST leaves. The average content of 20HE was 0.56% per dry extract. Thus, the optimization of extractive method in ST leaves increased the concentration of 20HE in crude extract, and a reliable method was successfully developed according to validation requirements and in agreement with current legislation.

Key words: Sida, phytoecdysteroid, 20HE, UHPLC, validation, Box-Behnken Design

1. Introduction

A significant number of Sida species have been investigated as potential therapeutic source such as *S. cordifolia* [1], *S. rhombifolia* [2], *S. acuta* [3], *S. spinosa* [4] and *S. cordata* [5]. The genus Sida (Malvaceae), consisting of over 200 species widely found in tropical and subtropical regions, has different plant parts used by traditional medicine [6].

In this context, *Sida tuberculata* (ST), characterized as a perennial or annual, erect, fibrous, branched sub-shrub species, is a widespread Malvaceae in southern Brazil and commonly used in folk medicine. Traditionally, *S*T is used to treat hyperglycemia, hypercholesterolemia, inflammatory process and some infections [7]. Recently, extracts from ST leaves and roots presented a significant antioxidant activity, anti-biofilm and anti-microbial action against *Candida krusei* strains [7, 8]. These properties described for ST can be attributed, in part, to the chemical composition from your leaves and roots, which is mainly composed of sterols, alkaloids and flavonoids. Among these, phytoecdysteroids are present in large amounts in ST leaves and roots [7,8]. As describe in literature, these compounds have already been detected for other Sida species [9, 2, 4]

Ecdysteroid is the general name for a class of arthropods steroidal hormones, responsible for moulting control (ecdysis) and others metamorphosis processes in this animal group [10]. However, in the 60's an analogous class was discovered in a variety of plant species, they were designated as phytoecdysteroids [11]. This class is derived from cholesterol or other plant sterols and it presents a polyhydroxylated chain with four-ringed skeleton, commonly composed between 27 and 29 carbon atoms [12]. More than 300 phytoecdysteroids have been identified in terrestrial plants, being the 20-Hydroxyecdysone (20HE) the most widely found. Although the phytoecdysteroids roles in plants remains unclear, the probable hypothesis is that they offer protection against phytophagous insects and/or soil nematodes [13, 14]. In vertebrates, studies have been showed that phytoecdysteroids exert numerous effects, especially anabolic actions [15-18].

The analytical methods applied to phytoecdysteroids analysis, especially interfaces including liquid chromatography and mass spectrometers, allow the successful in detection [19]. High performance liquid chromatography (HPLC) method has been an important analytical tool for phytoecdysteroids analyses in complex mixture as plant extracts [21]. In this sense, Zimmer et. al. [22] validated a method and quantified ecdysterone in *Pfaffia glomerata using a RP-18 column* with acetonitrile:water isocratic elution and detection with UV at 242 nm. Wang et al. [9] performed HPLC experiments, using reversed phase system, coupled with mass spectrometry and identified seven phytoecdysteroids in *S. rhombifolia*. Chen et al. [23] developed an HPLC method for quality control and detection of, among others, 20HE as a marker in medicinal herbs mixture. Gallo et al. [19] developed a HPLC method to detect 20HE in *Vitex polygama* using a reversed phase system. In addition, Da Rosa et. al. [7] previously described an efficient HPLC method to determinate the chemical composition of ST using a gradient chromatographic conditions, which were intended to be validated in this work.

Therefore, the present study aimed to determine the most effective condition to extract 20HE and validate an UHPLC-PAD method to analyze and quantify this chemical marker in leaves extracts from *Sida tuberculata*.

2. Materials and Methods

2.1 Chemicals

The chemical standard 20HE was purchased from Sigma Aldrich. Acetonitrile and phosphoric acid were purchased was purchased from Merck and Vetec, respectively. Purified water was obtained using the Milli-Q Plus® system from Millipore (Milford, MA, USA). All other reagents used in this study were of analytical or HPLC grade.

2.2 Plant material

Whole plant specimens were collected in Uruguaiana (Rio Grande do Sul, Brazil) in the western Brazil, border with Argentina, under geographical coordinates 29º50'06.3''S and 57 º05'50.5''W. The plant taxonomic classification was identified by botany department from Universidade Federal do Rio Grande do Sul, and voucher specimens were deposited at the Herbarium ICN (ICN 167493). The plant material was separated into leaves and oven dried at 40º C for 5 days.

2.4 Determination of Loss on drying

To determine the amount of volatile substances in the ST leaves, loss drying assay was performed according Brazilian Pharmacopoeia [24]. For this, 1g of samples (180 µm mesh) were transferred for weighting bottles (previously dried), and were placed in the heater at 105°C for 2 hours. After that, weighting bottles containing samples were cooled at room temperature in desiccator. To calculate the percentage of loss drying, the weighting bottles were weighed on analytical balance and the difference between the initial weight and final weight of the sample plus “weighting bottles” was estimated. The analysis was performed in triplicate.

2.3 Extraction experimental design

In order to obtain effective results in extract content of 20HE (Fig. 1), ST leaves were ground and submitted to different extraction conditions. The optimization of extractive method was performed in two phases: extractive method selection and optimization by BBD using the statistical software MiniTab 17®. The solvent utilized, ethanol 40%, was determinate according previous analyses described by Rosa et al. [8].

2.3.1 Extractive methods tested

The extractive methods tested were static and dynamic maceration, ultrasound, ultra-turrax and reflux extraction. These conditions were employed under different times according Table 1.

2.3.2 Optimization of extractive method

The optimization of the 20HE extraction process in dried leaves of ST was performed from the experiments carried out in the BBD . Three parameters were evaluated in three levels (-1, 0, +1), particle size, time and plant:solvent ratio, as shown in Table 2.

The peak area, corresponding to 20HE, in the chromatograms, was evaluated as a response in each BBD experiment by UHPLC (conditions described below). Before analyses, the extracts were added to 25 mL with 40% ethanol and filtered through a 0.22 µm membrane filter (Millipore) and injected directly.

2.4 Apparatus and chromatographic conditions

The UHPLC analyses were conducted using a Acquity UPLC® (Waters Co., MA, USA) PDA detector. A Acquity UPLC® BEH C-18 (1.7 µm x 2.1 mm x 50 mm) was used. Data acquisition was performed with Empower 3 software. The mobile phase consisted of water containing 0.05% phosphoric acid (A) and acetonitrile (B) at a flow rate of 0.08 mL/min using the following gradient: 0.01–16 min, 10–40% solvent B; 16.01–25 min, a fixed proportion of 10% solvent B and 90% solvent A. The injection volume was 5 µL, and the analysis was at 250 nm using a PAD detector. The mobile phase was prepared daily, filtered through a 0.22-µm membrane filter (Millipore) and sonicated before use.

2.5 Validation

The method was studied according to the validation of analytical methods from regulation RE 899/2003 of National Health Surveillance Agency, Brazil, and per International Conference on Harmonization (ICH Q2(R1) [25, 26].The

sample consisted of ethanolic extract (40%) from ST leaves obtained by static maceration as preconized by extract optimization.

2.5.1 Specificity/Selectivity

Illustrate the method ability to discern between the interest analyte and other interference components in the sample. Thus, the selectivity of the method was verified by the analysis of the ultraviolet (UV) profiles of the sample and standard, peak pipe reference to 20HE and by the analysis of the sample solution with and without addition of standard solution.

2.5.2 Linearity

The linearity test was performed from three analytical curves, constructed with 10.42, 31.25, 52.08, 72.91, 93.74 µg/mL, in order to evaluate the linear correlation between the chemical standard marker of ST, 20HE, and the areas obtained in the UHPLC method. Data from each concentration level (injected in duplicate) was evaluated by the value of coefficient correlation (r) of the calibration curve. From data obtained, it was constructed standard plots, which were evaluated statistically by linear regression analysis through least square method and Analysis of Variance (ANOVA).

2.5.3 Limit of detection (LOD) and Limit of quantitation (LOQ)

Calibration curves of the 20HE standards and signal to noise ratio were used for calculation of LOD and LOQ. The ratios 3:1 and 10:1 were used to determinate LOD and LOQ respectively, from the study of linearity [25].

2.5.4 Precision

The method precision was analyzed with respect to intraday repeatability and intermediate precision (inter-day). The precision test (repeatability intraday)

was performed by six injections within a day, with samples obtained from six extractions. The inter-day precision was determined through three injections in two different days in order to determine the dispersion between two days of samples extractions. The content of 20HE in each sample was determinate through UHPLC method described above, and relative standard deviation (RSD%) of average levels determined for the 20HE.

2.5.5 Accuracy

The accuracy was determined by means of recovery test. For this, known amounts of the 20HE standard was added to the sample, at three different levels (80%, 100% and 120%) of initial sample concentration. Thus, 20HE recovery evaluation presented final concentration at 24.0, 30.0 e 36.0 µg/mL. Each sample was injected in triplicate into UHPLC (conditions described previously) and the average recoveries were calculated by formula recovery (%) = {(amount found - original amount)/amount spiked} x 100.

2.5.6 Robustness

The robustness was evaluated by small and deliberate variations on analytical parameters and the obtained results were compared with the results of solutions analyzed under the usual conditions described in the method, by means of the relative standard deviation (RSD%) [20]. The parameters assessed were wavelength (245 and 255 nm), mobile phase flow (0.075 and 0.085 mL/min) and column (other lot).

2.6 Statistical analysis

Results are expressed as mean ± RSD% from extraction optimization and method validation. A BBD design was used to determine an extraction condition. Average calibration curve and the equation obtained from the standard linear regression were used to quantify 20HE in ST extract. Data from linearity were subjected to ANOVA.

3. Results and Discussion

3.1 Plant material characterization

Data obtained from loss of drying determination presented 11.07 % of water content in leaves from ST. The value found agrees to the maximum and minimum limit (14 and 8 % respectively) of water content for plant sample established by Brazilian Pharmacopoeia [24].

3.2 Optimization of the extraction procedure

Before analytical method validation, we assessed different extractive methods on 20HE obtainment. First, each method tested was considered by 20HE peak area compared to the standard substance. The extraction techniques evaluated were the follow: static and dynamic maceration, ultrasound, ultra-turrax and reflux method. The results are showed in Table 3. All these parameters used ethanol 40 % as solvent.

According to Table 3, maceration static during 7 days produced satisfactory results. In a view of it is a classic and not damage method, maceration was selected for validation study. This extractive method has already been used as important tool in the obtaining active compounds from Sida species [27-29]. It is important to emphasize that different studies report ethanol [30-32] and mainly methanol [33-35, 9] in phytoecdysteroids extraction. However, Dinan et al [37] in a review about chromatographic procedures for the isolation of plant steroids, including phytoecdysteroids, report that the both solvents can be used for this purpose due the moderate polarity of phytoecdysteroid. In addition, here we performed an extraction using methanol under magnetic stirring during four hours, and the data showed that 20HE extraction is about 48% less than that used in this study.

An optimum extraction process should be defined in order to obtain a high 20HE content. Thus, in sequence, we evaluated through BBD three parameters in extraction experiments in order to verify the effect of these variables on the mean peak area of 20HE in ST extract. BBD is an efficient mathematical tool applied for the optimization of several chemical and physical processes, very

useful in to determine extraction parameters [38, 39]. The conditions assessed by BBD range among the particle size (x1), time (x2) and plant:solvent ratio (x3). Table 4 presents the experimental design used and the experimental results obtained. 20HE yield (%) was selected as the response. As can be seen, 15 analyses generated by BBD using software MiniTab® 17 were performed. Results predicted the variables from 355 to 710 mesh, 7 to 10 days, and 1:20 to 1:60 are the most effective in 20HE extraction.

Table 5 describe the analysis of variance (ANOVA) for the response surface quadratic model applied in this work. According Table 5 the quadratic polynomial model used in this study presented $R^2 = 0.85$, ensuring that model was adequate. Le Man et al. [40], describe a model appropriate when R^2 shows values > 0.75. The ANOVA showed that this regression model was significant with $p < 0.05$, being to be able of influence the process evaluated in a significant way. In addition, the lack-of-fit for parameters assessed were not significant ($P > 0.05$) with F-value of 8.74, confirming the model reliability within variables processed in the conditions of this study. As shown in Table 5, the variable with the greatest linear ($p < 0.05$) and quadratic ($p < 0.1$) effect within the parameters investigated was particle size (codes x1 and $x1^2$). The others term coefficients did not influence the 20HE extraction significantly. Thus, considering the response surface graphic (Fig. 2), the software predicted optimized conditions as particle size 710 µm, 9 days and 1:54 (w/v) the plant/solvent ratio. These results are the first described in the literature for the present species.

In sequence, we performed five extraction from ST leaves on the optimized conditions presented by BBD. The data achieved revealed that the content extracted of 20HE was similar to that predicted by the software (1709293 with RSD% at 4.5)

3.2 Chromatographic profile

In the quality control of raw material and plant extracts, UHPLC fingerprint analysis is a very useful technique. Thus, in order to separate and become easy the compounds identification, we developed an UHPLC method for identification

and quantification of 20HE in ST hydroethanolic extract. Due its complex mixture, a gradient system was employed for extract analyses (specifications described above). Fig. 3 shows the chromatographic profiles obtained from ST extract pointing main constituent and chromatographic profile from 20HE standard solution. 20HE eluting at 10.8 min proved to be the major phytoecdysteroid peak in ST extract, and was selected as the species chemical marker. Previous studies of our group, using a similar methodology, allowed to find phytoconstituents from ST and detect 20HE as major compound, including roots extract [7, 8]. Gallo et al [19] also used reversed phase system and gradient elution in a developing and validation HPLC method for 20HE in detection in *Vitex polygana*.

3.3 Validation of the UHPLC method for *S. tuberculata* extract

As integral and necessary part in quality control of the plant material, the validation of an efficient method support the safety and effectiveness of the interest compound [41]. Therefore, in sequence, we describe a validation of analytical method for 20HE detection in ST extract.

3.3.1 Specificity/Selectivuty

Was determined checking UV profiles of the sample and standard, peak pipe reference to 20HE and by the analysis of the sample solution with and without addition of standard solution . The assessed method showed specificity at 250 nm detection for the leave extract of *S. tuberculata,* demonstrating reliability in the 20HE quantification. As can be seen in this Fig. 4, the UV periphents of the sample and pattern are similar, the 20HE peak slices shows no impurity and the addition of pattern to the sample results in incrementing only in the 20HE area.

3.3.2 Linearity, LOD and LOQ

Three calibration curves obtained on three different days were used to determine the linearity between peak area and concentration of 20HE. The solutions of reference standards with five points ranging from 10.42 to 93.74 µg/mL were injected in triplicate each day. The curves obtained were plotted in Fig. 3. The correlation coefficient (r) and the linear regression equation were determined on the experiments; (1) y= 34406x – 18240, r = 0.9998, (2) y = 34567x – 29090, r = 0.9995 and (3) y = 35427x – 11659, r= 0.9984. The data for the linearity parameter are shown in Table 6. The ANOVA showed the proposed method was adequate and has significant regression ($p<0.05$), since the *calculated F* value (5,631) was greater than the *critical F* value (5.21) for a confidence level of 95% ($p = 0.05$).

These results indicate that the linearity in the concentrations tested, for 20HE reference standard quantification, is in agreement with the RE 899 specifications (ANVISA Validation Guide) and ICH [25, 26].

The LOD and LOQ values were determined from the 20HE calibration curves as recommended by official guidelines [25, 26]. As shown in Table 6, the LOD and LOQ values were 2.631 and 7.973 µg/ml respectively, which indicate the sensitivity of the method.

3.3.3 Precision

The precision was investigated on two different levels: intraday and inter-day precision. In the intraday analysis, six extractions were performed and assessed in sequence. For inter-day assay, the analysis were performed in triplicate in two consecutive days .The 20HE amount in repeatability assay was 0.5614 ± 0.024 % (RSD = 4.29), while in intermediate precision that marker was found in concentration of 0.5656 ± 0,012 (RSD = 0.76) (Table 7). The precision showed a RSD% less than 5% in both analyses, intraday and inter-day demonstrating the ability of the method to obtain reliable results.

3.3.4 Accuracy

In order to investigate the method accuracy, a recovery test was carried out on ST hydroethanolic extracts that were spiked with known amounts of

20HE standard solution (three concentrations at 24.0, 30.0 and 36.0 µg/mL). The analytical data for three determinations are shown in Table 8. The mean recovery for ST extract was 95.4% with 0.32 %RSD. Thus, the results obtained demonstrate that method was considered accurate for 20HE determination in ST hydroethanolic extract according to criteria from official guidelines [25, 26].

3.3.5 Robustness

Robustness was assessed by introducing small but deliberate variations in flow mobile phase, wavelength and column during the chromatographic analysis. However these alterations did not affect significantly the analytical results (Table 7). Comparing the data from original chromatographic conditions and the variations introduced, the RSD was less than 5 %, evidencing the method robustness.

4. Conclusion

In the present study, initially the influences of extraction techniques, maceration, reflux, ultrasound and ultra-turrax, under different times, were evaluated in 20HE extraction from ST leaves. The classical maceration method presented satisfactory results and was selected for an extractive optimization. Therefore, the effect of granulometry, time and plant/solvent ratio was assessed by response surface methodology using Box-Behnken design. The better 20HE extractive condition predicted was granulometry of 710 nm, 9 days and plant/solvent ratio of 1:54 (w/v). Under these conditions, the experimental values were in agreement with the predicted values. Moreover, we performed an UPLC method validation with 20HE compound as a marker in ST extract. Thus, the method showed effective specificity, linearity, accuracy, precision and robustness, being useful for the analysis of this marker in ST leaves extracts.

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Figures

Figure 1 – Representative 20-Hydroxyecdysone (20HE) structure.

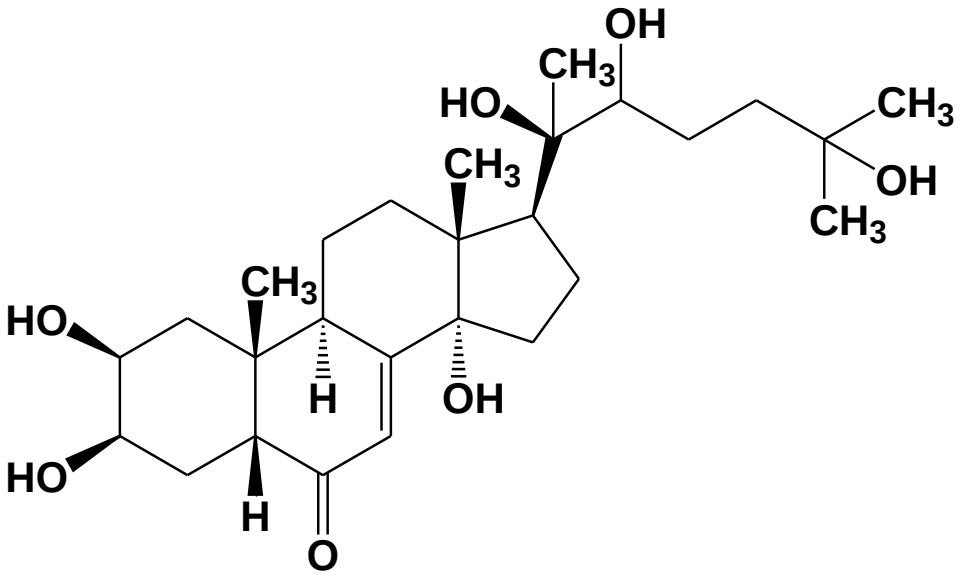


Figure 2 – Response surface graph in *S. tuberculata* referring to the area of 20HE

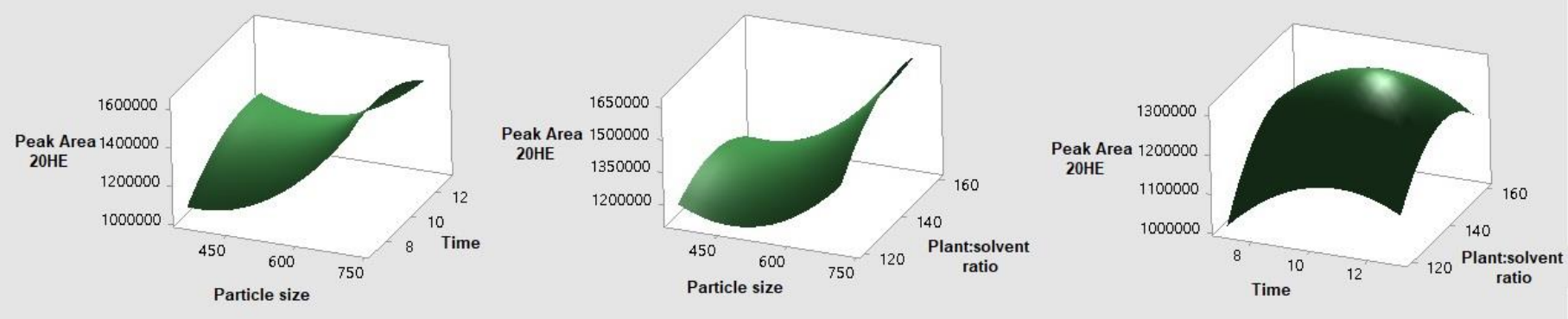


Figure 3 – UPLC profile obtained from *S. tuberculata* leaves extract, indicating the major compound 20-Hydroxyecdysone (A). Chromatogram from 20-Hydroxyecdysone standard (B). Analyses achieved at 250 nm.

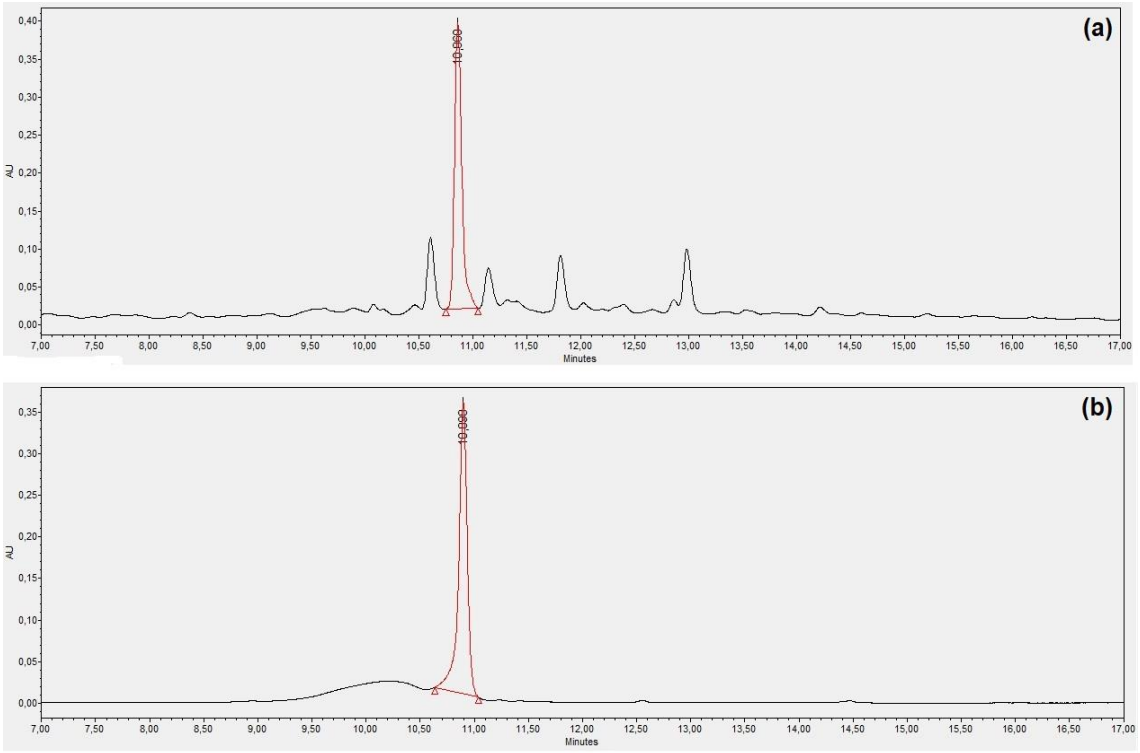
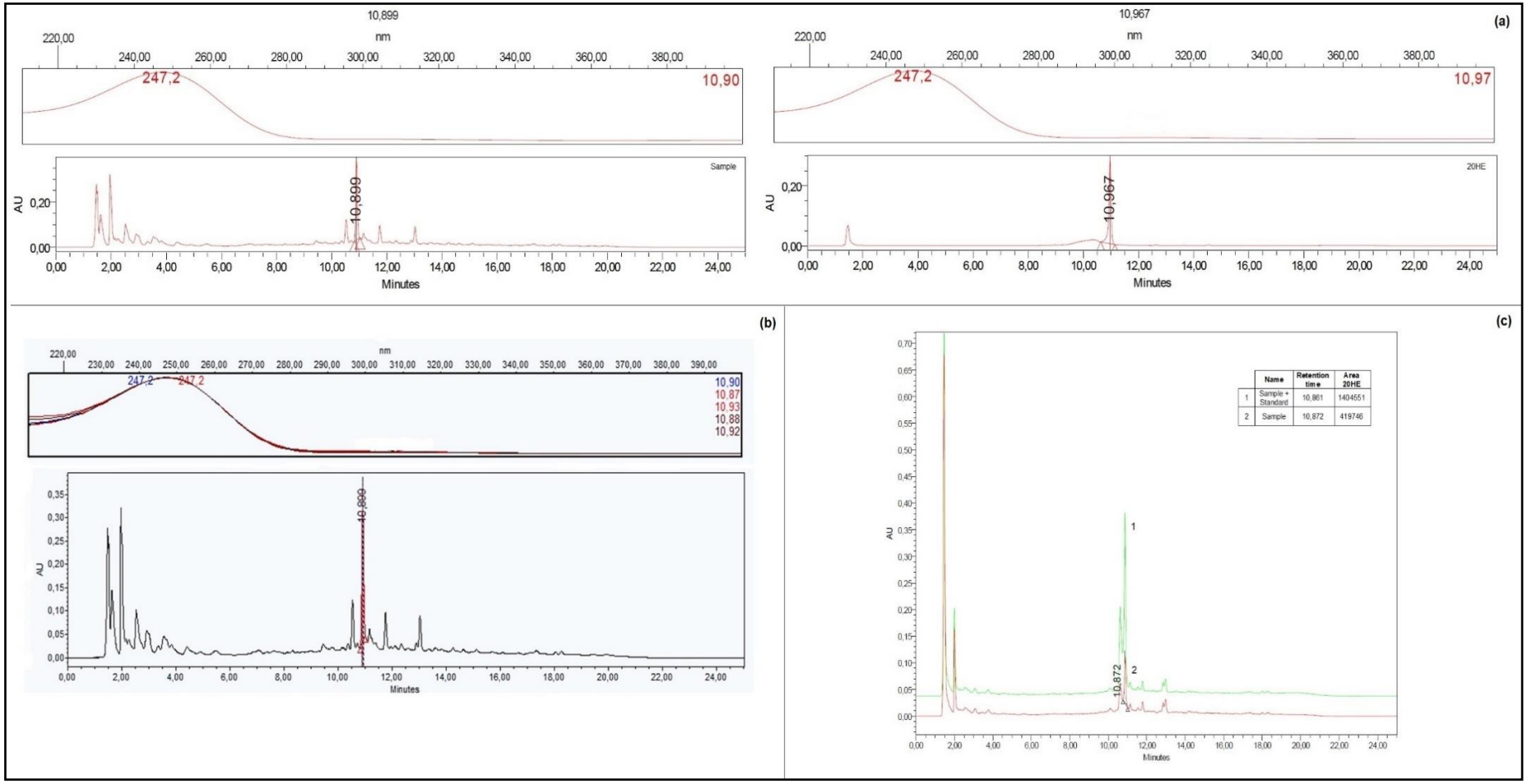


Figure 4 – Results of Specificity/Selectivity: (a) Comparative UV profiles and sample standard; (b) Peak shedding; (c) Sample with and without addition of standard



Tables

Table 1 – Extractive methods tested for 20HE extraction from *S. tuberculata* leaves.

Predetermined parameters	**Parameters evaluated**	
	Technique	***Time***
Leaves *Ethanol 40 %* *Plant:solvent ratio (w/v) 1:10* *Particle size 180 µm*	Maceration static	7 days
	Maceration dynamic*	45 min
	Ultrasound	30 min
	Ultra-turrax*	15 min
	Reflux*	30 min

* Maceration dynamic with constant agitation. * Ultra-turrax used at 600 rpm.
* Reflux condition at 40ºC.

Table 2 - Variables assessed by BBD in the extraction optimization of 20HE in *S. tuberculata* extract.

Variables	**Codes**	**Levels**		
		- 1	**0**	**+ 1**
Particle size (µm)	*x1*	180	355	710
Time (days)	*x2*	7	10	13
Plant:solvent ratio (w/v)	*x3*	1:20	1:40	1:60

Table 3 – 20HE achievement under different extraction techniques in *S. tuberculata* leaves.

Parameters evaluated			
Technique	***Time***	***20HE peak area***	***RSD%***
Maceration static	7 days	7334518	0.19
Maceration dynamic	45 min	1856054	2.63
Ultrasound	30 min	1761624	0.40
Ultra-turrax	15 min	1867487	0.06
Reflux	30 min	1264573	0.89

Table 4 – Parameters assessed by BBD design on extraction from *S. tuberculata* leaves and response in 20HE peak area prediction.

Order	x1 (µm)*	x2 (Days)	x3 (w/v)**	20HE peak area
1	355	7	140	**1140241**
2	710	7	140	**1535429**
3	355	13	140	**1336830**
4	710	13	140	**1424020**
5	355	10	120	**1214881**
6	710	10	120	**1533663**
7	355	10	160	**1018621**
8	710	10	160	**1626109**
9	532,5	7	120	**925325**
10	532,5	13	120	**1032093**
11	532,5	7	160	**1246137**
12	532,5	13	160	**1262513**
13	532,5	10	140	**1330892**
14	532,5	10	140	**1234698**
15	532,5	10	140	**1327879**

* 532,5 = 500 µm ** 120 = 1:20; 140 = 1:40; 160 = 1:60

Table 5 – Analysis of variance (ANOVA) for the response surface quadratic model in the optimization of parameters for 20HE extraction from *S. tuberculata* leaves.

Variable	Degrees of Freedom	Sum of squares	Mean square	*F*-value	*p*-value
x1	1	2.48036	2.48036	14.6	0.012*
x2	1	5424861122	5424861122	0.32	0.595
x3	1	25022914268	25022914268	1.48	0.278
$x1^2$	1	79303819056	79303819056	4.70	0.082#
$x2^2$	1	26832332605	26832332605	1.59	0.263
$x3^2$	1	34070043559	34070043559	2.02	0.215
x1 x2	1	23715692001	23715692001	1.40	0.289
x1 x3	1	20837716433	20837716433	1.23	0.317
x2 x3	1	2042723612	2042723612	0.12	0.742
Lack-of-fit	3	78445802990	26148600997	8.74	0.104
Pure error	2	5981750647	2990875324		
Total	14	5.61075			

x1= particle size; x2= time; x3= plant/solvent ratio; *p<0.05; #p<0.1

Table 6 – Linearity assay for validation of quantitative UPLC method to determine phytoecdysteroid 20HE, with LOD and LOQ values.

Concentrations tested (µg/mL)	**Peak area (AU)***	**RSD %**
10.42	371536 ± 5722,57	1.54
31.25	1130544 ± 31395,78	2.77
52.08	1838689 ± 1427,26	0.07
71.91	2516387 ± 9269,89	0.36
93.74	3303004 ± 71431,32	2.16
Calibration curve equation	y = 34800 x + 19663	
Correlation coefficient (r^2)	0.9995	
LOD	2.631 µg/mL	
LOQ	7.973 µg/mL	

*Mean ± SD (n = 3).

Table 7 – 20HE average concentration detected in *S. tuberculata* extract during intraday and inter-day precision and robustness evaluation.

Validated conditions	**Concentration tested (µg/mL)**	**Average concentration detected (%/g)**	**RSD (%)**
Precision			
Intraday*		0.5614	4.29
Inter-day (day 1)	56.0	0.5559	3.08
Inter-day (day 2)		0.5794	0.76
Inter-day (repeatability)		0.5656	2.17
Robustness[#]			
Normal conditions		0.5363	0.57
Flow 0.75 mL·min^{-1}		0.5333	0.78
Flow 0.85 mL·min^{-1}	56.0	0.5291	1.93
Wavelength (λ = 245 nm)		0.5427	1.05
Wavelength (λ = 255 nm)		0.5493	2.05
Different column batch		0.5468	2.71

* 6 analyses; [#] n = 3

Table 8 – Recovery assay performed during validation of UHPLC method for quantitation of 20HE in *S. tuberculata* extract.

Concentration*		Recovery (%) ± SD	Average Recovery (%) ± SD	RSD (%)
added (µg/mL)	recovered (µg/mL)			
20	35.0	95.6 ± 0.20		
30	40.7	95.5 ± 0.27	95.4 ± 0.30	0.32
36	46.2	95.0 ± 1.01		

*n=3.

5 DISCUSSÃO GERAL

Este estudo objetivou investigar o efeito antinociceptivo e anti-inflamatório dos extratos das folhas de ST em modelos de dor aguda e crônica em camundongos tratados por via oral. Além disso, o trabalho caracterizou fitoquimicamente os extratos das folhas e raízes de ST e avaliou o potencial antioxidante e citotóxico *in vitro* dos mesmos. Assim, este tópico se propõe a uma abordagem geral não cronológica dos principais resultados encontrados.

ST é classificada como uma espécie infestante, pioneira, caracterizando-se como plantas que iniciam a cobertura de um solo, com crescimento rápido, agressivo e tolerante a condições adversas (CARVALHO & PITELLI, 1992; KISSMANN & GROTH, 2000). Na medicina popular, é utilizada para tratar processos inflamatórios, diabetes, hipercolesterolemia e alivio da dor em picadas de inseto (ROSA et al., 2015; 2016). Portanto, baseado no conhecimento etnofarmacológico surge o interesse em investigar uma espécie pouco exigente a condições de solo e adaptada a região do Pampa.

Em trabalhos prévios do nosso grupo (ROSA et al., 2015) identificou-se uma diversificada composição química nos extratos de ST, formada por fitoecdisteróides, alcaloides e flavonoides. Neste trabalho, utilizando técnicas analíticas de maior performance (HPLC; UHPLC, LC-MS), determinou-se os fitoecdisteróides como classe majoritária nas diferentes técnicas extrativas testadas. Além disso, o composto 20-hidroxiecdisona (20HE) foi proposto como o mais abundante, tanto em folhas quanto em raízes de ST. Esses achados corroboram com estudos realizados em outras espécies do gênero *Sida*, os quais mostram uma diversificada presença de fitoconstituintes, composta principalmente por fitoecdisteróides (ARCINIEGAS et al., 2017; CHAVES et al., 2017 e 2013; WANG et al., 2008; SILVA et al., 2006; DARWISH & REINECKE, 2003).

Dinan et al. (2001), estudando 11 espécies de *Sida*, concluíram que *S. acuta*, *S. filicaulis* e *S. rhombifolia* possuem significativas quantidades de 20HE entre outros derivados ecdisteróides, e que por essa razão essa classe de compostos possui um valor quimiotaxonômico relevante dentro do gênero *Sida*. Vale ressaltar que mais de 300 ecdisteróides têm sido identificados e isolados de fontes animais e vegetais, onde 20HE é o mais representativo ecdisteróide em plantas (LAFONT & DINAN, 2003). Por ser

um polihidridroxilado esteroidal, algumas propriedades farmacológicas tem sido encontradas para o composto 20HE, tais como neuroprotetor, imuno-modulador (inibidor NF-κB), anti-inflamatório e principalmente um efeito anabólico (ativador da via Akt/Nrf2) (LIU et al., 2016; CSÁBI et al., 2015; PESCHEL et al., 2011; BÁTHORI et al., 2008). Entretanto, ainda é necessário ter uma melhor compreensão das ações farmacocinéticas, metabólicas e toxicológicas destes fitoecdisteróides em mamíferos.

Dentre as etapas de um estudo de plantas com potencial medicinal, além das análises de propriedades farmacológicas, existe a necessidade de garantia da identidade botânica com base em seus constituintes químicos (SIMÕES et al., 2003). Dessa forma, a identificação de uma espécie vegetal em estudo pode ser padronizada através de marcadores químicos, que podem ser característicos de cada espécie, ou estar presente em grandes quantidades no espécime (BARNES, 2003). Assim, deve se estabelecer e desenvolver um método cromatográfico que sirva como alternativa para assegurar identidade de um material vegetal, além da identificação botânica. Por isso, o chamado "*fingerprint"* cromatográfico de uma planta medicinal é uma técnica cromatográfica padrão que pauta características químicas comuns dos componentes de uma amostra vegetal (GONG et al, 2003; SIMÕES et al., 2003).

Assim, no presente trabalho foi desenvolvido e validado um método cromatográfico (UHPLC) para a determinação de 20HE em um extrato hidroetanólico das folhas de ST (Manuscrito III). Os dados obtidos mostram que o método desenvolvido por UHPLC (usando acetonitrila e ácido fosfórico a 0,05% em modo gradiente) é adequado para análise de quantificação e reconhecimento de 20HE em extratos de ST, se mostrando seletivo, específico, linear, exato, preciso e robusto conforme as normas estabelecidas pela ANVISA, resolução RE 089, e ICH (2005).

Nossos dados também mostraram que tanto extratos das folhas quanto das raízes apresentam um significativo ($p<0.05$) efeito neutralizador (*scavenger*) de espécies reativas. Esses achados estão de acordo com Arciniegas et al. (2017), Pawa et al. (2011) e Shah et al. (2013), os quais reportaram atividades *scavenger* de radicais livres para *S. acuta, S. rhombifolia, S. cordifolia e S. cordata*. Os ensaios utilizados nesse trabalho permitiram sugerir que a atividade antioxidante do extrato se dá por meio de duas possíveis ações: transferidora de átomos de Hidrogênio (ensaios que geram radicais peroxila termicamente) e/ou doadora de elétrons (ensaios que medem o poder redutor frente a um oxidante) (SHAHIDI & ZHONG, 2015).

Esta capacidade antioxidante é uma ação importante, uma vez que radicais livres e espécies reativas do oxigênio e do nitrogênio (EROs e ERNs respectivamente) estão envolvidas em diversos processos patológicos e no envelhecimento (VALKO et al., 2007). Quando ocorre uma geração em excesso dessas espécies reativas ou uma depleção das defesas antioxidantes (SOD, Catase, Glutationa redutase e Glutationa peroxidase), pode se estabelecer o estresse oxidativo, o qual pode contribuir na etiologia e na progressão de diversos agravos, entre estes cardiopatias, aterosclerose, diabetes, mutagênese e carcinogênese (HALLIWELL & GUTTERIDGE, 1999; MOSKOVITZ et al., 2002). Dessa forma, compostos antioxidantes são aliados importantes na prevenção, tratamento de várias enfermidades. Além disso, observou-se que o efeito antioxidante foi mais pronunciado no extrato das folhas do que nas raízes. O que, pelo menos em parte, se explica pelo maior teor de fenólicos totais e flavonoides totais nas folhas dentro das condições extrativas avaliadas (folhas 2.13 mgEAG/mL e 2.02 mgER/g de material vegetal; raízes 0.48 mgEAG/mL e 1.90 mER/g de material vegetal) (ROSA, 2013).

Também conforme análises fitoquímicas do nosso grupo foi identificado nas folhas de ST o composto Canferol-(*E*-p-coumaroyl)-glicopiranosídeo, outro majoritário junto com 20HE (ROSA et al., 2015 e 2016). Estes compostos fenólicos, como os flavonoides, são conhecidamente neutralizadores de radicais livres e quelantes de íons metálicos (FIRUZI et al., 2007; PIETTA et al., 2000), podendo estar entre os responsáveis pela ação *scavenger* observada. Entretanto, em vista da diversidade estrutural presente nas folhas e raízes de ST, estas propriedades antioxidantes dos extratos brutos pode, em certas situações, ser atribuída a um sinergismo entre as moléculas presentes no extrato e não necessariamente a um composto ou classe únicos.

Para o screening toxicológico, os dados mostraram que os extratos metanólicos obtidos das folhas (STLE) e raízes (STRE) quando incubados com leucócitos humanos, não apresentaram significativo potencial genotóxico e mutagênico. Além desse dado, foram gerados dados *in silico* sobre o potencial toxicológico do composto majoritário 20HE, e os mesmos também apontaram uma baixa predição para genotoxicidade, cardiotoxicidade, toxicidade reprodutiva e irritante de pele e olhos. Entretanto, STLE mostrou uma significativa redução na viabilidade celular a partir da dose de 1 µg/mL. Mesmo que o maior efeito tenha atingido aproximadamente 23% de redução, gerou-se

perspectivas para um possível efeito antiproliferativo de STLE e STRE frente a células tumorais.

Assim, STLE e STRE foram avaliados frente a duas linhagens tumorais, HepG2 e MCF-7, comparado a linhagem não tumoral 3T3. De acordo com os dados, os dois extratos avaliados apresentam um moderado grau antitumoral, pois apresentaram, na maioria, valores de IC_{50} superiores a 500 µg/mL. Esses dados estão de acordo com a maior parte dos resultados encontrados na literatura que mostram uma ação antiproliferativa classificada entre moderada a baixa para diferentes extratos de outras espécies de *Sida* (JOSEPH et al.,2011; PIEME et al., 2010; JANG et al., 2003). Porém ressaltamos que nesses experimentos, o extrato da raiz (STRE) mostrou maior redução frente as linhagens celulares testadas em comparação a ao extrato das folhas, principalmente frente a HepG2 (IC_{50} = 378,97 ± 37,88 µg/mL). Além disso, o conjunto de dados mostrou que a citotoxicidade foi mais pronunciada sobre células tumorais do que a linhagem não tumoral, revelando uma possível especificidade de ação de STLE e STRE. Consequentemente, embora com uma atividade moderada, mais investigações necessitam ser feitas para elucidar o mecanismo de ação antiproliferativo e citotóxico dos extratos de ST frente a células tumorais.

Sob outra perspectiva baseada no núcleo esteroidal da classe majoritária (fitoecdisteróides), surgiu a possibilidade de avaliar o potencial analgésico e anti-inflamatório dos extratos dos extratos de ST. Vale ressaltar que, devido ao maior poder antioxidante detectado no extrato das folhas, selecionamos o extrato destas (abreviados como STLE) para as investigações *in vivo*.

A dor é uma característica comum nas mais variadas condições patológicas (lombalgia, fibromialgia, artrite reumatoide, enxaqueca entre outras). Assim, a dor apresenta vias de ações moleculares complexas, que apesar do atual conhecimento e investimento farmacêutico na pesquisa de novas drogas, as classes de fármacos disponíveis no mercado ainda apresentam limitações no tratamento e/ou graves efeitos colaterais (tais como complicações gastrointestinais, cardíacas e circulatórias) (WOODCOCK et al., 2007; BOURINET et al., 2005). Por esse motivo, estudos que visem conhecer e desenvolver compostos farmacologicamente efetivos e com menores efeitos adversos é contínuo e necessário. Portanto, as plantas constituem uma fonte molecular importante para o estudo da dor e processos inflamatórios. Haja vista que o marco mais importante no desenvolvimento de medicamentos a partir de produtos

naturais (com as espécies *Salix alba* e *Papaver somniferum*) originou-se sobre estudos no tratamento da dor (VIEGAS et al., 2006).

Nossas avaliações começaram primeiramente pela investigação da possível atividade antinociceptiva, utilizando o modelo de injeção (i.pl.) de formalina em camundongos. Nesse modelo é possível avaliar duas fases da dor: neurogênica (decorre da ativação direta dos terminais nervosos nociceptivos) e inflamatória (decorrente da combinação da sensibilização periférica e da medula) (TJØLSEN et al., 1992). Os resultados encontrados mostraram que STLE administrados por v.o. possuem uma ação significativa nas duas fases avaliadas, sugerindo uma possível ação a tanto periférica quando a nível de SNC (MALMBERG & YAKSH, 1995).

Para aferir uma possível ação bloqueadora da dor inflamatória, optou-se por analisar o efeito de STLE no modelo de contorções abdominais induzidas pelo ácido acético em camundongos. Os dados encontrados mostraram um efeito bastante significativo ($p<0.001$) dos extratos (v.o.), sugerindo que o efeito antinociceptivo de STLE pode estar relacionado à inibição da produção de mediadores pró-inflamatórios induzido pelo ácido acético. Os resultados nesses modelos experimentais agudos estão em acordo com dados da literatura que mostram uma ação antinociceptiva para outras espécies do gênero *Sida*. Entre estas estão *S. cordifolia* e *S. rhombifolia* com ação analgésica detectada nos mesmos modelos de ácido acético e formalina (MOMIN et. al., 2014; KONATÉ et al., 2012; BONJARDIM et al., 2011).

Ressalta-se que, embora considerado pouco específico, o ácido acético injetado na cavidade peritoneal do animal promove a liberação vários mediadores como a bradicinina (BK), a substância P (SP) e as prostaglandinas (PGs), bem como algumas citocinas pró-inflamatórias como o IL-4, IL-6, IL-8, TNF-α e a IL-1β (IKEDA et al, 2001; RIBEIRO et al., 2000). Assim, após a constatação de que o STLE se mostrou significativo em modelos de dor aguda, mais especificamente no modelo de ácido acético, buscamos avaliar possíveis mecanismos de ação pelo qual o extrato estaria exercendo este efeito descrito.

O primeiro mecanismo avaliado foi a participação do sistema opióide pela utilização de agonista/antagonista. Foi utilizado naloxona (antagonista não seletivo), e morfina como agonista. Os resultados mostraram que STLE (100 mg/kg^{-1}) tem sua ação antinociceptiva, pelo menos em parte, mediada através de receptores opióides, uma vez que naloxona inibiu parcialmente o efeito do extrato. Acredita-se que os opióides

produzam analgesia primariamente por sua ação no SNC, porém também perifericamente (WHITESIDE et al., 2005; JAFFE & MARTIN, 1990;). Além disso, os opióides estão entre as drogas mais utilizadas no manejo da dor. Seus principais efeitos analgésicos decorrem da interação sobre receptores do tipo MOP ou mu (μ), KOP ou kappa (κ) e DOP ou delta (δ), os quais estão extensamente distribuídos pelo SNC e em células da periferia (PATHAN & WILLIAMS, 2012; KIEFFER & EVAN, 2009). Portanto, compostos de STLE teriam uma suposta interação com algum(s) desses receptores.

Objetivando apontar qual dos principais componentes do extrato (STLE) poderiam estar interagindo sobre receptores opióides, o presente estudo investigou por modelagem computacional o envolvimento dos majoritários 20HE e Canferol-(E-p-coumaroil)-glucopiranosídeo sobre receptores opióides. Os dados revelaram que ambos podem interagir mais especificamente com o receptor MOP. E que ao contrário da hipótese inicial, o derivado flavonoídico (Canferol) teve uma predição maior de interatividade. Esse dado corrobora com o estudo de Ruiu et al. (2015) que demonstrou, com avaliações computacionais, possível ação de flavonoides, como o canferol, em receptores opióides e interação com os mesmos aminoácidos observados neste estudo.

Outro mecanismo estudado, foi o envolvimento do sistema adenosinérgico pela administração do antagonista adenosinérgico A1 (DPCPX) i.p., e o agonista CHA (ciclohexil adenosine). Novamente foi mostrado que o efeito de STLE também envolve a participação do receptor adenosinérgico A1. Nesse sentido existem estudos que indicam que a ativação do receptor A1 produz antinocicepção em modelos de dor pós-operatória (ZAHN et al., 2007), neuropática (SCHADDELEE et al., 2005; CURROS-CRIADO & HERRERO, 2005) e inflamatória (POON & SAWYNOK, 1998; DOAK & SAWYNOK, 1995). Além disso, as evidências apontam que a ação do mecanismo antinociceptivo dos receptores A1 envolvem, em partes, a via de sinalização NO/cGMP/PKG/K^+ATP (óxido nítrico, monofosfato cíclico de guanosina, proteína quinase G e ATP sensível a potássio) (LIMA et al., 2010). Essa via é sensibilizada por NO gerado através da nNOS (oxido nítrico sintase neuronal) ativando diretamente cGMP ou indiretamente PKG, os quais levam a abertura dos canais iônicos de K^+ sensíveis a ATP, que por sua vez levam a uma hiperpolarização da célula, diminuindo a excitabilidade neuronal (SACHS et al., 2004). Esse mecanismo diminui a nocicepção periférica e central tanto de agentes ativadores de A1, como também opióides.

Segundo dados da literatura, a ação analgésica de opióides endógenos durante a inflamação periférica aguda se dá por ativação de receptores de opióides periféricos pela mesma via de NO/cGMP (TODA et al., 2009; POL, 2007; SACHS et al., 2004). Portanto, sugere-se que STLE contenha compostos com capacidade, pelo menos parcial, de se ligar a esses receptores e modular a resposta analgésica e anti-inflamatória por essa via.

Uma vez que o STLE possui uma ação antinociceptiva em modelos agudos de dor inflamatória, e que essa ação em parte envolve sistemas opióides e adenosinérgicos, foi proposta a investigação de STLE sobre parâmetros oxidativos, de migração celular e liberação de mediadores pró-inflamatórios sobre um processo inflamatório agudo. Para isso utilizou-se o modelo de peritonite induzida por carragenina (Cg) i.p. em camundongos pré-trados com STLE (v.o.). O modelo de peritonite induzida por Cg é caracterizado por uma inflamação aguda, e é amplamente empregado para testar compostos com ação anti-inflamatória, uma vez que permite a quantificação e correlação da migração celular e do exsudato inflamatório (LONGHI-BALBINOT et al., 2012; SHERWOOD & TOLIVER-KINSKY, 2004).

Os resultados mostraram que a indução da peritonite com carragenina aumentou os níveis de TBARS, e diminuiu NPSH no tecido peritoneal, e também acresceu a migração celular, atividade da MPO, além aumentar os níveis de IL-1β, IL-6 e TNF-α, e diminuir IL-10 no fluído o peritoneal. A administração prévia do STLE preveniu quase todos os parâmetros alterados, com exceção dos níveis de IL-10 (citocina anti-inflamatória), que não foram restaurados no exsudato peritoneal. Porém, mesmo assim, sugere-se que a modulação do estresse oxidativo, e a inibição das citocinas inflamatórias IL-1β, TNF-α e IL-6, pode ser uma das maneiras de STLE reduzir a migração celular e demais parâmetros inflamatórios.

Também destacamos, que a ação neutralizante de ST sobre espécies reativas derivada de nitrogênio, pode ser um mecanismo de minimização do dano inflamatório. Uma vez que o NO liberado por macrófagos e células endoteliais no sítio da inflamação atua como agente quimiotático para migração de neutrófilos (MARCHAND et al., 2005). Estudos com outras espécies de *Sida*, também mostraram uma ação anti-inflamatória em modelos *in vivo* usando Cg. Entre estes, extratos aquosos obtidos de *S. cordifolia* inibiram em 38% o edema de pata induzido por Cg na dose oral de 400 $mg.kg^{-1}$ (FRANZOTTI et al., 2000). Sutradhar et al. (2008) verificou que flavonoides

isolados de *S. cordifolia* inibiram em 22% o edema de pata por Cg, contra 28% do controle positivo. Outros trabalhos com o mesmo modelo de edema de pata, testaram extratos de *S. rhombifolia* e encontram efeitos significativos em doses que variaram de 100 a 500 $mg.kg^{-1}$ (LOGESWARI et al., 2013; VENKATESH et al., 1999; RAO & MISHRA, 1997). Assim, é possível sugerir um efeito anti-inflamatório para o gênero *Sida*, entretanto a elucidação do mecanismo ainda é um passo limitante.

O próximo passo do presente estudo foi avaliar se o efeito de STLE ocorre frente a um modelo de dor inflamatória crônica. Para tanto, foi empregado o modelo de inflamação periférica induzida pela injeção (i.pl.) de CFA, no qual a hipersensibilidade mecânica foi avaliada por meio da aplicação de filamentos de von Frey. Vale ressaltar que o CFA injetado na pata traseira é um importante agente álgico e comumente utilizado para mimetizar uma inflamação crônica com dano tecidual (MERSKEY & BOGDUK, 1994). Nossos resultados demonstraram que a aplicação de CFA foi efetiva em desencadear uma hipersensibilidade mecânica significativa, ocorreu a diminuição do limiar de resposta dos animais frente as aplicações dos filamentos de von Frey. Os dados obtidos demonstraram que o tratamento prolongado dos animais com o STLE (100 mg/kg^{-1}) reduziu a hipersensibilidade mecânica, porém a significância somente foi observada após o sexto dia de tratamento. Além disso, a administração de STLE uma vez por dia não revelou uma tolerância dos animais. Foi observado também uma diminuição do edema de pata após o quinto dia de tratamento.

Esses resultados corroboram com a ação anti-inflamatória de STLE, provavelmente por inibição de mediadores pró-inflamatórios no sítio danoso. Em concordância, pelo menos em parte, com nossos resultados, outros trabalhos usando CFA na indução de artrite em ratos, mostraram efeitos significativos na redução do processo inflamatório para extratos etanólicos de *S. rhombifolia*, juntamente com incremento das defesas antioxidantes (SOD, CAT e GSH) nos tecidos lesados (NARENDHIRAKANNAN & LIMMY, 2012; GANGU et al., 2011; GUPTA et al., 2009).

Portanto, baseado nos resultados obtidos pelos ensaios *in vitro*, e as avaliações antinociceptiva e anti-inflamatória *in vivo*, em suma, neste estudo foi demonstrado que o tratamento por v.o. de STLE possui um significativo efeito antinociceptivo e anti-inflamatório em modelos de dor inflamatória aguda e crônica em camundongos. O qual

pode ser em parte atribuído a sua diversidade química e ação antioxidante. A figura 7 ilustra os principais alvos de ação de STLE.

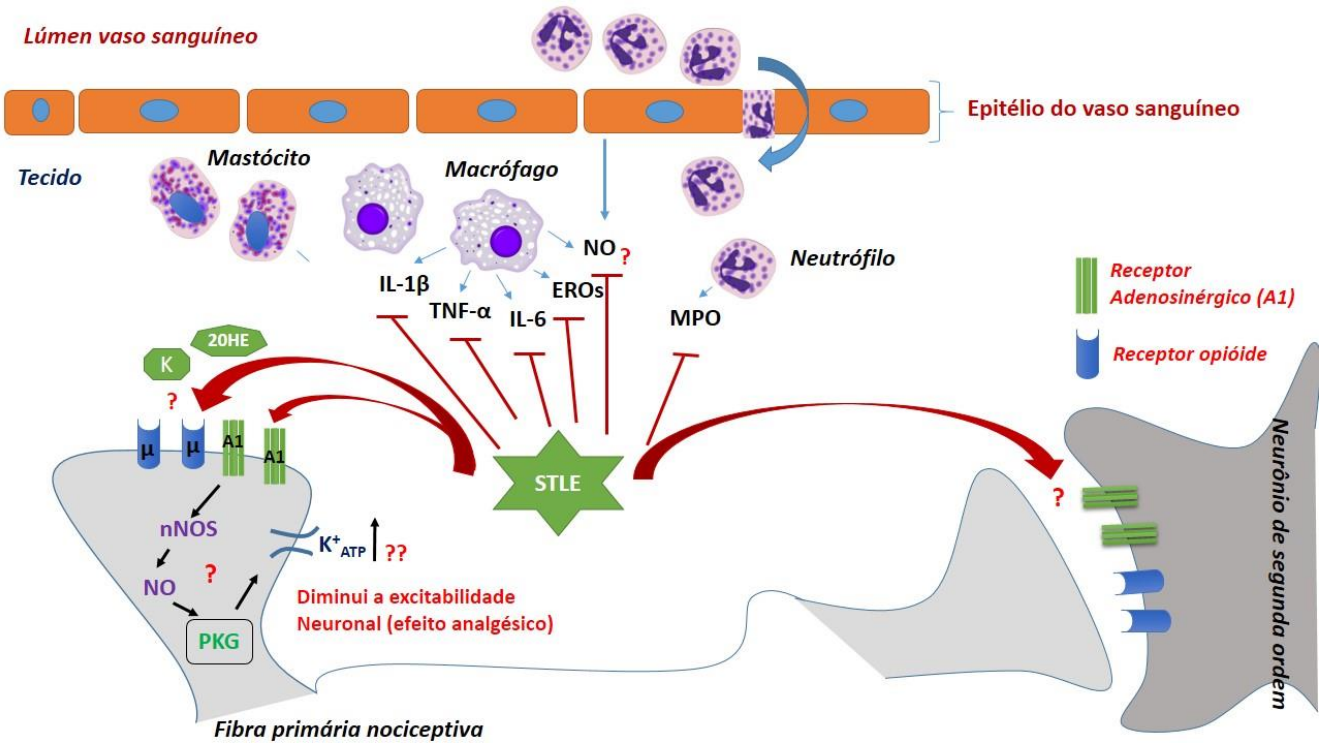


Figura 7. Possíveis ações para o efeito antinociceptivo e anti-inflamatório de STLE sobre sistema nervoso periférico e central. O efeito anti-inflamatório detectado para o extrato pode ser pela a inibição de citocinas pró-inflamatórias (IL-6, TNF-α e IL-1β), neutralização de EROs e consequente diminuição da migração neutrofílica. Ou ainda pela mimetização da ação de opióides, ligando-se a receptores tipo MOR. Também STLE pode interagir com receptores adenosinérgicos A1, ativando assim a via de sinalização NO/cGMP/PKG/ levando a abertura de canais K^{+}ATP hiperpolarizando a membrana, diminuindo, assim, a excitabilidade neuronal e levando a ação antinociceptiva. (NO) óxido nítrico, (MPO) mieloperoxidase, (nNOS) óxido nítrico síntase neuronal, (PKG) quinase da proteína G, (EROs) espécies reativas de oxigênio. *K = Kaempferol derivado; 20HE = 20-hydroxyecdysone.
Fonte: Autor (2017).

6 CONCLUSÕES

Por conseguinte, com base nos dados experimentais das avalições fitoquímicas, ensaios biológicos *in vitro* e dos modelos farmacológicos de dor inflamatória aguda e crônica *in vivo*, concluímos que:

- O composto 20HE é o fitoecdisteróide majoritário e pode ser usado como marcador químico da espécie ST;

- A capacidade antioxidante dos extratos, verificada pelos modelos "*free radical scavenger*" representa uma importante atividade a favor homeostase celular, e por isso sugerimos que pode estar envolvida no efeito antinociceptivo e anti-inflamatório verificado;

- A ação antiproliferativa com significativa especificidade para linhagens celulares tumorais representa um potencial alvo de estudo para busca de compostos antitumorais nos extratos;

- O efeito antinociceptivo e anti-inflamatório *in vivo* dos extratos de ST ocorre, em parte, por meio da interação com receptores opióides e adenosinérgicos, bem como a capacidade bloqueadora de mediadores pró-inflamatórios, podendo agir tanto perifericamente como a nível de SNC;

- Após ensaios *in vitro* e a administração diária dos extratos por 15 dias, os dados também apontam que o potencial toxicológico sistêmico de ST pode ser considerado de baixo, porém mais estudos precisam ser feitos para garantir o uso seguro na medicina tradicional;

- As atividades biológicas *in vitro* e *in vivo* detectadas corroboram com o uso popular da planta.

PERSPECTIVAS

Tendo em vista o efeito antinociceptivo e anti-inflamatório dos extratos de ST e a capacidade do mesmo em interagir com sistemas moduladores da dor, bem como a diversidade química detectada, o estudo realizado gera outras argumentações que poderiam ser contempladas com os seguintes objetivos:

- Analisar o envolvimento de outros sistemas, como serotoninérgico, glutamatérgico, colinérgico e dopaminérgico, no mecanismo de ação de ST;

- Estudar o envolvimento dos extratos de ST com receptores opióides específicos;

- Investigar se ST boqueia outros mediadores pró-inflamatórios (histamina, bradicinina e interleucinas);

- Determinar se ST ou seus compostos agem na via de sinalização NO/cGMP/PKG/;

- Averiguar o envolvimento da via L-arginina-óxido nítrico na ação de ST;

- Investigar se extratos de ST agem sobre os canais iônicos receptores de potencial transitório (TRP)

- Analisar se administração de ST aumenta o pool das enzimas antioxidantes nos tecidos avaliados dos modelos testados;

- Averiguar se os compostos majoritários de ST, isoladamente, apresentam ação antinociceptiva e anti-inflamatória;

- Investigar a ação antiproliferativa dos extratos e/ou seus isolados se dá por apoptose ou outro mecanismo a nível de membrana.

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