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Ritiel Corrêa da Cruz

ANÁLISE FITOQUÍMICA, BROMATOLÓGICA, GENOTÓXICA E ANTIMICROBIANA DE TUBÉRCULOS DE *Tropaeolum pentaphyllum* LAM.

Santa Maria, RS
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Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Ciências Farmacêuticas**.

Orientadora: Profª. Drª. Marli Matiko Anraku de Campos

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Santa Maria, RS
2016

DEDICATÓRIA

A meu pai, Brunel Duran da Cruz, e minha orientadora e amiga, professora Margareth Linde Athayde, exemplos de vida que precocemente nos deixaram durante a realização desse trabalho. Não puderam estar fisicamente presentes nos passos finais dessa jornada, mas certamente estarão acompanhando, de onde as boas almas repousam após a existência terrena, bem como vão estar eternamente presentes em nossos corações e mentes.

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RESUMO

ANÁLISE FITOQUÍMICA, BROMATOLÓGICA, GENOTÓXICA E ANTIMICROBIANA DE TUBÉRCULOS DE *Tropaeolum pentaphyllum* LAM.

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ORIENTADORA: Marli Matiko Anraku de Campos

A *Tropaeolum pentaphyllum* Lam. pertenceteà família Tropaeolaceae, tem seus tubérculos comumente conhecidos como crem, e utilizados popularmente no sul do Brasil para o tratamento de dermatoses e como tempero. Afim de confirmar suas propriedades e utilização popular foi realizado um estudo fitoquímico e bromatológico dos tubérculos, bem como uma avaliação de seu potencial antimicrobiano e genotóxico. Segundo normas internacionais de análise, foram realizadas determinações de macronutrientes e da composição mineral dos tubérculos, utilizando material fresco. Adicionalmente, foi extraído o óleo essencial do material fresco, por hidrodestilação (Clevenger), posteriormente submetido a análise por cromatografia gasosa com espectrometria de massas (GC-MS), e avaliação de atividade antimicrobiana. A partir da matéria vegetal seca foi obtido extrato bruto hidroalcoólico (70%), que por partição líquido-líquido deu origem as frações clorofórmio, acetato de etila e n-butanol. Juntamente com o extrato foram submetidos a determinação de polifenois totais (Folin-Ciocalteau), capacidade antioxidante*in vitro* (DPPH) e investigação de compostos fenólicos por HPLC-DAD, bem como aos testes de atividade antimicrobiana e genotoxicidade. A avaliação da atividade antimicrobiana foi realizada por microdiluição em caldo, frente a uma gama de bactérias Gram-positivas e negativas, e fungos leveduriformes e filamentosos, afim de se obter os valores de concentração inibitória, fungicida e bactericida mínimas. A genotoxicidade foi testada em cultura de células mononucleares do sangue periférico isolados, após incubação com os extratos, e os parâmetros avaliados foram a contagem total (câmara de Neubauer), viabilidade (azul de Tripan), formação de micronúcleos (Giemsa) e dano ao DNA (ensaio Cometa).As análises bromatológicas revelaram que os principais macronutrientes dos tubérculos são água (69,73%) e carboidratos (26,06%), e os principais constituintes minerais são potássio, magnésio e cálcio (450,05; 79,36 e 56,88 mg/100g de massa seca, respectivamente). A análise por GC-MS revelou que o componente majoritário do óleo essencial é o isotiocianato de benzila (ITB) (98,51%), e que a fração clorofórmio é composta pelo mesmo, amidas a este estruturalmente relacionadas, ácidos graxos e seus ésteres, enxofre elementar e um fitoesteroide. Na análise por HPLC-DAD não foi encontrado nenhum dos flavonoides e ácidos fenólicos pesquisados, em concordância com o baixo teor de polifenois totais e pequena capacidade antioxidante. O óleo essencial e a fração clorofórmio apresentaram potente atividade antimicrobiana, com valores de concentração inibitória mínima abaixo de 200 µg/mL, relacionado a presença do ITB, ácidos graxos e enxofre. A fração clorofórmio e o extrato bruto apresentaram efeitos genotóxicos, possivelmente em função da presença de ITB e ácido palmítico. Os resultados apresentaram a quantificação dos principais nutrientes dos tubérculos, revelaram o metabólito secundário responsável por suas propriedades organolépticas e uso como tempero, demonstraram a atividade antimicrobiana, que está relacionada com seu uso popular e demonstraram genotoxicidade nas condições testadas.

Palavras-chave: *Tropaeolum pentaphyllum.* Crem. Isotiocianato de benzila. Atividade antimicrobiana. Genotoxicidade.

ABSTRACT

PHYTOCHEMICAL, BROMATOLOGICAL, GENOTOXIC AND ANTIMICROBIAL ANALYSIS OF *Tropaeolum pentaphyllum* LAM. TUBERS

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Tropaeolum pentaphyllum Lam., belonging to Tropaeolaceae family, possess tubers commonly known as ‘’crem’’, popularly used in South of Brazil to the treatment of dermatosis and as spice. In order to verify its properties and popular applications, a phytochemistry and proximate analysis were made, as well as an evaluation of its antimicrobial and genotoxic potential. The quantification of macronutrients and mineral composition was executed according to international analytical guidelines, with fresh material. Also from fresh material, an essential oil extraction was made through hydrodistillation (Clevenger), lately submitted to gas chromatography-mass spectrometry (GC-MS) analysis and antimicrobial activity evaluation. From dried material, a crude hydroalcoholic extract (70%) was obtained, which through liquid-liquid partition generated the chloroform, ethyl acetate and n-butanol fractions, that along with the extract were submitted to total polyphenolics quantification (Folin-Ciocalteau), *in vitro* antioxidant capacity (DPPH) and polyphenolic compounds investigation by HPLC-DAD, as well as the biological activity tests. Antimicrobial activity was performed by broth microdilution, against a wide range of Gram-positive and negative bacteria, and yeasts and filamentous fungi, to obtain the inhibitory, bactericidal and fungicidal minimal concentrations. The genotoxicity was tested with culture of peripheral blood mononuclear cells isolated from blood, the evaluated genotoxic parameters were cell counting (Neubauer chamber), cell viability (Trypan blue), micronuclei formation (Giemsa) and DNA damage (Comet assay). Proximate and mineral analysis revealed that the main macronutrients of the tubers are water (69.73%) and carbohydrates (26.06%), the major mineral components are potassium, magnesium and calcium (450.05, 79.36 and 56.88 mg/100g of dry mass, respectively). GC-MS analysis revealed that the major constituent of the essential oil is benzyl isothiocyanate (BITC) (98.51%), and that the chloroform fraction is constituted by BITC, amides structurally related to it, fatty acids and its esters, elemental sulfur and a phytosterol. No flavonoid or phenolic acid were found through HPLC-DAD, which is in accordance with the low amount of total polyphenols and reduced antioxidant capacity. The essential oil and the chloroform fraction presented strong antimicrobial activity, with minimal inhibitory concentration values under 200 µg/mL, which is related to the presence of BITC, fatty acids and sulfur. The chloroform fraction and the crude extract presented genotoxic effects, possibly due to the presence of BITC and palmitic acid. The results presented a quantification of the major nutrients from the tubers, revealed the secondary metabolite responsible for its organoleptic properties and use as spice, demonstrated its antimicrobial activity, related to the traditional medicinal use, and revealed genotoxicity at the tested conditions.

Keywords: *Tropaeolum pentaphyllum*. Crem. Benzyl isothiocyanate. Antimicrobial activity. Genotoxicity.

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MANUSCRITO 1

LISTA DE ABREVIATURAS E SIGLAS

ANVISA	Agência Nacional de Vigilância Sanitária
CIM	Concentração Inibitória Mínima
CLSI	do inglês, Clinical & Laboratory Standards Institute
DNA	do inglês, deoxyribonucleic acid, ácido desoxirribonucleico
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
GC-MS	do inglês, Gas Chromatography-Mass Spectrometry, Cromatografia Gasosa-Espectrometria de Massas
HPLC	do inglês, High Performance Liquid Chromatography, Cromatografia Líquida de Alta Eficiência
IC50	do inglês, Inhibitory Concentration 50%
ITB	Isotiocianato de benzila
ITC	Isotiocianato

SUMÁRIO

1 INTRODUÇÃO

Durante o final do século XIX e início do século XX, os estados do Rio Grande do Sul e Santa Catarina receberam grandes grupos de imigrantes provenientes da Europa, especialmente italianos e alemães. Tais grupos populacionais trouxeram sua cultura e seus hábitos, e dentre estes se inclui as práticas gastronômicas e culinárias. Além da preparação de conhecidos pratos, também foi trazida para os referidos estados, o costume de utilizar diferentes condimentos, sendo bastante comum nas regiões onde predominam imigrantes destas etnias, a utilização do condimento conhecido como “crem”.

A etimologia da palavra “crem” está no vocábulo eslavo *chren*, denominação popular das raízes da espécie *Armoracia rusticana*, em diversos idiomas eslavos. A espécie é nativa da Europa Oriental, e conhecida há séculos por supostas propriedades medicinais e pelo uso como condimento. Comumente conhecida, em inglês, por *horseradish*, é amplamente consumida em todo o hemisfério norte, sendo muito popular sua comercialização na forma de molhos, disponível inclusive sob forma industrializada (AGNETA; MÖLLERS; RIVELLI, 2013). O hábito de consumir as raízes da planta foi introduzido nos estados do sul do Brasil por imigrantes, e por aqui é conhecida como “raiz-forte” ou “raiz-brava”, além da denominação "crem". Atualmente suas raízes são bastante apreciadas em diversas regiões destes estados como tempero, de forma que a planta é aqui produzida e comercializada em feiras e até mesmo redes de mercados (SOARES et al., 2004).

Entretanto, outra espécie também é conhecida pelo nome popular “crem”, e utilizada com a mesma finalidade, a planta *Tropaleoum pentaphyllum* Lam., nativa da América do Sul. Seus tubérculos, devido a propriedades físico-químicas e organolépticas semelhantes às raízes de *A. rusticana,* passaram a ser utilizados como condimento pelas mesmas populações de imigrantes que trouxeram a última (KINUPP, 2007).

Tendo em vista consumo dos tubérculos de *T. pentaphyllum* no Sul do Brasil, seja como condimento ou para fins medicinais (MENTZ, LUTZEMBERGER, SCHENKEL, 1997), bem como a confusão popularmente feita entre os mesmos e o *horseradish*, o presente trabalho foi executado no intuito de avaliar seu potencial nutricional, fitoquímico, antimicrobiano, cito e genotóxico, a fim de gerar esclarecimentos, melhor entender as propriedades e potencialidades da planta, bem

como diferenciá-la das raízes de *A. rusticana*. Os resultados obtidos nas análises e sua discussão, bem como os métodos utilizados no desenvolvimento da pesquisa serão apresentados no item 4 desta tese, na forma de artigos científicos.

2 REVISÃO BIBLIOGRÁFICA

2.1 *Tropaeolum pentaphyllum* E GÊNERO Tropaeolum

Tropaeolum pentaphyllum pertence a família *Tropaeolaceae*, pequena família composta por dois gêneros, dentre eles o Tropaeolum, exclusivos da América do Sul, e de ocorrência natural no Brasil. A espécie *T. pentaphyllum* foi descrita pela primeira vez por Monet de La Marck, e possui as características típicas da família: planta herbácea, de flores grandes e vistosas, trepadeira de folhas inteiras e alternas, como pode ser visto na ilustração abaixo (Figura 1) (JOLY, 2005; RIX, 2010). A espécie, bem como diversas outras pertencentes ao gênero Tropaeolum, apresentam a modificação de caule conhecida como tubérculo, estrutura vegetal de armazenamento de nutrientes (RAVEN; EVERT; EICHHORN, 2007).

Figura 1 - Ilustração de partes áreas de *Tropaeolum pentaphyllum* Lam.

Fonte: Rix (2010).

Segundo Kinupp (2007), os tubérculos da espécie (Figura 2), conhecida por "crem" ou "batata-crem", são de tamanho variado, e podem alcançar até 1,5 Kg. Estes tubérculos, de grande pungência, são bastante apreciados nas regiões de colonização italiana e alemã do interior dos estados do Rio Grande do Sul e Santa Catarina, sob a forma de conservas (ralados e curtidos em vinagre tinto), adicionados a carnes e sopas (KINUPP, 2007; ZUCHIWSCHI et al.,2010).

Figura 2 - Tubérculos formados por *T. pentaphyllum.*

Fonte: Belli Plantas, 2016

Na tese “Da flora medicinal do Rio Grande do Sul”, de D’Ávila (1910 apud MENTZ, LUTZEMBERGER, SCHENKEL, 1997) é relatado que os tubérculos da espécie são recomendados para tratamento de escorbuto e que sua decocção é indicada para dermatoses. Anos depois o potencial antifúngico da planta foi realçado por Fenner e colaboradores (2006). Já no levantamento etnobotânico de Ritter e colaboradores (2002), realizado no município de Ipê, RS, onde a planta é popularmente denominada de ‘’men’’, é relatado que os tubérculos (identificados pela população local como raízes) são popularmente consumidos para tratamento de gripe. Já às flores da planta são atribuídas propriedades antidiabéticas (RITTER et al., 2002; TROJAN-RODRIGUES et al., 2012). Além disso, há indicação popular para o uso dos tubérculos para redução e controle dos níveis sanguíneos de colesterol (SANTOS et al., 2013).

Até o momento não há estudos fitoquímicos e farmacológicos publicados descrevendo constituintes químicos ou propriedades do óleo, extratos ou compostos isolados, seja dos tubérculos ou de qualquer outra parte de *T. pentaphyllum*, que forneçam explicação, subsídio ou segurança para justificar suas formas de uso

popular. Não obstante, a espécie *Tropaeolum majus* L., da mesma família e gênero, e de ocorrência no mesmo ecossistema e região geográfica, já foi relatada quanto a estas potencialidades, como será demonstrado numa breve revisão abaixo.

O *T. majus,* popularmente conhecida por “capuchinha” ou “nastúrcio”, entre outros, é bastante apreciada na alta culinária, devido a suas flores e folhas comestíveis. Dentre os compostos conhecidos nas folhas de *T. majus* está a glucotropaeolina, presente não só nas folhas, mas em todas as partes da planta, das sementes às folhas maduras, e que após processamento do material vegetal dá origem ao composto ativo isotiocianato de benzila (ITB) (LYKKESFELDT; MØLLER, 1993).

Pintão e colaboradores (1994) demonstraram a atividade citotóxica *in vitro* do ITB de *T. majus* frente a diferentes linhagens de células tumorais. Atividade citotóxica promissora foi obtida na faixa de concentração de 0,86 a 9,4 µM para diversos tipos celulares, sendo mais sensíveis as células L-1210 (leucemia). Uma avaliação *in vivo* também fez parte do estudo, porém com resultados menos favoráveis, onde ratos portando tumores do tipo plasmocitoma subcutâneo (células PC6) sofreram efeitos tóxicos do isotiocianato, após administração de uma dose de 200 mg/Kg, sem haver redução na massa tumoral.

No trabalho de Zanetti e colaboradores (2003) foi verificada a atividade antibacteriana das frações hexânica e clorofórmica do extrato etanólico de folhas de *T. majus,* pelo método de bioautografia, frente a diversas bactérias Gram-positivas e Gram-negativas. No mesmo trabalho os autores apresentam a ausência de efeitos tóxicos em camundongos, após administração via oral, em dose única, dos extratos aquoso e hidroalcoólico (70%), que não foram tóxicos mesmo na dose considerada elevada, de 5000 mg/kg de peso dos camundongos.

As folhas de *T. majus* são também popularmente utilizadas para problemas cardiovasculares e infecções urinárias, o que levou Gasparotto Júnior e colaboradores (2009) a demonstrar a capacidade diurética do extrato hidroetanólico das folhas da planta em ratos. O mesmo grupo citado anteriormente reforça este uso popular das folhas de *T. majus*, demonstrando em estudo *in vivo* o efeito diurético do flavonoide isoquercitrina, presente no extrato (GASPAROTTO JÚNIOR et al., 2011a). Este mesmo composto, juntamente com o extrato de *T. majus*, apresentou atividade anti-hipertensiva, em modelo *in vivo*, como exposto em outro estudo de Gasparotto Júnior e colaboradores (2011b).

Além disso, extratos de folhas e flores de *T. majus* também exibiram, *in vitro*, atividade anticoagulante (SANTO et al., 2007), antioxidante (GÁRZON; WROLSTAD, 2009) e antimicrobiana (BUTNARIU; BOSTAN, 2011), bem como anti-inflamatória*in vivo*, neste último estudo citado. Como se percebe, os relatos para *T. majus* se referem a suas folhas e flores, enquanto o objeto de estudo para *T. pentaphyllum* foram os tubérculos, de forma que uma relação direta não pode ser feita. Ainda assim é possível que compostos das partes aéreas desta espécie, bem como as propriedades descritas possam estar presentes em menor, ou maior grau nos tubérculos de *T. pentaphyllum.*

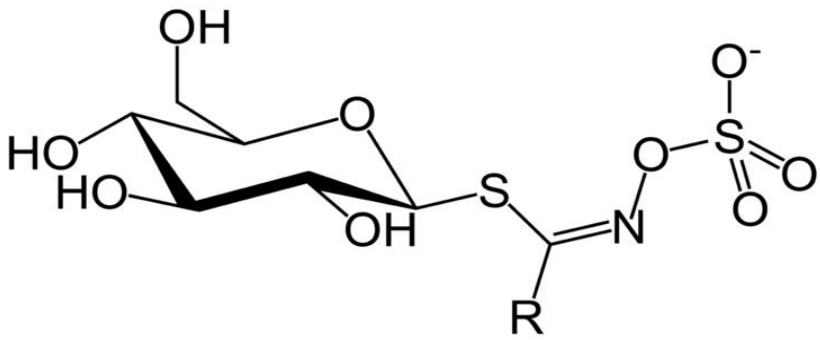
Outra espécie do gênero *Tropaeolum* há muito conhecida, consumida e que vem sendo estudada é a espécie *Tropaeolum tuberosum* Ruíz & Pavón. Planta de origem andina, seus tubérculos, conhecidos como *mashua* entre outros nomes, são utilizados pelas populações da Cordilheira dos Andes como alimento e para fins medicinais desde tempos remotos, que antecedem o surgimento da civilização Inca até os dias atuais, nos modernos Estados da Bolívia, Peru, Colômbia e Equador (HODGE, 1946). Em termos nutricionais, relata-se a notável presença de vitamina C nos tubérculos, perfazendo 77,37 mg a cada 100 g de produto integral. A planta também é rica em pró-vitamina A, expressos em equivalentes de retinol, perfazem um teor médio de 73,56 mg por 100 g de tubérculo, sendo considerados uma rica fonte de carotenos em comparação a outras espécies tuberosas (BARRERA et al., 2004).

Diversas propriedades farmacológicas são popularmente atribuídas a *mashua*, como as atividades antifúngica, antinematoide, antibacteriana, inseticida e anti-afrodisíaca. Tais propriedades têm sido estudas e relacionadas à presença de compostos do grupo dos isotiocianatos nos tubérculos, mais especificamente ao isotiocianato de p-metoxibenzila (JOHNS; TOWERS, 1981; JOHNS et al., 1982). Mais recentemente, outro conjunto de substâncias que tem sido explorado na *mashua* são os compostos polifenólicos. Estas substâncias já foram quantificadas como grupo, e diversos foram identificados e quantificados individualmente, sua capacidade antioxidante já foi verificada em testes *in vitro,* sendo inclusive sugerido o uso dos polifenois extraídos do tubérculo como um conservante de função antioxidante na composição de óleo de soja (CAMPOS et al., 2006; CHIRINOS et al., 2008; BETALLELUZ-PALLARDEL et al., 2012).

2.2 GLUCOSINOLATOS E ISOTIOCIONATOS

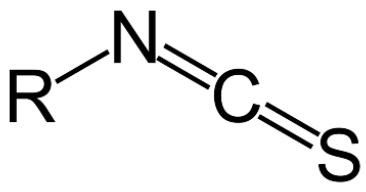
Os isotiocianatos (ITCs), citados no item anterior desta revisão como metabólitos secundários presentes em *T. majus* e *T. tuberosum*, e aos quais se atribuem diversas propriedades biológicas, não são encontrados nos vegetais originalmente sob esta forma, mas sim sob a forma de glucosinolatos (também conhecidos como tioglicosídeos). Segundo Heinzmann (2010) estes compostos são caracterizados por apresentar uma estrutura aglicona ligada a uma unidade de açúcar (Figura 3), e que quando há dano ao tecido vegetal (como no processamento dos alimentos e condimentos que contém tais substâncias) os glucosinolatos entram em contato com a enzima mirosinase (também presente na planta, porém em compartimento celular diferente) e sofrem hidrólise, originando β-D-glicose e uma aglicona instável. Esta aglicona instável, por rearranjo espontâneo perde um grupamento sulfato originando um ITC como produto majoritário, cuja estrutura é apresentada na Figura 4.

Figura 3 - Estrutura geral dos glucosinolatos.



Fonte: Cortesia do usuário Ben (Benjah-bmm27) do site colaborativo WikiProject Chemistry.

Figura 4 - Estrutura geral dos isotiocianatos.



Fonte: Cortesia do usuário Fvasconcellos do site colaborativo WikiProject Chemistry.

Os glucosinolatos são encontrados em diversas plantas consumidas como alimento como a *mashua* (JOHNS; TOWERS, 1981), a capuchinha (LYKKESFELDT; MØLLER, 1993) e plantas da família Brassicaceae como brócolis, repolho e nabo (FAHEY; ZALCMANN; TALALAY, 2001). Também estão presentes em vegetais que assim como a batata-crem são consumidos como tempero, onde os ITCs que liberam são responsáveis pela pungência e o odor característico, a exemplo do que acontece com a mostarda, a ‘’raiz-forte’’ *horseradish* (*A. rusticana*) e com o popular tempero japonês *wasabi* (*Wasabia japonica* (Miq.) Matsum.), cuja composição e usos são extensamente abordados por Fahey, Zalcmann e Talalay (2001).

Em linhas gerais, após a ingestão de alimentos cujos glucosinolatos já foram convertidos à ITCs, estes últimos são absorvidos passivamente pelas células epiteliais do trato gastrintestinal e ao atingirem a circulação e os tecidos, sua biotransformaçãoé iniciada pela via do ácido mercaptúrico, levando àexcreção urinária na forma do ácido mercaptúrico correspondente (LAMY et al., 2011).Tomando o ITB como exemplo, as reações de biotransformação iniciam com a conjugação com glutationa, esse conjugado é então transformado aos conjugados isotiocianato-cisteinilglicina e isotiocianato-cisteína, e por fim tem-se uma etapa de acetilação formando o conjugado isotiocianato-*N*-acetil-L-cisteína, a forma a ser excretada(BRUSEWITZ et al., 1977; ZHANG et al., 1995).

Diversas propriedades biológicas e farmacológicas são atribuídas aos ITCs, provenientes de plantas ricas em glucosinolatos, como citotoxicidade (PINTÃO et al., 1994; MIYOSHI et al., 2006), atividade anti-inflamatória (LEE et al., 2009) e pesticida (WU et al., 2009; YUN et al., 2012). De maior interesse no contexto dessa tese está a atividade antimicrobiana e os efeitos quimiopreventivos (a capacidade de inibir ou retardar o desenvolvimento de tumores quimicamente induzidos) relacionados aos ITCs, a serem abordados na sequência.

Os ITCs apresentam potente atividade antimicrobiana, send sua atividade antibacteriana, seja contra bactérias Gram-positivas como Gram-negativas, bem exemplificada pelos trabalhos de Kim e Lee (2009), Sofrata e colaboradores (2011) e Dias, Aires e Saavedra (2014). O mecanismo da ação antibacteriana dos ITCs ainda não é bem compreendido. Uma série de possíveis mecanismos são propostos, como a interação com membranas, inibição de sistemas regulatórios das bactérias, inibição de enzimas respiratórias, indução de choque térmico e estresse oxidativo (DUFOUR; STAHL; BAYSSE, 2015).

A atividade antifúngica dos ITCs, tanto frente a leveduras como fungos filamentosos, também é bem estabelecida (DROBNICA et al., 1967). Os mecanismos de ação propostos para tal efeito incluem a inibição de enzimas intracelulares por quebra de pontes dissulfeto, inibição de enzimas do metabolismo pelo radical tiocianato (indicado como produto de degradação dos isotiocianatos) e desacoplamento da fosforilação oxidativa (MANICI; LAZZERI; PALMIERI, 1997).

Os ITCs também interagem com as células dos organismos multicelulares de formas variadas, frente a células tumorais humanas podem apresentar efeito citotóxico conforme estudos *in vitro* atestam (PINTÃO et al., 1994; TSENG; SCOTT-RAMSAY; MORRIS, 2004; MIYOSHI et al., 2006). Esta capacidade de inibir o crescimento de células tumorais é um dos mecanismos para a sua propriedade quimiopreventiva. O consumo de ITCs na dieta pode prevenir ou retardar a formação de pequenos tumores quimicamente induzidos, antes de seu desenvolvimento(ZHANG; TALALAY, 1994;KUMAR et al., 2015).A inibição de enzimas do metabolismo de fase I necessárias para a bioativação de agentes carcinogênicos, ou indução da atividade de enzimas de fase II, que em geral favorecem a detoxificação, são também mecanismos de ação pelos quais os ITCs exercem a quimioprevenção (GOOSEN; MILLS; HOLLENBERG, 2001; HIGDON et al., 2007). Um outro mecanismo proposto é a indução de apoptose pela via mitocondrial (NAKAMURA et al., 2002; ZHANG, 2012)

Entretanto, os ITCs, sob determinadas condições, podem causar danos às células saudáveis de organismos vivos, como demonstrado pelos experimentos de genotoxicidade de Kassie e colaboradores (1999; 2003). Todavia, há um consenso de que a ingestão diária de ITCs na dieta não oferece maiores riscos toxicológicos, devido as baixas concentrações plasmáticas e teciduais alcançadas em comparação com a concentração utilizada em experimentos laboratoriais, seja *in vitro* ou *in vivo* (FIMOGNARI et al., 2012).

De suas propriedades biológicas às físico-químicas, temosque os ITCs são compostos bastante reativos. Sob diferentes condições de temperatura, umidade e presença de outros compostos (ácidos carboxílicos, por exemplo), diversos produtos podem ser obtidos a partir das reações com o grupo –NCS de sua estrutura (NOAA, 2016). Como exemplo, a estabilidade do isotiocianato de alila (o ITC característico das raízes de *A. rusticana*, o *horseradish*), em meio aquoso, foi investigada por Kawakishi e Namiki (1969) e Pecháček, Velíšek e Hrabcová (1997), e foi observado

que sob diferentes condições de temperatura e pH, diversos produtos derivados do ITC foram obtidos, como ureias, tioureias, tiocarbamatos, mercaptans, sulfetos e aminas, formados majoritariamente por uma reação de adição ao grupo –NCS. Até mesmo enxofre elementar (S_8) foi observado entre os produtos de degradação no experimento de Kawakishi e Namiki (1969).

Em relação aos procedimentos e cuidados laboratoriais, segundo Costa (2002), os ITCs podem ser obtidos do material vegetal por destilação sob vapor d'água (utilizando um extrator de Clevenger, por exemplo), ou maceração com solventes de caráter apolar tais como *n*-hexano e éter etílico, em ambos os casos o material vegetal deve ser previamente moído, ralado ou triturado para que haja a conversão enzimática dos glucosinolatos a ITCs. Diversas técnicas instrumentais se prestam a separação e análise quantitativa dos ITCs, como a titulometria volumétrica após derivatização (WILDERSPIN; GREEN, 1983), a cromatografia gasosa para os compostos voláteis e óleos (HEINZMANN, 2010), e mais recentemente a cromatografia líquida de alta eficiência (HPLC, do inglês *High Performance Liquid Chromatography*) para extratos purificados, a exemplo da técnica proposta por Herzallah e Holley (2012), que permite a separação e identificação tanto de glucosinolatos como de ITCs.

2.3 TESTES DE ATIVIDADE BIOLÓGICA

Os testes de atividade biológica são um componente de rotina no desenvolvimento de trabalhos de pesquisa em farmacognosia e fitoquímica, e podem servir a diferentes propósitos, dependendo do contexto da pesquisa. Em estudo bioguiado servem de ponto de referência, onde a investigação vai ser conduzida com a planta, o extrato, a fração, e assim por diante, que apresentar a atividade biológica mais pronunciada. Podem servir também de parâmetro em estudos de triagem em maior escala, onde diversas plantas e extratos serão testados para uma mesma atividade farmacológica, de uma forma mais simples, no intuito de se identificar a amostra mais promissora para uma investigação detalhada, a ser realizada em outro momento, tanto do ponto de vista fitoquímico como biológico (HAMBURGER; HOSTETTMANN, 1991; CECHINEL; YUNES, 1997).

Outra função em que os testes de atividade biológica podem ser empregados é uma triagem no sentido oposto ao que foi apresentado no parágrafo anterior.

Nesse caso, o extrato de uma espécie vegetal de aplicações desconhecidas seria avaliado frente a uma gama de testes de atividade, visando identificar as potencialidades da planta, de forma a nortear a futura investigação fitoquímica, na forma de um estudo bioguiado (HAMBURGER; HOSTETTMANN, 1991; CECHINEL; YUNES, 1997).

Contrariamente à busca por atividades em uma planta desconhecida, a avaliação de uma atividade biológica em um estudo de farmacognosia pode ter por objetivo confirmar ou buscar um maior entendimento das formas de uso popular de uma planta medicinal. Desse modo, a escolha da atividade a ser testada será mais criteriosa, representando um ou mais modelos que ofereçam uma simulação mais apurada do uso farmacológico que é feito da espécie vegetal em questão (HAMBURGER; HOSTETTMANN, 1991; CECHINEL; YUNES, 1997).

De maneira geral, as técnicas de avaliação de atividade biológica podem ser divididas em modelos *in vitro*, *in vivo* e *ex vivo*, expressões latinas que, respectivamente, representam os experimentos que são realizados de forma externa ou interna a organismos vivos, e no último caso, em tecidos vivos removidos de um organismo. Mais recentemente passou-se a utilizar a expressão *in silico*, que se refere a predições realizadas através de simulações computacionais. No contexto da farmacognosia, diversos *softwares* podem ser utilizados a fim de se obter previsões do comportamento, atividade e toxicidade de um composto isolado frente a organismos vivos. Os *softwares* específicos também pode oferecer informações a respeito de propriedades físico-químicas, perfil de reatividade e caminhos para semi-síntese de metabólitos secundários isolados (ELLISON; ENOCH; CRONIN, 2011; CASAVOTTO, 2015).

No presente trabalho foram utilizadas técnicas de avaliação de atividade antimicrobiana e de genotoxicidade. Testes de atividade biológica que representam etapas iniciais na pesquisa de novos fármacos com potencial antimicrobiano, a primeira consiste numa busca da atividade em si, e a segunda representa um modelo de avaliação de toxicidade.

Testes de atividade antimicrobiana *in vitro* são rotineiramente empregados, não somente na busca por novos compostos ativos, mas também com propósitos epidemiológicos, e para fins de acompanhamento e prognóstico do tratamento de infecções. Na busca de novos compostos ativos as técnicas *in vitro* representam um ponto de partida, seja quando aplicados numa triagem ou para amostras em que

suspeita-se da atividade, onde dessa forma servirão para confirmar e alavancar experimentos *in vivo*, onde o comportamento de um candidato a princípio ativo ou fitoterápico pode ser diferente (COS et al., 2006; BALOUIRI; SADIKI; IBNSOUDA, 2016).

Diferentes métodos podem ser aplicados na determinação antimicrobiana, entretanto, devido a custos e praticidade, a ampla maioria dos estudos realizados se valem da disco-difusão em ágar e/ou da microdiluição em caldo. A disco-difusão em ágar foi inicialmente desenvolvida em 1940, e é hoje um método oficial bastante utilizado, com formas padronizadas descritas nos manuais do Clinical and Laboratory Standards Institute (CLSI), para bactérias e leveduras (CLSI, 2004, 2012a; BALOUIRI; SADIKI; IBNSOUDA, 2016). Esta técnica consiste em inocular as placas contendo ágar, com inóculo padronizado do microrganismo a ser testado. Na sequência, discos de papeis impregnados com o composto, ou extrato a ser avaliado, em concentração previamente estabelecida, são colocados na superfície do ágar nas placas, que então são incubadas nas condições apropriadas. A amostra teste, presente no papel, irá se difundir radialmente na superfície do ágar, e se exercer atividade irá formar um halo onde o crescimento microbiano não será visualizado. De acordo com o tamanho do halo de inibição, e por comparação a padrões estabelecidos, a atividade da amostra será então categorizada, usualmente, em sensível, intermediária ou resistente. Dentre as desvantagens da técnica está a impossibilidade de se diferenciar se o efeito é de inibição da reprodução, ou se também leva a morte do microrganismo, bem como a impossibilidade de se calcular valor exato de concentração inibitória mínima (CIM) (JORGENSEN; FERRARO, 2009; BALOUIRI; SADIKI; IBNSOUDA, 2016).

Tais desvantagens da disco-difusão podem ser contornadas pelo uso de técnicas de diluição em caldo, que permitem o cálculo da CIM no meio líquido onde é testada, bem como a determinação da concentração letal mínima (usualmente desmembrada em concentração bactericida e fungicida mínimas). A microdiluição em caldo é executada em placas de microtitulação de 96 poços, onde cada poço contém o meio líquido, inoculado com o microrganismo teste, e as diluições seriadas da amostra a ser testada. Após a incubação nas condições apropriadas, o menor valor de concentração do composto, ou extrato, onde não é visualizado o crescimento microbiano é definido como a CIM. Com o desenvolvimento da técnica, a visualização do resultado, inicialmente feita a olho, é hoje feita com auxílio de

corantes, como os sais de tetrazólio, e leitura espectrofotométrica do meio (COS et al., 2006; BALOUIRI; SADIKI; IBNSOUDA, 2016).

Tendo em vista as variações decorrentes do tamanho e preparação do inóculo, meio utilizado e tempo de incubação, a técnica é utilizada segundo padronização do CLSI, ou do European Committee on Antimicrobial Susceptbility Testing (para leitura espectrofotométrica da CIM) (GOMEZ-LOPEZ et al., 2005; ARIKAN, 2007; CLSI, 2008a, 2008b, 2012b). A diluição em caldo é hoje bastante popular em sua forma miniaturizada (microdiluição), devido a vantagens como melhor reprodutibilidade, ganho de espaço e economia de reagentes (BALOUIRI; SADIKI; IBNSOUDA, 2016).

Além da disco-difusão e da diluição em caldo, a atividade antimicrobiana pode ser determinada por outras técnicas como a bioautografia (DEWANJEE et al., 2015), ensaio de tempo de morte (CLSI, 1998), bioluminescência de ATP e citofluorimetria de fluxo (BALOUIRI; SADIKI; IBNSOUDA, 2016). Técnicas mais elaboradas, que porém oferecem diferentes vantagens como a rapidez, possibilidade de se verificar sinergismo-antagonismo, diferentes mecanismos, entre outros (BALOUIRI; SADIKI; IBNSOUDA, 2016).

Contudo, independente da técnica escolhida, alguns critérios e recomendações devem ser seguidos para a execução de uma avaliação sólida, e confiável, da atividade antimicrobiana de produtos naturais. Cos e colaboradores (2006), em trabalho de referência na área, listam os seguintes critérios: uso de cepas de referência e isolados clínicos bem caracterizados; modelos *in vitro* que contemplem o todo dos organismos modelos; avaliação de seletividade em paralelo, através de teste de citotoxicidade ou pela inclusão de microrganismos não-patogênicos e não relacionados ao estudo; teste em diferentes concentrações de forma a estabelecer uma relação dose-resposta; um critério rigoroso na determinação da atividade, IC_{50} de no mínimo 100 µg/mL para extratos e 25 µM para isolados; adequado tratamento e processamento dos extratos; uso de formas apropriadas de controle; e por último, o prosseguimento do trabalho com os extratos e isolados mais ativos com uso de testes *in vivo*.

Também de interesse nessa tese está a genotoxicidade, definida como a propriedade de um agente químico de causar dano à informação genética contida nas células. O termo é muitas vezes confundido com mutagenicidade, visto que todo agente mutagênico é genotóxico, porém nem todo agente genotóxico é considerado

mutagênico. Mutagenicidade consiste na capacidade de um dado composto causar alterações no DNA, gerando mutações transmissíveis em células germinativas, e transformações malignas em células somáticas. Por outro lado, genotoxicidade é um termo mais amplo que define os agentes que causam efeito deletério, que poderão ou não acarretar em mutações (PHILIPS; ARLT, 2009; ZHOU et al., 2013).

A avaliação da genotoxicidade é uma forma de determinação de toxicidade celular, sendo um complemento a outras formas de determinação desta. Busca um maior entendimento dos efeitos tóxicos de longo prazo, pois tumores decorrentes de agentes genotóxicos podem vir a se desenvolver anos após uma dada exposição. Ao contrário de outros mecanismos de toxicidade, a genotoxicidade ainda é menos compreendida, não existe, por exemplo, um consenso em relação a um limiar de exposição a estes agentes ou não, visto que em teoria uma simples exposição que acarrete em dano ao DNA pode levar a instabilidade genômica e demais consequências (PHILIPS; ARLT, 2009; ZHOU et al., 2013).

Atualmente uma determinação de genotoxicidade é uma exigência legal para o registro de diversos produtos como defensivos agrícolas, aditivos e suplementos alimentares, e medicamentos. Já os produtos vegetais com aplicação farmacêutica, historicamente vistos como mais seguros, mas que não são livres de efeitos adversos, têm sido gradualmente, alvo de maior regulamentação.Wu e colaboradores (2010) sumarizam a legislação vigente, e perspectivas, para o desenvolvimento de produtos de origem vegetal nos Estados Unidos, nação que é frequentemente tomada como referência no desenvolvimento de regulamentações sanitárias em outros países. No Brasil, a Resolução n° 90 da Agência Nacional de Vigilância Sanitária (ANVISA, 2004), que estabelece guia para a realização de estudos de toxicidade pré-clínica para fitoterápicos, preconiza a execução de ensaio de genotoxicidade quando houver indicação de uso contínuo ou prolongado do produto.

Experimentalmente, os métodos envolvem alguma forma de detecção ou medição do dano que o composto avaliado causa ao DNA, ou cromossomos, das células onde se realiza o teste. Dentre as formas de dano as quais o material genético está sujeito encontram-se quebra das fitas DNA, perda de suas funções de reparo de reparo, formação de ligações intramoleculares na estrutura de dupla hélice, alquilação e substituição de bases nitrogenadas e pareamento incorreto,

entre outros (HELLEDAY; ESHTAD; NIK-ZAINAL, 2014). Já os cromossomos podem sofrer alterações estruturais e numéricas (BERNSTEIN et al., 2013).

Atualmente, o ensaio Cometa realizado *in vitro*, é uma das técnicas mais populares e aceitas para observação de dano ao DNA, considerada de boa sensibilidade e precisão, e relativamente livre de artefatos. Resumidamente, o método consiste na incubação de células mononucleadas isolados do sangue com a amostra a ser testada, após, as células são submetidas a eletroforese, e na sequência visualizadas com auxílio de corantes e microscopia, de forma que os diferentes graus de deslocamento que serão observados na eletroforese são proporcionais ao dano causado ao DNA (SINGH et al., 1988; COLLINS, 2009). Com o desenvolvimento da técnica, diversas recomendações e normativas foram estabelecidas, com o intuito de padronizar e otimizar as condições do teste, como o pH, parâmetros da eletroforese, formas de visualização, etc (TICE et al., 2000; HARTMANN et al., 2003).

Juntamente à observação de dano direto ao DNA, outros parâmetros de genotoxicidade também podem ser analisados, como a influência na viabilidade celular (BUROW et al., 1998), a formação de micronúcleos (THOMAS et al., 2008; KIRSCH-VOLDERS et al., 2011), o índice mitótico e instabilidade cromossômica (YUNIS, 1976; MONTAGNER et al., 2010).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Realizar análise fitoquímica e bromatológica dos tubérculos, além de investigar os compostos majoritários e avaliar a atividade antimicrobiana e a genotoxicidade do óleo essencial, extrato bruto e suas frações.

3.2 OBJETIVOS ESPECÍFICOS

a) Executar análise da composição centesimal dos tubérculos;
b) Analisar a composição mineral dos tubérculos;
c) Realizar extração e identificação do óleo essencial dos tubérculos;
d) Preparar extrato bruto e fracioná-lo com solventes orgânicos, para posterior análise das frações;
e) Investigar a presença de compostos fenólicos no extrato bruto e suas frações;
f) Testar a atividade antimicrobiana do extrato bruto, frações e óleo essencial;
g) Avaliar a genotoxicidade do extrato bruto, frações e de componente majoritário do óleo essencial;
h) Identificar os compostos responsáveis, ou relacionados com relação às atividades biológicas testadas;
i) Comparar os resultados obtidos com os dados disponíveis para as raízes de *Armoracia rusticana*.

4 RESULTADOS E DISCUSSÃO

Os resultados e discussão, bem como os materiais e métodos utilizados na pesquisa, serão apresentados a seguir na forma de artigo científico, e dentro do conjunto de normas dos periódicos aos quais foram publicados ou estão submetidos. O primeiro artigo será denominado nesta tese como Artigo 1, e foi publicado no periódico científico *Molecules*, volume 21, n° 566, em 2016. Já o segundo artigo, ainda não publicado, denominado Manuscrito 1, está submetido ao periódico *Journal of Food Science*.

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Antimicrobial Activity and Chromatographic Analysis of Extracts from *Tropaeolum pentaphyllum* Lam. Tubers

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Abstract: Background: *Tropaeolum pentaphyllum* Lam. tubers (Tropaeolaceae) are known and used as a condiment and for the treatment of skin infections in Southern Brazil. However, its activity and composition has not yet been investigated. Thus, different extracts and the essential oil from the tubers were tested against a range of microorganisms. The most active extracts were submitted to chromatographic analysis. Methods: Hydroalcoholic extract (70%), fractions of it, and the essential oil from the tubers were tested against several bacteria, yeasts and molds, furnishing the correspondinginhibitory, bactericidal and fungicidal minimal concentration values. The most active extracts were submitted to GC-MS investigation. Results: The strongest effects against different strains of microorganisms, such as Gram-positive and negative bacteria, *Candida* spp. and dermatophytes were observed for the essential oil and the chloroform fraction, with minimal inhibitory concentrations (MICs) well below 200 μg/mL. GC-MS analysis revealed that the major essential oil constituent is benzyl isothiocyanate (BITC), while the chloroform fraction is constituted of BITC, amides, sulfur, fatty acids and its esters, all compounds that may be related to the demonstrated activity. Conclusions: Overall, the results support the popular use of the plant for the treatment of skin infections, and revealed the main active compounds.

Keywords: *Tropaeolum pentaphyllum*; essential oil; benzyl isothiocyanate; antifungal; antibacterial

1. Introduction

Tropaeolum pentaphyllum Lam. (Tropaeolaceae) is a summer-growing species, native to South America, specifically Uruguay, Northern Argentina and Southern Brazil. It is used as an ornamental, food and medicinal plant. Its flowers make it known and appreciated throughout the world as an ornamental plant, but they are also locally consumed as part of salads [1] and used as an antidiabetic drug [2].

While the flowers of *T. pentaphyllum* have their applications, the most known and consumed part of the plant is its tubers. These can reach up to 1.5 kg in weight in due time, and are commonly known in south of Brazil as "crem" or "batata-crem" (crem-potato) and also "raiz-amarga" (bitter-

root) [3]. "Crem" is a word derived from the slavic "chren", a common denomination of the roots of *Armoracia rusticana* (horseradish) in Eastern Europe. *T. pentaphyllum*'s tubers and horseradish are commonly mistaken, as both are used as spices, prepared in the same way, as homemade pickles with red wine vinegar [4]. *T. pentaphyllum*'s tubers have similar organoleptic characteristics to those of the horseradish, and its consumption became popular during the 19th century with the settling of immigrants from Europe in the southern region of Brazil.

In traditional medicine, the consumption of the tubers is indicated to prevent and aid in the treatment of flu and scurvy. The decoction of the tubers is recommended as an option for the treatment of dermatosis and dermatological affections [5]. To the best of our knowledge, there is no scientific evidence corroborating this use, nor there are any studies demonstrating the presence of compounds related to these properties.

Bacterial and fungal infections represent part of the known and reported dermatological afflictions. Their treatments can be lengthy and expensive, requiring topical and parenteral medication, and as with other microbial infections, the microorganisms responsible can develop resistance mechanisms [6]. Therefore, the aim of the present study was to evaluate the popular use and the potential of *T. pentaphyllum* tubers, through an assessment of its *in vitro* antimicrobial activity and an investigation of the major constituents. For that, phytochemical analysis and antimicrobial evaluation against a series of microorganisms were conducted with the hydroalcoholic extract, its derived fractions and the essential oil obtained from the tubers.

2. Results

2.1. Extraction Methods

The dried tubers, extracted with hydroalcoholic solution (70%), yielded 20.2% of dried crude extract (CE). Yields for the fractions of the CE were 20.2% for the butanol fraction (BuF), 13.4% for the chloroform fraction (CfF) and 3.1% for the ethyl acetate fraction (EaF). The hydrodistillation extraction of the tubers yielded 0.082% of a limpid and pungent oil.

2.2. Antimicrobial Activity

Antimicrobial activity results are summarized in the Tables 1–3, divided among bacteria, yeasts and filamentous fungi. The CE and BuF did not show activity at the highest tested concentration (1280 μg/mL) against any of the tested microorganisms (data not shown). Potent bacteriostatic and bactericidal activity were observed for the essential oil (EO) and CfF (Table 1), against both Gram-positive and negative bacteria. Stronger results were those obtained against the Gram-negative *E. coli* and *S. pullorum*, with MICs in the same range of the tested reference antimicrobial agent (azithromycin). In addition, bactericidal activity was also verified, albeit at higher concentrations.

Table 1. Antibacterial activity of the essential oil and fractions from the crude extract (CE) of *T. pentaphyllum* tubers.

Bacteria	MIC/MBC (μg/mL)				
	EO	**CfF**	**EaF**	**BITC**	**Azithromycin**
Enterococcus faecalis ATCC 91299	40/640	80/640	80/1280	40/640	8/-
Escherichia coli ATCC 5922	10/320	10/80	20/640	10/320	8/-
Klebsiella pneumoniae ATCC 700603	40/1280	40/640	320/-	40/640	2/-
Pseudomonas aeruginosa ATCC 27853	20/320	40/640	40/640	20/320	4/-
Salmonella pullorum ATCC 9140	40/640	10/-	80/1280	40/640	8/-
Staphylococcus aureus ATCC 29213	40/1280	40/640	640/-	40/320	2/-

EO: Essential oil; CfF: Chloroform fraction; EaF: Ethyl acetate fraction; BITC: Benzyl isothiocyanate; -: Not active.

Table 2. Antifungal activity of the essential oil and fractions from the CE of *Tropaeolum pentaphyllum* tubers, against yeast fungi.

Fungus	MIC/MFC (μg/mL)				
	EO	**CfF**	**EaF**	**BITC**	**Fluconazole**
Candida albicans ATCC 14053	40/320	20/20	40/320	40/160	4/-
Candida dubliniensis CBS7987	40/320	10/10	40/640	40/160	8/-
Candida dubliniensis CI FS	40/160	20/20	40/640	40/160	2/-
Candida dubliniensis CI FR	40/160	20/20	40/640	40/160	64/-
Candida glabrata ATCC 2001	20/80	40/40	40/320	20/80	32/-
Candida glabrata CI FS	20/160	40/40	40/640	20/160	16/-
Candida glabrata CI FR	20/160	40/40	20/640	20/160	128/-
Candida glabrata CI CR	NT	160/1280	NT	160/640	4/-
Candida guilliermondii CI	80/320	10/10	80/320	80/320	8/-
Candida parapsilosis ATCC 22018	80/320	10/20	80/640	80/320	16/-
Candida parapsilosis CI CR	NT	320/1280	NT	320/1280	8/-
Candida tropicalis CI	320/-	10/10	1280/-	320/-	8/-
Cryptococcus neoformans ATCC 90012	10/80	2.5/2.5	10/320	5/80	16/-
Sacharomyces cerevisiae ATCC 2601	40/320	2.5/2.5	80/320	40/160	4/-

EO: Essential oil; CfF: Chloroform fraction; EaF: Ethyl acetate fraction; BITC: Benzyl isothiocyanate; -: Not active; NT: Not tested; CI: Clinical isolate; FS: Fluconazole sensitive; FR: Fluconazole resistant; CR: Caspofungin resistant.

Table 3. Antifungal activity of the essential oil and fractions from the CE of *T. pentaphyllum* tubers, against dermatophytes and filamentous fungi.

Fungus	MIC/MFC (μg/mL)				
	EO	**CfF**	**EaF**	**BITC**	**Itraconazole**
Aspergillus fumigatus CI	80/1280	160/1280	320/-	80/1280	8/-
Aspergillus fumigatus EI	20/1280	80/640	40/-	20/1280	16/-
Aspergillus flavus CI	NT	1280/-	NT	160/1280	1/-
Aspergillus niger CI	NT	1280/-	NT	160/1280	2/-
Trichophyton rubrum CI	NT	2.5/320	NT	2.5/40	2/-
Microsporum canis CI	NT	20/160	NF	20/80	8-
Fonsecaea pedrosoi CI	NT	80/640	NT	40/320	1/-
Pseudallescheria boydii CI	NT	80/1280	NT	40/640	4/-
Fusarium solani CI	NT	320/-	NT	160/1280	>16/-
Sporothrix schenckii CI	NT	40/320	NT	80/320	4/-

EO: Essential oil; CfF: Chloroform fraction; EaF: Ethyl acetate fraction; BITC: Benzyl isothiocyanate; -: Not active; NT: Not tested; CI: Clinical isolate; EI: Environmental isolate; FS: Fluconazole sensitive; FR: Fluconazole resistant; CR: Caspofungin resistant.

Antifungal activity is presented on Tables 2 and 3, divided between yeasts and filamentous fungi. Strong activity was observed for the EO, benzyl isothiocyanate (BITC) standard and CfF, for both fungal forms. Fungicidal effect of these samples was observed for the majority of the tested fungi, the exceptions being some of the filamentous fungi. Fluconazole-resistant yeasts, such as *C. dubliniensis* and *C. glabrata*, were sensitive to both BITC and CfF, showing a lower MIC value, as well a fungicidal effect.

2.3. Chromatographic Analysis

The most active tested samples in the antimicrobial activity evaluation were the EO and the CfF, as presented above, and therefore these were submitted to gas chromatography-mass spectrometry (GC-MS) analysis. The chromatograms are shown in Figures 1 and 2. Table 4 complements Figure 2, presenting the composition of the CfF according to the peaks shown in the chromatogram.

As can be seen in Figure 1, the GC-MS analysis revealed that the major component of the oil is BITC. CfF is a more complex and varied extract, constituted by BITC, amides, sulfur, fatty acids and its esters, with some of the other major components described as a phytosterol and oleic acid (Table 4).

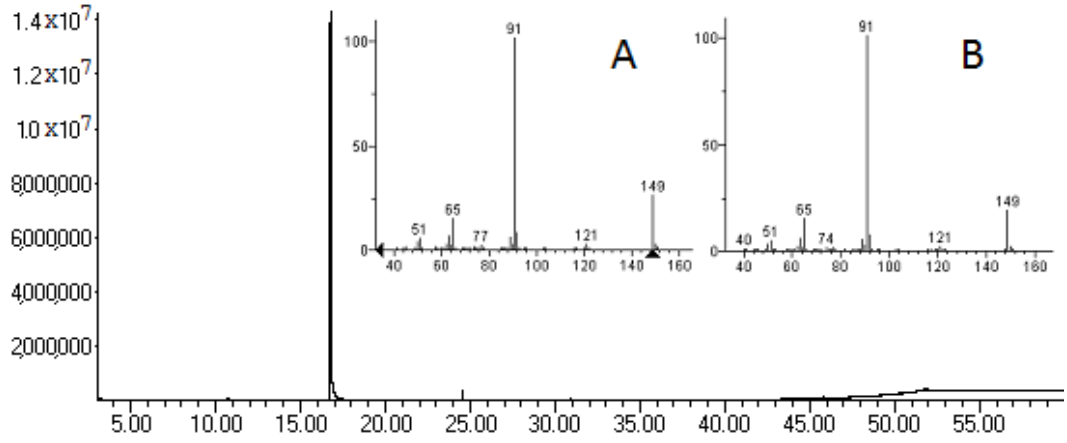

Figure 1. GC-MS chromatogram from the essential oil (EO) of *T. pentaphyllum* tubers, with mass spectra of benzyl isothiocyanate (BITC) standard from National Institute of Standards and Technology (NIST) library (**A**) and from peak with retention time of 16.76 min (**B**).

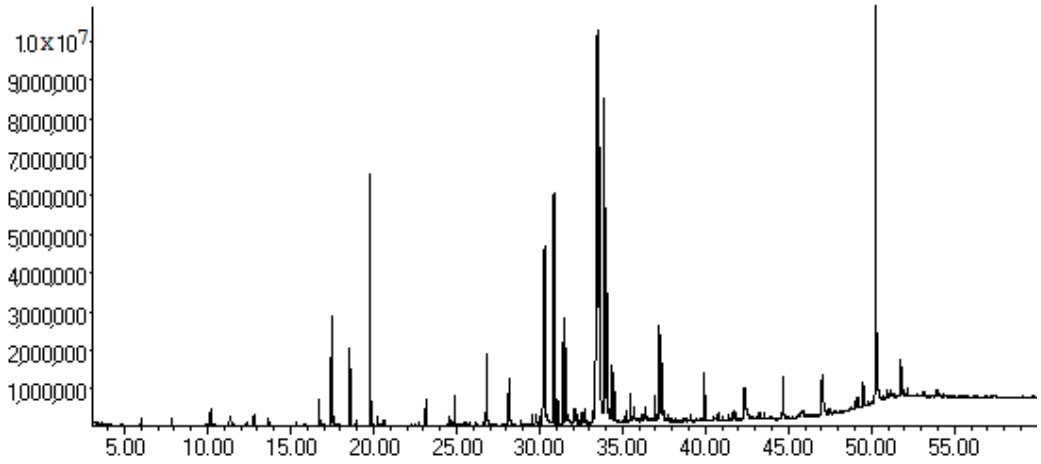

Figure 2. GC-MS chromatogram from the chloroform fraction of the CE from *T. pentaphyllum* tubers.

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Table 4. Identified compounds from chloroform fraction (CfF) of the CE from *T. pentaphyllum* tubers by GC-MS.

Compound	RT (min)	Relative Area (%)	(M^+), *m*/*z* (%)	Major Fragment Ions, *m*/*z* (%)
Benzyl isothiocyanate	16.72	0.65%	149 (16)	91 (100) 65 (14) 92 (7) 89 (5) 63 (5) 51 (4) 50 (3) 90 (3) 62 (2)
2-Phenylacetamide	17.49	4.37%	135 (18)	91 (100) 92 (91) 65 (20) 44 (12) 63 (8) 89 (7) 51 (5) 93 (5) 90 (4)
N-benzylacetamide	18.56	2.21%	149 (71)	106 (100) 91 (28) 43 (20) 107 (15) 77 (13) 79 (13) 51 (8) 65 (7) 150 (7)
Benzylcarbamide	19.80	6.23%	150 (50)	106 (100) 79 (41) 91 (34) 77 (34) 51 (23) 107 (20) 78 (15) 104 (15) 65 (13)
Palmitic acid	30.29	6.75%	256 (30)	73 (100) 60 (84) 43 (73) 57 (63) 55 (57) 41 (56) 129 (43) 71 (37) 69 (31) 83 (24)
Ethyl palmitate	30.86	4.66%	284 (7)	88 (100) 101 (58) 43 (26) 55 (22) 41 (20) 89 (17) 57 (16) 70 (16) 157 (15) 73 (15)
Elemental sulfur	31.61	1.62%	256 (70)	64 (100) 160 (53) 128 (52) 192 (41) 258 (25) 32 (21) 96 (21) 224 (18) 66 (11)
Oleic acid	33.60	10.25%	282 (1)	55 (100) 69 (72) 41 (67) 83 (58) 97 (49) 67 (45) 43 (45) 81 (37) 84 (34) 57 (32)
Ethyl-9,12-octadecadienoate	33.90	6.98%	308 (8)	67 (100) 81 (90) 95 (65) 55 (61) 41 (46) 79 (43) 82 (42) 68 (38) 96 (35) 54 (34)
Ethyl oleate	34.02	5.88%	310 (6)	55 (100) 69 (71) 41 (63) 83 (60) 88 (57) 97 (51) 84 (49) 43 (47) 96 (45) 101 (45)
Butyl palmitate	34.38	1.03%	312 (6)	56 (100) 57 (47) 257 (41) 43 (35) 41 (32) 55 (28) 73 (24) 60 (24) 239 (20) 129 (20)
Unknown phytosterol	50.26	11.35%	414 (100)	43(95) 107 (68) 105 (66) 145 (65) 95 (62) 55 (61) 81 (60) 329 (59) 303 (58)

RT: retention time.

3. Discussion

Overall the antimicrobial activity was strong for the EO and the CfF, with MICs below the cutoff point for promising activity (*i.e.*, lower than 200 μg/mL) [7]. In addition to the bacteriostatic effect demonstrated by the low MIC values of the aforementioned extracts, bactericidal activity was also verified, albeit at higher concentrations. Since mostly of the current antibiotics are growth inhibitors, this bactericidal effect can be a relevant improvement, which also highlights a difference between mechanisms of action, indicating some direct action against the bacteria cell instead of protein synthesis inhibition mechanism (*i.e.*, the mechanism of azithromycin)[8].

The antibacterial activity presented for the EO is due to its chemical composition. As presented in Figure 1, the GC-MS analysis revealed that the major component of the oil is BITC. While it is not a new compound to the genus, as it was already identified in *Tropaeolum majus* [9], this is the first report of its presence in *T. pentaphyllum*. The occurrence of BITC in *T. pentaphyllum* tubers explains their pronounced organoleptic properties, the reason behind its use as a condiment.

The antibacterial activity of standard BITC was tested (Table 1), with results highly corresponding those of the EO. This is in accordance with previous publications, which demonstrated BITC bactericidal activity against a range of Gram-negative bacteria, while the tested Gram-positive bacteria were less susceptible [10]. Kim and Lee [11] demonstrated BITC activity against some harmful intestinal bacteria such as *Clostridium difficile* and *E. coli*, while not inhibiting other intestinal bacteria, such as *Bifidobacterium* spp. and *Lactobacillus acidophilus*, indicating that *T. pentaphyllum* tubers consumption could have a similar effect against intestinal bacteria. Dias *et al.* [12] showed that BITC is a stronger growth inhibitor against methicillin-resistant *Staphylococcus aureus* (MRSA) than allyl and 2-phenylethyl isothiocyanate, with MICs ranging from 2.9 to 110 μg/mL against several MRSA strains, the closest comparison that can be made with our results is that both BITC and EO had a 40 μg/mL MIC against a standard non-MRSA strain in our work.

Antimicrobial mechanisms of action of isothiocyanates (and BITC as well) are not well established,nonetheless several modes of action are proposed such as effects on membranes, inhibition of regulatory systems (quorum sensing), inhibition of respiratory enzymes, induction of heat-shock response, oxidative stress and stringent response [13]. Studies specifically with BITC demonstrated that it can cause the loss of membrane integrity, conversely to what was observed for ampicillin (a reference antibiotic) [10], and it was also the most potent inhibitor of the quorum sensing system CviIR on *Chromobacterium violaceum* when compared to allyl isothiocyanate and 2-phenylethyl isothiocyanate [14]. Other mechanisms of action verified with BITC were the induction of a heat-shock-like response, reduction of O_2 consumption and protein aggregation on *Campylobacter jejuni* [15, 16].

While the EO verified antibacterial activity is solely due to BITC, it is not the case for the CfF, which is a mixture of low polarity compounds as seen in Table 4. BITC is in fact part of this mixture, and it is followed in the chromatographic run by three structurally related compounds: 2-phenylacetamide, *N*-benzylacetamide and benzylcarbamide. To this moment it is not clear if these compounds are of natural occurrence, or derived from BITC, during the processing and extraction of the tubers. Similar amides were already found in plant extracts [17]. On the contrary, there is some evidence on isothiocyanate reaction products in aqueous medium, including the formation of elemental sulfur [18,19] a minor bacterial growth inhibitor [20], which may be related to the observed effect, since it was encountered in the CfF at 31.61 min.

Additionally contributing to the demonstrated antibacterial activity of CfF are fatty acids and their esters. They comprise the bulk of the fraction and possess antimicrobial activity [21]. Recently Tamokou *et al.* [22] purified a fraction from the ethyl acetate extract of *Albizia adianthifolia*s stem bark containing only oleic and palmitic acid, two of the major constituents of the CfF, and it was active against *E. faecalis* and *S. aureus*, presenting MICs of 400 μg/mL and 200 μg/mL, respectively, which is less pronounced inhibitory activity than that exerted by CfF against the same bacteria (MICs of 80 and

40 μg/mL, respectively). In our study, we highlight the potent effect of the combined compounds of the fraction, rather than the action of individual constituents.

Not only was the antibacterial activity strong, but antifungal activity (Tables 2 and 3) was also remarkable, observed for the EO, standard BITC and CfF, for both fungal forms. *T. rubrum* (Table 3), one of the major causes of dermatophytosis, was highly sensitive to both BITC and CfF, with a MIC value of 2.5 μg/mL, almost the same value obtained for the tested antifungal agent, itraconazole (MIC of 2.5 μg/mL). However, itraconazole is a fungistatic agent, whereas BITC and CfF also exerted a fungicidal effect, presenting MFCs of 40 μg/mL and 320 μg/mL, respectively. Another common causative agent of dermatophytosis, *M. canis*, was also very sensitive to BITC and CfF (MICs of 20 μg/mL). These data corroborates the widespread use of the tubers' decoction to treat dermatophytosis [5]. Other filamentous fungi that causes cutaneous and subcutaneous infections, such as *F. pedrosoi* (chromoblastomycosis causative agent) and *S. schenckii* (sporotrichosis causative agent) were also sensitive to both BITC and CfF, while *Aspergillus* spp. presented mixed results, *A. flavus* and *A. niger* clinical isolates were resistant to CfF (MIC of 1280 μg/mL and with no observed fungicidal activity at the same concentration).

Antifungal activity of isothiocyanates against few fungi species was reported by Drobnica *et al.* [23] who showed that BITC is in general 3.6 time more potent than allyl isothiocyanate. Equivalent results can be seen, as the authors obtained a MIC of 26.86 μg/mL against filamentous fungus *A. niger* after four days of incubation, while we observed a MIC of 160 μg/mL for our clinical isolate of *A. niger*, with two days of incubation. More recently Manici *et al.* [24] showed isothiocyanates have fungitoxic activity against plant pathogenic fungi, and the proposed mechanisms of action were inactivation of intracellular enzymes by breakdown of disulfide bonds, inhibition of metabolic enzymes by thiocyanate radical (indicated as degradation product of isothiocyanates) and uncoupler action on oxidative phosphorylation.

While the antifungal activity of BITC is more established and explains the EO antifungal properties, the CfF presents a different situation, where activity can be ascribed to the whole set of compounds, instead of selected ones. Elemental sulfur (RT of 31.61 min), at 1.62% of the CfF, can play a major role in the observed antifungal activity. In fact, it is regarded as the oldest of all pesticides, with well-established antifungal action, whetherin its inorganic or organic forms [25].

There are no reports in the literature regardingthe activity of the amides of the CfF composition (eluted between 17.49 min and 19.80 min). However there are some indications that they can present some inhibitory properties. Antimicrobial activity of the ethyl acetate extract from the algae *Trichodesmium erythraeum* [26], containing, among other compounds, 2-phenylacetamide (benezeneacetamide) at 17.48%, inhibited the growth of some fungi such as *T. rubrum* and *Trichophyton simii*, with MICs of 500 μg/mL and 16.2 μg/mL. CfF was stronger against *T. rubrum*, with a MIC of 2.5 μg/mL, and 4.37% of 2-phenylacetamide in its composition, suggesting that these amides do not play a prominent role in the antifungal activity of the CfF, albeit a synergistic contribution is possible.

In addition to the BITC and elemental sulfur contribution to the observed antifungal activity, the fatty acids and its esters, which comprise the bulk of the CfF, may also have an important role. Extracts and fractions containing fatty acids have been reported to possess at least fungistatic activity. The results of Tamokou *et al.* [22] for a purified mixture of oleic and palmitic acid obtained from ethyl acetate extract of *Albizia adianthifolia* were active against different *Candida* sp. and *C. neoformans*, with MICs ranging from 100 μg/mL to 400 μg/mL, higher values than those observed for CfF against a wide range of tested yeasts.

Palmitic and oleic acid, major constituents of CfF (6.75% and 10.25%, respectively), are among the fatty acids reviewed for their antifungal properties [27]. A proposed possible mechanism of antifungal action of fatty acids indicates that they can alter fungal membrane fluidity, causing cell membrane disorganization and leakage of vital components, eventually leading to cell disintegration [28].

The importance of fatty acids in the plant, and in the extract, may not be restricted only to the antimicrobial activity, but also for their action as penetration enhancers, a supporting role in the utilization of the plant against skin infections. Fatty acids and their esters can penetrate the skin, but in

addition they could also promote the penetration of other active compounds such as the hydrophobic BITC, the amides and sulfur, to deeper layers of the skin, by disruption and alteration of the stratum corneum lipid strucure [29, 30]. An extract from the algae *Botryococcus braunii* rich in palmitic and oleic acid [31], fatty acids we identified in the CfF, enhanced the skin penetration of flurbiprofen, and while the extract were less effective than the purified fatty acids, it was also less irritating to the skin. Another work [32] demonstrated that fatty acids can penetrate and accumulate to different degrees in the skin, and, in addition, the authors have shown that oleic acid significantly enhanced tolnaftate penetration.

To the moment, of the compounds encountered in the EO and CfF of the tubers from *T. pentaphyllum*, only the fatty acids have an established relation with the skin. We have not found specific studies concerning BITC, amides and elemental sulfur. Their hydrophobic, and low molecular weight structures put them among candidates for skin penetration and enhancement by combined use along with fatty acids, although experimentation with these compounds is necessary to a better understanding of the behavior towards skin.

4. Materials and Methods

4.1. Plant Material and Extraction Methods

Tubers of *T. pentaphyllum* were acquired from local farmers in the municipality of Gaurama (Rio Grande do Sul, Brazil), in August 2011, along with the material required for identification. A dried voucher specimen, containing leaves and flowers, is preserved in the herbarium of the Biology Department at the Federal University of Santa Maria under the registration code SMDB-13137.

About 1.9 kg of fresh tubers were initially shredded, after that an amount of 200 g was separated for essential oil extraction and the remaining material was submitted to drying, at 40 °C, in an oven with air flow, during seven days. These shredded and dried tubers were then ground in a knife mill. The resulting dried tuber powder (517 g) was submitted to maceration, at room temperature during a week, with 70% ethanol in water, with a proportion of 8 mL of solvent for each g of plant material. The obtained crude extract (CE) was filtered and the alcoholic solution was evaporated under reduced pressure, in order to eliminate the ethanol. The remaining aqueous solution was successively partitioned with chloroform, ethyl acetate and *n*-butanol until depletion of the color visible components, which was achieved extracting 300 mL of the aqueous crude extract three times with 300 mL of chloroform, followed by three extractions with 300 mL of ethyl acetate and three extractions with butanol, using 300 mL, 200 mL and 150 mL of solvent. Each organic extract was subsequently dried under reduced pressure, resulting in chloroform (CfF), ethyl acetate (EaF) and *n*-butanol (BuF) dried fractions from the CE. In addition, an aliquot of the referred crude extract was fully dried, and stored for further use.

Essential oil (EO) from the tubers was obtained from the shredded fresh material (200 g), separated as mentioned above. Extraction was performed through hydrodistillation at 60 °C during four hours, using a Clevenger apparatus. Separation of the oil from the water was achieved in a separatory funnel.

4.2. Antimicrobial Assays

The essential oil, the hydroalcoholic extract and its fractions, as well as standard benzyl isothiocyanate (BITC) were tested against bacteria, yeasts and molds. Susceptibility tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) microdilution technique, M07-A9 [33] for bacteria, M27-A3 [34] for yeasts and M38-A2 [35] for molds. Compounds were solubilized in dimethyl sulfoxide (DMSO) to obtain stock solutions with final concentration of 128,000 μg/mL. The solutions were used immediately after preparation, and all the analysis were executed in triplicate. Final concentration of DMSO in the testing wells was of 0.01%, experimentally determined to not interfere with the analysis. The tested concentrations ranged from 2.5 μg/mL to 1280 μg/mL for all of the microorganisms. The results of the tests were expressed as the minimal

inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and minimal bactericidal concentration (MBC).

4.2.1. Antibacterial Activity

The following six bacteria strains were tested: *Enterococcus faecalis* ATCC 91299, *Escherichia coli* ATCC 5922, *Klebsiela pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella pullorum* ATCC 9140, *Staphylococcus aureus* ATCC 29213. The isolates were stored in 10% glycerol solution at −70 °C and revived by subculturing in Mueller-Hinton Agar (MHA). The compounds were tested by microdilution broth assay using Mueller-Hinton Broth (MHB) medium in 96-well microplates. The inoculum was prepared by growing bacteria at 37 °C for 24 h in MHA. Colonial growth was suspended in 2 mL of saline, to an approximate 0.5 McFarland turbidity or 1×10^8 colony-forming unit per mL (CFU/mL). The inoculum was diluted (1:20) in MHB and added to all wells, except negative control. The plates were incubated at 37 °C during 24 h. Antibacterial activity was detected by adding 20 μL of 0.5% TTC (triphenyltetrazolium chloride, Merck, Darmstadt,Germany) solution. MIC was defined as the lowest concentration of compounds that inhibited visible growth, as indicated by the TTC staining. Bacterial suspensions from all tests wells were subcultured in sterile agar medium in order to evaluate bactericidal activity.

4.2.2. Antifungal Activity

Dilutions of the compounds were prepared in RPMI-MOPS 1640 medium, in order to obtain two times the final concentrations, and 100 μL of each concentration of extracts was added to columns 1 to 10 of each 96-well microplates. After, 100 μL of standard inoculum was added to all wells, except negative control. The plates were incubated for 48 h at 35°C.

Strains of fourteen species of yeasts were used, including clinical isolates, caspofungin/fluconazole sensitive or resistant species and standard ATCC/CBS strains. The clinical isolates were obtained from the private collection of the Mycology Research Laboratory, Federal University of Santa Maria, Santa Maria, Brazil (LAPEMI-UFSM) and identified based on carbohydrate assimilation profiles using by ID32-C test (bioMérieux). The strains were grown in Sabouraud Dextrose Agar (SDA) for 48h at 35 °C to inoculum preparation. The following yeast were used (*Candida albicans* ATCC 140053, *Candida dubliniensis* CBS 7987, *Candida dubliniensis* fluconazole-sensitive, *Candida dubliniensis*fluconazole-resistant, *Candida glabrata* ATCC 2001, *Candida glabrata* fluconazole-sensitive, *Candida glabrata*fluconazole-resistant, *Candida glabrata* caspofungin-resistant, *Candida guilliermondii*, *Candida parapsilosis*ATCC 22018, *Candida parapsilosis* caspofungin-resistant, *Candida tropicalis*, *Cryptococcus neoformans* ATCC 90012, *Saccharomyces cerevisiae* ATCC 260).

Strains of ten species of molds were used, including clinical isolates (CI) and environmental isolates (EI) as follows (*Aspergillus fumigatus* (CI), *Aspergillus fumigatus* (EI-isolated from maize), *Aspergillus flavus* (CI), *Aspergillus niger* (CI), *Trichophyton rubrum* (CI), *Microsporum canis* (CI), *Fonsecaea pedrosoi* (CI), *Pseudallescheria boydii* (CI), *Fusarium solani* (CI), *Sporothrix schenckii* (CI)). The clinical and environmental isolates were obtained from the private collection of the Mycology Research Laboratory, Federal University of Santa Maria, Santa Maria, Brazil (LAPEMI-UFSM) and identified by macroscopic and microscopic examination. The strains were grown in Potato Dextrose Agar (PDA) from 48h until seven days at 35 °C for inoculum preparation.

The MIC is defined as the lowest concentration of the compound that inhibits the visible growth of a microorganism after 48 h of incubation, as indicated by the TTC staining. Fungal suspensions from all tests wells were subcultured in sterile Sabouraud Dextrose Agar medium in order to evaluate fungicidal activity.

4.3. Chromatographical Analysis

The essential oil and the chloroform fraction of the crude extract were submitted to separation through gas chromatography, with detection by mass spectrometry (GC-MS). The ethyl acetate and *n*-

butanol fractions were not suitable for this analysis due to their physical and chemical properties, moreover, chloroform fraction exhibited better results in the antimicrobial activity assay making it the logical choice for a more in-depth investigation.

The analysis was performed in a model 6890N chromatograph, coupled with a 5975B model mass detector, both from Agilent Technologies. Chromatographic conditions were as follows: oven initial temperature was 50 °C, during one minute, followed by heating at a rate of 5 °C/min until 300 °C, remaining this temperature during 9 min, of a total of 60 min run; separation was achieved in a DB-5MS column (30 m × 320 μm × 0.25 μm), at a constant rate of 1.5 mL/min of helium; detection was performed in the quadrupole equipment using electron ionization. Peak identification was carried by comparison of the experimental mass spectrum with those of the National Institute of Standards and Technology (NIST) standard library and published papers.

5. Conclusions

The *in vitro* antimicrobial activity tested and reported corroborates the popular use of the plant's tubers against cutaneous infections, signaling a possible alternative for the treatment of resistant skin infections, especially of fungal origin. The chemical findings suggest that more than one compound may be related to this effect, with the pro-eminence of benzyl isothiocyanate. Its presence not only explains and is related to the antimicrobial activity, but it is also relevant to its edible aspects. The reported results should pave the way for more investigations, aiming at determining the *in vivo* toxicity and pharmacological effects, and a more in-depth evaluation of the plant composition.

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Author Contributions: Ritiel Corrêa da Cruz, Sydney Hartz Alves and Marli Matiko Anraku de Campos conceived and designed the experiments; Ritiel Corrêa da Cruz, Laura Bedin Denardi, Natalia Jank Mossmann and Mariana Piana performed the experiments; Ritiel Corrêa da Cruz and Laura Bedin Denardi analyzed the data; Sydney Hartz Alves and Marli Matiko Anraku de Campos contributed with materials, equipment and tools for the execution of the experiments; Ritiel Corrêa da Cruz wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of all the extracts and the essential oil are available from the authors.

Genotoxic Evaluation and Centesimal, Mineral and Phenolic Composition of *Tropaeolum pentaphyllum* Lam. Tubers

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ABSTRACT: *Tropaeolum pentaphyllum* is a native species from South America, it grows tubers that are popularly used for the treatment of dermatologic diseases, and more commonly, as spice. Considering these applications, sample of the tubers were submitted to proximate, mineral composition and phenolic analysis, as well as a genotoxic evaluation of its extracts. With the fresh tubers, proximate and mineral composition analysis were performed, according to international guidelines. From dried material, a crude hydroalcoholic extract (70%) was prepared, and from it, organic fractions were obtained (chloroform, ethyl acetate and n-butanol). These four extracts were submmited to an investigation of polyphenolic compounds (by spectrophotometry and HPLC), *in vitro* antioxidant capacity measurement (DPPH radical assay) and genotoxic evaluation (viability, Comet assay and micronucleus formation), along with standard of benzyl isothiocyanate (BITC). Proximate analysis revealed that the tubers are mainly composed of water (69.73%) and carbohydrates (26.06%). Major minerals quantified were potassium, magnesium and calcium (450.05, 79.36 and 56.88 mg/100g of fresh weight, respectively). Antioxidant capacity and phenolic content was low, and none of the common phenolics present in plants were identified. Genotoxicity was observed for the crude extract, chloroform fraction and BITC, with increased effects at higher concentrations. Results showed the nutritional value of the tubers and indicate that its excessive consumption could pose a toxicological risk.

Keywords: *Tropaeolum pentaphyllm*; proximate analysis; minerals; genotoxicity; benzyl isothiocyanate

Introduction

The *Tropaeolum pentaphyllum* Lam. is a perennial plant of the Tropaeolaceae family, native to South America, first described by Monet de La Marck, from specimens collected near Buenos Aires and Montevideo. Today, around the world the different parts of the plant are used for many purposes, such as food, ornamental and medicinal uses. Its flowers are valued for ornamental uses, but are also edible, as part of salads, and as a drug, are regarded for antidiabetic properties (Rix 2010; Trojan-Rodrigues and others 2012).

Among the features that the plant shares with other Tropaeolum species is the development of tubers. These can grow up to 1 Kg in weight and are commonly known in South of Brazil as “crem’’, “crem potato’’ and “bitter-root’’. The decoction of the tubers are indicated for the treatment of skin diseases, however, they are widely used in the region as a condiment, thinly shredded and prepared as homemade pickles with red vinegar, which is consumed along salads and meat preparations (Fenner and others 2006; Kinupp 2007; Zuchiwschi and others 2010). This utilization of the tubers began with European settlers, which occupied south of Brazil during 19th century, and at the time consumed *Armoracia rusticana* roots (horseradish) in a similar way. This historical background has generated confusion nowadays, *T. pentaphyllum* tubers and horseradish are popularly mistaken, identified and consumed by common folk as being the same thing, since are both underground parts with similar organoleptic characteristics. The historical confusion can be traced by the common name “crem’’, a derivation of the slavic word “chren’’, one of the designations of horseradish in Eastern Europe (Agneta and others 2013).

The organoleptic characteristics of the tubers are due to the presence of benzyl isothiocyanate (BITC), previously reported by our group (Da Cruz and others 2016). BITC

shares the pungency and lachrymatory properties with allyl isothiocyanate (AITC), the major secondary metabolite from horseradish, which leads to the popular misunderstanding and misuse of both spices in South of Brazil (Agneta and others 2013). BITC is also one of the compounds related to the traditional medicinal use of the plant for the treatment of skin infections, as we have previously discussed (Da Cruz and others 2016).

To the best of our knowledge, there is no published nutritional or chemical evaluation of *T. pentaphyllum* tubers, neither toxicological study. Also, there is no data comparing and clarifying the differences between *T. pentaphyllum* tubers and *A. rusticana* roots. Therefore the present study aims to quantify several macro and micronutrients of the tubers as well as to determine the amount, and nature of phenolic compounds from the hydroalcoholic extract. Furthermore, *in vitro* antioxidant capacity and genotoxic activity of the extract, and fractions, were assessed.

Materials and Methods

Plant material and extraction method

Tubers of *T. pentaphyllum* were acquired from local farmers in the municipality of Gaurama (State of Rio Grande do Sul, Brazil) in August 2011. Dried voucher specimen containing leaves and flowers is preserved in the herbarium of the Biology Department, at the Federal University of Santa Maria, under the register code SMDB-13137.

The fresh tubers were initially shredded, after that an amount was separated for the centesimal and mineral analysis, and the remaining material was submitted to drying, at 40ºC in an oven with air flow, during seven days. These shredded and dried tubers were

then grinded in a knife mill. The resulting dried powder of the tubers was submitted to maceration, at room temperature, with ethanol 70% in water with a proportion of 8 mL of solvent for each g of dried material, during a week. The obtained crude extract (CE) was filtered and the alcoholic solution was evaporated under reduced pressure, in order to eliminate ethanol. The remaining aqueous solution was successively partitioned with chloroform, ethyl acetate and n-butanol until depletion of the color visible components. Each organic extract was subsequently dried under reduced pressure, resulting in chloroform (ChE), ethyl acetate (AcE) and n-butanol (BuE) fractions. Also, an aliquot of the crude extract was fully dried, and stored for further use. The dried CE, and its three derived fractions, were later used in the polyphenol determination, antioxidant capacity and genotoxic assessment.

Centesimal analysis

Proximate composition was determined following the official methods of sampling and analysis from Instituto Adolfo Lutz (IAL 2005). Briefly, the moisture was determined by drying the tubers sample to constant weight at 105°C. Loss in weight was reported as moisture. Crude fat (lipids) were extracted from the fresh tubers with petroleum ether, using a Soxhlet extractor, followed by solvent elimination and weighing. The percentage was determined according to the following equation:

Protein content was determined using Kjeldahl method (modified) for nitrate-free samples, and crude protein was calculated using a nitrogen-to-protein conversion factor of 5.75. Lastly, the ash content was determined by incineration of the sample at 550°C, remaining material was weighted and reported as ash. After these experimental

136 determinations, the total carbohydrate content (%) was calculated by difference method. All
137 the analysis were carried out in triplicate, and the results were expressed as g of constituent
138 by 100g of fresh weight (FW) (mean ± standard deviation).
139
140 *Mineral analysis*
141
142 The digestion processe was based on EPA 3050B + HCl with modifications (USEPA
143 2010). Around 0.25 g of the fresh tubers were accurately weighed in a 250 mL Pyrex flask
144 and 10 mL of 8.5 M HNO_3 were added. The solution was heated on a digestion block to circa
145 95°C without boiling and this temperature was maintained until nitric vapors formation
146 ceased. After cooling to less than 70°C, 3 mL of 8.8 M H_2O_2 was slowly added on the flasks.
147 The solution was then heated until effervescence reduced. It was then allowed to cooling,
148 after room temperature was achieved, 10 mL of H_2SO_4 was added and the solution was
149 heated again for 2 hours. After that, the digested samples were diluted with destilled water
150 to 50 mL on falcon tubes.
151 All measurements were carried out using an Analytik Jena AG™ (Jena, Germany)
152 model novAA 300 atomic absorption spectrometer, equipped with SpectrAA™ (Varian,
153 Australia) hollow cathode lamps as the radiation source. An acetylene-air or acetylene-
154 nitrous oxide flame was used; the gas flow rates and the burner height were adjusted in
155 order to obtain the maximum absorbance signal for each element. Analyzed elements
156 (wavelenght, slit, slope) were: calcium (422.7 nm, 1.2 nm, 4.0 mA), iron (248.3 nm, 0.2 nm,
157 8.0 mA), magnesium (285.2 nm, 1.2 nm, 2.0 mA), potassium (766.5 nm, 0.8 nm, 4.0 mA),
158 sodium (589.0 nm, 0.8 nm, 3.0 mA) and zinc (213.9 nm, 0.5 nm, 6.0 mA). Results are
159 expressed as mg of mineral by 100 g of FW.

Phenolic determination

Determination of the total phenolic content

The total phenolic content was determined in the crude extract and its fractions by the Folin-Ciocalteu method with minor modifications (Chandra and Mejia 2004). Briefly, 0.5 mL of 2 *N* Folin-Ciocalteu reagent was added to 1 mL of each sample solution (0.15 mg/mL). The mixture was allowed to stand for 5 min before addition of 2 mL of a 20% aqueous Na_2CO_3 solution. The resulting solution was then allowed to stand for 10 min before absorbance reading at 730 nm on a Shimadzu-UV-1201 (Shimadzu, Kyoto, Japan) spectrophotometer. Analyses were carried out in triplicate. The total phenolic content was expressed in milligram gallic acid equivalent (GAE) per gram of dry extract/fraction.

HPLC-DAD phenolic investigation

High performance liquid chromatography (HPLC-DAD) was performed with Prominence Auto-Sampler (SIL-20A) equipped with Shimadzu LC-20AT (Shimadzu, Kyoto, Japan) reciprocating pumps connected to a DGU-20A5 degasser and a CBM-20A integrator. UV-VIS detector DAD SPD-M20A and software LC Solution 1.22 SP1 were used. Reverse phase chromatography analyses were carried out with a Thermo Scientific C-18 column (4.6 mm x 250 mm) packed with 5 µm diameter particles. Injection volume was 40 µL and the gradient elution was conducted according to the Evaristo and Leitão (2001) method with modifications described in Da Cruz and others (2012). UV absorption spectra were recorded in the 200-400 nm range.

The crude extract and its three fractions were individually screened for the presence of the following polyphenolic compounds (retention time): pirogallic acid (7.95 min), gallic acid (8.13 min), catechin (16.78 min), chlorogenic acid (18.35 min), caffeic acid (20.06 min), orientin (25.87 min), vitexin (27.44 min), rutin (29.37 min), rosmarinic acid (31.82 min). 4-hidroxycoumarin (33.51 min), quercetin (37.71 min), luteolin (41.99 min), apigenin (46.21 min) and crisin (54.34 min). Identification of the compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. Stock methanolic solutions of standards were prepared at 0.050 mg/mL, for comparison. Chromatographic operations were carried out at ambient temperature and in triplicate.

DPPH radical scavenging capacity assay

The radical scavenging capacity of the CE and fractions of *T. pentaphyllum* tubers was assessed in the presence of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate)stable radical, according to method proposed by Mensor and others (2001). The four samples were tested at 7.81, 15.62, 31.25, 62.50, 125, 250, 500 and 1000 µg/mL. Briefly, 2.5 mL of each sample was mixed with 1.0 mL of 0.3 mM DPPH ethanolic solution, the mixture was allowed to stand for 30 min. After that, the absorption was measured at 518 nm on a Shimadzu-UV-1201 (Shimadzu, Kyoto, Japan) spectrophotometer. A solution of DPPH in ethanol was used as negative control, and ascorbic acid as positive control. The tests were performed in triplicate, and the scavenging capacity was calculated as the equation below:

$$\%\ Inhibition = 100 - \frac{(Abs_{sample} - Abs_{blank}) \times 100}{Abs_{control}}$$

206

207 Where: Abs_{sample} is the absorbance of CE or fraction tested; Abs_{blank} is the absorbance
208 of the samples without the addition of DPPH solution; $Abs_{control}$ is the absorbance the
209 solution of DPPH in ethanol.

210

211 *Cyto- and genotoxicity evaluation*

212

213 *Human blood samples*

214

215 Peripheral blood was collected by venipuncture into sterile vials containing 68 I.U. of
216 sodium heparin (BD Vacutainer®) per mL of blood, from healthy volunteers. The vials were
217 transferred to the laboratory, and a lymphocyte –rich peripheral blood mononuclear cell
218 (PBMC) fraction was isolated and separated using a Ficoll-Hystopaque gradient. The samples
219 were stored up to 24 h at 4 °C before culturing. This project was approved by the
220 University's Committee of Ethics in Research of Federal University of Pampa (Rio Grande do
221 Sul, Brazil) (authorization nº 27045614.0.0000.5323).

222

223 *Cell culture and extract samples preparation*

224

225 The lymphocytes cultures were prepared from PBMC fraction samples and
226 immediately transferred to 1 mL of culture medium containing RPMI 1640 supplemented
227 with 10% fetal bovine serum and 1% streptomycin/penicillin, as previously described
228 (Montagner and others 2010). The cells were then placed in a microaerophilic environment
229 at 37 °C for 72 h. The solutions under investigation were added with the PBMCs with a 10%

concentration. The analyzed samples were solutions of the crude extract of *T.pentaphyllum* tubers, its three fractions and standard BITC, a compound previously described for the species, all prepared in phosphate-buffered saline (PBS) at concentrations of 1, 10 and 100 µg/mL. We used 100 µM H_2O_2 as positive control (PC) and PBS buffer as negative control (NC). Each group consisted of three culture flasks. Genotoxicity assays were carried after 72 h of growth.

Analysis of cyto- and genotoxic parameters

To perform the cytotoxicity tests, we first counted the total number of PBMCs in a Neubauer chamber. In this technique, samples are mixed with Turk's solution (acetic acid 3% and 1% gentian violet in water) and placed in the chamber and counted in four quadrants (Montagner and others 2010). Viability was assessed by a loss of membrane integrity, which was indicated with trypan blue (Burow and others 1998). The differentiation of living/dead cells was observed by the blue coloration of dead cells. At least 300 cells were counted in this technique. Cell viability was expressed as a percentage of the control value. The genotoxicity test was conducted using Comet assay (Singh and others 1988), in accordance with the general guidelines (Tice and others 2000; Hartmann and others 2003). Although comet assay is not the only method for measuring oxidative DNA damage, it is one of the most sensitive and accurate, and is relatively free of artifacts (Collins 2009). After incubation, the PBMCs were mixed with low-melting point agarose and placed on a microscope slide pre-coated with normal melting point agarose. The slides were immersed in a lysis solution, and electrophoresis (20 min at 300 mA and 25 V) was performed. At the end, the slides were neutralized and left to dry overnight at room temperature. The dry

slides were re-hydrated and then fixed for 10 min, and left to dry again. The last stage was the staining with silver nitrate and the use of stop solution. The slides were analyzed under blind conditions.The cells were visually scored according to tail length, with scores ranging from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The tests were carried out in triplicate, and the data are presented as mean ± standard error.

In the micronucleus test (MN), the cells were fixed with acetic acid and methanol (75:25, v/v), transferred onto clean microscope slides in duplicates and stained with Giemsa 5%. The criteria for scoring cells with MN were described in a previous report (Thomas and others 2008). One thousand cells were counted for each sample, and the results were expressed as the micronucleus frequency per 100 cells.

Statistical analysis

Results were expressed as means±standard deviation (SD). Data from cell cultures exposed to the tested extracts were compared with those from untreated, non-exposed cell cultures (control). Statistical software GraphPad Prism 5.0 was used to perform all statistical analyses, which included an analysis of variance (ANOVA) followed by a post hoc Bonferroni test for the genotoxicity evaluation, and Tukey test was applied for the results of total phenolic content and DPPH assay. Values of $p < 0.05$ were considered significant.

278 **Results and Discussion**

279

280 Results from the proximate analysis are expressed in Table 1. As expected from a
281 tuber, a modified stem dedicated to energy storage, the major nutrients are water (69.73%)
282 and carbohydrates (26.06±0.02%). When compared to a most popular tuber, the widely
283 known and consumed potato (*Solanum tuberosum*), which according to USDA database
284 (USDA 2013) possess 79.25% of water and 19.59% of carbohydrates (including fiber), the
285 "crem'' presents itself as a richer source of carbohydrates. *Tropaeolum tuberosum*, an
286 Andean tuber classified in the same genus and that also can be consumed as a condiment,
287 possess even lower levels of carbohydrates (10.50%, including fiber), being mainly
288 composed of water (87.00%) (Sperling and King 1990).
289 The most relevant comparison to be made is nonetheless with *Armoracia rusticana*
290 roots (horseradish), since it is mistakenly cultivated and consumed by local population as
291 the same condiment as *T. pentaphyllum* tubers, under the popular name ''crem''.
292 Horseradish contains roughly 11.5% less carbohydrates and 2% less protein than *T.*
293 *pentaphyllum* tubers (USDA 2013). This highlights the nutritional value of the tuber, which is
294 not only consumed as condiment, but can also be consumed in salads, after coction (a less
295 popular form of use) (Kinupp 2007).
296 The data of six elements, obtained from mineral composition analysis, are presented
297 in Table 2. The major mineral constituent of the tubers is potassium, followed by
298 magnesium and calcium. Sodium content of the tubers (6.50 mg/100 g) is low, in the range
299 of potato sodium content (6.00 mg/100 g), which is a positive finding considering the
300 ubiquity of sodium among foods and the diseases associated with its excessive consumption
301 (Strazzullo and others 2009; He and others 2013).

Potassium and sodium content of the analyzed tubers are in the same range than those of potato, according to USDA National Nutrient available data (USDA 2013). However, *T. pentaphyllum* presents, roughly, 4.5 times more calcium (56.88 mg/100 g compared to 12.00 mg/100 g from potato) and 3.5 times more magnesium than potato (23.00 mg/100 g). Iron and zinc content are also slightly higher than those of potato, data that along with protein and carbohydrate content makes the tubers richer and more nutritious than potato.

The tubers from *T. pentaphyllum* also excels when compared to horseradish, with higher levels of the six evaluated elements. Potassium content is about two times that of horseradish (246.00 mg/100 g), and magnesium amount is approximately three times bigger (27.00 mg/100 g) (USDA 2013).

While *T. pentaphyllum*'s tubers proved to be a remarkable source of nutrients, such as carbohydrates, calcium and potassium, the same was not verified for its total phenolic content, presented in Table 3 along with an assessment of its antioxidant potential.

Total polyphenol content obtained for the CE and fractions of the tubers was low. For comparison, two well established sources of this group of secondary metabolites, *Camellia sinensis* (green tea) and *Ilex paraguariensis* (maté), leaves extracts possess 148.92 and 186.76 mg GAE/g (Chandra and Mejia 2004), while we determined an amount of 46.45 mg of gallic acid equivalent/g in ethyl acetate fraction, the highest value among our extracts. This finding is in accordance with the results from the chromatographic analysis, where none of the fourteen investigated phenolic acids and flavonoids were encountered in the extracts. Figure 1 presents the resulting chromatograms from the HPLC-DAD analysis of phenolics for the CE and its three fractions. While the retention time of some peaks in these samples matched those of the phenolics standard solutions, the spectral data did not correspond (data not shown), indicating the absence of the investigated polyphenols.

The results from the *in vitro* antioxidant screening, shown in Table 3, are in agreement with the low levels of phenolics in the extracts. The three fractions possess IC_{50} about half of the CE's IC_{50} value, due to the phenolic compounds concentration obtained with the fractionation process, however, it did not mean a remarkable antioxidant capacity, when compared with ascorbic acid, the tested antixodant standard for the reaction.

Published and available data for other plants that share similarities with the tubers are in agreement with the absence of phenolics. USDA database reports no polyphenols for horseradish and wasabi, underground plant parts also used as spice (USDA 2013). Potato, another good base of comparison, possess only small amounts of quercetin and kaempferol (USDA 2013). However, at the beginning of this investigation we expected to find a promising phenolic profile, due to published data showing the presence of several phenolics in mashua, the tubers from *Tropaeolum tuberosum*, largely consumed in the Andean region (Campos and others 2006; Chirinos and others 2008).

Besides macronutrients, minerals and polyphenols, we have also evaluated the genotoxic effects of the crude extract and fractions from the tubers (Figures 2 and 3). In the present study, cytotoxicity was assessed, through counting of the total number of PBMCs (Figure 2A), and the measurement of cell viability (Figure 2B). The most aggressive samples to the PBMCs were the CE, ChE (100 µg/mL and 10 µg/mL) and the standard BITC (dose-response relationship). These samples killed about 60-80% of the PBMCs, a cytotoxicity capacity in the same degree of the positive control (H_2O_2 100 µM). To complement these findings, of the surviving cells, about 10-20% were no longer viable after incubation with the CE and BITC.

The genotoxicity of the extracts, assessed by two parameters, is presented in Figure 3. The Comet and micronucleus assays are widely used in experimental toxicological studies

to evaluate genotoxicity and DNA damage (Thomas and others 2008; Collins 2009). Strong genotoxicity was observed for the BITC (dose-response relationship), followed by CE (dose-response relationship) and ChE (100 µg/mL), where the formation of micronuclei was significantly increased compared to the positive control. Damage to DNA followed a similar pattern, with the index ranging from 30 to 40 for the same samples, however they were significantly less aggressive than positive control (40-50).

In a previous work (Da Cruz and others 2016) we demonstrated that one of the major secondary metabolites present in the tubers is BITC, the hydrodistillation of the tubers yielded 0.082% of essential oil, constituted of 98.51% of BITC, leading us to evaluate the genotoxicity of standard BITC. Our results are in agreement with published reports, which demonstrates that the isothiocyanate possess genotoxic effects at the usual concentration of *in vitro* assays (Kassie and others 1999; Kassie and others 2003). However, with *in vivo* models the opposite was verified, BITC and isothiocyanates are being regarded as chemopreventive agents (Kumar and others 2015). There is evidence that prior to biotransformation, isothiocyantes have a different behave in biological systems, Kassie and others (1999) showed that BITC ability to interact with proteins (present in fluids such as saliva and gastric juice) reduces the DNA damage it causes. BITC pharmacokinetics, which involve reactions from the mercapturic acid pathway, starting with conjugation with glutathione, and finishing with excretion as a mercapturic acid, could be also a factor decreasing the risk of *in vivo* genotoxicity (Brusewitz and others 1977; Zhang and others 1995; Lamy and others 2011).

Furthermore, there is a consensus that concentrations achieved with the ingestion of vegetables containing isothiocyanates are far lower than the genotoxic concentrations of the laboratorial tests (Fimognari and others 2012). Similar circumstances was also reported

for other non-isothiocyanate essential oil compounds from different spices, they present some degree of genotoxicity at higher concentrations on some of the tested models, however there are no clear indication that these compounds pose a risk at amounts usually ingested in daily nutrition (Llana-Ruiz-Cabello and others 2015).

Standard BITC genotoxicity, discussed above, was followed by the CE and ChE genotoxic effects. As our results before have showed, the CE and the fraction possess low amounts of phenolics compounds, and the chromatographic analysis revealed none of the investigated compounds, such as rutin and quercetin (Figure 1). The low amount of phenolics coupled with the absence of flavonoids is certainly a factor that contributes to the genotoxicity of the extracts, since phenolics have been implied as genoprotective agents (Hayder and others 2004; Wilms and others 2005; Min and Ebeler 2009). For a direct comparison, an aqueous extract from horseradish, containing small amounts of kaempferol and quercetin was able to protect human PBMCs DNA's from hydrogen peroxide oxidative damage (Gafrikova and others 2014).

BITC is also present in the CE and ChE (0.65%) (Da Cruz and others 2016), albeit at low concentrations, it still may contribute to the observed genotoxicity. Although, other compounds can be exerting effect, in the ChE we previously encountered 2-phenylacetamide, N-benzylacetamide, benzylcarbamide, palmitic and oleic acid, along with some fatty acids esters (Da Cruz and others 2016). The three amides are structurally related to BITC, and may be degradation products of it. However, it is unlikely that they are responsible for the genotoxicity of the fraction along with BITC, to the moment, there is no data available on the genotoxicity of these compounds, and a simulation on OSIRIS Property Explorer (Organic Chemistry Portal 2014) did not predicted toxicity potential for these three compounds. On the case of these substances be experimentally proved to be genotoxic,

their formation is unlikely to happen *in vivo* after the ingestion of the tubers, as can be seen by the discussed biotransformation pathways of BITC. Even if these amides could be formed during cooking treatment of the tubers, such as boiling or heating (moisture and heat conditions similar to extract preparation), in the presence of other reactive food nutrients, they still would be in low concentration to cause damage, as it is the case with the ingestion of isothiocyanates in general, as discussed before.

Along with BITC and the amides, fatty acids and their esters comprise about 35.55% of the fraction mass, among them is palmitic acid (4.66% of ChE) (Da Cruz and others 2016), which in addition to BITC, may be responsible for the genotoxicity. According to Beeharry and others (2003) palmitic acid can cause DNA damage and induce apoptosis, effects that the authors could prevent with polyunsaturated fatty acids (linoleic acid). Furthermore, Zeng and others (2008) demonstrated that saturated fatty acid can interfere with DNA damage response pathway, contributing to tumor development and progression. These data support our findings, where our extract lacking antioxidants, such as polyphenols and polyunsaturated fatty acids, but containing palmitic acid and BITC was genotoxic.

Conclusion

We present here for the first time a determination of macronutrients and minerals of the tubers from *T. pentaphyllum*, a vegetable consumed as a pungent spice. The results revealed that the tubers are mainly composed of carbohydrates and are rich in potassium and calcium. The investigation of polyphenols performed on the hydroalcoholic extract and fractions from the tubers revealed low amounts of it, and none of the investigated flavonoids or phenolic acids were found, at the tested conditions. The scarce levels of

polyphenols are in agreement with low *in vitro* antioxidant capacity, and the genotoxicity observed for the crude extract and chloroform fraction of the tubers. These extracts reduced cell viability and altered the test model PBMCs, through micronuclei formation and direct DNA damage, probably due to benzyl isothiocyanate and palmitic acid presence. Nonetheless, this likely poses no threath to health since it is ingested in low amounts as a food complement, and along with dietary natural antioxidants present in other foods, that would reduce the risk of damage caused by the tubers composition. However, *in vivo* studies emulating diary intakes are required for a better understanding and safe consumption. This study has also provided important contribution to differentiate *T. pentaphyllum*'s tubers (crem) from *A. rusticana*'s roots (horseradish), which are identified and consumed as the same pungent spice in South of Brazil, showing important differences on nutritional and toxicological data.

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Author Contributions

Ritiel C. da Cruz, Michel M. Mansur and Marli M. A. de Campos conceived and designed the experiments; Ritiel C. da Cruz, Amanda L. Gindri, Jonathaline A. Duarte and Gabriel M. Reis performed the experiments; Ritiel C. da Cruz and, Luis Flávio S. de Oliveira and Carine Viana analyzed the data; Marli M. A .de Campos, Carine Viana and Michel. M. Mansur contributed with materials, equipment and tools for the execution of the experiments; Ritiel C. da Cruz and Marli M. A. de Campos wrote the paper.

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633 Table 1. Proximate composition of *T. pentaphyllum* tubers.

Constituent	Mean±SD (g/100g FW)
Moisture	69.73±0.05
Mineral	1.02±0.08
Protein	3.12±0.05
Lipid	0.07±0.01
Carbohydrate	26.06±0.02

634 FW: fresh weight

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652 Table 2. Mineral composition of *T. pentaphyllum* tubers.

Mineral	Mean±SD (mg/100g FW)
Calcium	56.88±2.60
Iron	1.25±0.01
Magnesium	79.36±0.63
Potassium	450.05±11.12
Sodium	6.50±0.08
Zinc	1.57±0.02

653 FW: fresh weight

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Table 3. Total phenolic content and calculated IC_{50} (DPPH) of crude extracts and fractions from *T. pentaphyllum* tubers.

Extract/Fraction	Polyphenol content[1,2,3]	IC_{50}[2,3](µg/mL)
Crude extract	20.01±0.49 [a]	961.60±12.86 [a]
Chloroform fraction	37.06±0.91 [b]	450.22±4.70 [b]
Ethyl acetate fraction	46.45±1.42 [c]	466.18±2.42 [b]
n-Butanol fraction	27.21±0.45 [d]	463.26±1.78 [b]
Ascorbic acid	-	15.98±0.02 [c]

1. Expressed as mg gallic acid equivalent/g of dry extract or fraction.
2. Values are expressed as mean (n = 3) ± standard deviation.
3. Numbers followed by different letters in each column are significantly different and differ by Tukey test at $p < 0.05$.

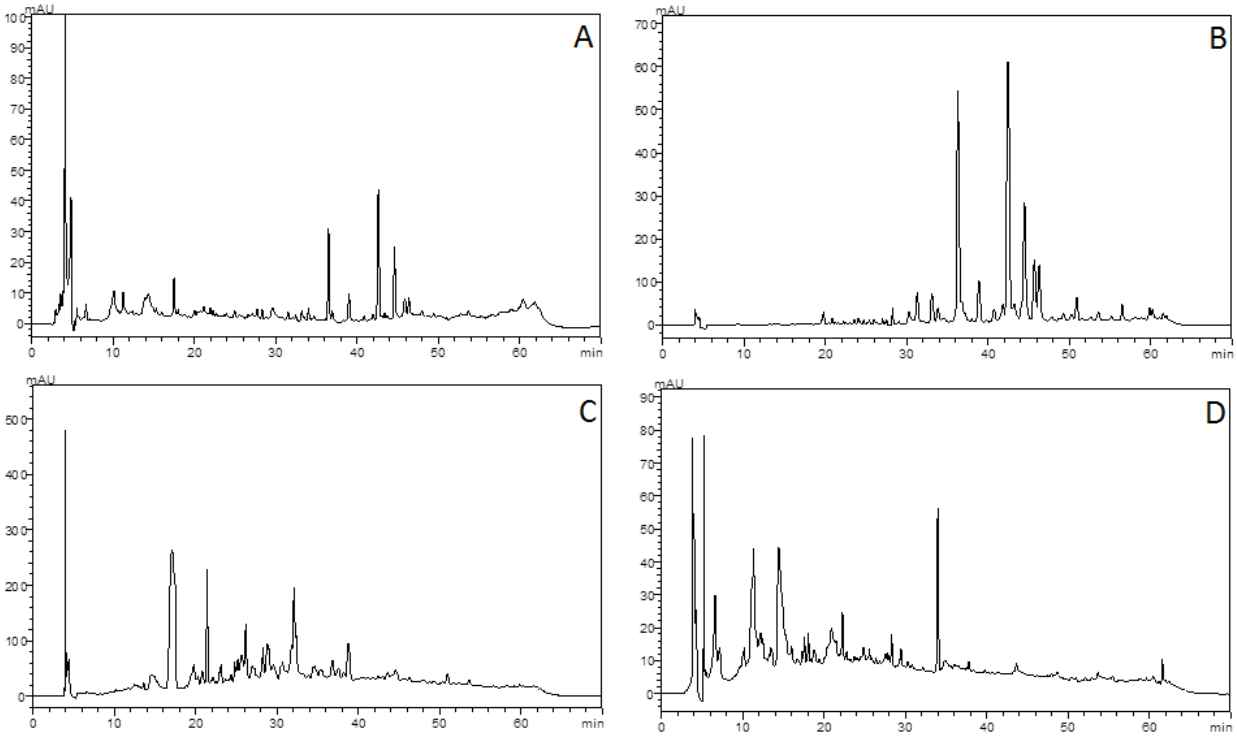
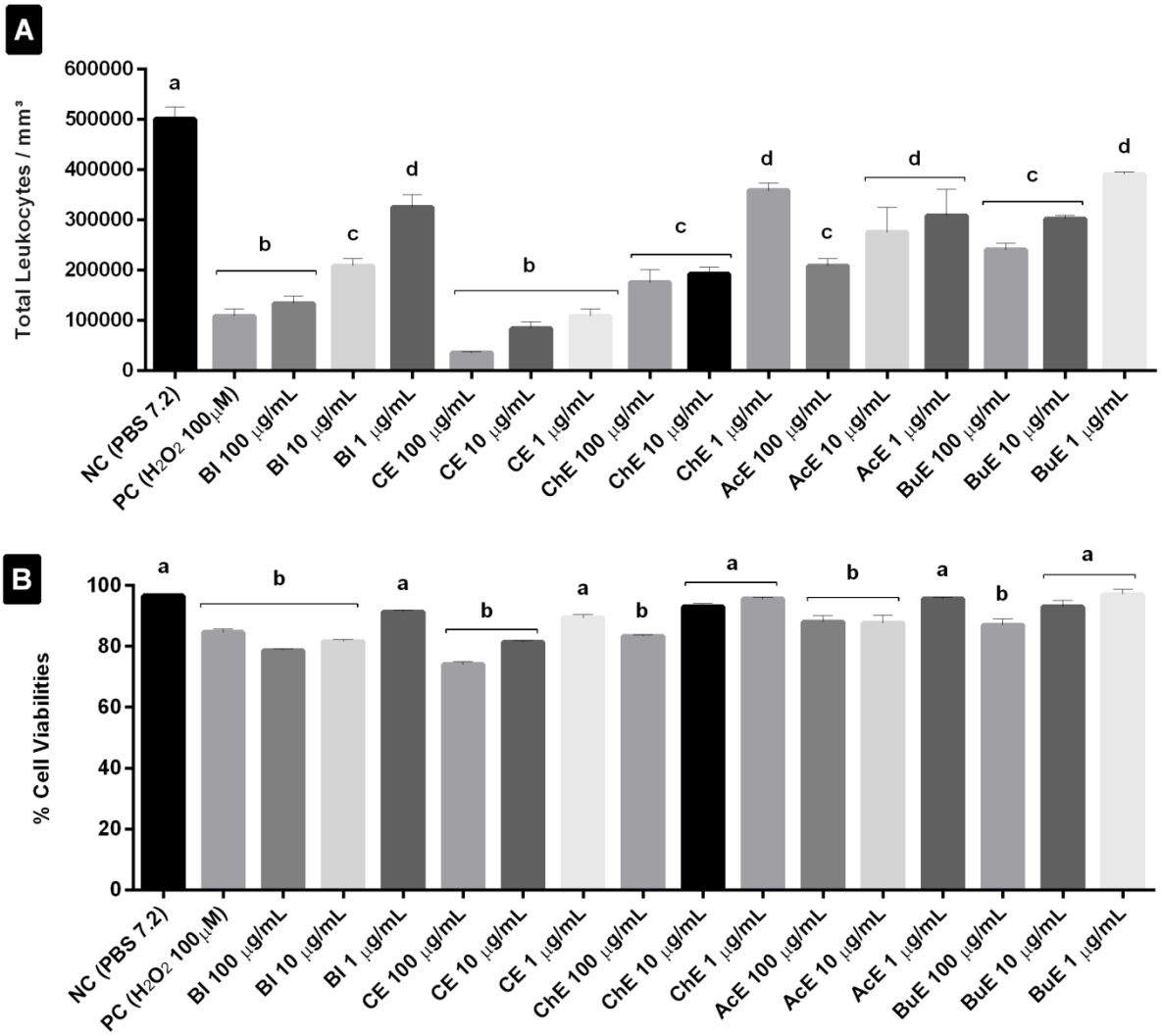


Figure 1. Chromatograms (254nm) of the CE (A), ChE (B), AcE (C) and BuE (D) from *T. pentaphyllum* tubers.



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700 Figure 2. Cytotoxicity of CE, fractions and standard BITC in different concentrations: total
701 PBMCs (A) and cell viability (B). Columns followed by different letters are significantly
702 different from control (untreated, non-exposed cell culture) by Bonferroni test at $p < 0.05$.
703 NC: negative control; PC: positive control; BI: benzyl isothiocyanate; CE: crude extract; ChE:
704 chloroform fraction; AcE: ethyl acetate fraction; BuE: *n*-butanol fraction.

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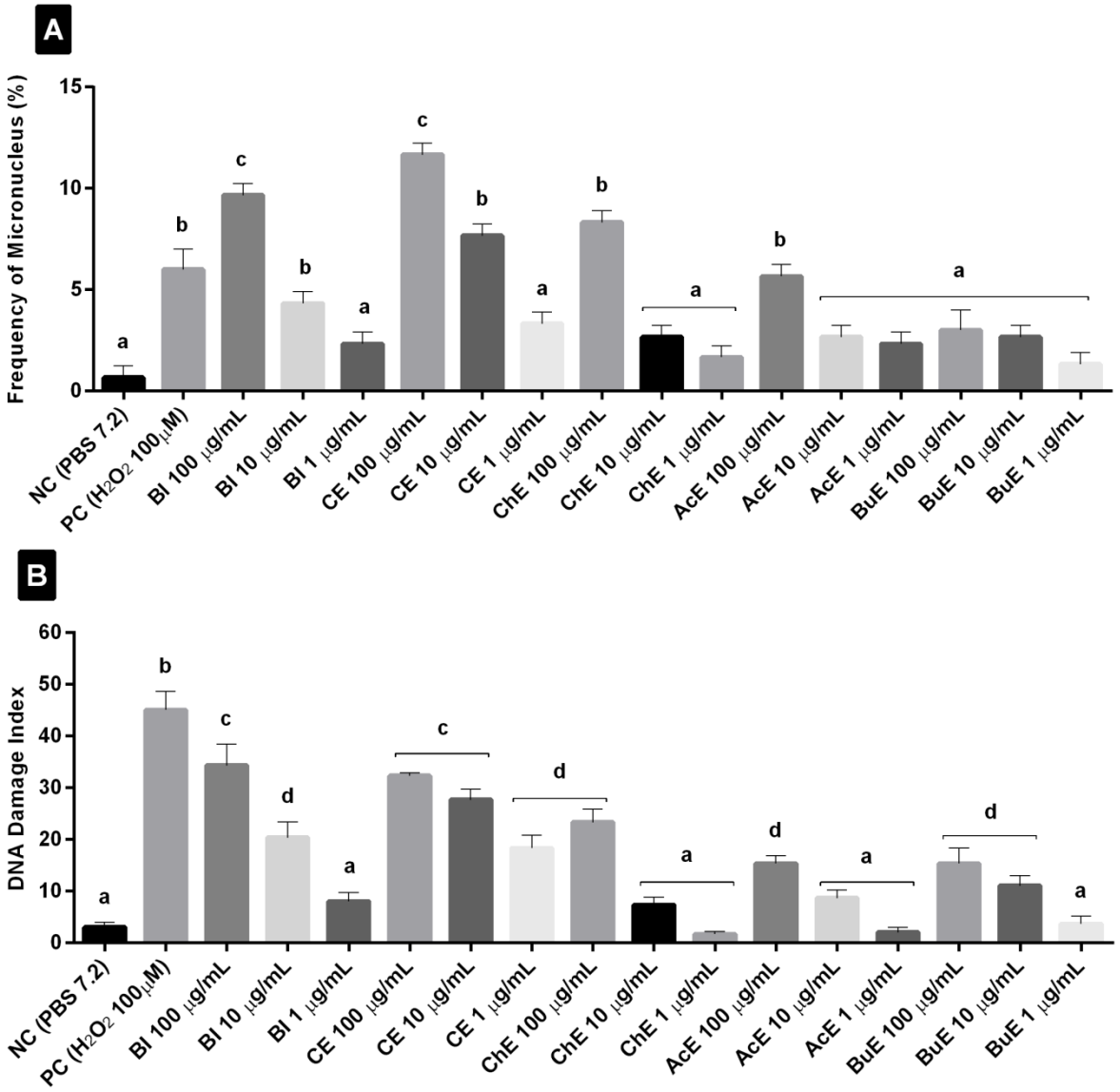

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709 Figure 3. Genotoxicity through micronucleus frequency (A) DNA damage (B) of CE, fractions
710 and standard BITC in different concentrations. Columns followed by different letters are
711 significantly different from control (untreated, non-exposed cell culture) by Bonferroni test
712 at $p < 0.05$. NC: negative control; PC: positive control; BI: benzyl isothiocyanate; CE: crude
713 extract; ChE: chloroform fraction; AcE: ethyl acetate fraction; BuE: *n*-butanol fraction.

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5 DISCUSSÃO

Os resultados do trabalho, apresentados nos dois artigos, representam uma investigação, até então inédita, realizada com os tubérculos de *T. pentaphyllum*, tendo em vista dois aspectos, seu uso farmacêutico segundo a medicina popular, e sua utilização como tempero, assumindo um caráter complementar na dieta.A intenção foi verificar a fundamentação em seu uso medicinal, e buscar informações nutricionais e toxicológicas em relação ao seu uso alimentar.

No Artigo 1 é dado um enfoque na forma de uso medicinal da planta, partindo das palavras usadas em Mentz, Lutzemberger e Schenkel (1997) de que ‘’as raízes, em decocção, são depurativas em certas dermatoses’’.Entretanto, sendo as dermatoses alterações patológicas da pele de causas variadas, de caráter alérgico ou não-alérgico (GARCIA; REY; BERND, 2005), a inclusão da planta na lista de Fenner e colaboradores (2006) de plantas da medicina popular brasileira com potencial antifúngico nos serviu de maior referência. As infecções fúngicas da pele, sejam por fungos dermatófitos (dermatofitoses), como por fungos do gênero Candida (candidíases) se enquadram entre as causas de dermatoses não-alérgicas.

Os resultados apresentados no Artigo 1 corroboram o uso das decocções do tubérculos para tratamento de dermatoses causadas por infecções fúngicas, pois potente atividade *in vitro*,tanto inibitória como fungicida foi observada, frente a uma série de fungos causadores de infecções da pele, para o óleo essencial e para a fração clorofórmio do extrato bruto. A análise da composição do óleo essencial demonstrou que o composto ativo por trás dessa atividade é o ITB, cuja presença é descrita pela primeira vez na espécie *T. pentaphyllum*, e com atividade antifúngica já estabelecida (DROBNICA et al., 1967). Já a fração clorofórmio, a despeito da baixa concentração de ITB, também apresentou potente atividade. Esse resultado indica que a estrutura íntegra de ITB pode não ser uma condição estritamente necessária para a atividade antifúngica que suporta o uso popular da planta, pois o preparo das decocções dos tubérculos envolve água e aquecimento, condições nas quais os isotiocianatos não retém estabilidade (KAWAKISHI; NAMIKI, 1969; PECHÁČEK; VELÍŠEK; HRABCOVÁ, 1997).

Somente um estudo de estabilidade poderá determinar com segurança se as amidas, e o enxofre elementar, descritos como constituintes da fração clorofórmio no Artigo 1 são componentes naturais da planta ou foram obtidos durante os

procedimentos laboratoriais. Porém, é possível estimar com base na reatividade e no comportamento físico-químico dos isotiocianatos (KAWAKISHI; NAMIKI, 1969; PECHÁČEK;VELÍŠEK; HRABCOVÁ, 1997; NOAA, 2016) que estes compostos foram formados em alguma etapa do processamento laboratorial, visto que condições que favorecem suas reações estavam presente, como a água na mistura hidroalcoólica extratora, o aquecimento no evaporador rotatório (50ºC) e a presença de ácidos carboxílicos que foram extraídos dos próprios tubérculos (na forma de ácidos graxos). Essas mesmas condições, ainda que em graus diferentes, estão também presentes no preparo de uma decocção, de forma que o ITB presente nos tubérculos pode sofrer alterações quando do preparo pelas populações usuárias, gerando produtos com atividade antifúngica própria, como o enxofre elementar (TWEEDY, 1981).

Os resultados e a situação discutida acima podem num primeiro momento parecer contraditórios, porém também podem indicar uma versatilidade no uso da planta, onde tanto o princípio ativo como seus possíveis produtos de degradação são responsáveis pelo efeito desejado. Essa situação reforça a necessidade de um estudo etnobotânico mais aprofundado junto às populações usuárias da planta, de forma a responder questões sobre o preparo das decocções, e aspectos como a forma, frequência e a intensidade do uso. De posse dessas informações, experimentos laboratoriais (*in vitro* e *in vivo*) utilizando uma forma padronizada da decocção poderiam resultar em mais esclarecimentos sobre a eficácia da mesma.

Além dos resultados mais diretamente ligados ao uso popular(a atividade antifúngica frente a dermatófitos e candidas) o óleo essencial e a fração clorofórmio também demonstraram atividade contra outros microrganismos, como bactérias, e fungos como o *Sporothrix schenkii* e *Fonsecaea pedrosoi.* Agentes infecciosos cujas patologias não se restringem somente ao tecido cutâneo, e cuja sensibilidade não é frequentemente avaliada em estudos de triagem com extratos e compostos de origem vegetal. Tais resultados podem servir de guia para futuros estudos envolvendo tratamentos *in vivo* de infecções causadas por estes fungos, de forma a avaliar um possível efeito após penetração na pele e/ou ingestão por via oral.

Outro grupo de microrganismos que foi sensível à fração, e ao óleo essencial, foram as cepas resistentes a agentes antimicrobianos (fungos resistentes a fluconazol e caspofungina). No contexto em que vivemos, em que rotineiramente na prática clínica novas cepas e mecanismos de resistência antimicrobiana surgem, a

busca por novos compostos antimicrobianos não precisa se restringir somente às suas fontes tradicionais (PFALLER, 2012). Desse modo, as plantas, que historicamente não têm grande relevância como fonte de antibióticos, não podem ser descartadas. Ainda da possibilidade de encontrar um equivalente vegetal da penicilina, com potencial para revolucionar a antibioticoterapia, seja remota, os compostos de origem vegetal podem vir a fornecer novas estruturas para modificação molecular, adjuvantes de efeito sinérgico aos agentes existentes, ou então podem fornecer alternativas de tratamento para infecções menos agressivas (COWAN, 1999; SAVOIA, 2012).

O Manuscrito 1 trata dos tubérculos de *T. pentaphyllum* tendo em vista seu uso como alimento (KINUPP, 2007). Do ponto de vista nutricional, foi determinado o teor de macronutrientes, composição mineral e compostos fenólicos. As diferentes técnicas utilizadas, nas condições descritas, revelaram um baixo teor de polifenois. Entretanto, uma técnica de extração otimizada para estes compostos, bem como variações nos parâmetros dos métodos (como a cinética das reações) podem vir a revelar informações adicionais sobre a composição fenólica dos tubérculos.

A avaliação de macronutrientes forneceu o teor de água, minerais, carboidratos, lipídios e proteínas presentes nos tubérculos. Essas determinações foram realizadas como uma caracterização básica dos tubérculos, pois na data de sua execução ainda não estavam relatadas em publicações científicas ou bancos de dados nutricionais. Além do propósito analítico imediato da realização dessas técnicas, também teve-se por intuito sedimentar futuros experimentos visando determinações bromatológicas mais específicas, como atividade de água, e perfil dos carboidratos e lipídios que compõem os tubérculos

Da mesma forma, os micronutrientes quantificados na avaliação da composição mineral (cálcio, ferro, magnésio, potássio, sódio e zinco) constituem um esforço inicial a fim de se traçar um perfil do valor nutricional dos tubérculos. Demais elementos como fósforo, enxofre, selênio, entre outros, deverão ser futuramente determinados.

Dentre as mais aprofundadas análises de micronutrientes a serem realizadas com os tubérculos também se inclui uma avaliação de seu perfil e teor de vitaminas.

Além da parte bromatológica, o restante do Manuscrito 1 trata de uma análise de toxicidade *in vitro* realizada com extratos da planta. Os resultados indicam que o principal metabólito secundário da planta, o ITB pode ser tóxico no nível celular,

assim como os extratos que o contém. Entretanto não há indicativo de que o consumo dos tubérculos, na forma como é feita, em pequenas porções, como tempero ou molho, ofereça risco. Uma ingestão de grandes quantidades dos tubérculos poderia levar a efeitos tóxicos decorrentes do ITB, porém tal circunstância é altamente improvável visto que às características organolépticas e pungência do tubérculos iriam naturalmente levar a repulsa em caso de consumo excessivo. Contrariamente, o consumo do crem, da forma como é feita, pode resultar em efeitos benéficos, através de sua propriedade quimiopreventiva, relatada para o consumo de plantas que contém isotiocianatos (FAHEY; ZALCMANN; TALALAY, 2001; FIMOGNARI et al., 2012).

Quantos a possíveis efeitos de toxicidade decorrentes do uso popular, a aplicação de decocção sobre pele infeccionada, não há relatos na literatura. O que indica que o ITB, ou seus produtos de degradação presentes nas decocções, ou não são agressivos a pele, ou as concentrações alcançadas com o preparo das decocções não são suficientemente altas para ocorrência de alguma forma de dano. De qualquer forma, esse é um ponto que ainda precisa ser mais explorado, bem como a possibilidade de absorção dos isotiocianatos pela pele, pois como visto no Artigo 1, dos compostos presentes nos extratos, somente há dados e informações para os ácidos graxos.

Em linhas gerais os resultados apresentados nessa tese discorrem sobre diferentes aspectos do uso dos tubérculos de *T. pentaphyllum*, porém apresentam alguns pontos em comum. Destaca-se o claro papel do metabólito secundário isotiocianato de benzila, responsável ao menos em parte por sua atividade antimicrobiana, fundamento do uso popular, e pelas características organolépticas marcantes, pelas quais os tubérculos são apreciados como condimento. Além disso, o composto constitui parte de uma dicotomia, em que seu consumo em grandes quantidades leva a efeitos tóxicos, e em menores quantidades apresenta efeitos benéficos a saúde.

Em termos mais amplos, os resultados se encaixam na busca de conhecimento, em termos de fundamentação e segurança, das forma de uso tradicional de uma planta com bastante popularidade no estados do sul do Brasil, e da qual até o momento dispomos de escassas informações.

6 CONCLUSÃO

Os resultados de composição centesimal revelaram que os principais macronutrientes presentes nos tubérculos são água e carboidratos, seguido de menor teor de proteínas, cinzas e lipídios, em ordem decrescente de proporção.

Os resultados de composição mineral revelaram elevado teor de potássio, magnésio e cálcio, e menores teores de sódio, ferro e zinco.

A análise da composição do óleo essencial obtido revelou que o constituinte majoritário é o isotiocianato de benzila.

A determinação quantitativa de polifenois revelou baixo teor, e dos 14 diferentes compostos investigados, nenhum foi encontrado. Além disso, a capacidade antioxidante *in vitro* também foi baixa.

A avaliação da atividade antimicrobiana demonstrou que o óleo essencial, e a fração clorofórmio do extrato bruto, possuem potente atividade, frente as bactérias e fungos leveduriformes e filamentosos.

O ensaio de genotoxicidade demonstrou que o extrato bruto, a fração clorofórmio e o padrão de isotiocianato de benzila, foram deletérios frente as células mononucleares de sangue perférico, nas condições testadas.

O conjunto de análises cromatográficas e espectrofotométrica realizadas revelou baixo teor de polifenois, o que está em acordo com a baixa capacidade antioxidante e com a genotoxicidade verificadas. As análises também revelaram que o principal constituinte do óleo essencial, o isotiocianato de benzila, pode ser extraído por maceração hidroalcoólica, juntamente a outros compostos, e é um dos constituintes da fração clorofórmio. Esta fração, de potente atividade antimicrobiana, e que apresentou propriedades genotóxicas, é também constituída de amidas estruturalmente relacionadas ao isotiocianato de benzila, enxofre elementar, ácidos graxos e um fitoesteroide. Desses constituintes, o isotiocianato de benzila e os ácidos graxos, possuem maior atividade antimicrobiana e possivelmente estão por trás dos efeitos genotóxicos verificados.

7 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Os resultados do trabalho demonstram que o óleo essencial, e diferentes extratos da planta, apresentam atividade antimicrobiana contra microrganismos causadores de infecções de pele, em concordância com o uso popular relatado. Entretanto, diversos pontos poderão ser melhor elucidados por futuros estudos, como detalhes da preparação e forma de uso popular, estudo de estabilidade da decocção/extrato e observação dos efeitos terapêuticos *in vivo*.

Já os resultados da parte nutricional e bromatológica, não revelaram significativo teor de compostos fenólicos nos tubérculos, nas condições testadas. Entretanto, a variação em diferentes parâmetros das técnicas utilizadas, ou mesmo a utilização de outros métodos, pode vir a revelar resultados diferentes.

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