UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA E TERAPÊUTICA

Avaliação da atividade anti-inflamatória e toxicidade de *Valeriana glechomifolia* Meyer (Valerianaceae)

Tielle Moraes de Almeida

PORTO ALEGRE, 2016.

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Dissertação de Mestrado apresentada como requisito parcial por Tielle Moraes de Almeida para obtenção de GRAU DE MESTRE em Farmacologia e Terapêutica

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William Shakespeare

RESUMO

Um estudo prévio de nosso grupo de pesquisa demonstrou que uma fração eriquecida em valepotriatos obtida a partir de partes aéreas e subterrâneas de *V. glechomifolia* submetida à extração com CO2 supercrítico (VAL) possui efeito antidepressivo e prevenção do comportamento de doente (*sickness behavior*) induzido por LPS. Além disso, alguns estudos revelaram propriedades anti-inflamatórias de *V.wallichii* e de *V.amurensis*. Estes dados da literatura sugerem que os valepotriatos podem ser utilizados no desenvolvimento de novos farmácos. Entretanto, dados sobre toxicidade, segurança e atividade anti-inflamatória de valepotriatos ainda são escassos. Considerando isso, o objetivo deste estudo foi investigar a atividade anti-inflamatória periférica e a toxicidade oral aguda e de doses repetidas de VAL. A atividade anti-inflamatória foi avaliada por meio do teste de formalina em camundongos CF1 e o ensaio de migração de leucócitos em ratos Wistar. Além disso, os estudos de toxicidade seguiram as normativas 423 e 407 da Organização para a Cooperação e Desenvolvimento Econômico (OECD). Diferentes grupos de camundongos foram tratados com VAL (1, 10 e 30 mg/kg), diclofenaco 50 mg/kg (controle positivo) ou saline (controle negativo) 1 h antes da injeção de formalina. No ensaio de quimiotaxia, os leucócitos foram tratados com concentrações de 0,1-1,0 µg/mL de VAL, indometacina ou diclofenaco (1 µg/mL). No estudo de toxicidade aguda, três camundongos CF1 machos foram tratados com uma dose única de VAL (2000 mg/kg, v.o.) e observados durante 14 dias. Já para o estudo de toxicidade de doses repetidas, diferentes grupos de animais (n = 10) receberam doses únicas diárias de VAL (30, 150 e 300 mg/kg, v.o.) ou veículo durante 28 dias. No teste de formalina, VAL inibiu o comportamento do tipo nociceptivo na segunda fase do teste de forma dose-dependente. O efeito da dose mais elevada de VAL foi comparável com o diclofenaco na dose de 50 mg/kg (v.o.). VAL (0,1-1 µg/mL) também inibiu a migração de leucócitos induzida por LPS (65 µg/mL) de modo dependente da concentração. Este efeito foi comparável ao efeito de indometacina (0,1 - 1 µg/mL) e superior ao efeito do diclofenaco (1 µg/mL). No estudo de toxicidade aguda apenas uma morte foi detectada, o que classifica VAL como segura (categoria 5), de acordo com a OECD-normativa 423. O estudo toxicidade de doses repetidas demonstrou que VAL na dose de 300 mg/kg retardou o ganho de peso e reduziu o consumo de ração dos animais deste grupo na primeira semana de tratamento,

provavelmente devido aos efeitos sedativos da mesma. As outras doses não alteraram o ganho de peso e ingesta de ração. Nenhuma das doses de VAL alterou qualquer parâmetro comportamental, urinário, bioquímico, hematológico, anatômico ou histológico. Em conclusão, estes resultados demonstram pela primeira vez que valepotriatos, uma classe especial de terpenos que ocorrem apenas no gênero *Valeriana*, apresentam atividade anti-inflamatória periférica e são seguros em doses pré-clínicas eficazes, por via oral.

Palavras-chave: Valeriana glechomifolia, Valerianaceae, valepotriatos, teste de formalina, migração de leucócitos, anti-inflamatório, toxicidade oral aguda, toxicidade oral de doses repetidas.

ABSTRACT

A previous study by our research group demonstrated that an enriched fraction obtained from the aerial and subterranean parts of *V. glechomifolia* submitted to supercritical CO_2 extraction (VAL) shows antidepressant-like effect and prevented LPS-induced sickness behavior. Also, some studies revealed anti-inflammatory properties of *V.wallichii* and *V.amurensis.* Altogether, these findings suggest that the valepotriates scaffold might be useful to develop new antidepressant and anti-inflammatory drugs. However, data about the toxicity, safety and anti-inflammatory activity of valepotriates from *V. gelchomifolia* are still scarce**.** Considering this, the aim of this study was to investigate the peripheral anti-inflammatory activity and the oral acute and repeated toxicity of VAL. The anti-inflammatory activity was assessed by using the formalin test in CF1 mice and Wistar rat's leukocytes migration assay. Besides, the toxicity studies followed the Organization for Economic Cooperation and Development (OECD) toxicity studies guidelines 423 and 407. Different groups of mice were treated with VAL (1, 10 and 30 mg/kg), diclofenac 50 mg/kg (positive control) or saline (negative control) 1 h before the formalin injection. In the chemotaxis assay, the leukocytes were treated with a range of 0.1-1.0 µg/mL of VAL, indomethacin or diclofenac (1 µg/mL). In the acute toxicity, three CF1 mice were treated with a single dose of VAL (2000 mg/kg, p.o.) and observed for 14 days. To perform the repeated toxicity study, separated group of animals (n=10) received single daily doses of VAL (30, 150 and 300 mg/kg, p.o.) or vehicle during 28 days. In the formalin test, VAL inhibited the nociceptive behavior in the late phase in a dose dependent manner at 30mg/kg dose. The effect of the VAL highest dose was comparable to diclofenac 50 mg /kg (p.o.). VAL (0.1 - 1 µg/mL) inhibited the leukocyte migration induced by LPS (65 µg/mL) in a concentration dependent manner. This antichemotatic effect was comparable to indomethacin (0.1 – 1µg/mL) and better than diclofenac (1 µg/mL) effect. In the acute toxicity study only one death was detected, which classify VAL as safe (category 5), according to OECD-guideline 423. The repeated dose toxicity study demonstrated that VAL 300 mg/kg delayed the weight gain and reduced the food consumption in the first week, probably due to sedative

effects. The other doses had no effect on weight gain and food consumption. None of doses altered any behavioral, urinary, biochemical, hematological, anatomic or histological parameters. In conclusion, these results demonstrate for the first time that valepotriates, a special class of terpenes occurring only in *Valeriana* genus, present peripheral anti-inflammatory activity and are safe at effective pre-clinical doses, by oral route.

Keywords: *Valeriana glechomifolia,* Valerianaceae, valepotriates, formalin test, leukocytes migration, anti-inflammatory, acute oral toxicity, repeated dose toxicity.

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ABREVIATURAS E SIGLAS

ANOVA - Análise de variância

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CCD – Cromatografia em camada delgada

CEUA – Comissão de Ética no Uso de Animais

CFMV - Conselho Federal de Medicina Veterinária

CIOMS – Conselho da Organização Internacional de Ciências Médicas (do inglês *Council for International Organizations of Medical Sciences*)

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

COX-2- Ciclo-oxigenase-2

CREAL - Centro de reprodução e experimentação de animais de laboratório

DL50 - Dose letal mediana

DNA – Ácido desoxirribonucléico

HBSS – Solução salina de Hanks balanceada (do inglês *Hanks' Balanced Salt solution*)

HPLC – Cromatografia liquida de alta eficiência (do inglês *High performance liquid chromatography*)

ikappaB-alpha – Factor nuclear de kappa polipéptido intensificador de gene inibidor em células B (do ingles *nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha*)

IL-1β – Interleucina 1β

IL-6 – Interleucina 6

iNOX- Isoforma da enzima óxido nítrico sintase

i.p. – Intraperitonial

IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

LPS – Lipopolissacarídeo (do inglês *Lipopolysaccharide*)

NaCl – Cloreto de sódio

OECD - Organização para a Cooperação e Desenvolvimento Econômico (do inglês *Organisation for Economic Co-operation and Development*)

PPGCF – Programada de Pós-Graduação em Ciências Farmacêuticas

PUC-RS – Pontifícia Universidade Católica do Rio Grande do Sul

SISBIO - Sistema de Autorização e Informação em Biodiversidade

S.D. – Desvio Padrão (do inglês *Standard Deviation*)

S.E.M. – Erro Padrão da Média (do inglês *Standard Error of the Mean*)

TNF-α – fator de necrose tumoral α (*tumor necrosis fator α*)

UFRGS – Universidade Federal do Rio Grande do Sul

v.o. – Via oral

VAL – Fração enriquecida em valepotriatos obtida por extração com CO2 supercrítico de *V. glechomifolia*

WHO – *World Health Organization*

SUMÁRIO

Revisão Bibliográfica

Um grande número de moléculas derivadas de produtos naturais em vários estágios de desenvolvimento clínico destaca a viabilidade e a importância da utilização de produtos naturais como fontes de novos candidatos a fármacos. Ainda hoje, as plantas são uma fonte importante de descoberta de novos compostos farmacologicamente ativos sendo derivados direta ou indiretamente de plantas (Veeresham C, 2012). Sendo assim, é importante estudar os efeitos biológicos de substâncias provenientes de plantas, bem como sua toxicidade, com vistas ao desenvolvimento de novos fármacos.

Dentre as espécies vegetais mais utilizadas com fins medicinais no mundo destaca-se a espécie *Valeriana officinalis,* cujo uso como sedativo e indutor do sono é aprovado pela Organização Mundial de Saúde (WHO, 1999). Além disso, *V. officinalis* vem sendo usada tradicionalmente como sedativo- hipnótico e ansiolítico (Hobbs, 1990; Hadley and Petry, 2003; Maurmann et al., 2011, Felgentreff et al., 2012). Além disso, estudos recentes têm indicado o efeito antidepressivo de espécies do gênero *Valeriana* (Hattesohl et al., 2008; Subhan et al.,2010; Holzmann et al., 2011; Sah et al., 2011; Liu et al., 2012; Müller et al., 2012a)

O gênero *Valeriana* L. compreende aproximadamente 400 espécies (Backlund e Moritz, 1998) e pertence à família Valerianaceae Batsch que abrange os gêneros *Valerianella* Miller, *Centranthus* DC, *Nardostachys* DC, entre outros (Hobbs, 1989). As espécies deste gênero mais utilizadas para a produção de medicamentos são *V. officinalis*, *V. wallichii* e *V. edulis* (Fugg-Berman e Cott, 1999; Herrera-Arellano *et al*., 2001). Sobre os constituintes neuroativos do gênero, um estudo demonstra que os valepotriatos parecem contribuir com as atividades farmacológicas relatadas (Patocka e Jakl, 2010).

No Brasil, foram descritas dezessete espécies de *Valeriana*. Destas, doze ocorrem no Rio Grande do Sul (Borsini, 1962): *Valeriana bornmuelleri, V. catharinensis, V. chamaedryfolia, V. eichleriana, V. eupatoria, V. glechomifolia, V. polystachya, V. salicarifolia, V. scandens, V. reitzania, V. tajuvensis* e *V. ulei* (Sobral, 1999a, 1999b, 1999c). Salles e colaboradores (2000) isolaram e identificaram nove iridoides das folhas, caules e raízes de *V. glechomifolia*, destes, sendo oito valepotriatos e um iridoide glicosilado. SILVA e colaboradores (2002) validaram uma

metodologia por cromatografia líquida de alta eficiência (CLAE) e quantificaram valepotriatos diênicos (acevaltrato, 1-β-acevaltrato, diavaltrato, diidrovaltrato e valtrato) em nove espécies de *Valeriana* coletadas no Rio Grande do Sul: *V. catharinensis, V. chamaedryfolia, V. eicleriana, V. eupatoria, V. glechomifolia, V. polystachya, V. salicarifolia, V. scandens* e *V. tajuvensis*. Dentre as espécies de *Valeriana* que ocorrem no Rio Grande do Sul, *V. glechomifolia* foi a espécie que apresentou os maiores teores de valepotriatos (2,05 %), sendo o caule a região onde há maior depósito destes (Silva, 2002).

Os valepotriatos são iridóides carbocíclicos não glicosilados cujo núcleo principal está apresentado na Figura 1.

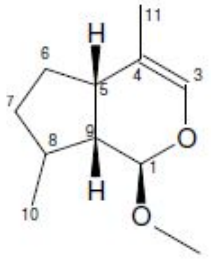

Figura 1. Estrutura geral do núcleo iridóide carbocíclico dos valepotriatos.

Sua diversidade estrutural é definida por diferentes substituintes ácidos esterificados com os grupos hidroxila, presença ou ausência de um grupo epóxido e pelo número e posição das ligações duplas no núcleo principal (Hobbs, 1989). Dividem-se em monoênicos e diênicos, os valepotriatos diênicos distinguem-se dos monoênicos por possuírem uma segunda ligação dupla entre C5 e C6. Os valepotriatos monoênicos mais comumente presentes em espécies de *Valeriana* são diidrovaltrato e homodiidrovaltrato, enquanto que os diênicos são valtrato, isovaltrato, diavaltrato, acevaltrato e 1-β-acevaltrato (Dossaji e Becker, 1981; Becker *et al*., 1983; Foerester *et al.*, 1984; Hölzl e Koch 1985; Bach *et al*.,1993; Tang *et al*., 2002). Estes compostos são muito instáveis; sob influência de acidez, umidade e calor, acabam se decompondo rapidamente em ácidos livres e núcleos monoterpênicos; também pode ocorrer a abertura do grupamento epóxido gerando baldrinal e homobaldrinal, produtos de degradação (Hobbs, 1989) e a quebra das ligações ésteres dos substituintes do núcleo principal, liberando ácido valérico, responsável pelo odor

característico deste gênero. Um estudo de nosso grupo de pesquisa demonstrou que as condições e o tempo de armazenamento de uma fração de *V. glechomifolia* obtida por dióxido de carbono supercrítico exercem influência no teor de valepotriatos dos mesmos, sendo que a fração seca armazenada em atmosfera de nitrogênio teve o núcleo iridóide carbocíclico conservado enquanto a fração armazenada em metanol apresentou produtos de degradação e a liberação do substituinte isovaleril do núcleo iridóide em maior proporção (Müller *et al*., 2012a).

Vários estudos demonstraram efeitos tóxicos de valepotriatos ou extratos enriquecidos em valepotriatos de espécies do gênero valeriana*, in vitro* e em modelos celulares. Em um estudo *in vitro*, foi demonstrado que a exposição de células de hepatoma de ratos a valtrato e diidrovaltrato em diferentes concentrações (0,14; 0,28; e 0,7 µM por 24, 48 e 72 horas, respectivamente) é capaz de induzir 100% de mortalidade nas culturas (Bouthan *et al*., 1981). Outro estudo demonstrou que valepotriatos são tóxicos *in vitro* às células medulares de ratos, porém não houve nenhuma alteração significativa nessas células após a administração oral de doses acima de 1500 mg/kg em ratos (Tortarolo *et al*.,1982). Lin e colaboradores (2009) demonstraram que valepotriatos e seus derivados acilados apresentam acentuada citotoxicidade sobre culturas celulares de adenocarcinoma de pulmão, linhagens celulares de hepatoma, câncer de próstata e cólon. Acevaltrato foi considerado com maior efeito tóxico dentro os valepotriatos testados. Outro estudo mostrou que valepotriatos extraídos de *V. walichi*, *V. edulis* e *V. officinalis* são tóxicos para linhagem de células de carcinoma de pequenas células de pulmão (GLC4) e linhagem de carcinoma de colo retal humano (COLO 320). Os valepotriatos diênicos apresentaram a maior toxicidade, com IC50 de 1-6µM, sendo que os valepotriatos monoênicos foram 2 a 3 vezes menos tóxicos. Os produtos de degradação baldrinal e homobaldrinal, foram de 10 a 30 vezes menos tóxicos que o valepotriatos diênicos (Bos *et al*., 1998). Hui-lian e colaboradores (2003) avaliaram o grau de dano ao DNA causado por um extrato diclorometano de *V. officinalis* em linhagem de células endoteliais humanas (ECV304) tratadas com diferentes concentrações do extrato (5-60 µg/mL) em ensaio cometa. Foi observado dano moderado ao DNA apenas nas células tratadas com 40 e 60 µg/mL após 48 horas.

Entretanto, a administração aguda de valtrato por via i.p. (45-65 mg/kg) e oral (45-1350 mg/kg) em ratos não foi tóxica a células precursoras hematopoiéticas quando comparado com o grupo controle (Braun *et al*., 1984). Além disso, a investigação do efeito da administração por 30 dias de uma mistura de valepotriatos (80% diidrovaltrato,15% valtrato e 5% acevaltrato) nas doses de 6, 12 e 24 mg/kg (v.o.) a ratas grávidas, não demonstrou alterações na fertilidade e nem nas ninhadas das fêmeas tratadas durante a gravidez (Tufik *et al*.,1994). Yao e colaboradores (2007) também demonstraram que um extrato etanólico de *V. officinallis* não teve efeitos adversos no desenvolvimento embrionário e na fertilidade de roedores. Além disso, foi demonstrado que um tratamento oral em camundongos com diferentes doses (500, 1000 e 2000 mg/kg/dia) de *Valeriana officinalis (*Produto comercial, cápsulas de 800 mg de raizes de *V. officinalis* e 220 mg de extrato seco de raízes padronizado para o ácido valerênico 0,8%) durante 7 dias, aumentou os níveis de malondialdeído e diminuiu os níveis de glutationa em células hepáticas e testiculares de camundongos. Além, disso observou-se aumento da freqüência de micronúcleos em eritrócitos policromáticos e redução da proporção destes em eritrócitos normocromáticos no fêmur de camundogos. Essas alterações são relacionadas a terpenoides (valepotriatos) e flavonoides (6-metilapigenina e 2S-hesperidina) encontrados no gênero *Valeriana* (Al-Majed *et al.,* 2006).

Salles (2010) realizou um estudo de toxicidade aguda com extrato diclorometano de *V. glechomifolia* (enriquecido em valepotriatos) nas doses de 10, 20, 30, 40 e 50 mg/kg por via intraperitonial (i.p.) 5, 10, 20, 40 e 80 mg/kg por v.o. Não houve morte nem sinais de toxicidade nos animais tratados pela via oral nas primeiras 24 horas após o tratamento; porém, nas doses de 20 - 80 mg/kg houve uma morte na primeira semana. A dose letal mediana (DL50) encontrada para a via i.p. foi de 42 mg/kg, atribuindo aos valepotriatos, segundo a OECD (2001), toxicidade de moderada a alta.

Maurmann e colaboradores (2010) demonstraram que uma fração enriquecida em valepotriatos obtida de *V. glechomifolia* apresentou efeito sedativo, ansiolítico e sobre a memória de roedores. Além disso, nosso grupo de pesquisa mostrou a atividade do tipo antidepressiva de um extrato de *V. glechomifolia* obtido por dióxido de carbono supercrítico ($SCCO_2$) enriquecido em valepotriatos em roedores (Salles, 2010; Müller *et al*., 2011). Estes achados estão em consonância com dados da

literatura que apontam o potencial antidepressivo de outras espécies do gênero, como *V. officinalis* (Hattesohl *et al.*, 2008; Trompetter *et al.,* 2013), *V. wallichii* (Subhan et al., 2010; Sah et al., 2011), *V. prionophylla* (Holzmann et al., 2011) e *V. fauriei* Briq (Liu et al 2012). Estudos pré-clínicos demonstraram um efeito antidepressivo de valepotriatos diênicos, que parece ser mediado pela neurotransmissão dopaminérgica e noradrenérgica (Müller et al., 2012a). De acordo com esta hipótese, um estudo de nosso grupo demonstrou que estes compostos apresentam interação sinérgica com antidepressivos clássicos (imipramina, desipramina e bupropiona), avaliados através da análise isobolar (Müller et al., 2015a). Além disso, uma fração enriquecida em valepotriatos diênicos de *Valeriana glechomifolia* aumenta a atividade da enzima Na+/K+-ATPase no córtex de camundongos, assim como a expressão protéica da isoforma α2 da Na+/K+-ATPase, o que pode contribuir para o seu efeito antidepressivo, uma vez que a diminuição da atividade da Na+/K+-ATPase tem sido associada à depressão (Müller et al., 2015b).

Um estudo recente de nosso grupo de pesquisa demonstrou que uma fração enriquecida em valepotriatos obtida por extração com CO2 supercrítico de *V. glechomifolia (VAL)* protegeu camundongos do desenvolvimento do comportamento de doente (*sickness behavior*) e do tipo depressivo induzido por LPS (lipopolisacarídeo de *Escherichia coli*), que relaciona a contribuição do sistema imune para o desenvolvimento da depressão. A ação desta fração parece ser mediada pela redução cortical da expressão de citocinas pró-inflamatórias, mais especificamente TNF-α e IL-1β (Müller et al., 2015c). Esses achados estão de acordo com estudos da literatura que demonstram a ação anti-inflamatória de outras espécies do gênero *Valeriana* (Subhan et al., 2010; Zhang et al., 2010; Khuda et al., 2013).

Levando em consideração o estudo citado anteriormente que investigou a toxicidade de um extrato diclorometano enriquecido em valepotriatos obtido de *V. glechomifolia*, torna-se necessária a investigaçãod a toxicidade aguda e de doses repetidas de uma fração enriquecida em valepotriatos obtidos a partir das partes aéreas e subterrâneas de *Valeriana glechomifolia* submetidas à extração por CO_2 supercrítico (VAL), seguindo as normativas 407 (1995) e 423 da OECD (2001). Além disso, considerando os dados da literatura que demonstram o possivel envolvimento do sistema imune no efeito antidepressivo de VAL, o presente estudo também

investigou a atividade anti-inflamatória periférica de VAL, usando o teste de formalina em camundongos e o ensaio de migração de leucócitos em ratos Wistar (quimiotaxia de neutrófilos induzida por LPS).

Objetivo Geral

Esta dissertação de mestrado teve por objetivo investigar a atividade anti-inflamatória e toxicidade de uma fração *Valeriana glechomifolia* enriquecida em valepotriatos.

Os objetivos específicos são:

Avaliação do efeito de uma fração enriquecida em valepotriatos de *V. glechomifolia* sobre o modelo experimental da formalina em camundongos;

Avaliação do efeito de uma fração enriquecida em valepotriatos de *V. glechomifolia* sobre a migração de leucócitos extraídos de ratos;

Avaliação da toxicidade oral aguda de uma fração enriquecida em valepotriatos de *V. glechomifolia* em camundongos.

Avaliação da toxicidade oral de doses repetidas de uma fração enriquecida em valepotriatos de *V. glechomifolia* em camundongos.

Capítulo 1

Os experimentos apresentados nesta parte do trabalho foram realizados no Laboratório de Psicofarmacologia Experimental da Faculdade de Farmácia da UFRGS, sob orientação da Profª. Dr. Stela Rates e Co-orientação da Profª.Dr. Liz Girardi e no laboratório de Química Medicinal da UFRGS sob orientação da Profª. Dr. Miriam Anders Apel e estão apresentados na forma de um artigo científico que está submetido ao periódico *Planta Medica*.

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Valepotriates from Valeriana glechomifolia Meyer present peripheral anti-inflammatory activity in rodents

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Valepotriates from *Valeriana glechomifolia* Meyer present peripheral anti-inflammatory activity in rodents

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Keywords: *Valeriana glechomifolia,* Valerianaceae, valepotriates, formalin, neutrophil chemotaxis, anti-inflammatory.

Abbreviations

IBAMA: Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis; $SCCO_2$: supercrítical carbon dioxide; SISBIO-IBAMA: Sistema de Autorização e Informação em Biodiversidade - Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis; VAL: valepotriates enriched fraction

Abstract

A previous pre-clinical study by our research group demonstrated that valepotriates from *Valeriana glechomifolia* were effective against LPS-induced sickness behavior as well as significantly decreased the cortical expression of pro inflammatory cytokines IL-1β and TNF-α. Other studies revealed anti-inflammatory properties of *V.wallichii* and *V.amurensis.* These findings open up new perspectives for *Valeriana* genus pharmacology, once it has been commonly associated to sedative and anxiolytic properties. Considering this, the aim of this study was to investigate the peripheral anti-inflammatory activity of a valepotriates enriched fraction obtained from the aerial and subterranean parts of *V. glechomifolia* submitted to supercritical CO_2 extraction (VAL). The anti-inflammatory activity was assessed by means of formalin test in CF1 mice and Wistar rat's leukocytes migration assay (modified Boyden chamber method).VAL (1,10 and 30 mg/kg, p.o.) inhibited the nociceptive behavior in the late phase of the formalin test in a dose dependent manner. The effect of the VAL highest dose was comparable with that of diclofenac 50 mg /kg (p.o.). VAL (0.1 - 1 µg/mL) inhibited the leukocyte migration induced by LPS (65 µg/mL) in a concentration dependent manner. This antichemotatic effect was comparable with that of indomethacin (0.1 – 1µg/mL) and better than diclofenac (1 µg/mL) effect. In conclusion, these results demonstrated for the first time that valepotriates, a special class of terpenes occurring only in *Valeriana* genus*,* present a peripheral anti-inflammatory activity.

Introduction

The *Valeriana* genus belongs to the family Valerianaceae Batsch, and comprises approximately 400 species worldwide distributed [1]. Several species from this genus are recognized by their mild sedative, antispasmodic and relaxing properties, and constitute the most popular herbal remedies for treating anxiety and insomnia [1, 2, 3]. Pharmaceutical companies use *V. officinalis*, *V. wallichii* and *V. edulis* as raw material to produce sedative phytomedicines [4, 5]. Additionally, some authors have already shown the antidepressant potential of this genus [2, 6, 7]. In this sense, our research group has been studying the pharmacological properties of one *Valeriana* species endemic to southern Brazil, *Valeriana glechomifolia* Meyer, which is the one that presents the highest valepotriates content among the south Brazil native species [8,9].

The pharmacological properties of the *Valeriana* genus plants are assigned to different constituents, including the monoterpene valerenic acid [10], flavonoids [11], and valepotriates [1,12-14], which comprise a family of terpenes [15] that is only found in the Valerianaceae family [7,8]. The valepotriates are no glycosylated carbocyclic iridoids, divided in monoenic and dienic types, the latter presenting a second double bond between C5 and C6 [16]. The diene valepotriates frequently found in *Valeriana* species are valtrate, isovaltrate, diavaltrate, acevaltrate and 1-β-acevaltrate [17,18, 19, 20].

Pre-clinical studies demonstrated an antidepressant-like effect of diene valepotriates, which seems to be, at least in part, due to the activation of noradrenergic and dopaminergic neurotransmission [2]. In line with this assumption, these compounds displayed a synergistic interaction with classical antidepressants (imipramine, desipramine and bupropion), assessed by isobolographic analysis [21]. Moreover, the diene valepotriates from *Valeriana glechomifolia* increased Na+/ K+-ATPase activity in the cortex of mice, as well as the cortical protein expression of the α2 Na+/K+-ATPase isoform, which may contribute to its antidepressant-like effect, since decreased Na+/K+-ATPase activity has been associated with depression [7]. Furthermore, some studies revealed anti-inflammatory properties of *V. wallichii* [22, 23] and *V. amurensis* [24]. In addition, pre-clinical studies have shown that *V.officinalis* and *V. glechomifolia* are able to prevent the sickness and depressive-like behavior induced by LPS in rodents, which have been related to neuroinflammation [7, 25].

Altogether, these findings open up new perspectives for *Valeriana* genus pharmacology.

Considering the above mentioned data, the aim of the present study was to investigate the peripheral anti-inflammatory activity of a valepotriates fraction obtained from the aerial and subterranean parts of *Valeriana glechomifolia* submitted to supercritical CO_2 extraction (VAL), by using the formalin test in mice and the Wistar rat's leukocyte migration assay (neutrophil chemotaxis induced by LPS).

Results

The characterization of the VAL fraction by HPLC demonstrated that valtrate is the valepotriate present in largest quantity, followed by acevaltrate, 1β-acevaltrate, 1β-aceacevaltrate, and isovaltrate. Table 1 depicts the concentration of each valepotriate. The chemical structures of the valepotriates shown in Table 1 have already been published elsewhere [16].

Figure 1 shows the effects of VAL in the nociceptive phase of the formalin test. The One way ANOVA [F (4. 25) = 0.772 p=0.5537] did not reveal any significant difference between the nociceptive behavior time of all groups.

Figure 2 depicts the results from the inflammatory phase of the formalin test. Kruskal-Walis analysis revealed that the groups treated with diclofenac (positive control) and VAL 30 mg/kg feature a time of nociceptive behavior significantly lower that the group treated with saline [H=10.11; p= 0.0386]. There was no significant difference between the nociceptive behavior of groups treated with diclofenac and VAL 30 mg/kg. It is also possible to observe that the effect of VAL 30 mg/kg was significantly higher than VAL 1 and 10 mg/kg ($p<0.05$), which indicates a dose dependent effect.

The results of chemotaxis assay are shown in Figure 3.One-way ANOVA [F (7. 49) = 41.93; P <0.0001] revealed that VAL significantly inhibited leukocyte migration at all concentrations tested when compared to negative control ($p< 0.0001$). Moreover, VAL at 1µg/ml and 0.5 µg/mL significantly inhibited leukocyte migration when compared to all concentrations of indomethacin and diclofenac as well as VAL at 0.1µg/mL. VAL and indomethacin displayed a concentration dependent response. Diclofenac was tested at a unique concentration because this drug is effective only at high concentrations in this assay [26].

Discussion

The present study demonstrated for the first time that a valepotriates fraction from *Valeriana glechomifolia* obtained by supercritical CO_2 extraction (VAL) was effective in the second phase of the mice formalin test and displayed antichemotactic activity in the leukocyte migration assay. These data point to a possible anti-inflammatory effect of valepotriates.

It is well established that the formalin produces a biphasic behavioral reaction with an initial phase within the first (0-5) minutes post injection, followed by a quiescent period (approximately 15 min), and a second phase of nociceptive behaviors lasting 15–30 min. The first phase relates to the direct stimulation of nociceptors and is sensitive to local anesthetics and opiates, while the second phase corresponds to inflammatory responses and central sensitization within the dorsal horn [27]. The second phase responds to various drugs with recognized analgesic and/or anti-inflammatory efficacy, such as steroidal or non-steroidal anti-inflammatory drugs, N-methyl-D-aspartate antagonists and gabapentin [28].

VAL produced antinociceptive effects only in the second phase of the formalin test, suggesting that it presents an activity similar to anti-inflammatory drugs (e.g, steroidal or non-steroidal anti-inflammatory drugs) [28], which suppress only the second phase of formalin test.

These results are in line with other studies on the anti-inflammatory effects of species of *Valeriana* genus. Khuda and colleagues (2013) demonstrated that a topical formulation of crude methanol extract of *V. wallichii* showed anti-inflammatory effect in the carrageenan induced hind paw edema model in rats. These authors suggested that a possible mechanism of the anti-inflammatory activity of *V. wallichii* is the inhibition of histamine, serotonin and prostaglandins synthesis. Another study demonstrated that an ethanolic extract of *V. amurensis* controlled the inflammatory reaction in the cortex and hippocampus of rats submitted to a model of Alzheimer's disease. The mechanism of anti-inflammatory action from *V. amurens* ethanolic extract appears to be the reduction of iNOS, COX-2 and ikappaB-alpha expression in cortical and hippocampal neurons [24]. *V. officinalis* prevented the development of sickness behavior and depressive-like behavior induced by LPS in rodents [25]. Our

research group has recently demonstrated that VAL was also effective against LPS-induced sickness behavior as well as significantly decreased the cortical expression of pro-inflammatory cytokines IL-1β and TNF-α [29].

During the acute phase of inflammation, there is a release of pro-inflammatory mediators including bioactive amines, lipid mediators and cytokines, typically TNF-α and IL-1β, which produces a chemotactic gradient to guide and activate recruited cells to the site of injury [30]. It is well accepted that cytokines constitute a link between cellular injuries or immunological recognition and the local or systemic signs of inflammation, e.g. cell migration, edema, fever, and hyperalgesia [31-33].

Considering that the suppression of neutrophil functions control inflammatory responses and it is part of the mechanism of action of some non-steroidal anti-inflammatory [34], we searched for the effects of VAL on leukocyte chemotaxis induced by LPS in the Boyden chamber *in vitro* method. Bacterial LPS has been extensively used in models studying inflammation as it mimics many inflammatory effects of cytokines, such as TNF-α, IL-1β or IL-6. This activity seems to be mediated by small G proteins facilitating the release of pro-inflammatory cytokines [35].

VAL significantly inhibited leukocyte migration at all concentrations tested when compared to negative control. In addition, at the highest concentrations (0.5 and 1μg/mL), VAL significantly inhibited leukocyte migration when compared to indomethacin and diclofenac (at the same concentrations). These results demonstrate that VAL presents a better effect than the reference drugs used on the inhibition of neutrophil migration. In line with these results, other studies also showed the antichemotatic activity of other terpenoids, such as 1,8-cineole [36], 28,28,30-trihydroxylupeol, 3,21,21,26-tetrahydroxylanostanoic acid, dehydroxybetulinic acid, taraxerone, ethyl palmitate and ursolic acid [37]. Notably, it has been shown that the systemic administration of the terpene rose-oxide inhibited key events related to inflammation, namely edema, local increase of IL-1β level, and leukocyte migration, producing consistent anti-inflammatory effects in different models of inflammation in mice and rats, including formalin test and leukocyte migration [38].

In conclusion, the results so far demonstrated for the first time that valepotriates, a special class of terpenes occurring in *Valeriana* genus*,* present

peripheral anti-inflammatory activity, corroborating with other studies showing the anti-inflammatory activity of *Valeriana* species. The anti-inflammatory activity of these compounds might be at least in part related to their ability to inhibit the expression of pro-inflammatory cytokines (IL-1β and TNF-α) and leukocyte migration. These results highlight the need for further investigations of valepotriates as prototypes for the development of new drugs to treat inflammatory conditions.

Materials and methods

Plant material

V. glechomifolia aerial and subterraneous, parts were collected from Aparados da Serra, in the state of Rio Grande do Sul – Brazil. The plant collection was authorized by IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) (29495-1). The identification was performed by Dr. M. Sobral (Universidade Federal de São João del-Rei, Minas Gerais, Brazil) and a voucher specimen (Sobral, 7733) was deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (ICN), Brazil.

Valepotriates fraction preparation

The VAL fraction was obtained according to Muller et al. (2012) [16]. Briefly, 100 g (dry weight) of powdered plant material were submitted to supercritical CO_2 ($SCCO_2$) extraction, using a Pilot Equipment as described elsewhere [39]. The conditions of the extraction were 40°C, 90 bar, $SCCO_2$ flow rate through the extraction vessel: 6.67×10^{-4}kg s^{-1}.The $SCCO_2$ extraction yield was 3.23 g%.

Valepotriates fraction characterization

The VAL fraction was dissolved in HPLC grade methanol and filtered (0.22 µm pore size, Merck) before the analysis by HPLC according to a method previously described [9,16], using Shimadzu HPLC system and Waters Nova-Pack C18 column (4 mm, 3.9 × 150mm i.d. with Waters Nova- Pack C-18 guard column, 60 ˚ A, 3.9 × 20 mm). The isocratic mobile phase (acetonitrile: water (50: 50 v/v)); flowed at 1 mL/min; UV detection at 254 nm. The samples were dissolved in HPLC grade methanol and filtered through a membrane filter (0.22mm pore size, Merck) before the analysis. Valtrate was isolated from the fraction, purified by column chromatography, identified by ^{1}H and ^{13}C NMR and used as external standard. For achievement of the calibration

curve, valtrate was diluted (in grade HPLC methanol) stepwise (250; 125; 62.5; 31.25; 15.62; 7.81mg/mL) and 20 µL were injected into the HPLC in triplicate. Linearity analysis of the calibration curve revealed R^2=0.9999. The valepotriates fraction was diluted (250 mg/mL) and 20 µL were injected into the HPLC in triplicate. The retention time of the valepotriates were: valtrate 26.5 min, isolvatrate 22 min, acevaltrate 14 min, 1-β-acevaltrate 11.5 min and for 1-β-aceacevaltrate 8.5 min. All valepotriates were quantified in mg of valtrate equivalent/g fraction.

Animals

Experiments were carried out using male CF1 mice (30–45g) and male Wistar rats (180-220g) from Centro de Reprodução e Experimentação de Animais de Laboratório - CREAL - UFRGS, Rio Grande do Sul, Brazil. Animals were housed in plastic cages at 23°± 1°C under a 12-hour light/dark cycle, with food and water provided *ad libitum*. Experiments were approved by Animal Care Local Ethical Committee (CEUA-UFRGS; 28603, April 16th, 2015) and were conducted in accordance with Brazilian law [40-42] and European Communities Council Directive of 24 November 1986 (86/609/EEC).

Formalin Test

Different groups of mice were treated with VAL (1.10 and 30 mg/kg), diclofenac 50 mg/kg (positive control) or saline (negative control) 1 h before the formalin injection. We based the doses range on previous studies by our group [6]. The animals were adapted to the apparatus (an acrylic squad chamber 20 cm wide x 30 cm high) for 20 minutes before receiving an intra-plantar (i.pl.) injection of formalin. Immediately, the animals were observed during the first 5 min (neurogenic phase) and between the15th and 30th min (inflammatory phase). The time that animals spent licking and biting the injected paw (during the first and second phases of the test) was recorded with a chronometer and was considered an indicative of nociceptive behavior [28,43].

VAL was suspended in saline (0.1; 1 and 3 mg/mL) with addition of 1% (v/v) polysorbate 80 and sonicated for 1 minute prior the administration. All treatments were administered by gavage at 10 mL/kg body weight. Twenty microliters of 2% formalin

(formaldehyde) diluted in saline were injected subcutaneously into the plantar surface (intraplantar administration, i.pl.) of the right hind paw of mice.

Chemotaxis Assay

Experiments were carried out according to the modified Boyden chamber method [44]. To obtain the rat polymorphonuclear neutrophils, 20 mL of sterile 1% glycogen (w/v) were injected into the peritoneum of Wistar rats and four hours later, the animals were euthanized by decapitation and the leukocytes collected. The cell pellets were washed, suspended in HBSS, in order to obtain a leukocyte density of about 1.5×10^6 cells/mL, and kept on ice until use. Plasma collected from rats was incubated at 37 ºC for 30 min with 65 µg/mL of LPS (lipopolysaccharide from *Escherichia coli*-k-235) and diluted in Hanks buffer to a 20% solution (v/v). Prior to the chemotaxis assay, the leukocytes were treated with a range of 0.1-1.0 µg/mL of VAL, indomethacin or diclofenac (1µg/mL) at 37° C for 30 min. The leukocyte/samples were added in the upper wells of the chamber, separated by an 8.0 µm nitrocellulose filter (Millipore, USA) from the chemotactic stimulant (LPS) present in the bottom compartment. Then, the chamber was kept at 37°C for 1 hour. The leucocytes migration through the filter was measured by using an optical microscope. The distance from the top of the filter to the farthest plane of focus containing two cells, in five microscopic fields of duplicate filters allowed the evaluation of leukocyte migration.

VAL stock solution (1mg/mL) was prepared by using Hanks' balanced salt solution (HBSS) with addition of 1% (v/v) polysorbate 80 and sonicated for 1 minute. The reference drugs indomethacin (0.1; 0.5 and 1 µg/mL) and diclofenac (1µg/mL) were also dissolved in HBSS. The HBSS plus polysorbate 80 was the negative control. The concentration of polysorbate 80 in all final working solutions was less than 0.01%.

Statistical analysis

The results from neurogenic phase of formalin test and from antichemotactic assay were analyzed by ANOVA followed by Tukey's test. Data from inflammatory phase were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test, once these data were not normally distributed; $p<0.05$ was considered

statistically significant. Data analysis was performed using the GraphPad Prism 7.0 software.

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Conflict of Interest

The authors declare no conflict of interest

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Figure legends

Figure 1 Effect of a valepotriates fraction from *Valeriana glechomifoliana (*VAL) in the nociceptive phase of the behavioral test of formalin. Mice were treated (p.o.) with VAL 1 hour before the formalin injection (i.pl.) (2%, 20µL). The nociceptive behavior time was recorded for the first 5 minutes after the formalin injection (i.pl.). One-way ANOVA (n = 6-10 mice / group). Data are expressed as mean ± S.E.M.

Figure 2 Effect of valepotriates fraction of *Valeriana glechomifoliana* (VAL) in the inflammatory phase of the formalin test. Mice were treated (p.o.) with VAL 1 hour before the formalin injection (i.pl. 2%, 20 µL). The nociceptive behavior was recorded during the final 15 minutes (from a 30 minutes session) after the injection of formalin. Kruskal-Wallis test followed by Dunn's multiple comparison test, * P < 0.05. The box plots represent the median and quartiles 25 % and 75 % as the lower and upper edges of the boxes, respectively (n = 6-10 mice / group)

Figure 3 Effect of a valepotriates fraction from *Valeriana glechomifolia* (VAL) on the polymorphonuclear neutrophil chemotaxis *in vitro*. The leukocytes were treated with a range of 0.1-1.0 µg/mL of VAL at 37° C for 1 h. Chemotaxisis expressed as mean ± SEM of leukocyte migration. * $P < 0.0001$ indicates a significant difference compared to reference chemoattractant (LPS); • $P < 0.0001$ indicates a significant difference compared to VAL 1µg/mL; ◦ $P < 0.0001$ significantly different from diclofenac; ≠ $P < 0.0001$ significantly different from VAL 0,5 µg/mL; % $P < 0.0001$ significantly different from VAL 0.1µg/mL; ≈ $P < 0.0001$significantly different fromindomethacin 1µg/mL; ∑ $P < 0.0001$ significantly different from indomethacin 0.5µg/mL .One way ANOVA followed by Tukey's test (n= 2).

Tables

Table 1: Valepotriates content (mg/g supercritical CO_2 *V. glechomifolia* fraction: VAL) determined through HPLC. The values are expressed as mean ± SD.

Valepotriate	**mg/g VAL**
Valtrate	230 ± 4.04
Acevaltrate	193 ± 4.06
1-β-Acevaltrate	58 ± 0.87
1-β-Aceacevaltrate	14 ± 0.98
Isovaltrate	13 ± 0.95

Figures

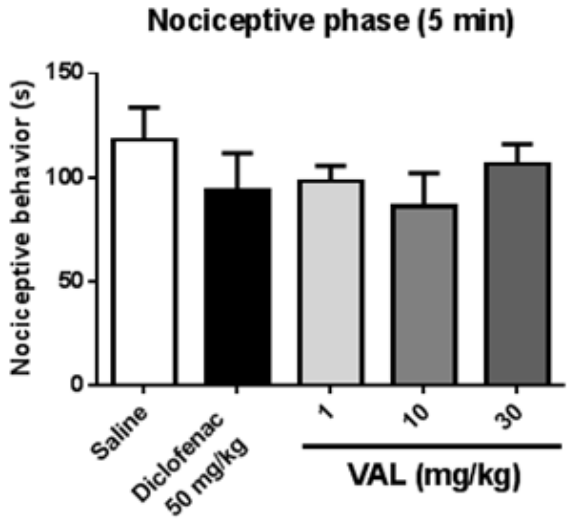

Figure 1

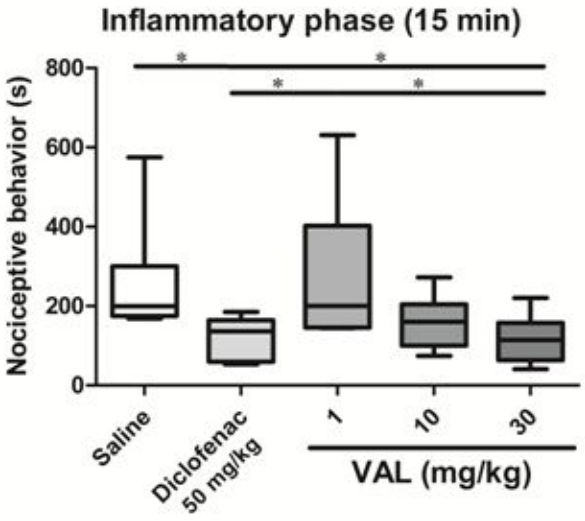

Figure 2

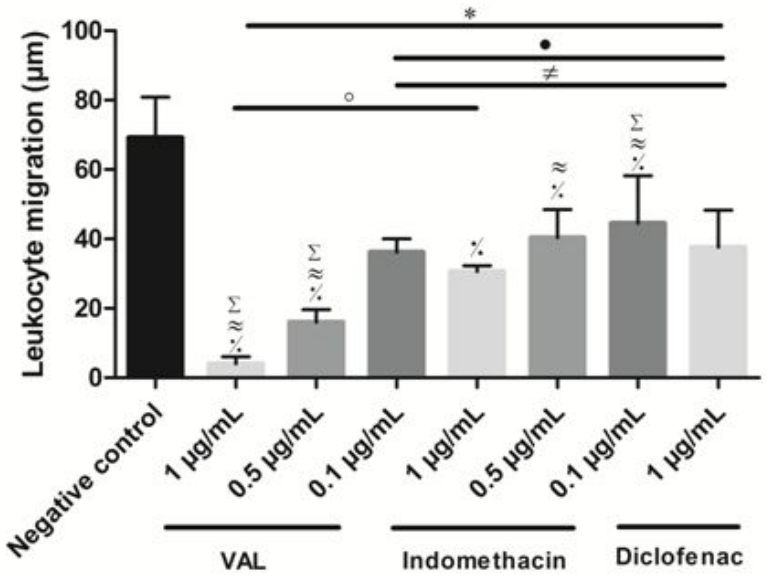
Leukocyte migration (µm)
100
80
60
40
20
0
*
•
≠
°
Negative control
1 µg/mL
0.5 µg/mL
0.1 µg/mL
1 µg/mL
0.5 µg/mL
0.1 µg/mL
1 µg/mL
VAL
Indomethacin
Diclofenac

Figure 3

Capítulo 2

Os experimentos apresentados nesta parte do trabalho foram realizados no Laboratório de Psicofarmacologia Experimental da Faculdade de Farmácia da UFRGS, sob orientação da Prof. Dr. Stela Rates e da Prof.Dr. Eliane Dallegrave, no Laboratório de Análises Clínicas Veterinário da Faculdade de Veterinária da UFRGS sob orientação da Prof. Dr. Stella Vale e no Laboratório de Patologia da Faculdade de Veterinária da UFRGS sob orientação do Prof. Dr David Dremeier estão apresentados na forma de um artigo científico que será submetido ao periódico *Food and Chemical Toxicology*.

Acute and repeated-doses toxicity study of valepotriates from *Valeriana glechomifolia* (Meyer) in mice

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Abstract

A valepotriates enriched fraction (VAL) from *Valeriana glechomifolia* Meyer (Valerianaceae), a native species to South Brazil, has shown antidepressant-like effect and prevented LPS-induced sickness behavior at 10 mg/kg (p.o.), as well as has exhibited anti-inflammatory activity at 30 mg/kg (p.o.), in mice. Altogether these findings suggest that the valepotriates scaffold might be useful to develop new antidepressant and anti-inflammatory drugs. However, data about the toxicity and safety of these compounds are still limited**.** Therefore, the aim of this study was to investigate the oral acute and repeated doses toxicity of VAL in CF1 mice. The acute toxicity study followed the Organization for Economic Cooperation and Development (OECD) guideline 423: three mice were treated with a single dose of VAL (2000 mg/kg, p.o.) and observed for 14 days. The repeated doses toxicity study was carried out based on OECD guideline 407: separated group of animals (n=10) received single daily doses of VAL (30, 150 and 300 mg/kg, p.o.) or vehicle during 28 days. Acute toxicity study detected only one death, which classify VAL in the hazard category 5, according to OECD-guideline 423. The repeated doses toxicity study has shown that VAL 300 mg/kg delayed the weight gain and reduced the food consumption in the first week, probably due to transient sedative effects. The other VAL doses had no effect on animals' ponderal evolution. At the end of the treatment all groups had the same body weight and food consumption. None of doses altered any behavioral, urinary, biochemical, hematological, anatomic or histological parameters. In conclusion, the results so far indicate that a valepotriates enriched fraction from *Valeriana glechomifolia* is safe at effective pre-clinical doses, by oral route.

Keywords: *Valeriana glechomifolia,* Valerianaceae, acute oral toxicity, repeated dose toxicity.

Abbreviations

IBAMA: Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

SCCO2: supercritical carbon dioxide

SISBIO-IBAMA: Sistema de Autorização e Informação em Biodiversidade, Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

VAL: diene valepotriates fraction

Highlights

Valepotriates mixture from *V. glechomifolia* should be classified in the hazard category 5, according to OECD guideline 423.

Oral repeated administration of valepotriates mixture from *V. glechomifolia* did not induce evident toxicity in CF1 mice.

Valepotriates from *V. glechomifolia* appear to be safe for use on pharmaceuticals products.

1.0 Introduction

Natural products have been used as a source of new pharmacological active compounds and to develop new pharmaceutical products, aiming at producing new efficient multitarget drugs (Veeresham, 2012). Therefore, it is important to study the biological effects of the substances derived from plants as well as their toxicity and safety. Among the species of plants commonly used for therapeutic purposes in the world, stands *Valeriana officinalis*, which has been traditionally used orally as sedative, hypnotic and anxiolytic (Hobbs, 1990; WHO, 1999). More recently, antidepressant (Hattesohl *et al*., 2008; Subhan *et al*.,2010; Holzmann *et al*., 2011; Sah *et al*., 2011; Liu *et al*., 2012; Müller *et al*., 2012a) and anti-inflammatory (Subhan *et al*., 2007; Zhang *et al.,*2010; Khuda *et al*.,2013) properties were also attributed to some species of *Valeriana*.

The pharmacological activities of species of *Valeriana* genus are attributed to several constituents, such as flavonoids (Marder, 2003), monoterpene valerenic acid (Benke *et al*., 2009) and valepotriates, which are a special class of terpenes found in *Valeriana* genus only (Andreatini *et al*., 2002; Maurmann *et al*., 2012; Müller *et al*., 2012a; Müller *et al*., 2015a; Müller *et al*., 2015b; Müller *et al*., 2015c).

Our research group has been studying the pharmacological activity of a *Valeriana* species found in southern Brazil, *Valeriana glechomifolia*, which presents high valepotriates content (Silva *et al*., 2002). These compounds were implicated in the antidepressant-like effect (Müller *et al*., 2012b) and seem to contribute to the prevention of the sickness and depressive-like behaviors induced by LPS in rodents (Müller *et al*., 2015b). However, data about the oral toxicity and safety of valepotriates are still limited.

Oral treatment of mice with different doses (500, 1000 and 2000 mg/kg/day) of *Valeriana officinalis* dietary supplement by Nature's way product – USA (each capsule containing 800 mg of valerian root and 220 mg of valerian root dried extract standardized to 0.8% valerenic acid) for 7 days increased the concentrations of malondialdehyde and decreased glutathione levels in hepatic and testicular cells. Besides, the frequency of micronuclei in the polychromaticerythrocytes was increased and the ratio of micronuclei in normochromatic erythrocytes in the femur was decreased. (Al-Majed *et al*., 2006).

In vitro studies have shown that valepotriates have cytotoxic, carcinogenic and mutagenic activity (Bounthanh *et a*l., 1981; Bounthanh *et al*., 1983; Hui-Lian *et al*. 2003; Vo *et al*.; 2003; Lin *et al*., 2009). However, a 30-day oral administration of a mixture of valepotriates (80% diidrovaltrate 15% valtrate and 5% acevaltrate) at doses of 6, 12 and 24 mg / kg (p.o) to pregnant rats did not change the fertility index, fetotoxicity or the development of the offspring (Tufik *et al.,* 1994).

Therefore, the aim of the this study was to investigate the oral acute and repeated doses toxicity of a valepotriates enriched fraction obtained from the aerial and subterranean parts of *Valeriana glechomifolia* submitted to supercritical CO_2 extraction (VAL).

2.0 Materials and methods

2.1 Plant material

The plant material (*V. glechomifolia* aerial and subterraneous parts) was collected from Aparados da Serra, in the state of Rio Grande do Sul – Brazil. The plant collection was authorized by IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) (29495-1). The identification was achieved by Dr. M.

Sobral (Universidade Federal de São João del-Rei, Minas Gerais, Brazil) and a voucher specimen (Sobral, 7733) was deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (ICN), Brazil.

2.2 Valepotriates enriched fraction preparation

To obtain an enriched fraction of valepotriates from *V. glechomifolia* (VAL), the aerial and subterraneous parts (100 g) were crushed and lyophilized and then submitted to supercritical CO_2 ($SCCO_2$) extraction (40°C, 90 bar, flow rate 6.67×10^{-4}kg s^{-1}), using a Pilot Equipment described by Cassel et al (2010). The $SCCO_2$ extraction had yield 2.89 g of VAL.

2.3 Valepotriates enriched fraction characterization

VAL was analyzed by HPLC according to a validated method previously described (Silva *et al*., 2002 ;Müller *et al*., 2012) using Shimadzu HPLC system and Waters Nova-Pack C18 column (4 mm, 3.9 × 150mm i.d. with Waters Nova- Pack C-18 guard column, 60 ˚ A, 3.9 × 20 mm). The mobile phase is isocratic (acetonitrile: water (50: 50 v/v)).The flow used was1 mL/min and the detection of valepotriates was performed with UV detector at 254 nm. The samples were dissolved (HPLC grade methanol) and filtered through a membrane filter (0.22mm pore size) and all valepotriates were quantified as mg of valtrate equivalent/g fraction.

2.4 Animals

Experiments were carried out using male CF1 mice (30–42g) from Centro de Reprodução e Experimentação de Animais de Laboratório - CREAL - UFRGS, Rio Grande do Sul, Brazil. Animals were housed by five in plastic cages at 23°± 1°C under

a 12-hour light/dark cycle, with food and water provided *ad libitum*. Experiments were approved by Animal Care Local Ethical Committee (CEUA-UFRGS; 28603, April 16th, 2015) and were conducted in accordance with Brazilian law (MCTI and CONCEA, 2013; MP, 2008) and European Communities Council Directive of 24 November 1986 (86/609/EEC)

2.5 Treatments

For the acute toxicity evaluation, VAL was suspended in saline (200 mg/ml) with 1% (v/v) of polysorbate 80, and sonicated by 1 minute immediately before using. VAL was administered once by gavage at 2000 mg/kg. For the repeated doses toxicity experiment, VAL was suspended in saline (3 mg/ml, 15 mg/ml or 30 mg/ml) with 1% (v/v) of polysorbate 80 every day, and sonicated by 1 minute immediately before using. Different groups of mice were daily treated by gavage with VAL single dose (30, 150 or 300 mg/kg) or vehicle (NaCl 0.9 % plus polysorbate 80 1% (v/v). All treatments volume of administration were 10 mL/kg body weight.

2.6 Experimental design

This study followed the guidelines of the Organization for Economic Cooperation and Development (OECD, 1995, 2001), adapted to our laboratory conditions, as described by Betti et al. (2012). The acute oral toxicity study was performed according to the Guideline 423 (1995) whereas repeated doses (28-day) oral toxicity study followed the Guideline 407 (2001).

To evaluate acute toxicity, the animals were treated with a single dose of VAL 2000 mg/kg by gavage (n = 3), observed 6 and 12 h after treating, and every day for 14 days. The signs and symptoms observed were piloerection, muscular tonus,

abdominal contortions, palpebral ptosis, motor activity, hypothermia, shacking, posterior paws paralysis, salivation, bronchial secretion, convulsions and death. The mouse body weight was registered across the whole period.

To perform the repeated doses toxicity study, VAL was given by gavage daily once for 28 days. The CF1 mice were divided in four groups (n = 10): vehicle treated group; group treated with VAL 30 mg/kg; group treated with VAL150 mg/kg; group treated with VAL 300 mg/kg. The 30 mg/kg dose was chosen because it is the maximal effective dose tested in the mouse paw formalin test, according to previous unpublished results by our group. The doses 150 and 300 mg/kg were extrapolated 5- and 10-fold respectively according to Guideline 407- OECD. Mice gross behavior was observed throughout the 28-day treatment. Body weight and food consumption were verified once a week. Mortality, body weight gain and food consumption were recorded throughout the repeated treatment. On the 15th day in acute study and 29th day in repeated study, the mice were anesthetized with thiopental sodium (50 mg/kg, i.p.) and the abdomen was incised. Prior to barbiturate anesthesia the animals were treated with lidocaine (10mg/kg, i.p) to avoid local pain due to thiopental administration. The caudal cava vein was punctured for blood collection and urinary vesicle for urine collection. The euthanasia was processed by diaphragm perforation and the following organs were collected: liver, kidneys, adrenal glands, spleen, lungs, heart and brain. All organs were surveyed for macroscopic alterations; gently dried under filter paper; weighted on digital balance and stored in plastics pots containing neutral buffered 10% formalin for further anatomic and histological analysis. The relative organ weight was calculated using the following equation:

$$\text{Relative weight organ (\%)} = \frac{\text{organ weight} \times 100}{\text{body mice weight}}$$

2.7. Hematological parameters

Mice blood was collected into polypropylene tubs (BD®) containing 3,6 mg of EDTA. The following hematological parameters were analyzed in mice blood: hemoglobin (Hb), red blood cells counts (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell corpuscular hemoglobin concentration (MCHC), eosinophils (E), monocytes (M), neutrophils (N) , linfocytes (L), white blood cell counts (WBC) and platelet counts were evaluated. The analyses were performed on the equipment Idexx Procyte Dx. Blood coagulation has occurred in some samples, thus the number of mice blood samples analyzed varied between 8 and 10.

2.8. Biochemical parameters

Blood was collected into polypropylene tubs (BD®). The following Biochemical parameters were analyzed trough the equipment CM200 Wiener: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkalinephosphatase (AP), total protein (PRO), blood glucose (GLU), total cholesterol (COL), triglycerides (TRI), uric acid (UAC), creatinine (CRE) and phosphokinase (CK) levels. Because in some cases it was not possible to collect appropriate volumes of blood, the final number of samples varied between 6 and 10.

2.9. Urinalysis

The qualitative urine analyses was performed by using Multistix -10 SG, Siemens and the parameters analyzed were urobilinogen, glucose, ketonic bodies, bilirubin, total protein, nitrite and pH.

2.10. Histopathology

Five animals from each treatment group were randomly chosen for further histological analysis. The organs collected (adrenals glands, kidneys, spleen, liver, heart, lungs and brain) were fixed in neutral buffered 10% formalin, dehydrated with alcohol, cleared in xylene, embedded in paraffin, sectioned and finally stained with hematoxylin and eosin. After these processes, samples were examined by optical microscopy.

2.11. Statistical analysis

Results from biochemical analyses, hematological analyses and relative percentage of organ weight were analyzed by one way ANOVA. Food consumption and animals' body weight were analyzed by using two way RM ANOVA. Tukey's pos hoc test was used when appropriate. $P < 0.05$ was considered as significant for all analyses. Data analysis was performed using the GraphPad Prism 7.0 software.

3.0 Results

3.1. Valeptoriates enriched fraction characterization

The main valepotriates found in the supercritical CO2 fraction were the dienic valepotriates valtrate, acevaltrate, 1β-acevaltrate, 1-β-aceacevaltrate, and isovaltrate. The table 1 shows the concentration of each main dienic valepotriate. The chemical structures of these compounds already have been published by Müller and colleagues (2012).

Table 1: Main dienic valtrates from supercritical CO2 *V. glechomifolia* fraction (VAL) determined through HPLC. The values are expressed as mean ± SD.

Valepotriate	**Concentration** (mg of valtrate equivalent/g fraction)
Valtrate	239 ± 3.52
Acevaltrate	198 ± 3.26
1-β-Acevaltrate	57± 1.78
1-β-Aceacevaltrate	9 ± 0.1
Isovaltrate	8 ± 0.27

3.2. Acute toxicity

One out of three mice showed significant change in the gross behavior and evident toxicity signs throughout the experimental period, as weight loss and piloerection, and finally died after the single oral administration of VAL 2000 mg/kg. The other animals did not show any behavioral or physical alterations. Thus, the study was repetead at the same conditions described above, and the result was replicated. Therefore, the oral acute toxicity of this fraction can be classified in category 5 (the lethal acute toxicity is greater than 2000 mg/kg) according to the Globally Harmonized Classification System of OECD.

3.3. Repeated-doses toxicity

3.3.1. Body weight gain

Two way repeated measures ANOVA revealed that treatments did not affect mice gain weight [$F_{treatement}(3.33) = 0.235$; $P = 0.8724$; $F_{day}(4.44) = 45.53$; $P< 0.001$](Fig. 1). Besides, there was a significant interaction between day and treatment factors [treatment x day interaction F(12.132) = 2.45 $P< 0.01$). The body weight of the groups vehicle, VAL 30 mg/kg and 150 mg/kg on days 7, 14, 21 and 28 was significantly higher than the body weight verified on day 1 [P=0,05] . The group VAL

300 mg/kg increased the weight only from day 14 when compared to day 1 [P=0,001]. At the end of the period (fourth week), there was no difference on body weight between the different groups [P=0,950].

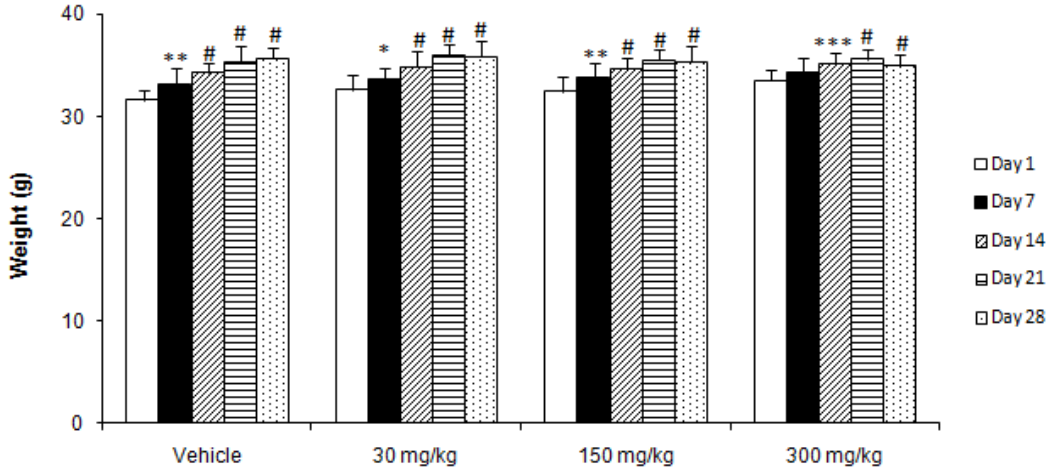

Fig.1.
Status of body weight gain in mice treated with different doses of VAL (valepotriates enriched fraction from *Valeriana glechomifoliana*) and vehicle (saline + polysorbate 80 1%) treated groups. Data are expressed as mean + S.E.M. (n =10). * P<0,05; ** P<0,005 *** P<0,001 # P<0,0001 represent differences in relation to the first measure (day 1) in the same group treatment.Two-way repeated measures ANOVA post hoc Tukey's test.

3.3.2. Food consumption

Two way repeated measures ANOVA revealed that both factors, treatment and week, have affected the mice food consumption [$F_{treatment}$ (3.6) = 27.38 P<0.01; F_{week} (3.60) = 7.533 P<0.05] (Fig. 2). There was a significant interaction between week and treatment factors [$F_{treatment\ x\ week}$ (9.18) = 7.391 P<0.001)]. At the first week, the group treated with VAL 300 mg/kg ingested less food than the vehicle group [P<0.001]. Mice treated with VAL 30 mg/kg [P< 0.01], VAL 150 mg/kg [P< 0.05] or vehicle [P< 0.01] decreased their food consumption in the second week when compared to the first week while those treated with VAL 300 mg/kg increased the food consumption in the same period [P< 0.05]. At the end of the period (fourth week), there was no difference on food consumption between the different groups [P=0,3916].

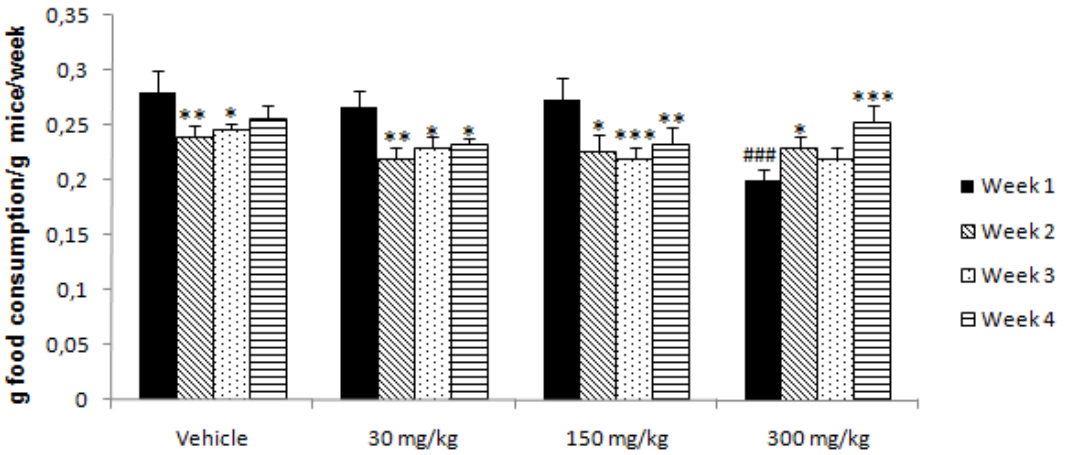

Fig. 2.
Status of food consumption per g of mice treated with different doses of VAL (diene valepotriates fraction from *Valerianaglechomifoliana*) and vehicle (saline + polysorbate 80 1%) treated groups. Data are expressed as mean + S.E.M. (n =10). Two-way repeated measures ANOVA post hoc Tukey's test. ***P < 0.001; **P < 0.01; *P < 0.05: difference in relation to week 1 with the same treatment; ###P < 0.001 difference in relation to vehicle at the same week.

3.3.3. Hematological parameters

One way ANOVA demonstrated that VAL treatments did not significantly alter any parameters evaluated in hematological analyses (Table 2).

Table 2
Effect of VAL (valepotriates enriched fraction from *Valeriana glechomifoliana*) repeated doses on hematological parameters in mice. Data are expressed as mean ± S.D. One way ANOVA (n = 8–10). Hemoglobin (Hb), red blood cells counts (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell corpuscular hemoglobin concentration (MCHC), reticulocytes, leukocytes, neutrophils, lynfocytes, monocytes and platelets.

	Vehicle	VAL 30 mg/kg	VAL 150 mg/kg	VAL 300mg/kg
Erythrocytes(x10^6/µ)	9.022 ± 0.53	8.93 ± 0.52	9.11 ± 0.44	8.87 ± 0.51
HCT (%)	43.3 ±2.54	42.38± 4.00	42.78 ± 2.77	41.33 ± 2.92
Hb (g/dL)	14.46 ± 0.72	14.11± 1.11	14.32 ± 0.88	13.92 ± 0.80
MCV (fL)	48.03 ± 2.23	47.37± 1.98	46.91 ± 1.16	46.57 ± 1.20
MCHC (%)	33.44 ±1.48	33.36± 1.11	33.49 ± 0.83	33.73 ± 1.37
Reticulocytes(%)	3.81 ±0.39	3.59± 0.41	3.43 ± 0.87	3.54 ± 0.79
Leukocytes (/µL)	3995 ±2037	4741 ± 1819	4105 ± 1569	4813 ± 1987
Neutrophils (%)	2.68 ±1.13	1.05± 1.00	2.73 ± 1.23	2.31 ± 1.33
Lymphocytes (%)	80.83 ±7.10	65.06± 40.29	81.46 ± 2.64	82.89 ± 5.21
Monocytes (%)	6.2733 ±6.69	3.04 ± 2.56	3.44 ± 1.76	5.48 ± 4.61
Platelets (x10^3 µL)	785.8 ±185.26	754.63 ± 267.16	939.55 ± 217.97	844.44 ± 180.04

3.3.4. Biochemical parameters

One way ANOVA demonstrated that VAL at different doses did not induce any significant alterations in biochemical parameters evaluated (Table 3).

Table 3
Effect of VAL (valepotriates enriched fraction from *Valeriana glechomifoliana*) repeated doses on biochemical parameters in mice. Data are expressed as mean ± S.D. One way ANOVA (n = 6–10). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), total protein (PRO), creatinine (CRE), blood sugar levels (GLU), total cholesterol (COL), triglycerides (TRI), uric acid(UAC), creatinine (CRE) and phosphokinase (CK).

	Vehicle	30 mg/kg	150 mg/kg	300 mg/kg
ALT (U/L)	42.44 ± 18.71	43.2 ± 21.96	54.22 ± 37.92	61.1 ±36.93
AST (U/L)	61.44 ± 15.02	89 ± 67.65	72.62 ± 30.26	101 ±74.07
COL (mg/dL)	122.29 ± 21.82	121.25 ± 13.47	121.28 ± 12.07	107.71 ±13.16
CRE (mg/dL)	0.021 ± 0.05	0.12 ± 0.21	0.08 ± 0.13	0.05 ±0.08
AP (U/L)	109 ± 19.14	100.25 ± 31.14	90.28 ± 41.96	114.6 ±7.21
GLU (mg/dL)	229.75 ± 9.66	235.86 ± 23.50	237 ± 30.87	240.33 ±29.37
UAC (mg/dL)	2.25 ± 1.77	1.75 ± 0.95	2.24 ± 1.39	1.99 ±0.68
TRI (mg/dL)	155.92 ± 34.79	160.09 ± 54.49	154 ± 48.68	131.92 ±41.23
PRO (g/L)	53.2 ±2.30	51.71 ± 2.21	52 ± 3.00	50.71 ±2.29
CK (U/L)	96.63 ± 47.73	78.5 ± 37.10	115.33 ± 72.00	226.5 ±207.68

3.3.5. Relative organ weights

One way ANOVA showed no significant difference in the relative organ weights (Table 4) between the VAL groups and when compared to vehicle group.

Table 4
Relative (%) organ weights of mice treated with VAL (valepotriates enriched fraction from *Valeriana glechomifoliana*) repeated doses for 28 days. Data are expressed as mean ± S.D. One way ANOVA (n = 10).

	Vehicle	Val 30 mg/kg	Val 150 mg/kg	Val 300 mg/kg
Adrenal	0.02 ± 0.002	0.017 ± 0.026	0.019 ± 0.018	0.021 ±0.002
Spleen	0.32 ± 0.019	0.31 ± 0.016	0.29 ±0.009	0.31 ±0.014
Brain	1.08 ± 0.021	1.12 ± 0.024	1.08 ± 0.02	1.08 ±0.06
Heart	0.47 ± 0.014	0.52 ± 0.021	0.48± 0.016	0.49 ±0.016
Liver	5.50 ± 0.222	5.60 ± 0.240	5.47 ±0.26	5.49 ±0.24
Lungs	0.54 ± 0.015	0.54 ± 0.023	0.54 ±0.023	0.57 ±0.056
Kidney	1.88 ± 0.08	1.85 ± 0.09	1.85 ±0.076	1.92 ±0.09

3.3.6 Anatomic and histopathological analyses

Organs from mice treated during 28 days with VAL did not present significantly anatomic or histopathological alterations at any dose when compared to organs from control group.

3.3.7 Urinary parameters

The urinary analyses (data not show) did not show any significantly alteration in the parameters evaluated in the groups treated with VAL when compared to vehicle treated group.

4. Discussion

In this study, the toxicity of a valepotriates enriched fraction from *Valeriana glechomifolia* was investigated for the first time, based on the OECD criteria. The guidelines of OECD used to perform the oral acute toxicity (guide 423-2001) and oral repeated doses toxicity (guide 407-1995) are worldwide accepted and are used as a standard model to test the toxicity of chemical compounds. In the oral acute toxicity study, the VAL fraction was administrated to CF1 mice (2000 mg/kg, p.o) and after 14 days of observation, only one death was detected, which allows us to classify VAL in the hazard category 5, according to OECD-guideline 423. It means that the VAL LD_{50} is greater than 2000 mg/kg. Criteria for hazard Category 5 are intended to enable the identification of test substances which are of relatively low acute toxicity hazard but which, under certain circumstances may present a danger to vulnerable populations (OECD, 2001).

In order to perform the repeated administration study, the range doses was chosen on the basis of previous studies of our research group. The effective dose of

VAL in preventing the sickness and depressive-like behaviors induced by LPS in rodents was 10 mg/kg (Müller *et al*., 2015b), and another study by us demonstrated that 30 mg/kg is the maximal effective dose tested in the mouse paw formalin test (Almeida et al., unpublished results). Therefore, in the present study the highest above mentioned dose was extrapolated 5- and 10-times, according to the Guideline 407-OECD.

VAL 30, 150 and 300 mg/kg did not present evident toxicity to mice treated by oral route for 28 days. The weight gain and food consumption were the only parameters altered; however these results do not seem to be related to toxicity. The body weight gain of animals repeatedly treated with VAL 300 mg/kg was observed only on day 14, in relation to day 1. The other groups, including the vehicle group, gained weight on day 7. At the end of the treatment, all groups treated with VAL reached the same weight of the group treated with vehicle. Interestingly, the groups treated with vehicle, VAL 30 and 150 mg/kg presented a decrease in food consumption in the second week, which could be related to the stress or physical trauma provoked by the repeated oral administration (gavage), which t was already described as responsible for the reduction of food consumption in mice (De Meijer et al., 2010). On the other hand, the group that received VAL 300 mg/kg increased the food consumption in the second week, when comparing with its own food consumption in the first week. However, it is noteworthy that when compared to the vehicle group, the group treated with VAL 300 mg/kg had a significant lower food consumption in first week, which could explain the delay in the weight gain. These findings may be related to the sedative effects of valepotriates (Hobbs, 1990; Maurmann et al., 2011), which could impair the animals' ability to eat and also protect against the stress induced by the gavage procedures. Considering that all groups reached the same food

consumption and body weigh at the end of the treatment, we suppose that the animals developed tolerance to the possible sedative effect of VAL 300 mg/kg. This hypothesis deserves further studies.

Altogether, the results indicate that VAL is safe at pre-clinical doses when administered by oral route, which are in accordance with other studies demonstrating that different extracts and valepotriates of *Valeriana* genus did not present any significant toxic effect after the oral administration (Braun et al., 1984; Tufik et al., 1994; Yao et al., 2007). One study revealed that the oral administration of *V. officinalis* extract was toxic to mice (Al-Majed et al., 2006), but the authors have used higher (500 – 2000 mg/kg/day) doses than the ones used in our study.

Conversely, several studies on the toxicity of valepotriates as well as *Valeriana* extracts demonstrated toxic effects after the intraperitoneal administration (Lin et al 2009; Bos et al., 1998; Hui-lian et al., 2003).

Salles and colleagues (2010) carried out an acute toxicity study using a dichloromethane extract of *V. glechomifolia* (containing valepotriates) at 10, 20, 30, 40 and 50 mg/kg (i.p), and 5, 10, 20, 40 and 80 mg/kg (p.o). The median lethal dose (DL_{50}) estimated by intraperitoneal route was 42 mg/kg, which classifies the extract as moderate to high toxic, according to the OECD (2001). However, this study revealed no toxic signs within the first 24 hours after oral administration. On the other hand, during the first week after treating, it was possible to observe one death in the groups orally treated with 20, 40 and 80 mg/kg.

In vitro studies have already demonstrated that valepotriates present marked cytotoxicity when directly exposed to hepatoma (Bounthan et al., 1981; Lin et al., 2009), lung carcinoma and medullary cells (Tortarolo et al., 1982). Bounthanh and

colleagues (1983) considered that the cytotoxicity of the valepotriates is due to the double bond between C5-C6 while other authors attribute the cytotoxicity to the epoxy group bond to C8, which would act as an alkylating agent (Braun et al., 1982), that may target the DNA, causing cell death (Zong et al., 2004).

Valeptoriates are very unstable in acid and alkaline medium, under these conditions they are degraded into homobaldrinals and baldrinals, which do not present the epoxide group bond to C8 (Hobbs et al., 1989). Herein, we did not detected any toxicity signs, which might be related to the instability of the valepotriates and degradation of these compounds when suffering acidification and alkalinization processes (Hobbs et al., 1989) after the oral administration, Thus, the valepotriates may be acting as pro-drugs, and wich is really having an effect are their degradation products such as baldrinal and homobaldrinal, wich are 10 to 30 times less toxic than diene valepotriates (Bos et al., 1998).

In conclusion, according to the OECD acute toxicity parameters the valepotriates enriched fraction from *Valeriana glechomifolia* can be classified as safe (category 5) at effective pre-clinical doses, by oral route. The repeated doses study showed that only body weight and food consumption were altered by VAL treatment and these alterations are probably not related to toxicity, but to its sedative effects. Considering the studies regarding the pharmacology and toxicity of valepotriates, we may conclude that the valepotriates might represent a promising scaffold for developing safe and effective new drugs, may be acting as pro-drugs.

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Conflict of Interest

The authors declare no conflict of interest.

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Discussão

Estudos anteriores do nosso grupo de pesquisa apontam para o potencial anti-inflamatório de valepotriatos e para a necessidade de mais informações sobre o perfil toxicológico deste grupo de metabólitos secundários vegetais (Müller *et al.,* 2015c, Salles *et al.,* 2010). Desta maneira, o presente trabalho visou investigar o potencial anti-inflamatório e a toxicidade de uma fração enriquecida em valepotriatos por extração com CO_2 supercrítico (VAL). Esta fração foi obtida a partir das partes aéreas e subterrâneas de *Valeriana glechomifolia*, espécie endêmica do Rio Grande do Sul (Sobral, 1999a, 1999b).

Os resultados desse trabalho demonstraram, pela primeira vez, que VAL apresenta atividade anti-inflamatória no teste da formalina, em camundongos, e atividade antiquimiotática, no ensaio de migração de leucócitos obtidos de ratos. A avaliação da toxicidade aguda e de doses repetidas demonstrou que VAL pode ser classificada como segura, de acordo com as normativas da OECD (407 e 423). A dose letal mediana (DL_{50})foi estimada como superior a 2000 mg/kg e não foram observados sinais de toxicidade no estudo de toxicidade de doses repetidas.

O efeito anti-inflamatório dose-dependente de VAL no teste de formalina sugere que a mesma pode apresentar atividade semelhante a fármacos anti-inflamatórios, os quais também suprimem a segunda fase do teste da formalina (Holanda *et al.,* 2015). Esses resultados estão em consonância com outros estudos que demonstram efeito anti-inflamatório de espécies do gênero *Valeriana*. Khuda e colaboradores (2013) demonstraram que uma formulação tópica de extrato bruto metanólico de *V. wallichii* apresentou efeito anti-inflamatório, no modelo de edema de pata induzido por carragenina em ratos, através da inibição da síntese de histamina, serotonina e prostaglandinas. Outro estudo demonstrou que um extrato etanólico de *V. amurensis* controlou a reação inflamatória no córtex e hipocampo de ratos submetidos a um modelo da doença de Alzheimer. O mecanismo de ação anti-inflamatória do extrato etanólico de *V. amurensis* parece estar relacionado com a redução da expressão de iNOS, COX-2 e lkappaB-alfa, o que pode inibir a atividade da glia e, consequentemente, reduzir a lesão inflamatória em neurônios corticais e hipocampais (Zhang *et al.,* 2010). Neamati e colaboradores (2014) demonstraram que *V. officinalis* preveniu o desenvolvimento do comportamento de doente (*sickness behavior*) e o comportamento do tipo depressivo induzido por LPS (lipopolissacarideo de *Escherichia coli)* em camundongos, sendo este um modelo comportamental de

depressão relacionada à neuroinflamação. O nosso grupo de pesquisa também demonstrou recentemente que VAL previniu o desenvolvimento de comportamento de doente e do tipo depressivo induzido por LPS, assim como inibiu o consequente aumento da expressão cortical das citocinas pró-inflamatórias IL-1β e TNF-α (Müller *et al.,* 2015c).

Os efeitos de VAL sobre a quimiotaxia de leucócitos induzida por LPS, através do método da câmara de Boyden *in vitro,* também foram investigados. Os resultados obtidos demostraram que VAL inibiu significativamente a migração de leucócitos em todas as concentrações testadas no ensaio quando comparado com o controle negativo. Além disso, nas maiores concentrações, VAL inibiu significativamente a migração de leucócitos quando comparado com os controles positivos indometacina e diclofenaco (nas mesmas concentrações), indicando que VAL apresenta melhor efeito do que os controles positivos utilizados na inibição da migração de leucócitos. Tendo em vista que os valepotriatos são uma classe especial de terpenos somente encontrados no gênero *Valeriana* (Nagara *et al.,* 2015), nossos resultados somam-se a outros estudos que demonstraram a atividade antiquimiotática de terpenos (Ahumada *et al.,* 1997; Mawa *et al.,*2016; Nonato *et al.,* 2012) , ,

Na fase aguda da inflamação, mimetizada pelo teste de formalina, há a liberação de mediadores pró-inflamatórios, dentre estes as citocinas, tipicamente TNF-α e IL-1β, as quais produzem um gradiente quimiotático que guia e ativa as células recrutadas para o local da lesão (Coutinho *et al.,* 2011). É bem aceito que as citocinas possuem relação com as lesões celulares, com o reconhecimento imunológico, os sinais locais e sistêmicos da inflamação, por exemplo, migração de células, edema, febre e hiperalgesia (Ferreira *et al*., 1988; Faccioli *et al.,* 1990; Dinarello *et al.,* 2000). Essas citocinas são responsáveis pela síntese de proteínas de fase aguda, aumento da permeabilidade vascular e de moléculas de adesão, além da indução da liberação de quimiocinas e IL-6 (responsável por ativar células T e B com síntese de imunoglobulinas) (Kindt, 2008; Abbas *et al.,* 2010). Assim, sugerimos que a atividade inibitória de VAL sobre a expressão das citocinas TNF-α e IL-1β, relatado por Müller e colaboradores (2015c), seja responsável, pelo menos em parte, pelo efeito antiquimiotático e anti-inflamatório de VAL, aqui demonstrado.

Tendo em vista o potencial farmacológico de VAL e que há poucos estudos sobre a toxicidade de valepotriatos administrados pela via oral, a toxicidade de uma

fração enriquecida em valepotriatos foi investigada. Para tanto, foram seguidas as normativas da OECD para execução dos estudos de toxicidade oral aguda (normativa 423-2001) e toxicidade oral de doses repetidas (normativa 407-1995), as quais são aceitas internacionalmente e utilizadas como um modelo padrão para testar a toxicidade de compostos químicos.

No estudo de toxicidade oral aguda, VAL foi administrada a 3 camundongos CF1 machos (2000 mg/kg, v.o.) e estes foram observados durante 14 dias, onde foi detectada a morte de um animal, o qual apresentou sinais de toxicidade como perda de peso e piloereção. O estudo foi repetido nas mesmas condições e o resultado encontrado foi de apenas uma morte novamente. Assim, VAL pode ser classificada como segura (categoria 5) – DL_{50} superior a 2000 mg/kg, segundo normativa OCDE 423).

Para o estudo de toxicidade de doses repetidas de VAL, a dose inicial foi escolhida com base nos resultados de eficácia no teste da formalina. A dose anti-inflamatória máxima testada, 30 mg/kg, foi extrapolada em 5 e 10 vezes. Desta maneira, as doses utilizadas de VAL no estudo da toxicidade foram 30, 150 e 300 mg/kg (v.o).

O grupo tratado com VAL 300 mg/kg ganhou peso apenas a partir da segunda semana de tratamento, enquanto os tratamentos com VAL 30 e 150 mg/kg não alteraram a evolução ponderal dos animais, em relação ao grupo tratado com veículo. Esse dado é coerente com o consumo de alimento que, na primeira semana, foi menor no grupo tratado com VAL 300 mg/kg em relação ao grupo tratado com o veículo. Porém, já na segunda semana de tratamento o grupo VAL 300 mg/kg consumiu alimento normalmente, de forma comparável ao grupo tratado com veículo, e ao final do perído de tratamento (28^0 dia) todos os grupos atingiram o mesmo peso e consumiram a mesma quantidade de alimento. Por outro lado, os grupos tratados com veículo ou VAL nas doses de 30 e 150 mg/kg apresentaram uma redução no consumo alimentar na segunda semana de tratamento, quando comparado ao próprio consumo observado na primeira semana. Esse achado pode estar relacionado com o estresse ou eventual trauma físico provocados pela administração oral repetida (gavagem), o que já foi relacionado com a redução do consumo alimentar e ganho de peso em ratos (de Meijer *et al.,* 2010). O conjunto desses resultados nos permite sugerir que o retardo no aumento de peso dos animais no grupo tratatado com VAL

300 mg/kg não se deve uma toxicidade de VAL, mas pode estar relacionado com o efeito sedativo já relatado para os valepotriatos (Hobbs, 1990; Maurmann *et al.,* 2011), o qual teria reduzido a busca de alimentos pelos animais e, ao mesmo tempo, protegido os mesmos do estresse - e eventualmente de um processo inflamatório - causados pela gavagem repetida, permitindo o aumento do consumo de alimentos pelos animais tratados com 300 mg/kg na segunda semana, em relação à primeira semana, contrariamente ao que aconteceu com os animais tratados com o veículo e as menores doses de VAL. A normalização do consumo de alimento na segunda semana de tratamento com VAL 300 mg/kg também sugere o desenvolvimento de tolerância ao efeito sedativo de VAL. Essas hipóteses necessitam de experimentos adicionais para serem comprovadas.

Nos demais parâmetros avaliados no estudo da toxicidade de doses repetidas (parâmetros hematológicos e bioquímicos, peso relativo dos órgãos, análise de urina e parâmetros histológicos), não houve diferença significativa entre os grupos tratados com VAL em diferentes concentrações e o grupo tratado com veículo. Assim, pode-se concluir que o tratamento repetido com VAL (30, 150 e 300 mg/kg, v.o) não apresentou efeitos tóxicos. Esses resultados estão de acordo com outros trabalhos que demonstram que administração via oral de valepotriatos e diferentes extratos do gênero *Valeriana* não apresentam efeitos tóxicos (Braun *et al*., 1984; Tufik *et al*., 1994; Yao *et al.,* 2007). Entretanto, Al-Majed e colaboradores (2006) demonstraram que a administração oral de um extrato de *V. officinalis* em doses elevadas (500, 1000 e 2000 mg/kg/dia) aumentou os níveis de malondialdeído e diminuiu os níveis de glutationa em células hepáticas e testiculares de ratos. Além disso, Salles (2010) demonstrou a toxicidade aguda de um extrato diclorometano de *V. glechomifolia* (enriquecido em valepotriatos), administrado pelas vias oral e intraperitoneal. Esse estudo revelou que embora nas primeiras 24 horas após a administração não tenham sido observados efeitos tóxicos nos animais tratados por via oral, durante a primeira semana após a administração houve uma morte nos grupos tratados com as doses 20, 40 e 80 mg/kg. Para a administração por via i.p, a DL_{50} encontrada foi de 42 mg/kg, o que classifica o extrato como de toxicidade moderada a alta, de acordo com a OECD (2001). Outros autores também demonstraram efeitos tóxicos de valepotriatos, bem como de extratos de espécies de *Valeriana,* quando utilizada a via de administração intraperitoneal (Lin *et al.,* 2009; Bos *et al*., 1998; Hui-Lian *et al*.,

2003). Além disso, alguns ensaios *in vitro* demonstraram citotoxidade causada por valepotriatos em células de hepatoma (Bounthan *et al.,* 1981; Lin *et al*., 2009), carcinoma de pulmão e células medulares (Tortarolo *et al*., 1982).

O conjunto dos dados apresentados acima e os resultados do presente estudo demonstram que a magnitude da toxicidade de espécies do gênero *Valeriana* e de valepotriatos parece ser dependente do tipo de extrato e da via de administração empregados, sendo a via intraperitoneal mais tóxica. Isso é corente com a química dos valepotriatos, que são muito instáveis, e em meio ácido e alcalino podem ser degradados em compostos como homobaldrinal e baldrinal, os quais não apresentam grupamento epóxido, que tem sido relacionado com a toxicidade dos valepotriatos (Hobbs *et al*., 1989). A citotoxicidade dos valepotriatos tem sido relacionada à ligação dupla entre C5-C6 do núcleo iridoide (Bounthanh *et al.,* 1983) e também à ligação do grupo epóxido ao C8, que poderia agir como um agente alquilante do DNA (Braun *et al*., 1982), causando morte celular (Zong *et al*., 2004). Esses achados podem ajudar a explicar a ausência de efeitos tóxicos após a administração oral de VAL no ensaio de toxicidade de doses repetidas e sugerem que os valepotriatos podem estar atuando como pró-drogas, o que levanta uma interessante perspectiva de estudo, como, por exemplo, o estudo da farmacocinética dos valepotriatos e farmacodinâmica dos produtos de degradação.

Conclusão

A partir dos resultados do presente trabalho, conclui-se que uma fração enriquecida em valepotriatos de *V. glechomifolia* apresenta atividade anti-inflamatória e inibe significativamente a migração de leucócitos, o que pode estar relacionado à capacidade de redução da expressão de citocinas pró-inflamatórias por estes compostos.

Os estudos de toxicidade oral aguda e de doses repetidas demonstraram que a fração enriquecida em valepotriatos de *V. glechomifolia* pode ser classificada como segura nas doses efetivas utilizadas nos ensaios pré-clínicos, por via oral.

O conjunto de dados deste trabalho mostra a necessidade da realização de mais estudos utilizando valepotriatos como protótipos para o desenvolvimento de novos fármacos úteis no tratamento de condições inflamatórias e abrem novas perspectivas para a farmacologia do gênero *Valeriana.*

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Anexos

Parecer da Comissão de Ética no Uso de Animais em Pesquisa da UFRGS (CEUA-UFRGS):

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 28603
Título: AVALIACAO DA ATIVIDADE ANTI-INFLAMATORIA E TOXICIDADE DE VALERIANA GLECHOMIFOLIA MEYER (VALERIANACEAE)

Pesquisadores:

Equipe UFRGS:

STELA MARIS KUZE RATES - coordenador desde 01/03/2015
GILSANE LINO VON POSER - pesquisador desde 01/03/2015
DAVID DRIEMEIER - pesquisador desde 01/03/2015
MIRIAM ANDERS APEL - pesquisador desde 01/03/2015
Tielle Moraes de Almeida - Aluno de Mestrado desde 01/03/2015

Equipe Externa:

Eliane Dallegrave - pesquisador desde 01/03/2015
ANDRESA HEEMANN BETTI - pesquisador desde 01/03/2015

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 06/04/2015 - Sala 330 do Anexo I do Prédio da Reitoria - Campus Centro- UFRGS, em seus aspectos éticos e metodológicos, para a utilização de 92 camundongos CF1 machos, 7 ratos Wistar machos e 18 neonatos de ratos Wistar de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quinta-Feira, 16 de Abril de 2015

BRUNO CASSEL NETO
Vice Pró-Reitor de Pesquisa