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AVALIAÇÃO TOXICOLÓGICA DA FITORREMEDIAÇÃO, EXTRATOS E ÓLEO ESSENCIAL DE *Plectranthus neochilus*

TESE DE DOUTORADO

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Uruguaiana, RS, Brasil

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BRUNA PIAIA RAMBORGER

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Tese apresentada ao programa de Pós-graduação Stricto Sensu em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutora em Bioquímica.

Orientador: Prof. Dr. Rafael Roehrs

Coorientador: Dr. Jefferson de Jesus Soares

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RESUMO

A planta *Plectranthus neochilus* (boldo gambá) é uma espécie herbácea, rasteira e suas folhas e flores possuem um forte aroma. Na medicina popular seu chá é utilizado para dores de estômago e problemas de fígado. Além disso, as pesquisas têm mostrado diferentes atividades biológicas de seus extratos em diferentes modelos. Essa espécie promoveu a fitorremediação do herbicida 2,4-D da água e apresentou alterações no metabolismo fenólico e na atividade antioxidante após essa remediação. Por essa razão, o presente estudo avaliou se o metabolismo dos compostos voláteis também é afetado na exposição ao 2,4-D e a possibilidade da utilização do óleo essencial e do chá da planta, também após a fitorremediação. Devido ao forte odor de suas folhas e a utilização do chá da planta, foi verificado o efeito repelente e inseticida da folha, chá e óleo essencial de *P. neochilus*. Por fim, foi avaliado a toxicidade do seu chá em modelos biológicos. Os resultados mostraram que o metabolismo dos compostos voláteis (trans-tujeno e alfa-pineno) e o conteúdo do óleo essencial (1-octen-3-ol) é minimamente afetado pela exposição ao 2,4-D. O meio aquoso se tornou menos tóxico através da menor detecção do 2,4-D na água da fitorremediação (5,18 µg/mL sem a planta e 4,42 µg/mL com a planta), e para os modelos biológicos *Artemia salina* (sem mortalidade em ambos os grupos) e *Allium cepa* (maior toxicidade e citotoxicidade das raízes no grupo sem a planta). Em relação ao uso da planta após a fitorremediação, seu óleo essencial pode ser usado como antimicrobiano (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonie, Acinetobacter baumannii*, and *Enterococcus faecalis)*, pois não houve diferença significativa na faixa de concentração do crescimento inibitório mínimo da planta exposta e não exposta ao 2,4-D. Porém, seu chá apresentou maior toxicidade nas plantas expostas ao 2,4-D (mortalidade de 100% da *D. melanogaster* após ingestão). Em relação ao efeito repelente e inseticida (distância e morte da *D. melanogaster*), tanto a folha, quanto o chá da planta apresentaram efeito atrativo para as moscas. Apenas o óleo essencial apresentou tais atividades, que foram confirmados pela sua constituição química, sendo o cariofileno, 1-octen-3-ol e o linalol como os principais compostos com tais efeitos. Além disso, o óleo essencial de *P. neochilus* apresentou maior efeito inseticida (90 minutos de exposição) perante outros óleos que também possuem essas características: *P. neochilus> L. angustifolia> F. vulgare> C. paradisi*. Por fim, o chá apresentou alcaloides, flavonoides, taninos, glicosídeos, saponinas, fenóis e terpenoides em sua constituição. A atividade antioxidante chegou à 71,48% de inibição do radical DPPH (15 mg/mL) e houve um aumento linear dos compostos fenólicos e flavonoides (243,53 µgEAG/g e 103,16 mgER/g, até 21 mg/mL). A toxicidade do chá ocorreu apenas em

concentrações excessivamente altas nos modelos experimentais *A. cepa* (24 mg/mL), *A. salina* (4.94 mg/mL) e *D. melanogaster* (6.93 mg/mL). Esse estudo obteve dados valiosos da planta *P. neochilus* que ainda poderão ser explorados nas seguintes áreas: ambiental, pela fitorremediação e efeito inseticida (principal novidade do trabalho); medicina tradicional, pelas propriedades do seu chá.

Palavras-chave: fitorremediação; fatores abióticos; antibacteriano; inseticida; chá.

ABSTRACT

The plant *Plectranthus neochilus* (boldo gambá) is a herbaceous, creeping species and its leaves and flowers have a strong aroma. In traditional medicine, its tea is used for stomach pain and liver problems. Furthermore, research has shown different biological activities of its extracts in different models. This species promoted the phytoremediation of the herbicide 2,4-D from water and showed changes in phenolic metabolism and antioxidant activity after this remediation. For this reason, the present study evaluated whether the metabolism of volatile compounds is also affected by exposure to 2,4-D and the possibility of using essential oil and tea from the plant, also after phytoremediation. Due to the strong odor of its leaves and the use of tea from the plant, the repellent and insecticidal effect of the leaf, tea, and essential oil of *P. neochilus* was verified. Finally, the toxicity of its tea was evaluated in biological models. The results showed that the metabolism of volatile compounds (trans-tujene and alpha-pinene) and essential oil content (1-octen-3-ol) is minimally affected by exposure to 2,4-D. The aqueous medium became less toxic through the lower detection of 2,4-D in the phytoremediation water (5.18 µg/mL without the plant and 4.42 µg/mL with the plant), and for the biological models *Artemia salina* (no mortality in both groups) and *Allium cepa* (greater toxicity and cytotoxicity of the roots in the group without the plant). Regarding the use of the plant after phytoremediation, its essential oil can be used as an antimicrobial (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonie*, *Acinetobacter baumannii*, and *Enterococcus faecalis)*, as there was no significant difference in the concentration range of the minimum inhibitory growth of the plant exposed and not exposed to 2,4-D. However, its tea showed greater toxicity in plants exposed to 2,4-D (100% mortality of *D. melanogaster* after ingestion). Regarding the repellent and insecticidal effect (distance and death of *D. melanogaster*), both the leaf and the tea of the plant presented an attractive effect for the flies. Only the essential oil showed such activities, which were confirmed by its chemical constitution, with caryophyllene, 1-octen-3-ol and linalool as the main compounds with such effects. Furthermore, the essential oil of *P. neochilus* showed a greater insecticidal effect (90 minutes of exposure) compared to other oils that also have these characteristics: *P. neochilus* > *L. angustifolia* > *F. vulgare* > *C. paradisi*. Finally, the tea presented alkaloids, flavonoids, tannins, glycosides, saponins, phenols and terpenoids in its constitution. The antioxidant activity reached 71.48% of DPPH radical inhibition (15 mg/mL) and there was a linear increase of phenolic compounds and flavonoids (243.53 µgGAE/g and 103.16 mgRE/g, up to 21 mg/mL) . Tea toxicity occurred only at excessively high concentrations in the experimental

models *A. cepa* (24 mg/mL), *A. salina* (4.94 mg/mL) and *D. melanogaster* (6.93 mg/mL). This study obtained valuable data from the *P. neochilus* plant that could still be explored in the following areas: environmental, through phytoremediation and insecticidal effect (the main novelty of the work); traditional medicine, for the properties of its tea.

Keywords: phytoremediation; abiotic factors; anti-bacterial; insecticide; tea.

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LISTA DE ABREVIATURAS E SIGLAS

2,4-D - ácido 2,4-diclorofenoxiacético.

ANVISA – Agência Nacional de Vigilância Sanitária.

CL_{50} - Concentração Letal Mediana.

DL_{50} - Dose Letal Mediana.

DPPH• - Radical 2,2-difenil-1-picrilhidrazil.

EPA/USEPA – United States Environmental Protection Agency, Agência de Proteção Ambiental dos Estados Unidos.

ERO/ROS - Espécies reativas de oxigênio/do inglês, Reactive Oxygen Species.

CG-EM/GC-MS - Cromatografia a Gás acoplada à Espectrometria de Massas/do inglês, Gas Chromatography coupled to Mass Spectrometry.

CLAE-DAD/ HPLC-DAD – Cromatografia Líquida de Alta Eficiência com Detecção por Arranjo de Diodos/do inglês, High Performace Liquid Chromatography with Diode Array Detector.

$^1O_2$ - Oxigênio singleto.

H_2O_2 - Peróxido de hidrogênio.

OH• - Radical hidroxil.

O_2• - Radical ânion superóxido.

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APRESENTAÇÃO

A presente tese está organizada em seis capítulos que estão escritos em português, para a apresentação deste documento, e em inglês, quando se refere aos artigos científicos e manuscritos.

O Capítulo I está composto pelas seções Introdução, Referencial Teórico, Justificativa e Objetivos. As demais seções, Material e Métodos, Resultados e Discussão e as respectivas Referências, estão apresentadas sob a forma de artigos científicos e manuscritos, os quais compõem os capítulos II, III, IV e V deste trabalho e representam a íntegra deste estudo.

Dessa maneira, o Capítulo II apresenta um artigo de revisão referente a *Plectranthus neochilus*, que foi a planta utilizada em todos os experimentos da presente tese. Esta revisão tem como título "A review of anatomical, physiological, biological characteristics and uses of *Plectranthus neochilus*" que foi publicada na revista *Ciência e Natura*, e- ISSN 2179-460X, ISSN 0100-8307, doi: 10.5902/2179460X40157.

O Capítulo III está apresentado sob a forma do artigo científico intitulado "Toxicological parameters of aqueous residue after using *Plectranthus neochilus* for 2,4-D phytoremediation". Este artigo foi publicado na revista *Chemosphere*, ISSN: 0045-6535, doi: https://doi.org/10.1016/j.chemosphere.2020.128638.

O Capítulo IV está na forma de um manuscrito intitulado "Repellent and insecticidal effect of *Plectranthus neochilus* essential oil on *Drosophila melanogaster*". Este manuscrito foi submetido na revista *Chemosphere*.

O Capítulo V se refere aos resultados que fazem parte desta tese e estão apresentados sob a forma de um manuscrito intitulado: "Low toxicity of *Plectranthus neochilus* tea in alternative models" que será submetido na revista *Journal of Medicinal Food*.

No Capítulo VI está uma discussão geral desta tese, suas principais conclusões e perspectivas futuras. Ao final estão apresentadas as referências que foram utilizadas nas seções Introdução, Referencial Teórico e Discussão desta tese.

CAPÍTULO I

1 INTRODUÇÃO

A planta *P. neochilus* é conhecida como boldo-gambá, devido ao forte odor de suas folhas. Possui boa produtividade, fácil cultivo e adaptação climática (COUTO, 2006). É uma planta com caráter medicinal, pois é amplamente utilizada em forma de infusão (chá) para fins digestivos e problemas hepáticos (LAMBRECHTS, 2020; LORENZI; MATOS, 2002).

As pesquisas científicas com *P. neochilus* têm avaliado os benefícios de seus extratos de acordo com os compostos identificados, como ácido caféico, ferúlico, cumarínico e rosmarínico. Além de outros, também identificados em diferentes frações dos seus extratos (BRITO et al., 2018c; RAMBORGER et al., 2017a; VIANA, 2011). Os estudos mais recentes têm mostrado a importância de seu óleo essencial, que possui monoterpenos e sesquiterpenos (CAIXETA et al., 2011; LAWAL; HUTCHINGS; OYEDEJI, 2010; MOTA et al., 2010). Dessa maneira, as atividades biológicas identificadas em seu óleo essencial são: atividade antimicrobiana (CREVELIN et al., 2015), antifúngica (AGUIAR et al., 2018), inibição da oviposição da mosca do tomate (BALDIN et al., 2013) e antiesquistossomal (CAIXETA et al., 2011).

Essas atividades biológicas, tanto presentes nos chás adquiridos por decocções ou infusões, ou do óleo essencial, estão associadas ao seu metabolismo secundário. Os vegetais possuem o metabolismo primário, que corresponde à síntese de compostos essenciais para sua sobrevivência (açúcares, aminoácidos, ácidos graxos, nucleotídeos), e o metabolismo secundário. Neste último, diferentes compostos são produzidos em resposta aos fatores (ou estresses) ambientais, que podem ser físicos ou biológicos. Assim, a planta promove interação ou repulsão, sustentação ou destruição dos diferentes organismos que os cercam. Dependendo do fator ou estresse que a planta está exposta, ela altera a produção de algum composto ou classe de compostos em seu metabolismo secundário para promover essa defesa. Assim, poderá haver aumento de flavonoides, compostos fenólicos, compostos voláteis, entre outros (TAIZ; ZEIGER, 2002, 2017).

Um tipo de estresse ambiental recorrente é a vasta aplicação de pesticidas. Um herbicida que é mundialmente utilizado é o ácido 2,4-diclorofenoxiacético (2,4-D). Ele é um regulador de crescimento de plantas (auxina sintética) e é utilizado para a prevenir plantas daninhas de folha larga em ambientes agrícolas e não agrícolas, terrestres ou aquáticos (ANVISA, 2019). Portanto, quando os pesticidas são aplicados de forma indevida, ou em excesso, pode ocorrer contaminação de espécies não alvo e o desenvolvimento de toxicidade. Assim, há alteração no

metabolismo vegetal ou animal exposto ao 2,4-D (ATAMANIUK et al., 2013; OLIVEIRA JÚNIOR et al., 2007). Então, é necessário encontrar maneiras de remover esse contaminante do ambiente. Existem diversas técnicas que promovem a descontaminação ambiental e uma delas é através da utilização de plantas e este processo é denominado de fitorremediação. Nesse contexto, a espécie *P. neochilus*, dentre sua versatilidade de uso, mostrou promover a fitorremediação do herbicida 2,4-D da água. No entanto, essa planta mostrou ter sido afetada pelo pesticida, no que diz respeito ao seu metabolismo fenólico e atividade antioxidante (RAMBORGER et al., 2017a).

Considerando os benefícios do uso de *Plectranthus neochilus* tanto na medicina tradicional quanto no controle da contaminação aquática pelo 2,4-D, se faz necessário avaliar se: a fitorremediação deste agente estressor com essa planta possa contribuir para a menor toxicidade do meio aquático; essa interação é capaz de produzir compostos secundários que ficarão no chá ou óleo essencial extraído da planta para fins biológicos, e assim, se espera ainda utilizar a planta após a técnica; além disso, se é possível encontrar novas atividades biológicas com os extratos da planta (chá e óleo essencial) em modelos biológicos.

2 REFERENCIAL TEÓRICO

2.1. *Plectranthus neochilus*: espécie, usos e pesquisas

A planta *Plectranthus neochilus* é uma espécie herbácea cultivada e utilizada em diversas regiões. Possui cultivo rápido, promovendo cobertura de solo, e é de fácil adaptação à diferentes condições climáticas. Atinge aproximadamente 0,5 m de altura e 1,5 m de largura, suas folhas são verdes, suculentas e possuem um aroma característico, bem como suas flores, de coloração roxa (Figura 1). No Brasil, a *P. neochilus* é conhecida como “boldinho”, “boldo da folha miúda”, “boldo” ou “boldo-gambá” (COUTO, 2006; LAMBRECHTS, 2020).

Figura 1 - Fotos de *Plectranthus neochilus* em seu habitat natural, apresentando sua florescência e no processo de enraizamento em água.

Fonte: Autor.

A maior importância e uso desta planta é na medicina caseira, pois suas folhas são utilizadas em forma de chá para o tratamento de dores de estômago e problemas hepáticos (LAMBRECHTS, 2020; LORENZI; MATOS, 2002). Também, já se sabe que essa planta pode ser utilizada para problemas respiratórios (YORK; DE WET; VAN VUUREN, 2011). De acordo com a literatura, as espécies *Plectranthus* são comumente usadas para distúrbios gástricos, respiratórios, doenças de pele, atividades anti-inflamatórias, antimicrobianas e antitumorais. Porém seu maior uso é para os problemas gástricos (LUKHOBA; SIMMONDS; PATON, 2006). Existem outras espécies que também são denominadas de “boldo”, por serem utilizadas para dores de estômago e problemas hepáticos. Nesse sentido, um estudo analisou a anatomia do caule e da folha de *P. neochilus* e o distinguiu do "boldo brasileiro" (*P. barbatus*), do “boldo japonês” (*Vernonia condensata*) e do tradicional e mais conhecido, “boldo do Chile” (*Peumus boldus*) (DUARTE; LOPES, 2007).

Diversas pesquisas têm investigado as propriedades farmacológicas dos extratos de *P. neochilus* e a área que têm ganhado destaque é a de óleos essenciais. Neste sentido, já se sabe que o óleo essencial de *P. neochilus* comprovou atividade antifúngica (AGUIAR et al., 2018), antibacteriana (CREVELIN et al., 2015), contra o *Schistosoma mansoni* (CAIXETA et al., 2011) e na inibição da oviposição da mosca do tomate (BALDIN et al., 2013; FANELA et al., 2016). Porém, a utilização e as pesquisas com esta planta vão além do uso medicinal ou das atividades biológicas atribuídas. Algumas pesquisas mostraram que a *P. neochilus* pode ser

utilizada para jardinagem e cobertura de solo permitindo a retenção da umidade e fertilização (JAARSVELD; THOMAS, 2006; POOLEY, 1998). Também se investigou a capacidade de fitorremediação (RAMBORGER et al., 2017a, 2021) e utilização na tecnologia de telhados verdes (termo em inglês: "g*reen roof technology*") (MORAU; LIBELLE; GARDE, 2012).

Os detalhes sobre as características anatômicas, formas de utilização, compostos identificados nos diferentes tipos de extratos e atividades farmacológicas atribuídas à espécie *P. neochilus* estão apresentadas como forma de artigo científico na seção "Capítulo II" desta tese.

2.2. Remediação ambiental com plantas: Fitorremediação

As plantas possuem diversas contribuições para o ambiente, uma delas é a capacidade que algumas espécies possuem de realizar fitorremediação, como é o caso da *P. neochilus*.

O conjunto de técnicas e operações que visam anular os efeitos nocivos de elementos tóxicos, tanto para o ser humano quanto para o ecossistema é conhecido como remediação ambiental (GHASEMZADEH et al., 2014). As estratégias de remediação ambiental podem ser do tipo: física, química, biológica, ou, a combinação de duas ou todas estas técnicas (PASCAL-LORBER; LAURENT, 2011).

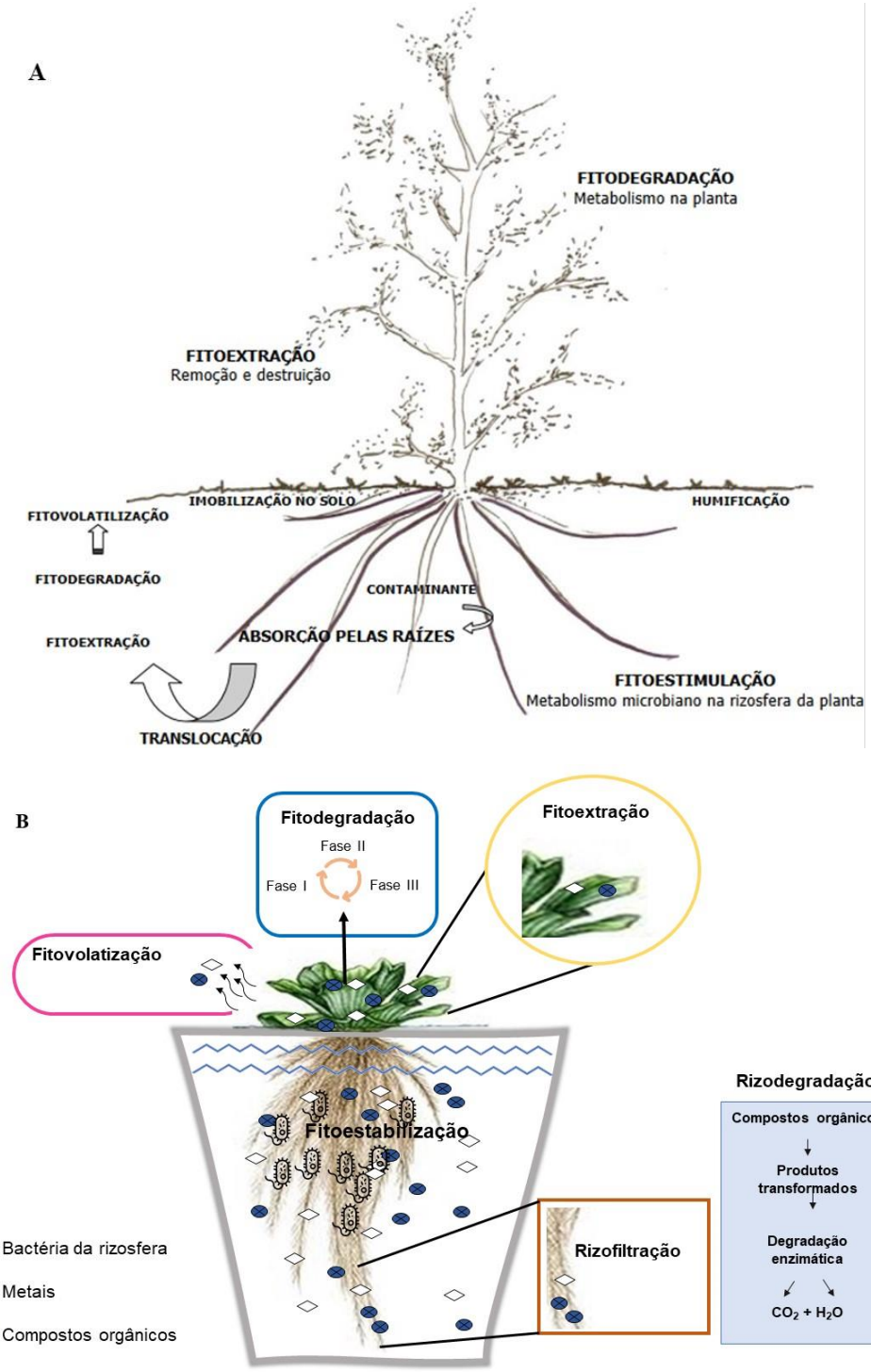
As técnicas de caráter físico e químico são conhecidas como tradicionais e promovem a descontaminação por meio dos métodos de bombeamento e tratamento/controle hidráulico ("*pump and treat"*, termo em inglês), extração de vapor no solo, dessorção térmica, aeração ("*air sparging"*, termo em inglês), barreiras reativas permeáveis, incineração, solidificação/estabilização química, e, lavagem do solo (TAVARES, 2009). Estas técnicas são relativamente caras, principalmente quando a contaminação abrange uma grande área, ou, quando é necessário transportar o material contaminado para ser descontaminado (DIJKGRAAF; VOLLEBERGH, 2004; JIN; WANG; RAN, 2006). Neste sentido, existem as estratégias de carácter biológico, que englobam a biorremediação e a fitorremediação. A biorremediação promove a conversão dos contaminantes de interesse em seus constituintes elementares pelos microrganismos, como bactérias e fungos. Ela é barata e fácil de ser implementada *in situ* (MEGHARAJ et al., 2011). Já fitorremediação, considerada uma técnica verde ("*green technology*", termo em inglês), consiste em utilizar plantas para a remediação ambiental por meio de suas atividades naturais ou em conjunto com microrganismos presentes na zona da rizosfera da planta. Ou seja, o mecanismo desta técnica está ligado aos processos biológicos do metabolismo vegetal para a eliminação das substâncias nocivas presentes no solo,

água ou ar. Portanto, também é mais barata em relação àquelas tradicionais, além de causar menor impacto no ambiente (AGNELLO et al., 2016; LAI et al., 2011; TANGAHU et al., 2011; WEI et al., 2021). Os diferentes métodos pelos quais a planta pode realizar a fitorremediação são:

- Fitoextração, fitoacumulação ou fitoabsorção: é a absorção dos contaminantes pelas raízes, seguida pelo transporte até as partes áreas da planta, onde são acumulados nas células vegetais (FAVAS et al., 2014).
- Fitodegradação ou fitotransformação: é a degradação dos contaminantes absorvidos pelas plantas por meio de seus processos metabólicos (TANGAHU et al., 2011).
- Fitoestimulação ou rizodegradação: é a estimulação da proliferação de microrganismos que são capazes de degradar contaminantes orgânicos. Esse processo ocorre na região da rizosfera, através crescimento das raízes (FAVAS et al., 2014).
- Fitovolatilização: é a absorção e conversão dos contaminantes em uma forma volátil, que é liberada na atmosfera (WATANABE, 1997).
- Fitoestabilização ou rizofiltração: é a imobilização ou lignificação dos poluentes nos tecidos radiculares (KHASHIJ; KARIMI; MAKHDOUMI, 2018).
- Fitofiltração: é a absorção, concentração ou precipitação dos contaminantes presentes em um meio aquoso (águas superficiais contaminadas ou águas residuais) (PRASAD; DE OLIVEIRA FREITAS, 2003).

Alguns destes processos podem ser visualizados na figura 2 (A e B).

Figura 2 - Mecanismos utilizados pelas plantas nos processos de fitorremediação. A: fitorremediação em solo. B: fitorremediação em água.



Fonte: A - Andrade; Tavares; Mahler (2007) B - Fonte: Autor, adaptado de Kumar; Shahi; Singh (2018).

A eficácia da fitorremediação varia de acordo com as características do contaminante, espécie de planta utilizada e condições ambientais. Assim, é possível realizar a fitorremediação do solo ou da água, no local contaminado ou, no laboratório. Os estudos na área da fitorremediação abrangem tanto compostos inorgânicos, como metais pesados, quanto orgânicos, como pesticidas e resíduos de indústrias (WEI et al., 2021). Inicialmente estas

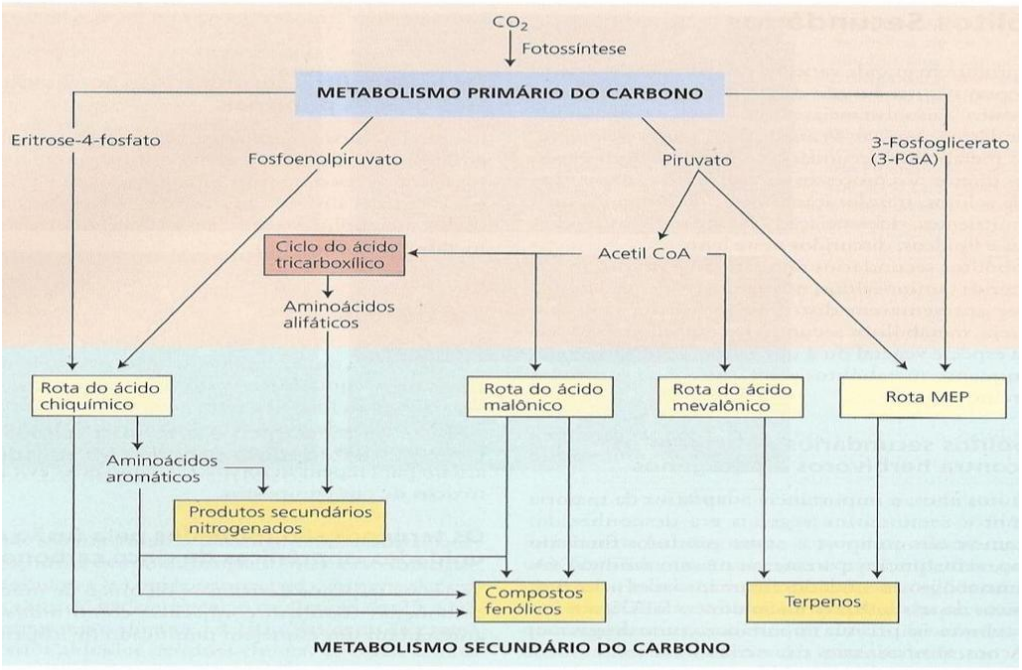
pesquisas são desenvolvidas em laboratório para testar a toxicidade do contaminante em diferentes espécies vegetais, como também as possíveis alterações metabólicas ou, se outros compostos podem melhorar a capacidade da fitorremediação da planta avaliada (HAN et al., 2021; RAMBORGER et al., 2017a, 2021; VIEIRA et al., 2021; XIAO et al., 2021). Algumas espécies, principalmente as que se reproduzem em larga escala, têm sido utilizadas para fitorremediação e destinadas para uso posterior. Por exemplo, a alface d'água (*Pistia stratiotes*), considerada uma planta daninha por formar densas camadas em águas calmas, já foi utilizada para promover a fitorremediação aquática de um resíduo industrial e, posteriormente, utilizada para a produção de biogás e bio-hidrogênio (MTHETHWA et al., 2018, 2019; PANTAWONG et al., 2015). Isso torna a fitorremediação uma técnica tanto atrativa e ecológica, quanto rentável.

Quando a planta fica exposta à um local contaminado, como em local com pesticidas, ou à algum fator ambiental estressante, ela tende a desenvolver mecanismos de adaptação para sua sobrevivência. Isso pode ocorrer no processo da fitorremediação, uma vez que a planta vai estar exposta à um agente nocivo que poderá desenvolver alterações em seu metabolismo. De acordo com Hassan, (2012), essa capacidade desenvolvida pelos vegetais para sobrevivência está vinculada à produção de seus metabólitos secundários e servem como base de muitos medicamentos, indústrias de perfumaria, agroquímica e cosmética.

2.3. Metabólitos secundários

Os vegetais produzem uma grande diversidade de compostos químicos que podem ser classificados como metabólitos primários e secundários. O metabolismo primário corresponde à síntese dos compostos essenciais para sua sobrevivência que são os açúcares, aminoácidos, ácidos graxos, lipídeos e nucleotídeos. Também se enquadram neste grupo as moléculas que são sintetizadas a partir deles, como proteínas, polissacarídeos, membranas, DNA e RNA. Os metabólitos secundários (ou metabólitos especializados), ao contrário, são altamente específicos para determinada espécie de planta, e em geral pertencem a uma das três principais classes de moléculas: terpenos (isoprenoides), compostos fenólicos (flavonoides e fenilpropanoides) e compostos contendo nitrogênio (glicosídeos, alcaloides e glucosinolatos) (FANG et al., 2011; TAIZ; ZEIGER, 2002). A visão geral dos dois metabolismos pode ser visualizada na figura 3.

Figura 3 - Rotas do metabolismo primário e secundário do carbono. Biossíntese dos metabólitos secundários.



Fonte: Taiz e Zeiger (2002).

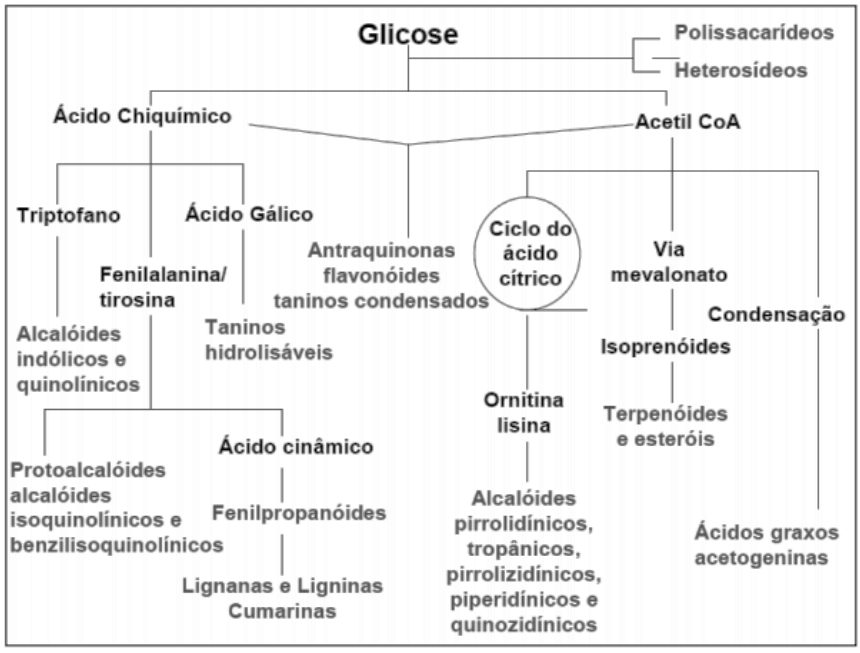
No reino vegetal existe cerca de 200.000 metabólitos secundários (HARTMANN, 2007), que são produzidos em resposta aos diferentes fatores ambientais (físicos ou biológicos). Assim, são capazes de promover a interação ou repulsão, sustentação ou destruição dos vegetais com os diferentes organismos e fatores que os cercam (GOTTLIEB et al., 1996; MARTINS et al., 2003). Dessa maneira, eles são fundamentais na defesa e proteção da planta contra qualquer dano ecológico (SAMUNI-BLANK et al., 2012) uma vez que são capazes de proteger contra herbívoros e patógenos, servir como atrativo através do aroma, cor e/ou sabor para polinizadores, e desempenharem ações como agentes de competição entre plantas e de simbiose entre plantas e microrganismos. Os hormônios vegetais citocininas, giberelinas, brassinosteroides, ácido abscísico e estrigolactona também respondem aos fatores abióticos, estão em baixas concentrações na planta e são derivados de uma dessas rotas. Entretanto, são considerados metabólitos primários, pois todas as plantas necessitam deles para seu crescimento e desenvolvimento. Os hormônios auxina e etileno também são sintetizados pelo metabolismo primário, uma vez que seus precursores são aminoácidos (TAIZ; ZEIGER, 2017).

Apesar da grande diversidade, toda essa gama de substâncias produzidas é sintetizada a partir de quatro vias metabólicas principais: via do acetato-malonato, via do acetato-

mevalonato, via do metileritritol fosfato e a via do ácido chiquímico (figuras 3 e 4), que por sua vez, são provenientes do metabolismo primário. Portanto, seus precursores são a acetilcoenzima A, o ácido chiquímico, o ácido mevalônico e o metileritritol fosfato. A biossíntese dos grupos de metabólitos secundários pode ser resumida da seguinte forma segundo Dewick (2009):

- Terpenos: são produzidos a partir do ácido mevalônico (no citoplasma) ou do piruvato e 3-fosfoglicerato (no cloroplasto);
- Compostos fenólicos: são derivados do ácido chiquímico ou ácido mevalônico;
- Compostos contendo nitrogênio são derivados de aminoácidos aromáticos (triptofano, tirosina), os quais são derivados do ácido chiquímico, e de aminoácidos alifáticos (ornitina, lisina).

Figura 4 - Esquema com as principais vias do metabolismo secundário de plantas que ocorrem no citosol.



Fonte: Simões (2013).

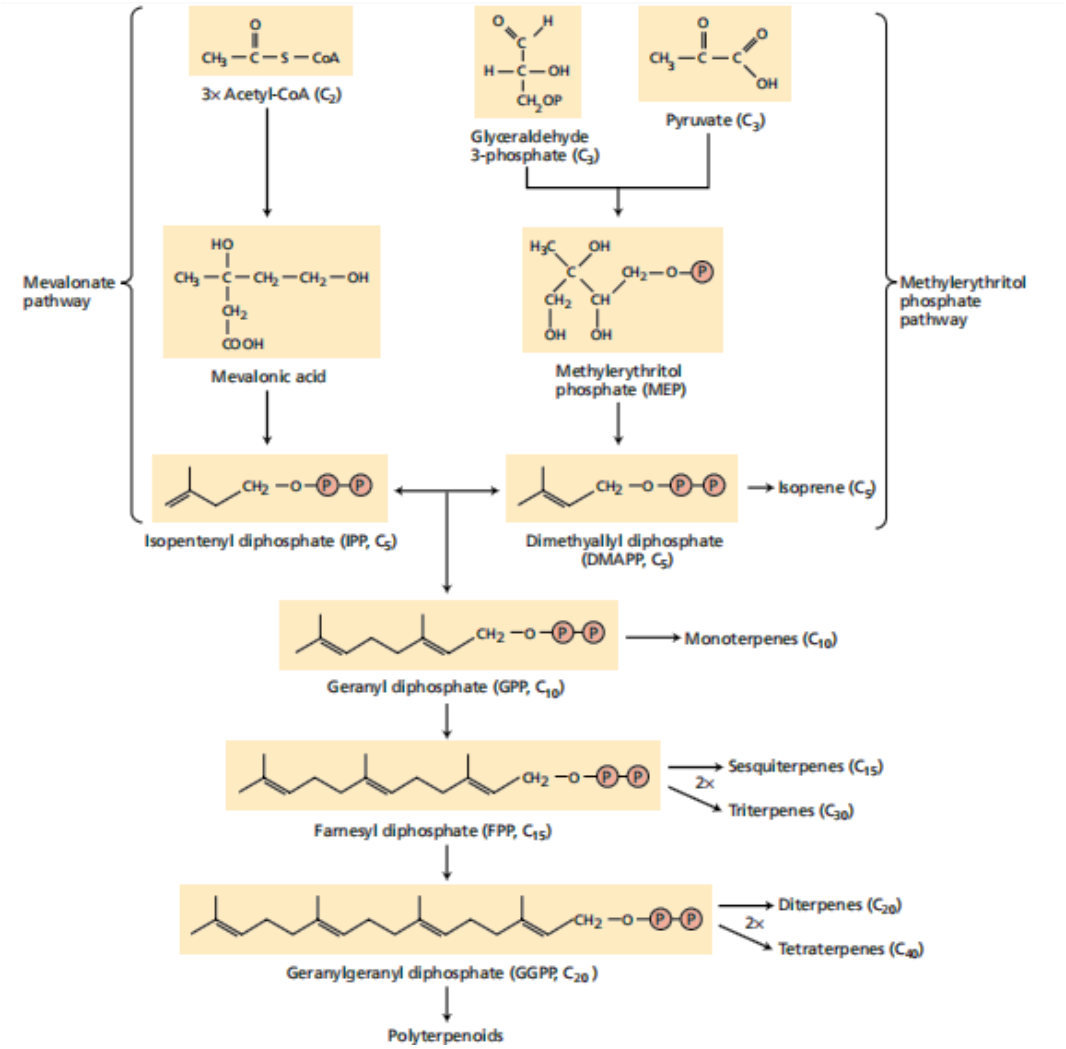
Cada classe de compostos secundários permite o crescimento, desenvolvimento e defesa da planta. Além disso, as vias específicas de biossíntese variam de acordo com a espécie vegetal e do ambiente que a circunda. Dessa forma, o metabolismo secundário varia de acordo com diversos fatores para manter a planta em boas condições de sobrevivência.

2.3.1 Terpenos

Os terpenos, também conhecidos como terpenóides, são produzidos pela via do acetato-mevalonato (ou ácido mevalônico) no citosol e pela via do metileritritol fosfato (MEP) nos cloroplastos ou plastídeos (figura 5). Este grupo é derivado da ligação de unidades pentacarbonadas que apresentam um esqueleto ramificado do isopentano (C5). Contudo, os elementos estruturais básicos dos terpenos também são chamados de "unidades de isopreno", porque em altas temperaturas os terpenos podem se decompor a isopreno. Através da via do acetato-mevalonato três moléculas de acetil-coA se unem para formar o ácido mevalônico, que é fosforilado, descarboxilado e desidratado até se transformar nas unidades básicas isopentenil difosfato (IPP), ou seu isômero (dimetilalil difosfato - DMAPP). Na via do MEP, as reações que ocorrem são partir de intermediários da glicólise ou do ciclo de redução de carbono fotossintético (Lichten thaler 1999). Nessa via, o IPP é formado após a formação de um intermediário gerado pela união do gliceraldeído-3-fosfato e dois átomos carbono derivados do piruvato. Após alguns rearranjos, essa molécula forma um intermediário que é convertido no IPP. A partir desta biossíntese, IPP e DPP reagem para produzir monoterpenos, sesquiterpenos e assim por diante (TAIZ; ZEIGER, 2002).

A través da condensação de outra unidade C5 ao IPP e/ou DMAP, o difosfato de geranil (GPP) é formado, formando os monoterpenos (C10). Ou seja, os terpenos são classificados pelo número de unidades de cinco carbonos que eles contêm, embora modificações metabólicas extensas às vezes possam dificultar a seleção dos resíduos originais de cinco carbonos. Terpenos com dez carbonos, que contêm duas unidades C5, são chamados monoterpenos; Terpenos de 15 carbonos (três unidades C5) são sesquiterpenos; e terpenos de 20 carbonos (quatro unidades C5) são diterpenos. Terpenos maiores incluem triterpenos (30 carbonos), tetraterpenos (40 carbonos) e politerpenóides ([C5]n carbonos, onde n> 8. Na figura 5, pode-se ver a biossíntese a partir destas duas vias.

Figura 5 - Biossíntese dos terpenos.



Fonte: Taiz e Zeiger (2002).

Nas células os terpenos participam de ações tanto no metabolismo primário quanto no secundário. Assim temos as seguintes substâncias: giberelinas (diterpenos), importante grupo de hormônios vegetais; esteróis (triterpenos), componentes essenciais das membranas celulares, os quais estabilizam ao interagir com os fosfolipídios; carotenóides vermelho, laranja e amarelo (tetraterpenos), que são pigmentos acessórios na fotossíntese e protegem os tecidos fotossintéticos da fotooxidação; ácido abscísico (sesquiterpeno), hormônio produzido pela degradação de um precursor de carotenoide; álcoois de politerpenos de cadeia longa (dolicois), que são transportadores de açúcares na parede celular e na síntese de glicoproteínas; cadeias laterais derivadas de terpenos, como a cadeia lateral de fitol da clorofila, elas ancoram certas moléculas nas membranas. Além disso, muitos terpenos funcionam como toxinas (inseticidas, larvicidas etc.) ou como impedimento alimentar de animais (TAIZ; ZEIGER, 2002).

Os autores trazem ainda que muitas plantas contêm misturas de monoterpenos voláteis e sesquiterpenos, chamados de óleos essenciais, que dão uma característica de odor em suas

folhagens. Eles repelem herbívoros ovipositadores e atraem inimigos naturais, que matam insetos que se alimentam das plantas e, assim, ajudam a minimizar os danos.

Os óleos essenciais são frequentemente encontrados em pelos glandulares e podem ser extraídos das plantas por destilação a vapor, sendo importantes comercialmente na aromatização de alimentos e na fabricação de perfumes. Entre os compostos anti-herbívoros não voláteis de terpenos estão os limonoides, um grupo de triterpenos (C30) que conferem o aspecto amargo em frutas cítricas. As fitoecdisonas, grupo de esteroides vegetais que têm a mesma estrutura básica dos hormônios de muda de insetos. Portanto, sua ingestão interrompe a muda e outros processos de desenvolvimento destes animais. Existem triterpenos que são ativos contra herbívoros vertebrados, como é o caso dos cardenólidos (glicerídeo) e saponinas (glicosídeo de esteróides e triterpenos). Os cardenólidos têm um sabor amargo e são extremamente tóxicos para animais superiores. Nos seres humanos, eles têm efeitos no músculo cardíaco por influência nas ATP-ases ativadas por Na+/K+. De acordo com a dose, eles diminuem e fortalecem os batimentos cardíacos, e, portanto, são prescritos a milhões de pacientes para o tratamento de doenças cardíacas. As saponinas, por sua vez, possuem propriedades detergentes, pois em sua estrutura há elementos solúveis em lipídios (o esteróide ou triterpeno) e solúveis em água (o açúcar). As saponinas podem interferir na captação de esterol do sistema digestivo ou perturbar as membranas celulares após serem absorvidas pela corrente sanguínea (TAIZ; ZEIGER, 2002).

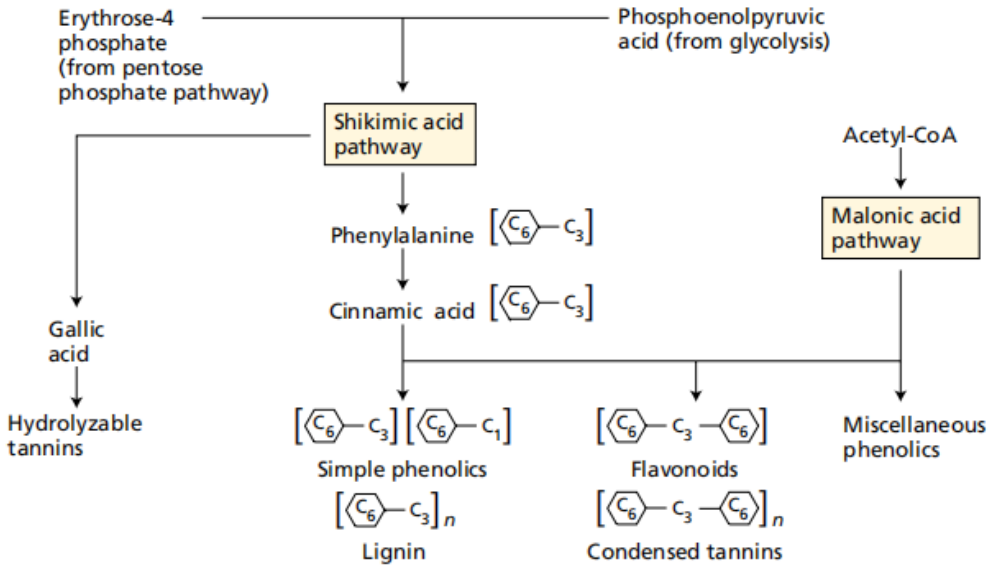
2.3.2 Compostos fenólicos

Os compostos fenólicos possuem em sua estrutura o grupo funcional hidroxila em um anel aromático e compreendem um grupo heterogêneo de mais de 8.000 compostos de plantas individuais, com grande variabilidade estrutural. De maneira geral, muitos servem como compostos de defesa contra herbívoros e patógenos, enquanto outros promovem suporte mecânico, atração de polinizadores e dispersores de frutos, absorção de radiação ultravioleta ou na redução do crescimento de plantas concorrentes próximas (DE LA ROSA et al., 2018; TAIZ; ZEIGER, 2002).

A biossíntese dos compostos fenólicos ocorre por meio de diferentes rotas sendo as rotas metabólicas básicas a do ácido malônico e a do ácido chiquímico. A via do ácido malônico, embora seja uma fonte importante de produtos secundários fenólicos em fungos e bactérias, é menos significativa em plantas superiores. A via do ácido chiquímico está presente em plantas, fungos e bactérias, mas não é encontrada em animais.

De acordo com as figuras 4 e 6, a rota do ácido chiquímico inicia pela via do ácido chiquímico inicia com o acoplamento do fosfoenolpiruvato (via glicolítica) e a eritrose 4-fosfato (ciclo da pentose fosfato). Após mecanismos complexos, esses compostos formam o ácido 3-dehidroquinínico, que pode formar o ácido quinínico, ou, o ácido 3-dehidrochiquímico produzindo tanto o ácido chiquímico propriamente dito, como o ácido quinínico e o ácido gálico. O ácido quinínico é precursor de alcalóides, como a quinina e o ácido gálico formará galotaninos e elagitaninos. A incorporação de outra molécula de fosfoenolpiruvato ao ácido chiquímico gerará o ácido corísmico. A partir do ácido corísmico são formados os ácidos fenólicos simples e, aminoácidos aromáticos, como o triptofano que promove a formação de alcalóides indólicos, fenilalanina e tirosina. As classes mais abundantes de compostos fenólicos secundários nas plantas são derivadas da fenilalanina, que é transformada em ácido cinâmico através da eliminação de uma molécula de amônia. Essa reação é catalisada pela fenilalanina amônia liase (PAL). As reações subsequentes à catalisada pelo PAL levam à adição de mais grupos hidroxila e outros substituintes. O ácido trans-cinâmico, o ácido p-coumarico e seus derivados são compostos fenólicos simples chamados fenilpropanóides porque contêm um anel benzeno e uma cadeia lateral de três carbonos. Os fenilpropanóides podem formar monômeros formadores de ligninas, lignanas e neolignanas e cumarinas. Os fenilpropanoides (ácido p-cumárico), juntamente com moléculas de malonil-CoA (via acetato-malonato) podem formar os flavonoides e estilbenos. Por fim, as catequinas que também são enquadradas como flavonóides, são as unidades formadoras de polifenois (taninos condensados) (DEWICK, 2009; TAIZ; ZEIGER, 2002). A figura 6 apresenta de maneira resumida a biossíntese de cada grupo dos compostos fenólicos.

Figura 6 - Biossíntese dos compostos fenólicos.



Fonte: Taiz e Zeiger (2002).

Cada composto fenólico tem um importante papel no metabolismo secundário da planta (TAIZ; ZEIGER, 2002). Então, de maneira resumida:

- A lignina fortalece mecanicamente as paredes celulares;

- Os pigmentos flavonóides funcionam como escudos contra a radiação ultravioleta prejudicial e como atrativos para polinizadores e dispersores de frutas;

- Lignina, flavonóides e outros compostos fenólicos servem como defesas contra herbívoros e patógenos.

2.3.3 Compostos nitrogenados

Os alcaloides, glicosídeos cianogênicos e os glicosinolatos são substâncias nitrogenadas biossintetizadas de aminoácidos (figuras 3 e 4). A maioria dos alcalóides são biossintetizado a partir de aminoácidos comuns (ornitina, lisina, ácido nicotínico, tirosina, triptofano, ácido antranílico e histidina). Além disso, pode haver incorporação às estruturas de porções provenientes de outras vias, como acetato-malonato, chiquimato e acetato-mevalonato. Os glicosídeos cianogênicos tem como aminoácido precursor de sua síntese a fenilalanina, tirosina, vanilina, isoleucina e leucina. Já os glicosinolatos são biossintetizados a partir da tirosina, fenilalanina e triptofano (DEWICK, 2009; TAIZ; ZEIGER, 2002).

Os alcaloides são uma grande família de mais de 15.000 metabólitos secundários e sua estrutura contém um ou mais átomos de nitrogênio, que geralmente faz parte de um anel heterocíclico, dispostos como aminas primárias, secundárias ou terciárias, o que confere o

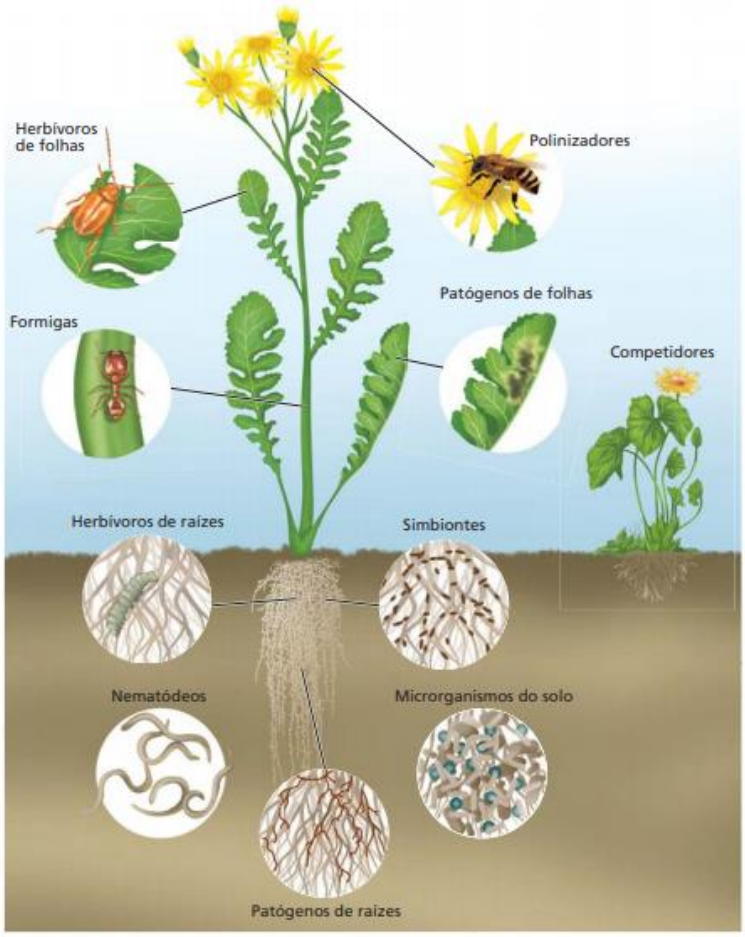
caráter básico desse grupo. Acredita-se que a maioria dos alcaloides promova defesas contra predadores, especialmente mamíferos, devido à sua toxicidade. Quase todos os alcaloides também são tóxicos para os seres humanos quando tomados em quantidade suficiente. Por exemplo, estricnina, atropina e coniina (de cicuta venenosa) são agentes clássicos de intoxicação por alcaloides. Em doses adequadas, morfina, codeína e escopolamina são alguns dos alcaloides vegetais atualmente usados na medicina (TAIZ; ZEIGER, 2002).

Os glicosídeos cianogênicos são hidrolisados por enzimas específicas e ocorre a liberação de ácido cianídrico (HCN) quando ocorre alguma lesão no tecido da planta contendo esses compostos. O HCN é um gás tóxico e impede a alimentação de insetos e outros herbívoros, como caracóis e lesmas, pois HCN é uma toxina de ação rápida que inibe metaloproteínas, como a citocromo oxidase contendo ferro, uma enzima chave da respiração mitocondrial. Da mesma forma, os glicosilados, conhecidos como óleo de mostarda, também conferem toxicidade à planta se seus tecidos forem lesionados. Nesse caso, ocorre liberação de substâncias pungentes como isotiocainatos e nitrilas que possuem odor característico, responsáveis pelo cheiro e sabor dos vegetais, como repolho, brócolis e rabanete (DEWICK, 2009; TAIZ; ZEIGER, 2002)

2.4 Variação no metabolismo secundário: fatores bióticos e abióticos

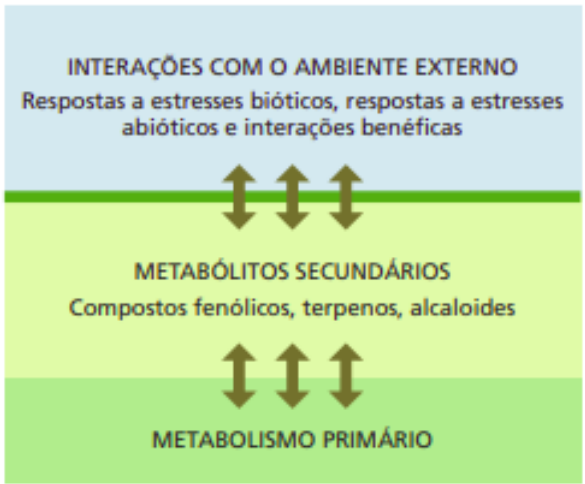
A síntese e acúmulo dos compostos secundários são afetados por diversas condições, tanto pelo desenvolvimento interno (circuitos genéticos: gene regulado, enzima), quanto por fatores externos relacionados ao ambiente (luz, temperatura, água, salinidade, organismos). Isso ocorre porque o local em que as plantas vivem são extremamente adversos e elas são seres sésseis (BARTWAL et al., 2013). A interação da planta com os organismos e o ambiente externo pode ser visualizada nas figuras 7 e 8.

Figura 7 - Correlações da planta e organismos.



Fonte: Taiz e Zeiger (2017).

Figura 8 - Relação dos metabólitos primários e secundários na resposta de defesa da planta contra o estresse biótico, abiótico e nas interações benéficas.

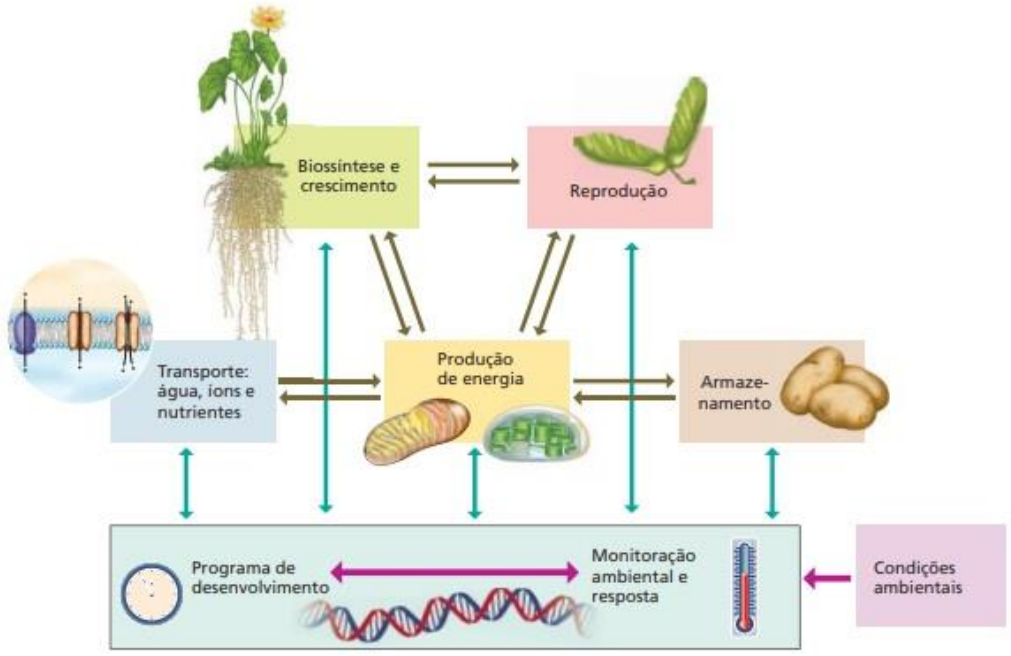


Fonte: Taiz e Zeiger (2017).

Portanto, as figuras 7 e 8 ilustram o que Taiz; Zeiger (2017) abordam sobre a existência de interações que são benéficas, se não essenciais, tanto para a planta quanto para outros organismos. Essa relação se denomina mutualismo. Na figura 7 estão apresentados os mutualismos nas interações bióticas de planta-polinizador, a relação simbiótica entre bactérias fixadoras de nitrogênio (rizóbios) e leguminosas, as associações micorrízicas entre raízes e fungos, e os fungos endofíticos de folhas. Porém, existem também as interações bióticas prejudiciais, como a herbivoria, infecção por patógenos microbianos ou parasitas e a alelopatia. Em resposta a esse estímulo as plantas podem desenvolver mecanismos de defesa complexos para se protegerem contra os organismos nocivos.

Existem alguns parâmetros ambientais abióticos primários que afetam o crescimento vegetal, como a luz (intensidade, qualidade e duração), água (disponibilidade e umidade), dióxido de carbono, oxigênio, conteúdo e disponibilidade de nutrientes no solo, temperatura e toxinas (metais pesados e salinidade). O desequilíbrio desses fatores ambientais geralmente leva a consequências bioquímicas e fisiológicas estressantes para o vegetal (RAMAKRISHNA; RAVISHANKAR, 2011; STEPHENSON; METCALFE, 2013). O equilíbrio entre a intensidade das condições ambientais impostas e os processos vegetais (desenvolvimento, crescimento, produção de energia, equilíbrio de íons e nutrientes e armazenagem) são controlados pelo genoma vegetal. Assim, o genoma pode codificar sensores e rotas de transdução de sinal que fazem o monitoramento e o ajuste dos parâmetros ambientais para direcionar o fluxo de energia entre os diferentes processos. Consequentemente, o genoma pode estabelecer um novo estado homeostático correspondente às condições específicas de estresse. Esse processo está resumido na figura 9.

Figura 9 - Interações entre condições ambientais e a homeostasia gênica nos vegetais.



Fonte: Taiz e Zeiger (2017).

Na figura 9, a caixa azul (parte de baixo) corresponde o genoma vegetal. O genoma codifica sensores e rotas de transdução de sinal que fazem o monitoramento e o ajuste dos parâmetros ambientais. As setas marrons correspondem ao fluxo de energia direcionado entre os diferentes processos para estabelecer um novo estado homeostático correspondente às condições específicas de estresse, que é desenvolvido pelo genoma.

Como todo sistema biológico, as plantas possuem rotas anabólicas e catabólicas associadas para direcionarem o fluxo de energia e recursos dentro das células e entre elas para sua sobrevivência e crescimento. Quando ocorre algum fator estressante, pode ocorrer o rompimento dessas rotas metabólicas, e as células podem produzir espécies reativas de oxigênio (EROs). As EROs são os grupos intermediários tóxicos mais comuns produzidos pelo estresse abiótico, pois, são moléculas altamente reativas (possuem ao menos 1 elétron desemparelhado em seus orbitais). Portanto, as EROs são capazes de reagir rapidamente com proteínas, DNA, RNA e lipídeos, oxidando-os. Estas espécies também podem oxidar as membranas celulares e promover a degradação de organelas, da membrana plasmática e levar à morte celular. As formas mais comuns de EROs em células vegetais são: radical ânion superóxido (O_2•–), oxigênio singleto ($^1O_2$), peróxido de hidrogênio (H_2O_2) e radical hidroxil (OH•). A acumulação de EROs nas células leva ao efeito negativo no crescimento, desenvolvimento e produtividade dos vegetais (PANDEY et al., 2017; SANCHITA; SHARMA, 2018; TAIZ; ZEIGER, 2002).

Porém, as células possuem mecanismos de aclimatação, ou seja, também desencadeiam homeostasia celular para evitar o dano fisiológico. Essa homeostasia/aclimatação, são capazes de neutralizarem os efeitos negativos do estresse, incluindo a acumulação de EROs. O mecanismo da produção das EROs e a resposta vegetal está ilustrado na figura 10.

Figura 10 - Formação e ação das Espécies Reativas de Oxigênio (EROs) pelos fatores abióticos nos vegetais.

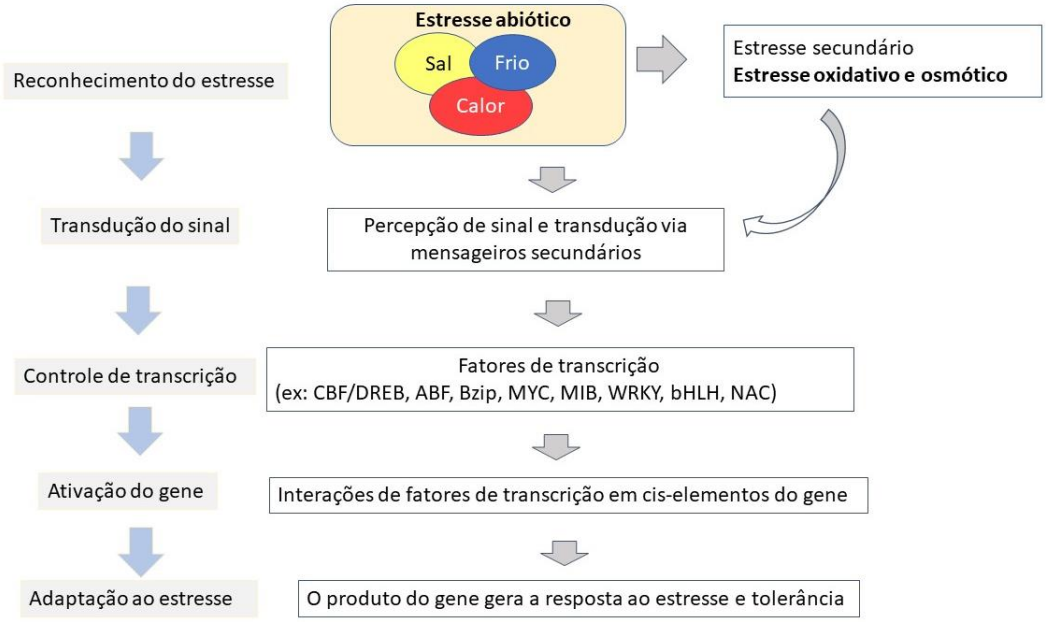
Fonte: Taiz e Zeiger (2017).

Quando algum fator abiótico se torna estressante para a planta, a célula vegetal promove uma cascata de eventos para tolerá-lo. Assim, a membrana celular vegetal percebe o sinal do estresse abiótico, através de seus receptores, e então o transduz nas células. As plantas usam diversos mecanismos sensores para gerar a resposta ao estresse abiótico. Existem pelo menos cinco tipos:

- Físicos: são efeitos mecânicos de estresse na planta ou na estrutura celular.
- Biofísicos: são mudanças na estrutura proteica ou na atividade enzimática.
- Metabólicos: promovem a detecção de subprodutos que se acumulam nas células devido ao desacoplamento de reações enzimáticas ou de transferência de elétrons.
- Bioquímicos: envolvem a presença de proteínas especializadas que se desenvolveram como sensores de um estresse em especial.
- Epigenéticos: referem-se a modificações da estrutura do DNA ou do RNA que não alteram sequências genéticas.

Após a ativação de um ou mais sensores, a planta irá transduzir o sinal mediante alteração ou ativação de rotas de resposta ao estresse. Essas rotas envolvem cálcio, proteínas quinases, proteínas fosfatase, sinalização de EROs, ativação de reguladores de transcrição, acumulação de hormônios vegetais, e assim por diante. Portanto, as respostas ao estresse não são lineares, mas circuitos complexos que envolvem múltiplas vias. As proteínas dessas vias de sinalização são ativadas e translocam mensageiros secundários, que por sua vez, estão envolvidos na ativação dos fatores de transcrição (FTs) (SANCHITA; SHARMA, 2018; TAIZ; ZEIGER, 2017). A cascata de eventos realizados pelas células após o estímulo do fator abiótico estressante está representada na figura 11.

Figura 11 - Representação esquemática da sinalização de estresse abiótico.



Fonte: Redigido de Sanchita e Sharma (2018).

O acúmulo desses metabólitos varia nas espécies vegetais de acordo com o perfil de expressão dos genes responsáveis (SANCHITA; SHARMA, 2018). A produção e acumulação desses compostos nas células vegetais conferem os efeitos das plantas medicinais na saúde e/ou tratamento de determinadas condições patológicas (CARVALHO; ANNE, 2010).

2.5 Plantas medicinais e suas aplicações

Há milhares de anos as plantas têm sido uma fonte de medicamentos tradicionais pelo mundo, pois produzem metabólitos secundários como alcaloides, terpenoides e fenilpropanoides, que em alguns casos podem ser benéficos para a saúde humana. Além destes compostos desempenharem um papel importante na adaptação das plantas às alterações ambientais, são ativos sobre outros organismos. Por esse motivo, possuem atividade medicinal, biológica ou farmacológica. Desta maneira, os compostos secundários das plantas despertam interesse no setor comercial, pois, por meio deles podem ser produzidos medicamentos, inseticidas, fungicidas, produtos farmacêuticos, fragrâncias, aromas e materiais industriais (TAIZ; ZEIGER, 2002). Os efeitos farmacológicos alcançados pelos compostos secundários compreendem muitas atividades como: antimaláricas, antidiabéticas, hepatoprotetoras, antiulcerosas, anti-inflamatórias, antimicrobianas, entre outras (SANCHITA; SHARMA, 2018).

Nesse sentido, a utilização destes metabólitos secundários provenientes das plantas para desencadear os efeitos farmacológicos só é possível através da extração destes compostos dos vegetais. Assim, é possível obter estes compostos bioativos para uso. As formas ou técnicas de extração variam conforme as características dos compostos a serem extraídos, espécie, parte da planta e solvente utilizado (BELWAL et al., 2018). Belwal et al. (2018) e Kharbach et al. (2020) resumiram as diferentes técnicas utilizadas compreendendo desde as técnicas clássicas quanto às técnicas alternativas e/ou não convencionais. Aquelas consideradas clássicas são: decocção, maceração, infusão, digestão, métodos de percolação, extração líquido-líquido, extração sólido-líquido, Soxhlet e hidrodestilação. Já aquelas alternativas e/ou não convencionais, são: extração por micro-ondas, extração por fluido supercrítico, extração líquida pressurizada, extração por ultrassom, extração por campo elétrico pulsado, extração por enzimas, líquido-líquido dispersivo, extração em fase sólida, microextrações, processamento térmico, entre outros. Dos diferentes extratos obtidos por estas técnicas, os que foram utilizados para o desenvolvimento desta tese foram os óleos essenciais e extratos aquosos (infusões).

2.5.1 Óleos essenciais

Os óleos essenciais geralmente são adquiridos por hidrodestilação, destilação a vapor, hidro-difusão, prensagem a frio, coobação ou *enfleurage*. Também podem ser utilizadas técnicas alternativas, que foram desenvolvidas para reduzir a duração da extração, consumo de energia e limitações de transferência de massa, como: hidrodestilação assistida por micro-

ondas, extração por micro-ondas sem solvente, extração líquida pressurizada, extração assistida por ultrassom, extração super e subcrítica de fluidos, hidrodestilação ôhmica e destilação a vapor acelerada por ôhmica (GAVAHIAN et al., 2020) .

Os óleos essenciais são misturas orgânicas voláteis que consistem em uma variedade de classes químicas, incluindo fenóis, terpenos, sesquiterpenos, álcoois, éteres, aldeídos, cetonas, ésteres, lactonas e éteres de fenóis e possuem características de serem compostos hidrofóbicos com densidades relativas menores que 1. Existem milhares de espécies de plantas aromáticas que sintetizam óleos essenciais em seus diferentes órgãos (folhas, flores e raízes). Estes óleos têm diferentes propriedades físico-químicas devido às variações na composição química. A Cromatografia a Gás acoplada ao Espectrômetro de Massas (CG-EM) é, provavelmente, o método analítico mais popular para analisar a composição química de óleos essenciais devido à resposta rápida e a dados precisos (GAVAHIAN et al., 2020).

Kharbach et al. (2020) compilaram os estudos já realizados cm os óleos essenciais e suas atividades farmacológicas, e encontraram as seguintes propriedades: atividade alelopática, antioxidante, antimicrobiana, antifúngica, antimicobacteriana, anti-helicobacter pylori, inseticida, inibição da acetilcolinesterase, efeito citotóxico, mutagênico, toxicidade de invertebrados, repelente e anti-inflamatória. Dessa forma, essas propriedades mostram a vasta área da utilização de óleos essenciais.

2.5.2 Decocções e Infusões (chás)

De acordo com Chandrasekara e Shahidi (2018), os chás são fontes ricas de compostos bioativos naturais (compostos secundários), pois podem extrair das plantas ácidos fenólicos, flavonoides, lignanas, ligninas, taninos, terpenoides, cumarinas, alcaloides, poliacetilenos, saponinas, entre outros.

A obtenção destes compostos também ocorre por métodos clássicos (tradicionais) ou métodos convencionais. De acordo com Zhao et al. (2013), os métodos clássicos são: decocção e infusão (também denominada maceração ou imersão), que se utiliza água como solvente. Essas extrações são eficazes para a extração de sacarídeos, aminoácidos e outros ingredientes ativos de alta polaridade, porém, ocasionalmente, soluções aquosas com aditivos (sais e ácidos, entre outros) podem ser usadas para melhorar a seletividade de extração de ingredientes ácidos ou básicos, como ácido ascórbico, polifenóis e alcaloides. Além disso, Zhao et al. (2013) relatam que as extrações de compostos de alta polaridade usando água como solvente também podem ser possíveis através do uso da agitação e extração por refluxo. Porém, quando se

pretende extrair compostos de média a baixa polaridade, como flavonoides, iridoides e saponinas, diferentes porcentagens de solventes orgânicos (metanol, etanol, acetona ou outros) podem ser usados na água ou como solventes orgânicos puros.

Como o objetivo de reduzir o consumo de solvente orgânico, eliminar etapas adicionais de limpeza e concentração da amostra antes da análise cromatográfica (que detecta e quantifica os compostos extraídos da planta), foram desenvolvidas técnicas de extrações otimizadas, conhecidas como técnicas não convencionais. Devido à alta quantidade de compostos que podem ser adquiridos dos chás e das diferentes partes das plantas, o estudo de Belwal et al. (2018), selecionou as técnicas avançadas de extração mais utilizadas e das partes da planta para a extração dos diferentes compostos. Os exemplos destas extrações são: extração por ultrassom, extração por micro-ondas, extração por líquido pressurizado, extração por fluido supercrítico, extração em fase sólida e microextração em fase sólida.

De acordo com Chandrasekara e Shahidi (2018), muitos estudos vêm demonstrando que estes compostos presentes nos chás produzem uma série de efeitos biológicos diversificados, como ações antioxidantes, antibacterianas, antivirais, anti-inflamatórias, antialérgicas, antitrombóticas e vasodilatadoras, além de efeitos de antimutagenicidade, anticarcinogenicidade e antienvelhecimento. Portanto, a utilização dos extratos das plantas, como, óleos essenciais (por hidrodestilação) ou chás (por decocção ou infusão), promovem muitos benefícios à saúde.

2.6 Contaminação ambiental por pesticidas

Como mencionado anteriormente, diferentes fatores causam estresse para as plantas e isso pode refletir na alteração de compostos secundários que, consequentemente, altera os efeitos biológicos/farmacológicos que uma planta pode ter, quando são extraídos do vegetal. No âmbito da contaminação ambiental, os pesticidas podem exercer um forte estresse no metabolismo vegetal.

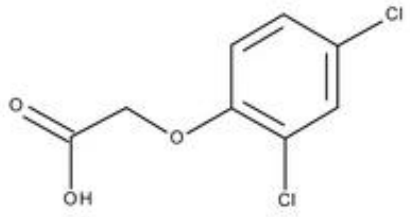
Os pesticidas pertencem a uma variedade de produtos químicos mundialmente utilizados, tanto em ambiente domiciliar quanto na agricultura. As pragas-alvo destes compostos incluem insetos, ervas daninhas, nematoides, animais roedores e microrganismos. Assim, é possível reduzir os danos que estas pragas causam aos seres humanos, seja pela redução da qualidade de alimentos, ou, pela propagação de doenças (ZAGANAS et al., 2013). A classificação dos pesticidas se dá pelo alvo a ser atingido (inseticidas, herbicidas, fungicidas, rodenticidas, acaricidas, hematicidas, bactericidas, vermífugos), ou, pelas características da

composição química (organoclorados, organofosforados, carbamatos, piretróides). Esta última abordagem é especialmente útil para fins didáticos, mas, sobretudo essencial para o estabelecimento de diagnósticos e posterior adoção do tratamento adequado em casos de intoxicações (YU; TSUNODA; TSUNODA, 2011).

2.6.1. Ácido 2,4-diclorofenoxiacético (2,4-D)

O ácido 2,4-diclorofenoxiacético (2,4-D) é um herbicida da classe dos desfolhantes que faz parte da família dos organoclorados (ácido fenoxiacético ou fenoxi) (figura 12). Ele é uma auxina sintética, portanto, regulador de crescimento de plantas. Ou seja, em pequenas dosagens é capaz de mimetizar a auxina natural das plantas e promover a divisão, diferenciação e alongamento das células vegetais (ISLAM et al., 2017; SONG, 2014). Contudo, em altas doses, ou até em baixas dosagens, é capaz de promover um efeito herbicida em dicotiledôneas devido à sua capacidade de produzir crescimento celular descontroladamente (STACKELBERG, 2013), danos aos vasos condutores e malformação das raízes, caules e folhas (EPA, 2005).

Figura 12 - Fórmula química da molécula de 2,4-D ($2,4\text{-}(Cl)_2C_6H_3OCH_2COOH$).



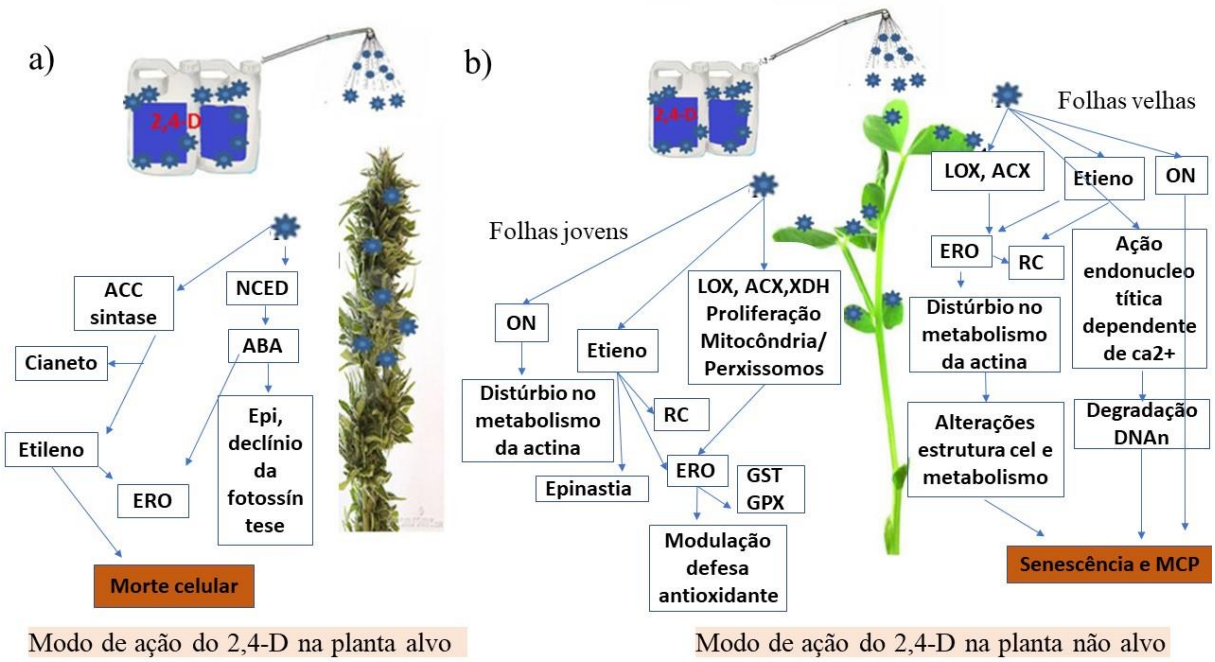
Fonte: Sigma-Aldrich.

Ao ser aplicado, este herbicida interage com o receptor de auxina solúvel na planta, TIR1 (Transport Inhibitor Response-1) ou em algum de seus vários análogos. Após, se inicia o aumento da expressão de genes responsivos à auxina, especialmente aqueles envolvidos na síntese de etileno e ácido abscísico (ABA). A consequência desse mecanismo gera os sintomas característicos do aumento da auxina natural que são: crescimento descontrolado da planta e epinastia seguido de inibição de crescimento e morte do vegetal (GOGGIN; CAWTHRAY; POWLES, 2016; GROSSMANN, 2010). O 2,4-D também pode desencadear respostas fitotóxicas, tanto pela produção de etileno, como pelo seu subproduto gerado, o cianeto (CHINALIA; REGALI-SELEGHIN; CORREA, 2007; GROSSMANN, 2010; WANG et al., 2016). A produção de etileno também aumenta a produção de EROs, pois promove a inibição da fotossíntese, causada pelo fechamento estomático mediado pelo ABA (GROSSMANN,

2010; WANG et al., 2016). As EROs também são formadas pela via de ativação da xantina oxidase e enzima acil-CoA oxidase (PAZMIÑO et al., 2011; RODRÍGUEZ-SERRANO et al., 2014; ROMERO-PUERTAS et al., 2011). Além disso, este herbicida também é capaz de aumentar peroxidação lipídica (ISLAM et al., 2016, 2017) devido sua possibilidade de se ligar a certos fosfolipídios, perturbando as interações físicas da membrana (BUKOWSKA, 2006).

A epinastia que ocorre na planta após a aplicação do 2,4-D é decorrente das alterações morfológicas násticas, onde ocorre o aumento do crescimento das células adaxiais das folhas em comparação com as células abaxiais. Estas alterações no formato celular das folhas são devido a: (i) modificação no citoesqueleto de actina; (ii) aumento no volume celular e inchaço na epiderme, bem como nas células mesofilas do lado adaxial e; (iii) encolhimento da epiderme no lado abaxial das folhas (RODRÍGUEZ-SERRANO et al., 2014; SANDALIO; RODRÍGUEZ-SERRANO; ROMERO-PUERTAS, 2016). O mecanismo de ação do 2,4-D na planta alvo e não alvo pode ser visualizado no esquema da figura 13 (a, b).

Figura 13 - Esquema mostrando o mecanismo de ação do 2,4-D na célula nas plantas alvo (a) e não alvo (b). Acil-CoA oxidase (ACX), xantina oxidase (XOD)**,** lipoxigenase (LOX), 1-aminocyclopropane-1-carboxylate (ACC) sintase, Espécies Reativas de Oxigênio (ERO), Respostas Citotóxicas (RC), ácido abscísico (ABA), Gene regulado pelo estresse (NCED), Óxido Nítrico (ON) e Morte celular programada (MCP).



Modo de ação do 2,4-D na planta alvo

Modo de ação do 2,4-D na planta não alvo

Fonte: Traduzido e adaptado de Islam et al. (2018).

Portanto, nas plantas daninhas alvo (figura 13 a), o 2,4-D induz a expressão de genes responsivos à auxina, especialmente aqueles envolvidos na síntese de etileno e ácido abscísico,

o que leva ao crescimento descontrolado das plantas, produção de cianeto e epinastia, seguido por inibição do crescimento e morte. E nas plantas não alvo (figura 13 b), as folhas jovens expostas ao 2,4-D aumentam as atividades da acil-CoA oxidase, (ACX), xantina oxidase (XOD) e lipoxigenase (LOX), que induzem as EROs, mediando o crescimento celular e o desenvolvimento da epinastia. As folhas velhas apresentam senescência e respostas programadas à morte celular devido à degradação do nDNA, distúrbio no citoesqueleto de actina, distúrbio na estrutura celular, produção de ERO e formação de etileno (ISLAM et al., 2018).

A utilização deste herbicida tem como objetivo a prevenção de plantas daninhas de folha larga em ambientes agrícolas e áreas urbanas e é aplicado diretamente no solo ou pulverizado sobre as culturas e, a partir daí, frequentemente atinge águas superficiais e sedimentos (CHINALIA; REGALI-SELEGHIN; CORREA, 2007). No Brasil, este composto tem registro para aplicações em pós-emergência das plantas daninhas das culturas do arroz, aveia, cana-de-açúcar, centeio, cevada, milho, sorgo, trigo, pastagens, soja (pré-plantio) e café (AGROFIT, 2021). Além disso, seu uso vem ganhando destaque, uma vez que, segundo os dados do Ministério Do Meio Ambiente, (2019), o 2,4-D foi classificado como o segundo pesticida mais vendido no país em 2019, após o glifosato.

De acordo com as características físico-químicas deste herbicida, ele pode estar presente em diferentes quantidades no solo, ar, poeira, comida e, principalmente, na água. Sua meia vida é de 20 – 312 dias, dependendo das condições ambientais, como microrganismos e oxigênio. Dessa forma, pode afetar o metabolismo de outros organismos e plantas presentes no solo, água ou ar (ISLAM et al., 2018). A capacidade do 2,4-D estar presente em áreas aquáticas se deve à polaridade do grupo carboxila desta molécula (pKa = 2,8) e baixa adsorção ao solo. Portanto, o 2,4-D pode ser transportado através dos processos de escoamento superficial, infiltração, lixiviação e/ou percolação do solo, tornando-se um problema de saúde ambiental e humana (GAULTIER et al., 2008; KEARNS et al., 2014; SHAREEF; SHAW, 2008).

O 2,4-D é moderadamente tóxico para o ser humano, por via oral, dérmica e inalatória (categoria de toxicidade III) e gravemente tóxico por via ocular (categoria de toxicidade I) (EPA, 2005). A preocupação com a contaminação com este composto é de extrema importância. O trabalho de Islam et al. (2018), reuniu os trabalhos sobre a detecção do 2,4-D no ambiente e agrupou os estudos com as reações tóxicas deste herbicida nos organismos. De maneira geral, a toxicidade do 2,4-D pode levar a respostas inflamatórias, afetar o sistema nervoso através da inibição da defesa antioxidante induzida por EROs, causar genotoxicidade e afetar o funcionamento normal das organelas, induzir autofagia e carcinogênese no nível celular. O 2,4-

D também interfere no funcionamento normal do sistema endócrino, modulando a produção de hormônios da tireoide e a expressão de hormônios esteroides sexuais. Como relatado anteriormente, o 2,4-D possui uma significativa importância agrícola. Contudo, quando não utilizado com os devidos cuidados pode ser tóxico e ocasionar a contaminação de espécies não alvo.

2.7 Modelos experimentais complementares para bioensaios

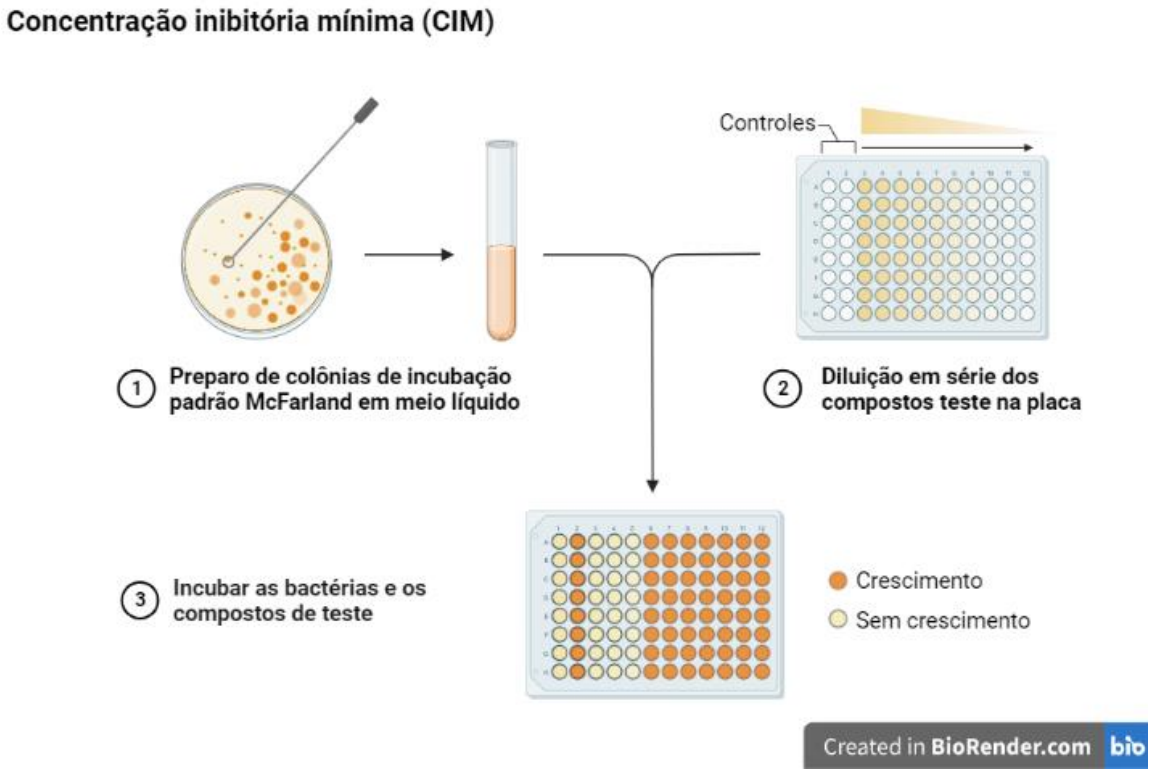
Para avaliar a toxicidade tanto de compostos secundários extraídos das plantas, quanto de amostras ambientais, os estudos têm utilizado modelos biológicos complementares *in vitro, in vivo* e/ou *ex vivo*, principalmente devido as restrições impostas pelos comitês de ética em estudo animal (FREIRES et al., 2017). Dentre eles, serão apresentados os seguintes modelos utilizados no desenvolvimento deste trabalho: Ensaio antimicrobiano, *Allium cepa*, *Artemia salina* e *Drosophila melanogaster*.

2.7.1 Ensaio Antimicrobiano

Devido ao elevado crescimento de patógenos resistentes aos medicamentos, as pesquisas têm buscado novos agentes antimicrobianos. Assim, as espécies de plantas medicinais levam destaque nesses estudos, pois são uma importante fonte de novos agentes (extratos ou compostos isolados) com tal propriedade.

Existem diversos métodos laboratoriais que podem ser empregados para medir a sensibilidade das bactérias aos agentes antimicrobianos. A avaliação de susceptibilidade de antibióticos utilizados pelos laboratórios é padronizada por normas estabelecidas por órgãos como o Clinical and Laboratory Standards Institute (CLSI), British Society for Antimicrobial Chemotherapy (BSAC) e European Committee on Antimicrobial Susceptibility Testing (EUCAST) e o Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST) (ROQUETE AMPARO et al., 2018). Tanto os métodos de diluição em caldo ou em ágar são aceitáveis. Eles são capazes de quantificar a atividade *in vitro* de um agente antimicrobiano contra um determinado isolado bacteriano. A figura 14 apresenta um esquema do método da concentração inibitória mínima (CIM) bacteriana.

Figura 14 - Avaliação da atividade antimicrobiana pela técnica do crescimento inibitório mínimo (CIM) por microdiluição seriada.



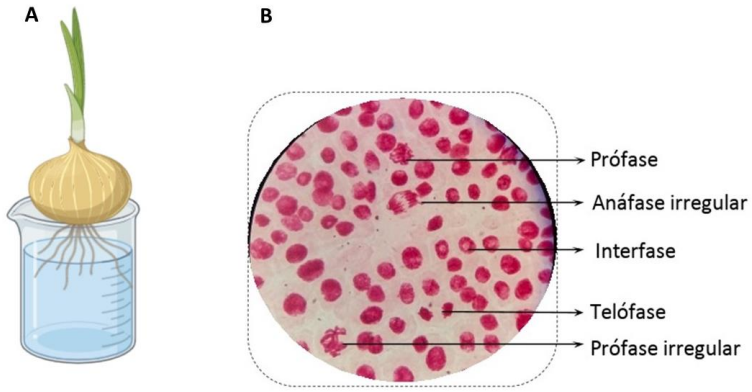
Fonte: Traduzido e adaptado de Biorender.com.

Para realização deste teste, são necessários tubos de ensaio ou placas com meio caldo ou ágar, aos quais são acrescentadas diversas concentrações dos agentes antimicrobianos. Esses tubos ou placas são inoculados com uma suspensão padrão do organismo a ser testado. A determinação da concentração inibitória mínima (CIM) é realizada após a incubação de um dia para outro (35ºC) e se verifica, através da coloração, se o crescimento das cepas foi inibido ou não com o extrato ou agente utilizado. O resultado é significativamente influenciado pela metodologia, que deve ser realizada cuidadosamente em laboratório com condições controladas, para evitar contaminação e poder obter resultados reprodutíveis (intra e interlaboratório) (CLSI, 2003).

2.7.2 *Allium cepa*

O bioensaio com as raízes da cebola (*Allium cepa*), é um teste sensível que tem sido usado para a determinação de efeitos mitóticos, citotóxicos e/ou genotóxicos de várias substâncias químicas e preparações com plantas (TIMOTHY et al., 2014) (figura 15). Este teste tem sido considerado favorável para avaliar danos e distúrbios cromossômicos no ciclo mitótico, devido à presença de boas condições cromossômicas, como grandes cromossomos e com um número reduzido (2n = 16) (FISKESJÖ, 1985). Além disso, este sistema de teste mostrou alta sensibilidade na detecção de produtos químicos ambientais.

Figura 15 - Bioensaio com as raízes de *Allium cepa* (A). Análise microscópica da divisão celular das células das raízes: teste de citotoxicidade, genotoxicidade e índice mitótico (B).



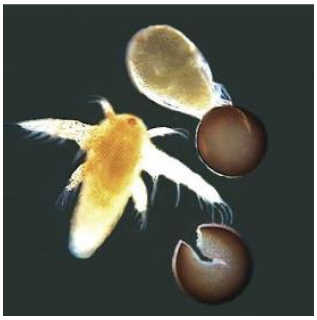
Fonte: Adaptado de Biorender.com (A). Autor (B).

O teste com *A. cepa* é considerado de baixo custo, fácil manuseio e sua principal vantagem é permitir o contato direto da solução (infusão, extrato, resíduo industrial) com as raízes de *A. cepa*. A rapidez do teste (24 a 96 h) permite observação direta ao microscópio dos possíveis danos celulares (figura 15 B). Leme e Marin-Morales (2009) compilaram os estudos já realizados com esse bioensaio e encontraram sua aplicação em análises de metais, pesticidas, hidrocarbonetos aromáticos, corantes de indústria têxtil, produtos usados para desinfetar a água potável, amostras de água e solo de áreas poluídas.

2.7.3 *Artemia salina*

A espécie *A. salina* é um microcrustáceo adaptável a ambientes hipersalinos (figura 16). Esta espécie serve como organismo teste em uma ampla variedade de ensaios e pesquisas toxicológicas. Ela pode ser usada como indicador para detecção de compostos antitumorais, toxicidade de extratos vegetais, toxinas de fungos, toxicidade de metais pesados, toxinas de cianobactérias e pesticidas (BLAISE, 1998).

Figura 16. Espécie *Artemia salina*. Náuplios recém eclodidos.



Fonte: Mayorga et al. (2010).

Esse modelo também é uma ferramenta útil nas pesquisas de isolamento de compostos bioativos a partir de extratos vegetais (OHIKHENA; WINTOLA; AFOLAYAN, 2016). A *A. salina* é muito utilizado em pesquisas ecotoxicológicas devido às seguintes características: ciclo de vida curto, alta adaptabilidade a condições ambientais desfavoráveis e alta fecundidade (NUNES et al., 2006).

A *A. salina* nasce de cistos e têm idade e genótipo semelhantes, portanto, garantem confiabilidade nos resultados. Como esta espécie vive em lagos de sal, também é capaz de tolerar altas concentrações de íons cloreto encontradas na maioria dos lixiviados. Portanto, é adequada para determinar a toxicidade devido a outras fontes que não o cloreto (SVENSSON et al., 2005). A toxicidade dos extratos vegetais na *A. salina* implica apenas em vida ou morte do microcrustáceo, sendo a ausência de toxicidade um ponto de partida para que os sistemas biológicos possam tolerar a planta (SANDOVAL et al., 2020). De maneira geral, esse bioensaio possui diversas vantagens, incluindo: disponibilidade comercial dos cistos; pode ser mantido indefinidamente no laboratório em sua forma de cisto e é facilmente induzido a eclodir; o ensaio é rápido, simples e realizado a baixo custo; requer pequeno volume de amostra e pode ser realizado com alto rendimento de amostras (microplacas); está em conformidade com as diretrizes de ética animal em muitos países (HASSAN et al., 2016).

2.7.4 *Drosophila melanogaster*

A *Drosophila melanogaster* é popularmente conhecida como a “mosca da fruta”, ela pertence à subespécie Diptera da família Drosophilidade (figura 17). O uso deste modelo experimental alternativo começou no início do século XX (STEPHENSON; METCALFE, 2013) e, atualmente, é um dos organismos modelo mais estudados e com ótimo custo-benefício para a pesquisa biológica. Esta espécie é utilizada em diferentes pesquisas, como na área da genética, desenvolvimento embrionário, comportamento, e estudos de sinalização relacionados a doenças (PANDEY; NICHOLS, 2011).

Figura 17 - *Drosophila melanogaster* macho e fêmea, armazenamento e exemplo de ensaio comportamental.

Fonte: Adaptado de Biorender.com.

A *D. melanogaster* é vastamente utilizada como modelo experimental pois possui baixo custo, fácil manuseio, requer pequeno espaço no laboratório (3 mm de tamanho de cada mosca) e permite o cultivo de muitas moscas em pequenos frascos (figura 17, armazenamento). É vastamente aplicada em estudos rápidos de doenças dependentes da idade, como doenças neurodegenerativas e análise dos efeitos dos extratos e/ou componentes ativos específicos de plantas, em condições normais e de doenças. Isto porque a *D. melanogaster* tem um ciclo de vida curto (10–12 dias a 25°C) e vida útil de 90–120 dias (FERNÁNDEZ-MORENO et al., 2007). Uma única fêmea pode depositar de 30 a 50 ovos/dia (BARNES et al., 2008). Além disso, possuem somente 4 pares de cromossomos que podem ser geneticamente manipulados. Seu genoma é totalmente sequenciado e já se sabe que elas possuem uma similaridade de 75% com genes relacionados a doenças em humanos (FERNÁNDEZ-MORENO et al., 2007).

Essa espécie pode ser facilmente alimentada com uma dieta misturada com plantas ou compostos derivados de plantas. Após essa exposição ao contaminante ou ao extrato/composto vegetal, se avalia o efeito dos mesmos através de alterações fenotípicas (olhos, anomalias), mudanças no desenvolvimento (ciclo de vida, vida útil), fecundidade, desenvolvimento (larval, pupal ou adulto) e alterações comportamentais (locomotora, escalada, fototaxia) (PANCHAL; TIWARI, 2017).

3 JUSTIFICATIVA

Considerando a capacidade de *P. neochilus* para a fitorremediação do herbicida 2,4-D, torna-se importante verificar a alteração do metabolismo secundário dos compostos voláteis desta planta, já que a espécie produz óleo essencial. Dessa forma, também se torna importante encontrar formas de utilizar a planta após a fitorremediação, visto que seu chá é utilizado na medicina popular e seu óleo essencial vem se destacando nas pesquisas com diferentes modelos biológicos. Além disso, se torna pertinente analisar a possível atividade repelente e inseticida deste óleo essencial, uma vez que possui um forte aroma e já se conhece sua atividade de inibição de oviposição. Por fim, a avaliação da toxicidade do chá desta planta é imprescindível, devido sua alta ingesta na medicina popular e a falta dessa informação.

4 OBJETIVOS

4.1. Objetivo geral

Avaliar as atividades toxicológicas dos extratos e óleo essencial de *P. neochilus* em diferentes modelos experimentais.

4.2. Objetivos específicos

- Identificar, por meio de uma revisão da literatura as características de *P. neochilus*, bem como a composição química de seus extratos e as formas de uso da planta.
- Investigar se a constituição do metabolismo secundário dos compostos voláteis presentes nas folhas da planta e dos compostos presentes no óleo essencial extraído dessas folhas são afetados após a exposição ao herbicida 2,4-D.
- Verificar se o óleo essencial de *P. neochilus* pode ser utilizado como antimicrobiano após a fitorremediação do 2,4-D.
- Examinar se após a fitorremediação do 2,4-D o chá de *P. neochilus* se torna tóxico por ingestão no modelo *D. melanogaster*.
- Analisar se o resíduo aquoso da fitorremediação do 2,4-D se torna menos tóxico para os modelos biológicos *A. salina* e *A. cepa*.
- Investigar o perfil químico do óleo essencial de *P. neochilus* e relacionar com os efeitos repelente e inseticida para o modelo *D. melanogaster*.
- Verificar a composição e atividade antioxidante do chá das folhas de *P. neochilus*, bem como sua toxicidade em modelos complementares (*A. salina*, *A. cepa* e *D. melanogaster*).

CAPÍTULO II

A review of anatomical, physiological, biological characteristics and uses of *Plectranthus neochilus*

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A review of anatomical, physiological, biological characteristics and uses of *Plectranthus neochilus*

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ABSTRACT

Plectranthus neochilus' tea is widely used for medicinal purposes like digestive disorders, liver problems, hangover and to a lesser extend, for treating respiratory infections. It is one type of "boldo" used in different corners of the world because it has a year-round growth, easy adaptation and environmental resistance, tolerating intense sun and demanding little manipulation. This review aims to compile the up-to-date information about *P. neochilus* obtained from scientific databases (ScienceDirect, Springer, Google Scholar and PubMed). The plant was recently identified and differentiated anatomically and microscopically from anothers also called "boldo". Many compounds were identified in their extracts (aqueous, ethanolic, hexane among others). The most recent studies point out the importance of its essential oil, rich in mono and sesquiterpenes. Some activities presented by these extracts or essencial oil are: antioxidant, insecticidal, antibacterial, antifungal, anticancer and antischistosomal activity. In relation to the non-traditional use of the plant, it is used to promote phytoremediation or to be used in green roof. *P. neochilus* has versatility use and high valuable biological character therefore, more studies are necessary with more extracts and formulations of their oil in order to verify activities in different experimental models.

Keywords: Extracts; Essential oil; Biological activity.

1 INTRODUCTION

The genus *Plectranthus*, of family *Lamiaceae*, belongs to the subfamily *Nepetoideae*, tribe *Ocimeae*, subtribe *Plectranthinae*. This genus has approximately 300 species of perennial

herbs and shrubs native to tropical regions of Africa, Asia and Australia (Codd, 1985; Rice et al., 2011). The name *Plectranthus* comes from the Greek words *plektron* (spur) and *anthos* (flower), describing a spur the flowers have at their base (figure 1) (Waldia et al., 2011).

About 85% of *Plectranthus* species are used for medicinal purposes (Rice et al., 2011). In Brazil, the use of these plants is mainly directed to digestive disorders and hepatic complaints (Bandeira et al., 2010; Bandeira et al., 2011; Lukhoba et al., 2006). Among the species identified, around 62 of the genus *Plectranthus* are used worldwide for medicinal or ornamental purposes, along with a rich diversity of ethnobotanical uses (Waldia et al., 2011). Popular uses of these species worldwide include: treatments for headaches, wounds, burns, dermatitis, allergies, insect and scorpion stings, and also as an antiseptic agent. Plants of the genus are important sources of new bioactive compounds and potential medicines, perfumery and cosmetics (Ascensão et al., 1998; Ascensão, Mota and Castro, 1999). The study of Abreu et al. (2015) about *Plectranthus amboinicus* (Lour.) Spreng, *Plectranthus barbatus* (Andrews) and *P. neochilus,* in Northeastern Brazil, showed that *P. neochilus* are the most known medicinal plants considering they were mentioned by 47% of the interviews.

The *P. neochilus* is a herbaceous plant known as “boldinho”, “boldo da folha miúda”, “boldo”, or, "boldo-gambá", according to traditional medicine in Brazil. This plant is an aromatic herb of easy territorial adaptation which grows during all year and its leaves are widely used in the form of tea in traditional medicine (Couto, 2006). In South Africa, this species is very cultivated mainly because it is a plant that grows rapidly by cuttings and requires little maintenance, it is widely used for the development of new gardens, as well as for ground cover, therefore, it assists in the accumulation of mulch that fertilizes the soil, retains moisture and competes with weeds (Pooley, 1998; Van Jaarsveld and Thomas, 2006). At the same time, in domestic use it works as an air purifier (Pooley, 1998).

This review will address the following subjects: 1) anatomical, morphological and microscopic characteristics of *P. neochilus*; 2) chemical composition of its extracts and essential oil; 3) traditional and non-traditional uses of this species.

2 CHARACTERISTICS AND MORPHOLOGY OF PLECTRANTHUS NEOCHILUS

2.1 Anatomy and development of plant

P. neochilus has an important role in human health due to its uses for healing purposes in the folk medicine through tea infusions, especially for the treatment of hepatic failure and dyspepsia (Couto, 2006; Lorenzi and Matos, 2002). However, its traditional use goes beyond that, an ethnomedical research in rural communities of South Africa showed that this species is

used to treat respiratory infections (chills, cough and a runny or blocked nose) taken orally (York et al., 2011).

In this sense, through the vast use of this medicinal plant, the identification of *P. neochilus* had already been done by some authors, although they focused only in the morphological characteristics of the plant. Whereas many species are called boldo because they are used for stomach aches and liver problems, Duarte and Lopes (2007) analyzed the stem and leaf anatomy of *P. neochilus*, making possible possible to distinguish more clearly "Brazilian Boldo" (*P. barbatus* Andrews), "Japanese Boldo" (*Vernonia condensata* Baker, Asteraceae) (Lolis and Milaneze-Gutierre, 2003) and the "Chilean Boldo" (*Peumus boldus* Molina, Monimiaceae) from *P. neochilus* (boldo-gambá). So, the union of the oldest researches with the new ones contributed with the creation of a list containing the peculiar characteristics (morphological, anatomic, and others) for *P. neochilus*, as can be seen in table 1:

Table 1 - Characteristics of growth and development, morphological, anatomic, and others of *P. neochilus*

Characteristic	Description	Reference
Growth and development	Perennial, sometimes annual plant, prostrate and erect, usually very branched and dense. The species does not produce seeds and its vegetative propagation by cuttings enables the production of seedlings in less time, with greater uniformity and standardization. The plant can be planted at any time of the year	Codd, 1985; Coelho et al., 2014; Couto, 2006; Lorenzi e Matos, 2002
Anatomical	Succulent grass measuring 0.12-0.5 m in height, with moderate to densely villous branches	Codd, 1985; Coelho et al., 2014; Couto, 2006; Lorenzi e Matos, 2002
Leaves	Petiolate and have juicy blades, usually viscous. It also tends to fold along the central vein, being egg-shaped, but with the apex wider than the base (20-50 x 15-35 mm), pubescent, with orange glands beneath the surface, obtuse apex, narrow, acute base and margin with some teeth	Codd, 1985, Duarte and Lopes, 2007
Odor	Leaves and flowers: are strong and the taste is bitter	Lorenzi e Matos, 2002
Flowers	Petiole measures 5-15 mm in length, has inflorescence racemosa with violet coloring, and	Codd, 1985

	tuberous roots. Inflorescence terminal racemic type (70-150 mm), bracts (25 ° angle), greenish with purple tip, early deciduous. Flowers in 3 sessions, forming whorls of six flowers, dense whorls above, flexible and 5-15 mm to the bottom and erect pedicels. Chalice 6 mm long in fruit, tube slightly geniculate on the middle and expanded to the throat. Upper lip bluish white, 2 mm long; boat-shaped lower lip (8-11 mm). Stamens 8-11 mm long, attached at the base 2-3 mm	

These characteristics can be seen in figure 1 (a) and (b).

Figure 1 – Photos of *Plectranthus neochilus* and its inflorescence. Figure 1 (a) Drawing of the anatomy of *P. neochilus*. Figure 1 (b) *P. neochilus* with inflorescence.

Source (a): Duarte and Lopes, 2007. Source (b): Author.

A peculiar characteristic in relation to its strong odor was that bees are the effective pollinators of this plant in South African region, according to the study of Stirton (1997). Among the genera of the bees, five species of *Megachile*, three species of *Xylocopa*, one species of *Anthophora* (now genus *Amegilla*) and *Apis mellifera* (all *Hymenoptera, Apidae*) were considered to be effective pollinators. The author also listed other insects known as non-effective pollinator: a family of flies *Diptera: Bombyliidae*, a specie flower flies (*Diptera: Syrphidae*) and a butterfly *Macroglossa trichilioides (Lepidoptera: Sphingidae*).

Regarding the best conditions of growth and development of the plant, the study of Lima et al. (2018) showed differences in the development of this plant when it was cultivated in two conditions of solar intensity (50% and 100%). Under 50% of the availability of sunlight, the "boldo" increases its leaf area and the internodes of distance for the best use of the luminosity

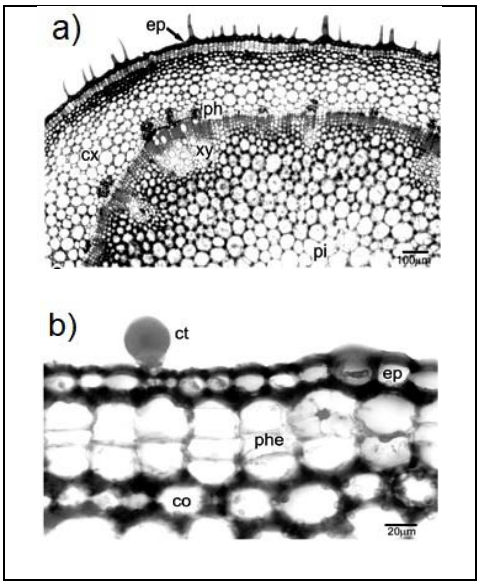
in the photosynthetic process, while under full sunlight the plant produces less leaf area and reduces the distance between leaves that are practically superimposed to avoid stress due to excessive light.

In order to produce plants with greater synthesis of secondary metabolites potentiated by traditional genetic improvement or genetic engineering, the technique of tissue culture in aromatic and medicinal plants should be considered (Swain et al., 2010). In this sense, Mota et al. (2010) aimed to evaluate the organogenic capacity of leaf explants of *P. neochilus* to obtain plants for future studies of metabolites and potential use in the genetic transformation of plants. So, through the protocol produced, the report demonstrates that shoots and full plants, in this case *P. neochilus*, could be efficiently induced leaves when plants are *in vitro* cultivated in medium supplemented with 0.2 mg/dm-3 NAA (α-naphthaleneacetic acid) and 4.7 to 5.3mg/dm-3 BAP (6-benzilaminopurine).

2.2 Histology

In relation to the microscopic anatomy of this plant, Duarte and Lopes. (2007) observed that *P. neochilus* has a stem with quadrangular rod in transection and reveals an incipient secondary growth at the analyzed level (Figure 2.a). The epidermis is unisserized with polygonal cells in the front shape, being more tangential than radially and, remains as the dermal system, although the phellogen has been established (Figure 2.b). In the cortex , below the phellogen, of this same organ, a continuous strand of collenchyma is found (Figures 2.a and 2.b). It is angular and comprises three or four rows. The multilayered cortical parenchyma has chloroplasts and shows small intercellular spaces. The innermost boundary of the cortex is represented by a single layer of large parenchymatous cells, whose tangential and radial walls are impregnated with lipophilic substances. The vascular cambia form xylem inward and phloem outward, being active mainly in the fascicular region and toward the xylem, although collateral bundles can be distinguished (Figure 2.a). The pith consists of thin walled parenchymatic cells, comparatively greater, containing many amyloplasts and forming small intercellular spaces (Figure 2.a).

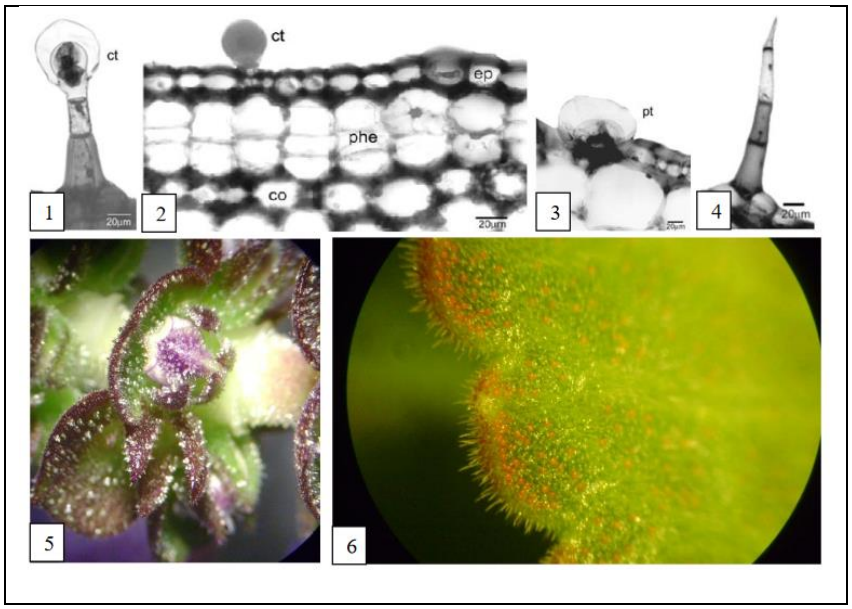
Figure 2 - Transection microscopy of *Plectranthus neochilus*. A) Transection of stem in secondary growth. B) Caulinar dermal system and collenchyma.



Legends: ep - epidermis; cx - cortex; ph - phloem; xy - xylem; pi - pith. Source: Duarte and Lopes (2007)

Viana (2011) observed that *P. neochilus* has some trichomes in the leafs that represent tissues that, in a functional level are the producers of essential oils. This production is responsible for both protection against herbivores and pathogens, and for attraction of pollinators in floral parts. These are checked for their internal anatomical characteristics and can be visualized in Figure 3.

Figure 3 - Transection microscopy of trichomes found in Plectranthus neochilus. 1 - trichome capped with short peduncle, 2 - tricohme capped with short peduncle, detail of the caulinar dermal system and collenchyma, 3 - peltate trichome, 4 - non-glandular trichome, 5 - trichomes in the inflorescence, 6 - trichomes in the leaf limbus.



Legend: ct - capitate trichome; ep - epidermis; phe - phellogen; co - collenchyma; pt - peltate trichome. Source: Duarte and Lopes (2007) and Viana (2011)

Briefly, more morphologically similar species (*P. neochilus*, *P. barbatus*, *P. boldus* and *Veronia condensata*) can be distinguished in the following manner: *P. neochilus* and *P. barbatus* resemble the stem organization, but differ in leaf characteristics. *P. barbatus* has leaves with polygonal epidermal cells in surface view, anomocytic stomata, comparatively more trichomes and dorsiventral mesophyll. *P. neochilus* differs from *P. boldus* because the latter exhibit hypostomatic leaf, stellate trichomes, hypoderm, secretory cells and dorsiventral mesophyll. And, finally, *P. neochilus* differs from *V. condensata* by chlorenchyma organization, that also occurs in *V. condensata* leaf, but the latter has tetracytic or anomocytic stomata, T-shaped non-glandular trichomes and calcium oxalate crystals (Duarte and Lopes, 2007).

Microscopic analysis are very useful, not only for identification purposes, but for toxicity studies of pollutants in plants. In this sense, the work of Campos, Azevedo and Sant'Anna-Santos (2010) characterized, visually and microscopically, the leaf damage caused by potassium fluoride. The authors showed that there was accumulation of fluoride in leaves of "boldo-gambá", but no visual symptoms were observed in the leaves, only microscopic changes. These modifications occurred in the form and turgidity of the epidermal cells, in the rupture of the cuticle. In addition, the apical cell of the secretory trichomes was deformed and in the stomata there was alteration of the cellular turgidity and rupture of the stomatal crest. The tectorial trichomes were flaccid and folded in the cell wall of the basal cell. Besides that, it was observed that the content of the pollutant present in the tea solution was 8.49 mg/g. Therefore, these results indicate that tea from this species can contribute significantly to the intake of fluoride, depending on the level of consumption of the beverage and the conditions in which the plants are exposed.

3 COMPOSITION AND CONSTITUENTS: ESSENTIAL OIL AND EXTRACTS

3.1 Compounds of essential oil

According to Correa, Scheffer and Ming (2006), the chemical compounds elaborated by the secondary metabolism of medicinal plants give them their value. A characteristic that stands out of *P. neochilus* is the composition of its volatile oils, as previously mentioned in the histology (section 2.1). In this way, several studies were carried out with the essential oil of this plant in different places, like Africa, Brazil and Portugal, and, *P. neochilus* showed to be rich in mono and sesquiterpenes constituents. However, these researches presented differences in

the constituents and/or concentrations of the major compounds according to the study site. The table 2 summarizes the relevance of compounds according to location.

These differences are considered accepted according to that Correa, Scheffer and Ming (2006) mentioned on environmental factors (altitude, latitude, temperature, relative air humidity, day length, soil, availability of water and nutrients) and techniques (season and planting form) that influence the production of secondary compounds by plants.

Table 2 - Places of study with *P. neochilus* and constitution of major compounds

Place	Major compounds	Reference
Southern Africa	Predominance of monoterpenes, with citronellyl formate, linalool and isomenthone	Lawal and Hutchings, 2010
Brazil	Predominance of sesquiterpenes, with α and/or β-caryophyllene, trans caryophyllene, germacrene D, or caryophyllene oxide; or half monoterpenes and half sesquiterpenes	Aguiar et al.2018; Baldin et al., 2012; Bandeira et al., 2011; Caixeta et al., 2011; Crevelin et al., 2015; Medeiros et al., 2016
Portugal	Predominance of monoterpenes, with α-thujene, α-pinene, and β-pinene	Mota et al., 2014

In the study by Lima et al., 2018, (cited in section 2.1), that analyzed the percentage of light/shadow in the growth of "boldo" and its development, the results showed that 50% of light had a good influence in biomass production and constituents of essential oil too. In the research of Rosal et al. (2011) the application of different organic fertilizer sources in a light-protected environment were compared, and it was observed the production of biomass, content and quality of essential oil. The following results were obtained: the plants were more responsive when submitted to fertilization with poultry manure than those that were not fertilized or fertilized with bovine and organic manure. The results were very significant, with the amount of essential oil with poultry manure being 11.36 times higher than with non-fertilized plants, 1.76 and 4.59 times higher than bovine and/or organic manure, respectively. In addition, differences were found in the chemical composition of the volatile compounds, being higher for the treatment without fertilizer (27 compounds) than with the treatments using bovine manure (12) and poultry manure (18). This research suggested that the plant suffered stress due to lack of fertilizer for its growth and development, which generated this variation in the composition of volatile compounds treatment without fertilizer. A similar study was found by Medeiros et al. (2016), which used liquid humus from bovine solid humus at concentrations of

5, 10 and 20%. In this case, the use of liquid humus in different concentrations was not related to the increment of dry and fresh matter, only a decrease in root length in the highest concentration of liquid humus.

3.2 Compounds of extracts

Regarding the analysis of the compounds present in the extracts with organic solvents with the different types of "boldos" there are many works in the literature, for example: Gupta et al. (2013) found caffeic acid, coumaric acid, rutin, quercetin and gallic acid in the acetone extract from the *Plectranthus amboinicus* Benth (Lamiaceae), although in the extract with ethyl acetate from the same plant, these authors found caffeic acid, coumaric acid, rutin, quercetin and gallic acid; Lara-Fernández et al (2013) efficiently extracted the main bioactive component of the "Chilean boldo" (*Peumus boldus*), the boldine, through a hydroethanolic extraction. However, there are few studies about the compounds founded in the *P. neochilus* extracts, but, in the work of Ramborger et al. (2017) it was possible to identify caffeic acid, ferulic acid and coumaric acid in the aqueous extract, and Brito et al. (2018) identified rosmarinic acid, which mimicked the use of traditional tea by decoction. In the study of Viana (2011) the following compounds were found in hexane extract from leaves and stems: friedelin, α-amirine fatty acid ester, sitosterol and stigmasterol. While flavone cirsimaritin could be isolated from the ethanolic extract. It can be seen in table 3 (section 4.2).

4 BIOLOGICAL ACTIVITIES OF P. NEOCHILUS

4.1 Effects of essential oil

The relevance of the studies with the extraction of its essential oil goes beyond the analysis of the constituents, since many studies have identified different biological activities attributed to it. In this way, the essential oil of the leaves of *P. neochilus* obtained a promising activity against causing cavities bacteria (moderate activity against *Enterococcus faecalis* - MIC = 250 μg/mL and *Streptococcus salivarus* - MIC = 250 μg/mL, significant activity against *Streptococcus sobrinus* - MIC = 62.5 μg/mL, *Streptococcus sanguinis* - MIC = 62.5 μg/mL, *Streptococcus mitis* - MIC = 31.25 μg/mL, and *Lactobacillus casei* - MIC = 31.25 μg/mL, and interesting activity against *Streptococcus mutans* - MIC = 3.9 μg/mL) (Crevelin et al., 2015). Besides being antibacterial, the oil has antifungal activity with MIC = 125 μg/mL against *R. stolonifera* (Aguiar et al., 2018). Other activities that have already been attributed to essential oil of "boldo" were the antischistosomal properties *in vitro*, where the concentration of 100 μg/mL killed 100% of the adult worms of *Schistosoma mansoni* (Caixeta et al., 2011), and caused reduction in female egg laying and oviposition inhibitor of *Bemisia tabaci* type B

whitefly in tomato (Baldin et al., 2013; Fanela et al., 2016). In relation to its essential oil of leaves and stem in the flowering phase, Rosal et al. (2011) analyzed the TBARS assay (thiobarbituric acid reactive substances) to verify the lipid oxidation prevention capacity. The results showed that the essential oil obtained 60% inhibition at the concentration of 1 mg/mL, meaning a reasonable antioxidant action. These researches are listened in table 3 (section 4.2).

4.2 Effects of extracts

By the fact that this plant is widely used in traditional medicine, in the form of tea in substitution of the *Peumus boldus* (Duarte and Lopes, 2007), one study of aqueous extract of leaves obtained good results concerning antioxidant activity (14.7%) (Rijo et al., 2014). This was very good in relation to others types of extracts, as the results found in the work of Viana (2011) which shows smaller antioxidant activity by DPPH method, in methanolic, ethyl acetate and hexane extracts of the leaves and stems. Viana (2011) found 11% of methanolic extract from leaves and stems, 7% of ethyl acetate extract from leaves and stems and, 4% of hexane extract from leaves and stems. In the work of Ramborger et al. (2017), when "boldo" was put in contact with the herbicide 2,4-D (in soil and water), the total antioxidant capacity of the leaves in aqueous extract was decreased in both environments indicating the toxicity of the compound to this plant. It corroborates with what Correa, Scheffer and Ming (2006) mentioned about the environmental and chemical conditions can alter the constituents of the plants, in this case, the antioxidant activity.

When the flower extracts were studied, the results obtained of its methanolic extract showed inferior antioxidant activity (5%) in relation to the leaves and stem which was confirmed by the phenolic content of the extracts. But, Viana (2011) obtained good results for Acetylcholinesterase (AChE) activity of leaves, stem and flower extracts of *P. neochilus* due to the inhibition of AChE enzyme for all extracts tested.

It is known that the most common ethnobotanical use of *Plectranthus* envolves digestive purposes and also, but not so expressive, the cure of hangover. In this sense, a very interesting study of Brito et al. (2018) investigated the compound that the species of *Plectranthus* possessed in common and that justified those traditional uses. For this justification, this study explains that the effects of inhibition of the enzyme AChE, at muscular level, are responsible for catalyzing the hydrolysis of the neurotransmitter acetylcholine (transmit information for muscle contraction, in this case, intestinal motility), and inhibition of alcohol dehydrogenase (ADH), the enzyme responsible for the metabolism of alcohol. The nine species of *Plectranthus* which were studied (among them *P. neochilus)*, all had the compound rosmarinic acid in

common. The inhibitory action for AChE by *P. neochilus* was IC50 430 ± 50 μg/mL and by ADH, IC50 95 ± 8 μg/mL.

Beyond this work, previous studies verified the effect of its extracts, like the study of Tempone et al. (2008) that showed methanolic extract of its leaves had a marked determination of the 50% effective concentration (EC50) of 20.51 μg/mL, killing 100% of yeast (*C. krusei*) at the highest concentration. In the work of Antinarelli et al. (2015), the methanolic extract of *P. neochilus* leaves presented promising leishmanicidal activity, exhibiting IC50 values below 20 μg/mL in the *L. chagasi* species. The ethanolic extract of *Plectranthus neochilus*, in study of Arcanjo et al. (2012), showed an indication of antitumor activity, due to the lethality of the crustacean *Artemia salina*. The artemia lethality bioassay (*Artemia salina* Leach) is used for quality control of botanical products. This test promotes a correlation with antitumor activity (cytotoxicity) and can be used to monitor the activity of natural bioactive products. Thus, the authors attributed this activity by association with antitumor and larvicidal.

Table 3 - Summary of compounds identified in *P. neochilus* extractions and their respective biological activities.

Extraction	**Part of plant and compounds identified**	**Biological activity**	**Reference**
Aqueous	Leaves: caffeic acid, ferulic acid and coumaric acid	Antioxidant activity (DPPH and Phosphomolibdenic complex)	Ramborger et al., 2017
	Leaves: chlorogenic acid and rosmarinic acid	Antioxidant activity (DPPH)	Rijo et al., 2014
	Leaves: rosmarinic acid	AChE at muscular level and ADH	Brito et al., 2018
Methanolic	Leaves: -	Antioxidant activity, AChE	Viana (2011)
		Antifungal (*C. krusei*)	Tempone et al., 2008
		Leishmanicidal activity	Antinarelli et al., 2015
Ethanolic	Leaves and stems: -	Cytotoxic (lethality *Artemia salina)*	Arcanjo et al. 2012

	Leaves: Caffeic acid derivatives	Cytotoxic (cell lines of epidermoid carcinoma of head and neck)	Borges et al. 2016
Hexanic	Leaves: friedelin and others), steroidal compounds (sitosterol, among others), diterpenes and sesquiterpenes	Cytotoxic (tongue carcinoma cells: SCC-25)	
	Leaves and stems: friedelin, α-amirine fatty acid ester, sitosterol and stigmasterol	Antioxidant activity (DPPH), AChE	Viana, 2011
Ethyl acetate	Leaves and stems: flavone cirsimaritin	Antioxidant activity (DPPH), AChE	
Essential oil	Monoterpenes and sesquiterpenes	Antibacterial (Enterococcus faecalis, Streptococcus salivarus, Streptococcus sobrinus, Streptococcus sanguinis, Streptococcus mitis, Lactobacillus casei, Streptococcus mutans)	Crevelin et al., 2015
		Antifungal (R. stolonifera)	Aguiar et al., 2018
		Antischistosomal properties *in vitro*	Caixeta et al., 2011
		Reduction in female egg laying and oviposition inhibitor of Bemisia tabaci type B whitefly in tomato	Baldin et al., 2013; Fanela et al., 2016
		Antioxidant action (TBARS)	Rosal et al., 2011

Note: "-" means that authors who did not mention the compounds in this extract.

5 NON TRADITIONAL USES OF P. NEOCHILUS: DOMESTIC AND INDUSTRIAL APPLICATIONS

Although the traditional use of *P. neochilus* is widely reported in various parts of the world to treat stomach and liver problems, this plant has been shown to have utilities that are not taxed in medical terms, but ecological. In this case, the studies related to this aspect are about the phytoremediation capacity and the green roof technology.

5.1 Phytoremediation

The phytoremediation is a low-cost technique and a solution with less environmental side effects, which utilizes plants for the purification or decontamination of aquatic and/or terrestrial environments contaminated (Cunningham and Berti,1993; Islam et al., 2007). The interesting thing about phytoremediation is that it allows the removal of both organic and inorganic pollutants. The organic ones are those produced by the man and they cause damages to the organisms for being, in the majority, toxic and even carcinogenic. Depending on their properties, the organic pollutants can be degraded in the root zone of the plants and when absorbed they can be degraded, sequestered or volatilized. Inorganic matter occurs as natural elements in the earth's crust or in the atmosphere and can not be degraded, but phytoremediated by stabilization or sequestration by the tissues of plants that are treated later (Pilon-Smits, 2005). In order to perform the phytoremediation process it is necessary that the plants meet one or more requirements, such as: deep and dense root system; accelerated growth rate; easy harvest; which have resistance to the pollutant, pests and diseases; be adaptable to the site to be remediated, easy control or subsequent erradication (Pilon-Smits, 2005, Procópio et al., 2009).

In this sense, since *P. neochilus* has the characteristic of being able to be planted and grow throughout the year (Couto, 2006), a study pointed out that for this reason and with its capacity to be strongly resistant to climate and temperature changes, it is able to promote phytoremediation (Ramborger et al., 2017). In this case, the plant promoted the remediation of water contaminated with the acid 2,4-dichlorophenoxyacetic (2,4-D) pesticide. Likewise, in the study of Pereira (2018) the “boldo” also demonstrated this peculiarity, as it promoted the phytoremediation of the herbicide sulfentrazone also in water. One interesting detail of these two works, which aimed at the environmental remediation with this plant, is that they also showed an adaptation of the “boldo” in the water. Although it is a terrestrial plant, it showed an ability to stay alive and healthy throughout the experiment, being in contact only with tap water.

5.2 Green roof technology

The green roof technology consists in building roofs made of waterproofed slabs, followed by a draining layer and substrate, where small and medium-sized plant species are planted. The green roof technology is a viable and sustainable alternative to the traditional roofs and slabs because it provides shade, removes heat from the air, and reduces temperatures of the roof surface and surrounding air (EPA, 2019).

In this way, the research of Morau, Libelle and Garde (2012) used plants to perform an extensive green roof on Réunion Island (Indian Ocean - influenced by a humid tropical climate). According to the authors, the criteria for selecting the plants of the study were succulent plants exhibiting a strong capacity to store water in their leaves and to be highly resistant to drought.

The plants listed for this study were *Plectranthus neochilus, Kalanchoe thyrsiflora* and *Sedum reflexum.* As result, the *P.neochilus* showed capacity to decrease the temperature in the surface of the roof and contributed for an exchange of flow of low heat in this green roof. Therefore, *P. neochilus* presented significant results for its use as green roof, however it did not obtain the best performance comparing to other plants studied for this purpose.

6 CONCLUSIONS

In conclusion, *Plectranthus neochilus* has peculiarities according to the location, culture and climate where it is inserted. Its main use is in the traditional way, in the form of tea, however, recent studies of its essential oil or from solvents extraction have shown a greater applicability like in medicine use, to combat certain bacterial strains, schistosomiasis and fly, as well as for environmental remediation (phytoremediation) and environmental temperature control (green cover). Although this plant is extremely versatile, more comprehensive studies of its compounds in extracts are indispensable in specific pharmacological activities for experimental studies, because *P neochilus* is a plant with increasing use and research, having a promising future.

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CAPÍTULO III

Toxicological parameters of aqueous residue after using *Plectranthus neochilus* for 2,4-D phytoremediation

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Toxicological parameters of aqueous residue after using *Plectranthus neochilus* for 2,4-D phytoremediation

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Highlights

- Boldo tea is toxic to *Drosophila melanogaster*.
- Bioassays are important to evaluate the toxicity of the waste phytoremediation process.
- Phytoremediation reduces the effects of 2,4-D in roots of *Allium Cepa*.

Abstract

Phytoremediation is a technique that reduces the impact and environmental toxicity of toxic agents. *Plectranthus neochilus*, a species of aromatic plant, has already promoted phytoremediation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). In addition, it was unclear whether the degradation of 2,4-D alone allows for a non-toxic environment (decontamination efficiency). Therefore, the aim of the present study was to verify the changes of the volatile compounds and concentrated essential oil of P. neochilus after phytoremediation of 2,4-D and the subsequent antibacterial activity of this essential oil concentrate. In addition,

the toxicity of the plant's tea and the aqueous medium (waste) after the decontamination of 2,4-D was analyzed. The exposure to 2,4-D did not cause many changes in the volatile compounds, nor in the essential oil concentrate from the plant. Therefore, this essential oil concentrate can be used as an antimicrobial after phytoremediation. Regarding the use of this plant in tea form, it was found to be unsafe, even after phytoremediation, as this tea was toxic to the *Drosophila melanogaster* model (death of up to 100% of flies). The aqueous medium after 2,4-D phytoremediation became less toxic than the initial one (bioassays with *Artemia salina* and *Allium cepa* in the waste groups). However, the efficiency of phytoremediation with this plant must be improved. Therefore, we are performing new studies with *P. necohilus* and 2,4-D in aqueous medium.

Keywords: Boldo gamba; Decontamination; Herbicide; Xenobiotic; Volatile compounds; Non-target organism.

1. Introduction

The production and use of pesticides of varied classes are applied to eliminate pests. They are mainly used in agriculture, to increase the quantity and quality of agricultural products. On a household scale, these compounds promote the reduction of diseases transmitted by vectors, such as insects and rodents (Diel et al., 2003). Although the use of pesticides has all these advantages, the environment is often affected by these compounds and not always beneficial for non-target organisms. For example, recent cases of bee death and human health problems after application or drift of pesticides used in crops have been reported (Nocelli et al., 2012; OPAS/OMS, 2018; Van Engelsdorp et al., 2009). These are extremely important, considering the pesticide market in Brazil grew at an alarming rate (190%) in the last decade compared to the global market (93%), placing Brazil in the top world ranking since 2008 (Rigotto et al., 2014).

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is widely used worldwide. This chemical compound is a growth regulator and is applied to several crops to control broadleaf plants (Njoku et al., 2015). It is considered moderately toxic to humans and has a toxicological classification I (extremely toxic product) and a potential hazardous environment III classification (dangerous to the environment; NPIC, 2019). After application, several studies detected phenoxyacetic acids, a group that includes 2,4-D, in surface and underground waters (Azevedo et al., 2004; De Armas et al., 2007; Ribeiro et al., 2013). This presence is due to the physical and chemical characteristics of these compounds, such as water solubility, low pKa values, low biodegradability, and low adsorption coefficients in the soil (Carter, 2000; Chao et al., 2008; Fontaine et al., 1991; Hermosin et al., 2006; Nelson and Faust, 1969). In addition, its use has been gaining prominence, since 2,4-D was classified as the second best-selling pesticide in the country in 2017 (after glyphosate), at 57,389.35 tons (IBAMA, 2018).

In this sense, novel techniques that promote the degradation or elimination of this pesticide in contaminated places is extremely important. An economically viable and environmentally effective technique is phytoremediation, which uses the metabolism of plants themselves to carry out environmental decontamination (Olette et al., 2008). However, it is often unclear whether the contaminant causes metabolic changes in the plant. Also, in cases where the plant species produces some biological compound, it is important to know if the stress caused by the application of pesticides affects this production and, consequently, its biological action. In the case of “boldo gambá” (*Plectranthus neochilus Schltr.*), which

produces essential oil in its leaves and is known for its antimicrobial activity (Crevelin et al., 2015), it becomes interesting to analyze whether the stress caused by 2,4-D is able to increase this biological effect on bacteria. In addition, while this plant is capable of promoting phytoremediation of 2,4-D (Ramborger et al., 2017), it was unclear whether tea made from this plant can be used after this process.

Because studies with phytoremediation generally emphasize only the absence of the contaminant in the environment, as previously described by Eevers et al. (2017), which evaluated a variety of plant species for pesticide decontamination, it is highly relevant to assess the toxicity of these environmental residues after applying the technique. For this, biological models are used to promote toxicological and genotoxic analysis of solutions and environmental samples. To achieve this, the bioassay can be used with the microcrustacean *Artemia salina* (Montanher et al., 2003) and/or with roots of an onion (*Allium cepa)* (Fiskesjö, 1985; Grover et al., 1990). Therefore, the present study aims to 1) verify the involvement of the contaminant 2,4-D (aminol, commercial formulation) in the metabolism of *P. neochilus*, regarding the content of its volatile compounds, the essential oil concentrate and its biological antibacterial activity after phytoremediation; 2) analyze the toxicity of the plant's tea after phytoremediation; and 3) evaluate the decontamination efficiency of the aqueous residue after phytoremediation.

2. Materials and methods

2.1. Standards, reagents and equipments

For analytical curves and quantification of 2,4-D in waste groups, the 2,4-D standard (Sigma Aldrich, Steinhein, Germany) was used. Further, the 2,4-D used for phytoremediation tests was a commercial formulation (Aminol 806, Adama, Londrina/Brazil). The composition of the commercial formula contained 806 g/L (80.6% w/v) of dimethylammonium (2,4-dichlorophenoxy) acetate (2,4-D dimethylamine), 670 g/L (67.0% w/v) acid equivalent of 2,4-D, and 429 g/L (42.9% w/v) of unspecified ingredients. The solvents, dimethyl sulfoxide (DMSO) and ethyl acetate, were purchased from Neon (SP, Brazil). The solvents used in the chromatographic analysis (methanol and acetonitrile) were of chromatographic grade and obtained from J.T. Baker (Netherlands). High purity water from a Milli-Q Millipore system (Milford, MA, USA) was used for conditioning of the extraction cartridges and as a solvent for

the mobile phase. Ethanol and hydrochloric acid were obtained from Neon (SP, Brazil), and 1% acetic orcein (Dynamic) was obtained from Prolab, SP, Brazil.

Gas Chromatography Mass Spectrometry (GC-MS) analyses were performed using a gas chromatograph coupled to a mass spectrometer (Shimadzu model QP-2010Plus, Shimadzu Corporation, Kyoto, Japan) for the analysis of volatile compounds and the essential oil concentrate. The GC was equipped with an RTX-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), comprising a stationary phase of 5% diphenyl and 95% dimethyl polysiloxane.

The equipment for high performance liquid chromatography (HPLC) used for the degradation analysis of 2,4-D was the Young Lin (YL 9100) model, equipped with quaternary pump, auto sampler, and diode-array detector (DAD). The guard column was an analytical guard cartridge system (Security Guard phenomenex®) and C18 column (Inertsil ODS-3.5 μm; 4.6 × 250 mm - GL Sciences).

2.2. Plant

P. neochilus seedlings were collected from the Federal University of Pampa, campus Uruguaiana, RS and were registered in the university under the number 108/2016. For phytoremediation treatment, the plant seedlings, already rooted in pots with tap water, were used. To verify changes of volatile compounds, concentrated essential oil, antimicrobial action, and toxicity of the tea, only leaves that were frozen after phytoremediation and maintained frozen until the moment of analysis were used.

2.3. Experimental groups

Plants group: Blank - plant that has not been exposed to 2,4-D and was only in water for 30 days. - Treatment - plant that was exposed to 2,4-D (0.604 g/mL, equivalent to 1.209 kg/ha) in water, to promote phytoremediation, for 30 days.

Waste group: Control - water with 2,4-D, without the plant, for 30 days. Treatment - water with 2,4-D (0.604 g/mL, equivalent to 1.209 kg/ha) and with the plant, 30 days.

2.4. Analysis of variation of plant volatile compounds (GC-MS) after phytoremediation

This test was performed to verify possible stress caused by 2,4-D in the metabolism of plant volatile compounds after exposure to the herbicide (after phytoremediation). For this, two plant seedlings were used per bottle, and the test was performed in quadruplicate in a greenhouse (30 days), based on the procedure from Ramborger et al. (2017). The plant groups used were blank and treatment. For that, 2 g of leaf were collected (n = 3) on day zero, the day the herbicide was added to the water, and 30 days after application. The samples were collected between 8 and 9 a.m., and the light/dark cycle was dictated by sunlight. The volatile compounds were analyzed by GC-MS in headspace injection mode.

2.5. Extraction of essential oil (essential oil concentrate)

To verify if there was a change in compounds present in the plant's concentrated essential oil (in the plant, blank and treatment groups) after 30 days, hydrodistillation was performed, based on the study by Rosal et al. (2011). First, chopped leaves (200 g) were placed in the Clevenger equipment with 1000 mL of distilled water. From the condensation of water vapor and the consequent accumulation of essential oil, manual collection of water and oil from the equipment was performed every hour after boiling began, totaling 5 h of extraction. After each collection, liquid-liquid separation was performed in a separating funnel using ethyl acetate as the extraction solvent. Then, anhydrous sodium sulfate was added to completely remove the remaining water in the mixture of solvent and oil. Then, the solvent was evaporated in a rotary evaporator at 30 °C. For GC-MS analysis, the essential oil concentrate was resuspended in ethyl acetate (1 mL) in vial-type bottles that were stored in a refrigerator (4 °C) until analysis. For the microbiological test, the essential oil concentrate was resuspended in 20% DMSO and 80% ultrapure water in bottles (Crevelin et al., 2015), which were stored in a refrigerator (4 °C) until analysis.

2.6. GC-MS conditions: volatile compounds and oil extract

For both analyses, the GC-MS equipment and Rtx-5MS chromatographic column were used. For the analysis of volatile compounds, "headspace" injection mode was used, with 2 g of leaf incubated for 15 min at 80 °C. Afterwards, the injection was performed in the chromatographic column with an injection temperature of 250 °C, split injection mode, and helium gas flow (0.95 mL/min). The total analysis time was 31 min. The spectrum acquisition mode was full scan, with a mass of 50–600 (*m*/*z*). Compounds were identified through their

retention times, and their fragmentations were compared with the spectral library in the equipment's data system. This test was performed in quadruplicate.

For the reading and quantification of compounds present in the essential oil concentrate, a direct injection of the split mode was performed in the chromatographic column. The conditions (temperature, heating ramp, gas, acquisition of the spectrum, and identification of the compounds) were the same as used for the "headspace" analysis. This test was performed in duplicate.

2.7. Antimicrobial test of the essential oil concentrate

The minimum inhibitory growth (MIC) technique was used by quantitative microdilution in broth for the different extracts (plant group: blank and treatment). Gram positive and negative bacterial strains, standardized by the American Type Culture Collection (ATCC), were used for MIC: *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonie* (ATCC 700603), *Acinetobacter baumannii* (ATCC BAA-747), and *Enterococcus faecalis* (ATCC 29212). This methodology was based on the broth microdilution technique (CLSI, 2016), where Muller Hinton broth (MH) was used in a 96-well "U" bottom plate and in duplicate for each tested microorganism. Two controls were used on each plate: a positive growth control, in which only the microorganism was inoculated, and a negative growth control, in which only the MH broth was added. The gradual range of concentration of the oil extract was from 64 mg/mL for plant groups. For the inoculum, a suspension in 0.9% saline solution was prepared until the turbidity reached an equivalent of 0.5 McFarland (108 UFC/mL) for each microorganism. Subsequently, this solution was diluted 1:100 in MH broth. The final volume in each well was 0.1 mL. The plates were incubated at 35 °C (±2 °C) for 18–24 h. The result was read through visual verification with the aid of light contrast to determine the MIC.

2.8. Toxicity tests of *P. neochilus* tea after phytoremediation in *Drosophila melanogaster (DM)*

2.8.1. Preparation of tea

The tea was prepared according to Ramborger et al. (2017) with brief modifications. The leaves of the two plant groups (blank and treatment) were placed in a beaker with boiling water and heated on a heating plate for 10 min. The tea was cooled to room temperature and

filtered with cotton. Then, the teas were subjected to water evaporation in a rotary evaporator (45 °C, 150 RPM) until a dry extract was obtained. Posteriorly, this extract was dissolved in distilled water with 1% sucrose at a concentration of 5 mg/mL for the DM toxicity test.

2.8.2. DM toxicity test

The fly species used was DM, of the wild type, obtained from the National Species Stock Center, Bowling Green, OH, USA. The flies were bred on a standard corn meal diet with yeast granules as the protein source at constant temperature and humidity (22 ± 1 °C; 60% relative humidity), under 12 h/12 h light/dark.

For the analysis of tea toxicity, the continuous liquid feeding method, described by Soares et al. (2017), was used, where flies (n = 20) of both sexes and aged 4 days were separated into the following groups: 1) control, which received only the solution of distilled water with 1% sucrose; 2) blank, which received the tea solution (5 mg/mL) with 1% sucrose of the blank plants group; and 3) treatment, which received the tea solution (5 mg/mL) with 1% sucrose of the treatment plants group. The flies received this feed every 24 h, and a count of surviving DM during this period was conducted. This treatment was continued for 7 days, and the concentration of tea used was based on the median lethal dose (LD_{50}) concentration of DM performed previously (data not shown). In addition, the same temperature, humidity, and light conditions were maintained during the experiment period.

2.9. Waste analysis with 2,4-D after 30 days

The percentage of degradation of 2,4-D in the residual water was verified with the herbicide aminol (2,4-D) from the control waste group (without the plant) and from the treatment waste group (with the plant), based on the work of Ramborger et al. (2017). Sample preparation consisted of extraction and concentration of 2,4-D from water through solid phase extraction (SPE; cartridge: Strata-X with pore size of 55 μm in 500 mg from Phenomenex) using 3 mL of methanol, 3 mL of ultrapure water, and 3 mL of ultrapure water pH 3 (phosphoric acid) for conditioning the cartridge. Afterwards, percolation of the sample (10 mL), acidified at pH 3, was carried out with subsequent washing of the cartridge with 3 mL ultrapure water (pH 3) and drying with 10 min of vacuum. To elute the compound (2,4-D), 10 mL of methanol was used, and the cartridge was dried again with 10 min of vacuum. The sample was evaporated on a rotary evaporator (40 °C) and resuspended in 1 mL of methanol for further

chromatographic analysis by HPLC-DAD. The methodology data by HPLC-DAD was a mobile phase with acetonitrile/methanol/ultrapure water (30:24:46 v/v/v) at pH 3 with a flow of 0.8 mL/min. Isocratic mode was used for 20 min, and 20 μL of sample was injected into the chromatographic system. The detection wavelength of 2,4-D was 220 nm. The line equation was $y = 60.6\times - 15.824$, $R^2 = 0.9975$, and the points on the curve were 2, 4, 6, 8, 10, and 12 mg/L. For the 2,4-D analysis method, we found an LOD and LOQ of 0.17 mg/L and 0.512 mg/L with the instrumental condition used in this experiment.

2.10. Waste toxicity bioassays with *Artemia salina* and *Allium cepa*

The toxicity of residual water (waste group) after 2,4-D phytoremediation was carried out using the bioassays *Artemia salina* and *Allium cepa*, which made it possible to verify the efficiency of decontamination by the plant. First, the LD_{50} of the microcrustacean *A. salina* was performed on the herbicide aminol (2,4-D), according to Meyer et al. (1982) with changes. For this, cysts were induced to hatch in saline (NaCl 0.9%) in a ventilated environment for 24 h. The nauplii were collected and transferred individually to a 96-well microplate containing different concentrations of herbicides, which was designated as a treatment group, and for the control group, saline solution was used. After 24 h, the number of dead nauplii were quantified to acquire the LD_{50}, which was the concentration necessary to kill 50% of the nauplii. The tests were performed in triplicate with n = 30 nauplii for each test. An $LD_{50} < 1000$ μg/mL was considered toxic. Afterwards, the toxicity of the aqueous residues was tested with aminol of the two residue groups (control and treatment).

The bioassay with roots of the onion (*A. cepa*) was used to estimate the toxicity of the herbicide aminol, and this methodology was based on the work of Bezerra et al. (2016). In this test, onion root growth was the parameter used to analyze the toxicity of the treatments, and cell divisions and chromosomal anomalies were verified as parameters of cytotoxicity and genotoxicity. The bulbs of *A. cepa* were obtained from a local supermarket, with a healthy appearance and not germinated. The bases of the onion bulbs were placed in contact with distilled water to develop roots (48 h). The outermost bark and the aged or dry roots of the bulb were removed to prevent rot. After a period of root growth, the onions were put in contact with the solutions of the following groups: 1) Positive Control Glyphosate 15% (CPG+); 2) Positive Control 2,4-D, 0.604 g/mL (CP2.4-D); 3) Treatment 1, waste group: control, water with 2,4-D, without the plant, for a period of 30 days (T1); 4) Treatment 2, waste group: treatment, water

with 2,4-D and with the plant, for 30 days (T2); 5) Negative Control, distilled water (NC); and 6) Blank Negative Control, water that was in contact with the plant for 30 days (BNC).

To analyze toxic effects of solutions from each group, the root lengths were measured during the 144 h of contact of the roots with these solutions. The roots were measured every 24 h for comparison. The cytotoxic effect of each solution was analyzed using the mitotic index, where cells in any division phase (prophase, metaphase, anaphase, and telophase) are added, dividing by the total number of cells counted, and multiplying by 100. All types of chromosomal aberrations found (irregular anaphase, chromosomal bridges, and micronuclei) were observed for the analysis of genotoxic effects. For both tests (cytotoxicity and genotoxicity), the following procedures were carried out: after 24 h of exposure of the onions' roots in these solutions, the root apexes were measured (2 mm), cut, and fixed in Carnoy I solution (95% ethanol and acid glacial acetic acid, 3:1 v/v) for 24 h. The roots were washed with distilled water and placed in 70% ethanol until staining and observation of the slides. To prepare the slides, the roots were hydrolyzed in HCl 2 N for 5 min, washed, and placed under the slides, where they were in contact with the acetic orcein solution (1%) for 15 min. Afterwards, coverslips were placed under the roots and acetic orcein (1%), wrapped in paper towels to remove excess dye, and crushed with the thumb. The slides were analyzed using an optical microscope (Nikon Elcipse E200, China) with a 1000× objective. For each treatment, three bulbs were used, and for each bulb, a slide was made. There were 1200 cells counted per group.

2.11. Statistical analysis

For quantification of volatile compounds and compounds present in the essential oil concentrate on different phytoremediation days, data are presented as means ± Standard Deviation (SD). Significant differences between the Blank group and treatment group were determined by one-way analysis of variance with Tukey's post-test. For tests with *D. melanogaster*, Anova one-way was used with a Bonferroni post-test, and the 2,4-D toxicity data for *Artemia salina* were calculated according to the LD_{50}. For cytotoxicity and genotoxicity data (root growth and chromosomal aberrations) of the bioassay with *Allium cepa*, mean ± SD was calculated, and significant differences were determined by the analysis of variance (one-way) with a Bonferroni post-test. For all tests, p values < 0.05 were considered significant, and Graph Pad Prism version 6.0 was used.

3. Results and discussion

3.1. Variations of volatile compounds and the essential oil concentrate from *P. neochilus* leaves

In this study, the stressful effect of 2,4-D on the plant, through the increase, decrease, or appearance of new compounds was verified. The *P. neochilus* antimicrobial activity is well documented, and here we determined whether the changes caused by 2,4-D would affect this beneficial effect. In Fig. 1, the data related to the changes of volatile compounds after exposure of the plant to 2,4-D are presented. This result aimed to verify if 2,4-D affected the production of volatile compounds in the leaf and to correlate to compounds of the essential oil extract of leaves, identified through GC-MS.

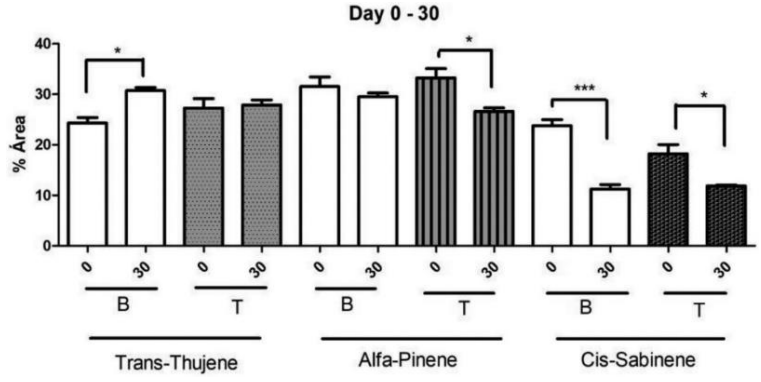

Fig. 1. Variation of the majority of volatile compounds by GC-MS (headspace mode) of *P. neochilus* leaves during 30 days of phytoremediation. Plant groups: blank (B) and treatment (T).

Of the 21 compounds identified in the groups during phytoremediation, only three were in a higher percentage (11.25–33.25%; trans-thujene, alpha-pinene, and cis-sabinene) compared to the others (isooctane, hexamethylethane, bicyclo [4.1.0] hept-2-ene 3.7.7-trimethyl, beta-cis-ocimene, beta-ocimene, 3-carene, cis-sabinene hydrate, alpha-cubebene, copaene, delta-cadinene, caryophyllene, 1,4,7-cycloundecatriene, 1,5,9,9-tetramethyl-, Z, Z, Z-, germacrene D, (+)-ledene, 10s. 11s-himachala-3 (12). 4-diene, isoledene, and caryophyllene oxidize), which were 0.03–7.99%. When comparing the variation profile of the compounds in 30 days of phytoremediation for the blank and treatment groups, trans-thujene was observed to increase significantly on day 30 for the blank group. This variation did not occur in the treatment group. Therefore, trans-thujene may be correlated with 2,4-D metabolism/degradation by the plant.

This assumption was based on the fact that the blank group used refers to a natural alteration of the plant in relation to abiotic conditions (light, temperature, and aqueous medium), and the treatment group, which was in the same conditions as the blank group, represented change caused by 2,4-D. The alpha-pinene compound showed a significant decrease on day 30, only in the treatment group, and this may be related to the stressor effect caused by 2,4-D. Finally, cis-sabinene showed a significant decrease both in the blank (day 30) and treatment (day 30) groups. In this case, there was no correlation with 2,4-D but rather with some abiotic factor. Data in Fig. 1 revealed that the stress caused by the herbicide can slightly affect the metabolism of biosynthesis or degradation of volatile compounds detected in fresh leaves. Data regarding the variation of volatile compounds present in the concentrate of the essential oil are shown in Fig. 2.

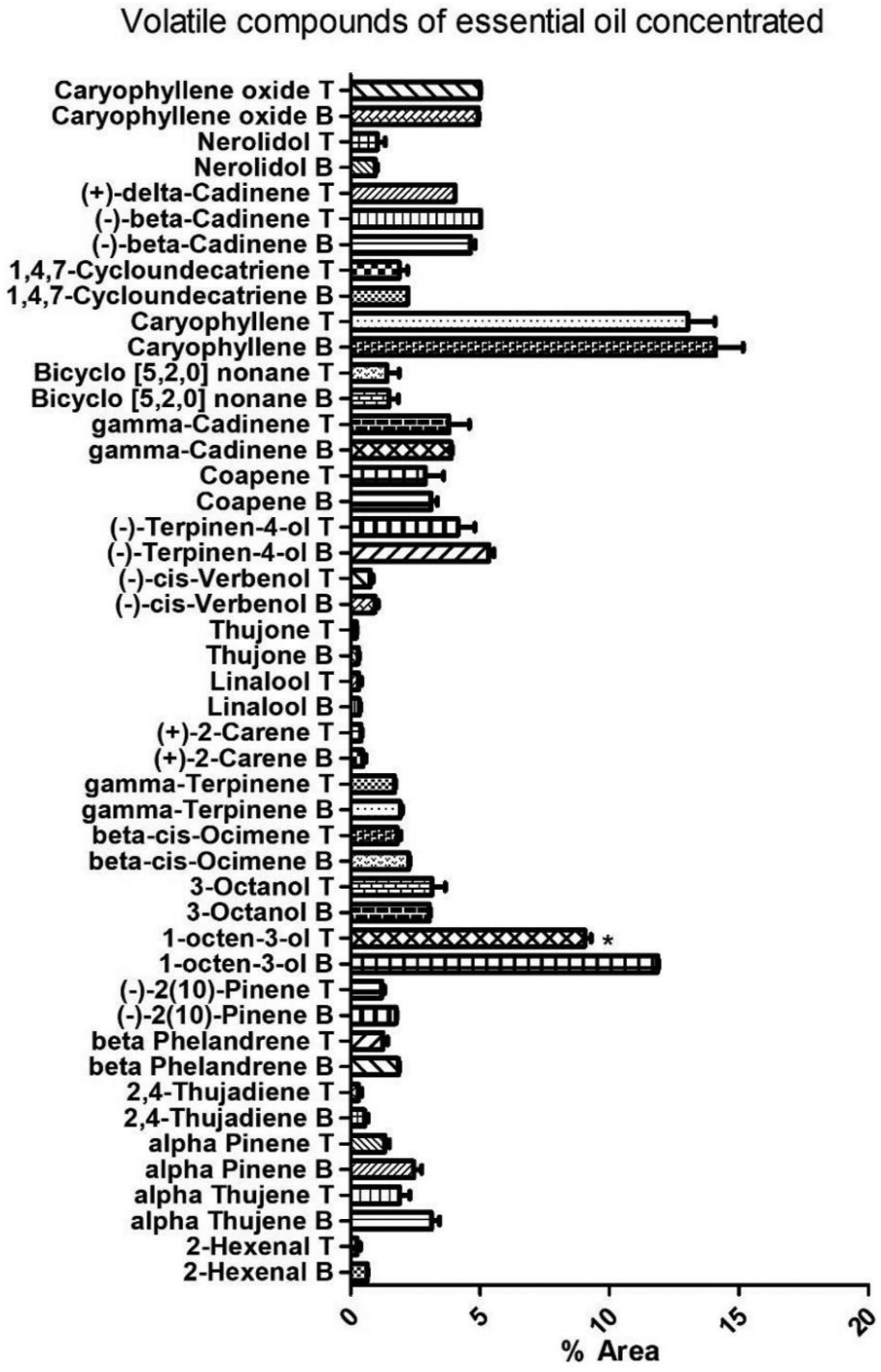

Fig. 2. Compounds present in the essential oil extract from the leaves of *P. neochilus.* Plants group: blank (B) and treatment (T) after 30 days of exposure.

Through the action of 2,4-D in plants, which leads to a change in the intracellular and extracellular redox state due to an overproduction of reactive oxygen species (Grossmann, 2010), the biosynthesis of many aroma compounds present in the treatment group were expected to be significantly affected. However, from the analyzed compounds, only 1-octen-3-

ol obtained a significant decrease (2.81%) in the treatment group compared to the blank group. This difference occurred in the same period (after 30 days of phytoremediation) and may be related to stress caused by 2,4-D in the plant, since 1-octen-3-ol activates defense genes in plants (Kishimoto et al., 2007). This behavior was similar to that shown by Ramborger et al. (2017), who also obtained a decrease in antioxidant defense activities of *P. neochilus* when exposed to 2,4-D. A compound that appeared only in the treatment group was (+)-delta-cadinene (4.04%), a sesquiterpene. This compound may have been biosynthesized due to the metabolic stress caused by 2,4-D; however, (+)-delta-cadinene was in a low percentage and belongs to the cadinene group (which was present in groups B and T). Therefore, it was, again, found that the herbicide minimally affects the metabolism of the aroma compounds of *P. neochilus*, and this is in accordance with the data in Fig. 1. Regarding research related to the toxicity of pesticides, which use 2,4-D for both toxicity and phytoremediation, the data presented are generally about parameters of inhibition rate or growth of plants after exposure, visual intoxication, number of leaves, and chlorophyll production (Peterson et al., 1994; Sota et al., 2003; Wong, 2000) or characteristics of the plant, to select a new phytoremediation plant (Aguiar et al., 2018). In this sense, studies that used 2,4-D and verified the production of secondary compounds were those that used 2,4-D in low concentrations to stimulate the production of these compounds, such as the production of alkaloids by *Hyoscyamus albus L* (Kadi and Yahi, 2007). Martínez-Ruiz and Martínez-Jerónimo (2018) evaluated parameters that match the production or alteration of compounds by microalgae and cyanobacteria (*Ankistrodesmus falcatus* and *Microcystis aeruginosa*), where it was observed that 2,4-D increased the concentration of pigments and macromolecules (carbohydrates, lipids, and proteins) and modified the activity of all evaluated enzymes (catalase, glutathione peroxidase, and superoxide dismutase), as well as ultrastructural changes. Most importantly, they observed a stimulation of the production of cyanotoxins under stress by 2,4-D, which affects the biota of aquatic environments, and this data corroborates our results. The work of Chrysargyris et al. (2019) observed that stress caused by exposure of salinity and copper concentrations in aqueous medium promoted changes in the metabolism of the primary compounds of *Mentha spicata L.*, in favor of the biosynthesis of the main volatile components of the essential oil of the plant. However, few studies focus on the biological production of secondary compounds from plants after 2,4-D phytoremediation, and this is a determining factor in the final destination of the plant.

3.2. Bacterial MIC

In Table 1, the results regarding the bacterial inhibitory concentration of the essential oil extract of the plant groups (blank and treatment) are presented. These results revealed that there was no significant difference after exposure, as both the blank and treatment groups showed the same inhibition range.

Table 1. MIC of bacterial strains in MH broth with oil extract. Test concentration range: 0.06–32 mg/mL. Both groups were solubilized in water/DMSO (80:20 v/v).

Microorganisms	MIC value in mg/mL	
	Blank	Treatment
Escherichia coli (ATCC 25922)	32	16
Pseudomonas aeruginosa (ATCC 27853)	32	32
Klebsiella pneumonie (ATCC 700603)	32	32
Acinetobacter baumannii (ATCC BAA-747)	16	16
Staphylococcus aureus (ATCC 29213)	16	8
Enterococcus faecalis (ATCC 29212)	4	4

The values found may be above those considered low antibacterial activity (1000 µg/mL; Ríos and Recio, 2005) in both groups. However, this essential oil was extracted from a small amount of viable leaves for the phytoremediation groups (blank and treatment), showing that it can be used as an antimicrobial. Studies with the pure essential oil of this plant showed that it was necessary to use 1200 g of leaves, with repetitions of this extraction, to extract the oil from it, since *P. neochilus* has little essential oil. However, its oil had an antimicrobial effect, which was considered medium to high by Crevelin et al. (2015). Due to the fact that *P. neochilus* is a terrestrial plant, where its root growth may be in water (vegetative propagation) (Codd, 1985), oil production was expected to be affected in this condition, as the plant remained in the water for 30 days. In the case of plants exposed to 2,4-D in the plant group (treatment), where a small difference was observed in some compounds, it was expected that there would be a change in the action of the essential oil extract on the bacteria. Taking into account the results of variations of volatile compounds and compounds present in the oil extract, along with those of biological activity, 2,4-D did not cause stress on the plant to the point of affecting the biological activity of the oil extract in the bacteria. Therefore, the plant can be used for phytoremediation purposes, and later, its oil can still be utilized.

3.3. Toxicity of tea from *P. neochilus* leaves in DM

The results obtained with the dry tea extract from the different plant groups (blank and treatment) after 30 days of the experiment (Fig. 3) showed that both were more toxic than the control group, and the dry tea extract from the treatment group was more toxic than the blank group at the same concentration. Therefore, the boldo tea is unfit for consumption after its use in phytoremediation.

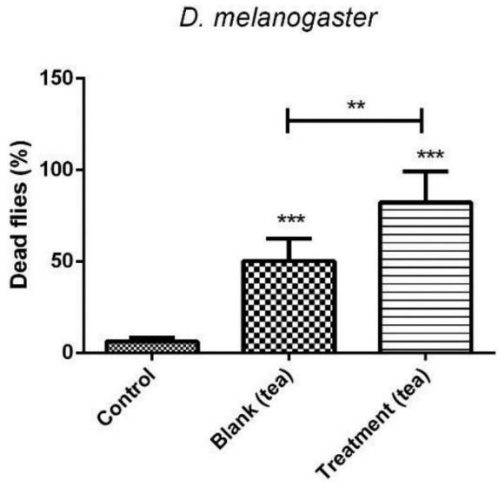

Fig. 3. Mortality of DM after receiving dried extract of boldo tea in the plant groups (blank and treatment) of phytoremediation. Values are considered significant in relation to the control group and between the blank and treatment groups.

Studies in the genus *Plectranthus* species describe compounds such as abietane-royleanone type, phenolic compounds (such as rosmarinic acid), essential oils and flavonoids as responsible for the antimicrobial activity (Pereira et al., 2015; Rijo et al., 2013; Mogib et al., 2002; Renee et al., 2010). Brito et al. (2018) shows in their work that rosmarinic acid present in the species *Plectranthus* is responsible for AChE inhibition, thus the mortality of DM in the Blank is higher than the control.

The fact that the treatment group showed greater toxicity may be due to the presence of 2,4-D in plants (stored or in excess) or due to the increase in oxidative stress caused by 2,4-D's mechanism of action in vegetable cells. This generated stress occurs due to the decrease in the performance of mitochondria and peroxisomes (Rodriguez-Serrano et al., 2014) and increased ethylene biosynthesis in cells (Grossmann, 2010). The decrease in the antioxidant capacity of

P. neochilus tea after 2,4-D phytoremediation was verified by Ramborger et al. (2017), evidencing this fact.

3.4. Degradation of 2,4-D after 30 days (HPLC-DAD) and toxicity bioassays: *A. salina* and *A. cepa*

The degradation analysis of 2,4-D was performed to verify the phytoremediation of 2,4-D by the plant over 30 days, a period that was chosen for all previous tests. The result obtained on day zero (0), the day on which aminol (2,4-D) was added to the water for both residue groups, was 5.18 mg/L of 2,4-D. In other words, this was the initial concentration. After 30 days, 4.42 mg/L (degradation equivalent to 14.8%) of 2,4-D was detected for the control residue group and, for the treatment residue group, 3.68 mg/L (degradation equivalent to 29.1%). These results showed that 2,4-D degradation occurred, and this degradation was greater with the use of the plant in this period, which is in accordance with the results of Ramborger et al. (2017). Other studies have obtained a higher rate of removal of this herbicide, as in the case of González et al. (2019), who obtained a reduction of up to 98% of 2,4-D (20 mg/L), using the set plant, microorganisms, and dissolved organic carbon. However, few studies have observed the efficiency of remediation used, considering the toxicity of the medium (waste solution) after remediation. One of the studies that verified the toxicity of the residue after removing 2,4-D was by Samir et al. (2015), who verified the germination and growth of *Lepidium sativum* seeds, which were considered sensitive to the herbicide after remediation of 2,4-D with pretreatment with Ultraviolet/Titanium dioxide (UV/TiO_2) and subsequent microbial inoculation. In addition to this, Nobre et al. (2019) verified the toxicity of the medium through the growth of *A. cepa* roots after using titanium dioxide ($nTiO_2$) nanoparticles with heterogeneous photocatalysis for the purposes of environmental remediation.

With these results, analyses of residual water toxicity were carried out in the control and treatment groups, referring to the waste with the experimental models *A. salina* and *A. cepa* (Fig. 4). Toxicological tests were performed using bioassays with *A. salina* and *A. cepa* in the water residue after phytoremediation, as a way to evaluate the efficiency of this technique. Although both the present study and work by Ramborger et al. (2017) confirmed that 2,4-D decreased its concentration in the phytoremediation group, it was not known whether it would make the aqueous medium even more toxic. This could happen through a process of self-degradation to a more toxic compound, such as 2,4-Dichlorophenol (2,4-DCP), for example, or by the elimination of this more toxic compound after metabolization of 2,4-D by the plant.

Therefore, the present study performed two techniques to resolve this question, and phytoremediation was found to be effective because it promoted the decrease of 2,4-D and made the aqueous medium less toxic.

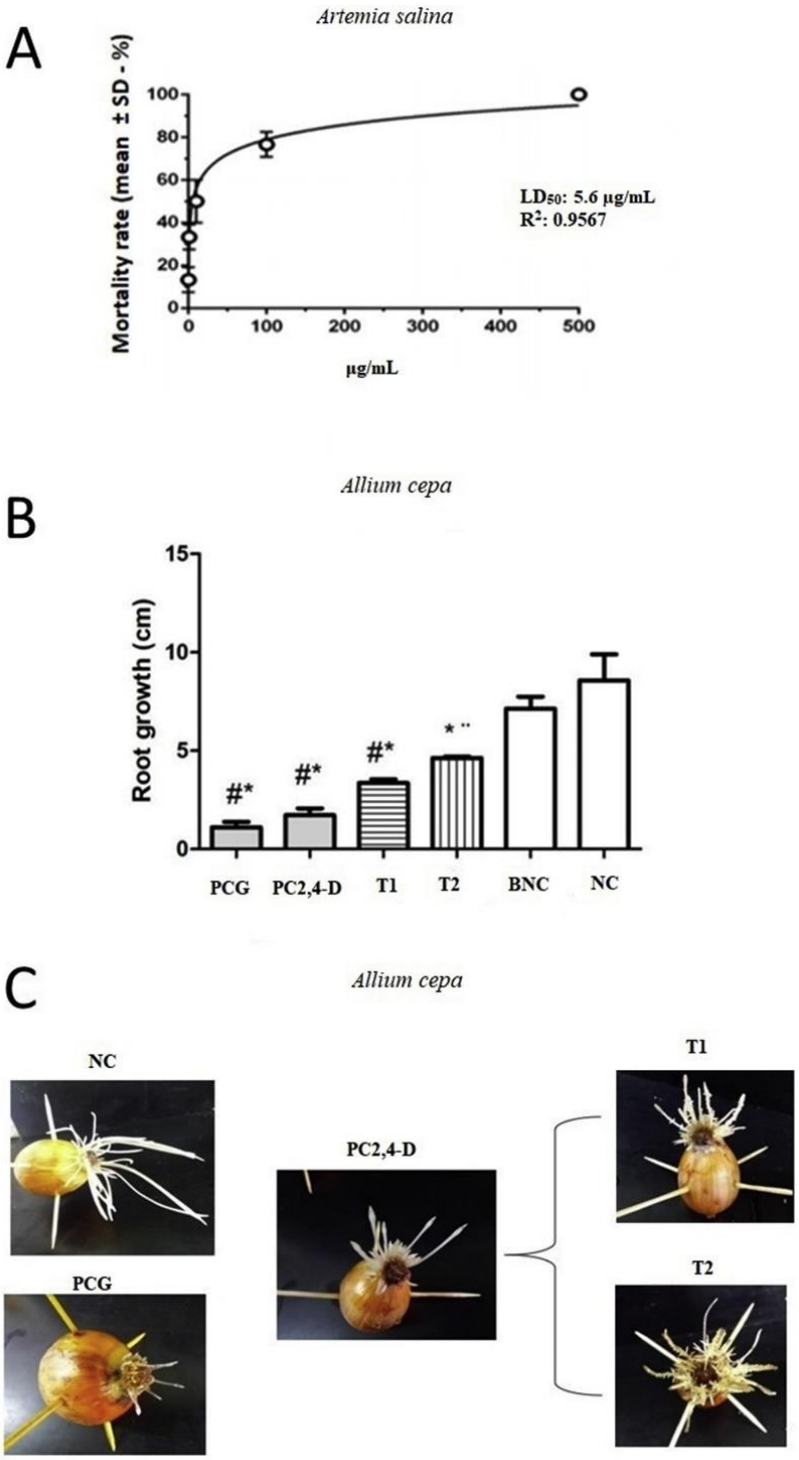

Fig. 4. Toxicity bioassays. **A**) LD_{50} of 2,4-D in *Artemia salina*. **B**) Growth of the roots of *Allium cepa*: Positive Control Glyphosate 15% (PCG); Positive Control 2,4-D 0.604 g/mL (PC2,4-D); treatment 1 (T1), aminol control waste, 2,4-D after 30 days in water without the plant; treatment

2 (T2), waste treatment (phytoremediation) of aminol, 2,4-D after 30 days in water with the plant; Blank Negative Control (BNC), water that was in contact with the plant for 30 days; and the Negative Control (NC), distilled water. # significant difference in relation to BNC; ∗ significant difference in relation to NC; ·· significant difference in relation to PCG. **C**) Photos of the size and shape of the roots of *Allium cepa* in the control and waste groups.

The bioassay with *A. salina* is an acute toxicity test (24–48 h), where the mortality of a specific number of the microcrustacean is verified, and the LD_{50} is calculated after 24 h. This technique can be used to assess the toxicity of pure compounds, contaminated sea water and estuarine/marine sediments, effluents, and biotoxins. (Kokkali and van Delft, 2004). Therefore, LD_{50} determination of the herbicide aminol (2,4-D) was carried out in the biological model of *A. salina*, and a value of 5.6 μg/mL (Fig. 4A) was obtained and classified as toxic, according to McLaughlin (1991).

After determining the LD_{50}, the toxicity of the aqueous medium in the residue groups (control and treatment) was verified after the 30 days of experiment, and normal growth of this microcrustacean was obtained in these media. Therefore, both aqueous residues were not toxic to *A. salina* (data not shown). Although 2,4-D, at a concentration of 5.6 μg/mL, which is equivalent to 5.6 mg/L, caused toxicity (Fig. 4A), the results pertinent to the waste groups (control and treatment) were not toxic, since they were below this concentration (4.42 mg/L for the control group and 3.68 mg/L for the treatment group). However, with this result, it can be concluded that the aqueous medium where the plant was inserted does not produce more toxic compounds after phytoremediation.

The onion root bioassay (*Allium cepa*) is a sensitive test that has been used for the determination of anti-mitotic, cytotoxic, and/or genotoxic effects of various chemical substances and preparation with plants (Timothy et al., 2014). This test has been considered favorable to assess damage and chromosomal disorders in the mitotic cycle, due to the presence of good chromosomal conditions, such as large chromosomes with a reduced number (2n = 16) (Fiskesjö, 1985). There are no studies in the literature specifically using *A. salina* exposed to 2,4-D. However, many studies use this biological model as a bioindicator of toxicity in waters contaminated with pesticides (Barahona and Sánchez-Fortún, 1999; Borba et al., 2019; Cruzeiro et al., 2017; Gambardella et al., 2018; Ivorra et al., 2019; Varó et al., 2002). Other works verified the acute LC_{50} of 2,4-D with *Astacus leptodactylus* (non-target organism), and the value obtained was considered highly toxic for this species (32.6 mg/L; Benli et al., 2007).

Other studies used *in vivo* models, such as zebra-fish (*Danio rerio*) and *Cnesterodon decemmaculatus*, where a decrease in survival rate was observed (IC_{50} = 46.71 mg/L and 1008 mg/L), among other damages observed in these models (Li et al., 2017; Ruiz de Arcaute et al., 2016).

Results regarding the toxicity of the roots of *A. cepa* in contact with the waste groups (control and treatment) after phytoremediation showed toxicity in the two waste groups (Fig. 4B), as in both was observed a significant decrease in relation to the negative control (distilled water, NC). However, the control residue (T1) had greater toxicity than the blank negative control (BNC), but not from the T2 group, referring to the treatment residue group. In addition, T2 showed a significant difference in relation to the Positive Control Glyphosate, and this did not occur in T1. Therefore, once again, the phytoremediation technique was more effective in decontaminating water than using no treatment. These root results can be seen in Fig. 4C.

A factor that may have contributed to the toxicity in treatment 2 (T2) as well as the reduction of root growth in Treatment 1 (T1; Fig. 4B) could be the products of degradation or extraction of the plants, from the leaves and roots found in the bottles after the treatment period. Allied to this, the observed pH values were different in the two treatments, as following: 7.4 in the BNC, 9.51 in the control waste group (T1), and 8.4 in the phytoremediation treatment waste group (T2).

The data related to cytotoxicity of the onion roots are presented in Table 2 and showed that, in both groups (control (T1) and treatment (T2)), there was a decrease in the cell cycle. This data is linked to the reduction in the number of mitoses, meaning a reduction in the number of dividing cells that occurred in these groups compared to the negative controls. A large number of cells were observed in the interphase, a period in which the cell is able to divide and originate daughter cells, in the PCG, T1, and T2 groups but were low in the other phases of the cell cycle (prophase, metaphase, anaphase, and telophase), indicating toxicity of the residues (control and treatment). The mitotic index refers to the percentage of total mitoses in each group and evidenced the same result, which was the toxicity of the residues.

Table 2. Number of cells in each cell cycle (interphase, prophase, metaphase, anaphase, and telophase) in *A. cepa* root meristems in the solution groups after 1 month of treatment.

Cell division	Treatments				
	PCG	T1	T2	BCN	NC
Interphase	1183	1162	1166	1031	973
Prophase	08	24	10	90	123
Metaphase	04	04	09	16	24
Anaphase	00	04	04	23	30
Telophase	05	06	11	40	50
Total mitosis	17#*	38#*	34#*	169*	227
Mitotic index (%)	1,41#*	3,16#*	2,83#*	14,08*	18,91

Positive control group (PCG): 15% glyphosate; treatment 1 (T1): control waste group; treatment 2 (T2): waste treatment group (phytoremediation); blank negative control (BNC): water that was in contact with the plant for 30 days; and negative control (NC): distilled water. # significant difference in relation to BNC; ∗ significant difference in relation to NC. 1200 cells were analyzed per treatment.

The results presented in Table 3 correspond to the capacity of these residues to cause changes in the genetic material (chromosomal abnormalities, genotoxicity) of *A. cepa* cells.

Table 3. Number of chromosomal aberrations in *A. cepa* root meristems in the solution groups after 1 month of treatment.

Aberration	PCG	T1	T2	BNC	NC
Retardation	00	03	05	02	00
Chromosomal bridges	05	00	01	00	01
Fragments	10	00	01	00	00
Loose chromosomes	06	00	00	00	00
Micronucleus	00	00	00	01	00
Total	21	03··	06··	03··	01··

Positive control group (PCG): 15% glyphosate; treatment 1 (T1): control waste group; treatment 2 (T2): waste treatment group (phytoremediation); blank negative control (BNC):

water that was in contact with the plant for 30 days; and negative control (NC): distilled water. # significant difference in relation to BNC; ∗ significant difference in relation to NC. # significant difference in relation to BNC; ∗ significant difference in relation to the NC; ·· significant difference in relation to PCG. 1200 cells were analyzed per treatment.

Structural chromosomal changes can be induced by several factors, such as DNA breaks, inhibition of DNA synthesis and altered DNA replication. The numerical AC, for example aneuploidy and polyploidy, are consequences of abnormal chromosome segregation, which can occur spontaneously or through the action of aneugenic agents. Chromosomal Aberrations (CA) are characterized by changes in any chromosome structure or in the total number of chromosomes, which can occur spontaneously and as a result of exposure to the body or chemical agents. A study to evaluate the genotoxic effects of the herbicides pentachlorophenol (PCP), 2,4-D and butachlor by the test of A. cepa showed significant values of chromosomal aberrations (CA) for all the tested chemicals. To assess chromosomal abnormalities by the A. cepa test, several types of AC are considered in the different phases of the dividing cell (prophase, metaphase, anaphase and telophase) (Leme and Marin-Morales, 2009). These data showed that no group was able to alter cell division significantly in relation to PC. Thus, residues T1 and T2 were considered toxic and cytotoxic only. The results obtained in the present study with 2,4-D are in agreement with Rambo et al. (2017) and Nobre et al. (2019), who used the roots of *A. cepa* as a model of cytotoxicity and genotoxicity, and Özkul et al. (2016), who obtained a reduced mitotic index with an increased concentration of 2,4-D and chromosomal aberrations, as well. In other models, the results were similar to the case of Ruiz de Arcaute et al. (2016), who obtained an increase in the frequency of micronuclei in erythrocytes from the peripheral blood of *C. decemmaculatus*, induced by the herbicide formulation based on 2,4-D. Laborde et al. (2020) observed the genotoxic and cytotoxic potential of 2,4-D in Chinese hamster ovary cells (CHO–K1), evidencing the toxicity of this compound and correlating with the findings of the present study. Finally, according to Zuanazzi et al. (2019), research on 2,4-D toxicology and mutagenicity has advanced rapidly and may focus on the area of molecular biology, such as gene expression, assessment of exposure in human bioindicators, or other vertebrate bioindicators, in addition to studies of degradation of pesticides.

4. Conclusions

This study has shown that *P. neochilus* can be used for antimicrobial activity after phytoremediation of 2,4-D, since the composition of the volatile compounds and the essential

oil concentrate were not changed in the blank and treatment groups (plant group). Although we have not achieved a promising effect in the tests with bacteria, we suggest using more plants for extracting the pure essential oil in future studies, since previous studies have linked the essential oil of *P. neochilus* to high bacterial activity. Consumption of plant tea after phytoremediation is not advisable because the plant tea extract (5 mg/mL) showed toxicity in the alternative *in vivo* model (*D. melanogaster*) from the plant treatment group. Regarding the efficiency of the aqueous medium after 2,4-D phytoremediation, the medium became less toxic than the initial one through the use of the *A. salina* and *A. cepa in vitro* models in the waste groups. Thus, to increase the effectiveness of 2,4-D phytoremediation process, studies are being performed in our laboratory with daily exchange cycle of *P. neochilus* seedlings in water with or without 2,4-D.

CRediT author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CAPÍTULO IV

Repellent and insecticidal effect of *Plectranthus neochilus* essential oil on *Drosophila melanogaster*

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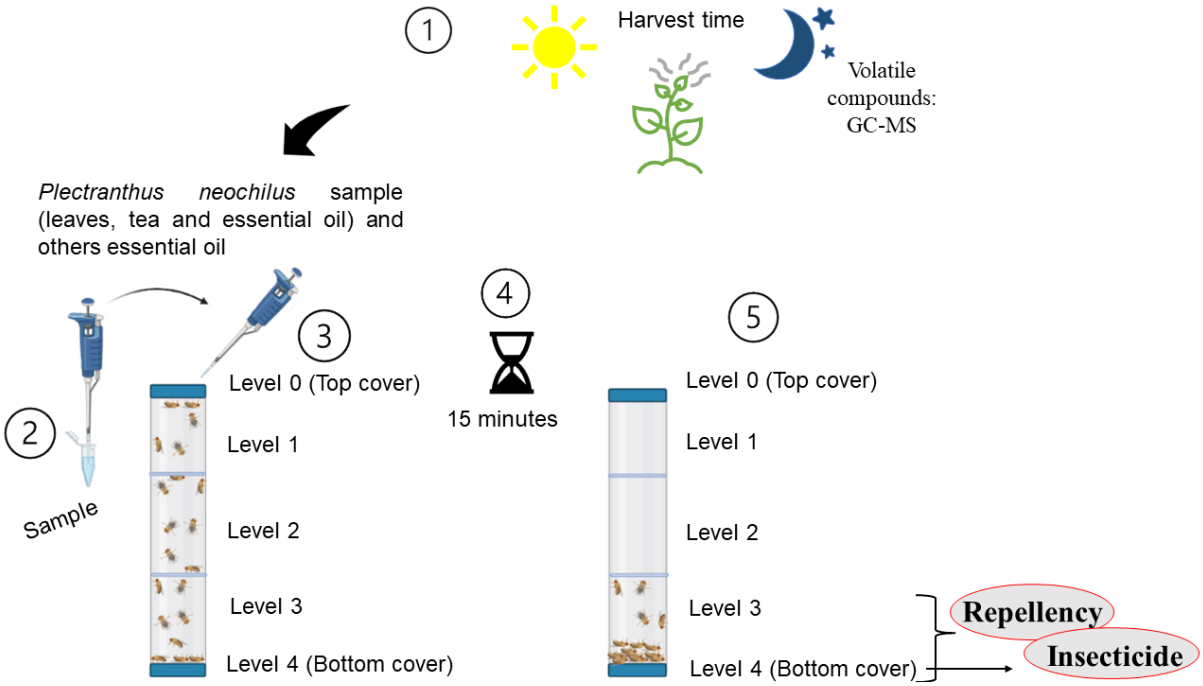
Abstract

The constituents of essential oils (EOs) and other plant extracts have been investigated for insecticidal and repellent purposes as a mode of replacing synthetic products. The production and metabolization of these compounds are influenced by biotic and abiotic conditions. So, the present study evaluated the volatile compounds present in *Plectranthus neochilus* leaves according to harvest hour (8 a.m., 1 p.m., and 6 p.m. - May) and the composition of *P. neochilus* EO by gas chromatography-mass spectrometry (GC-MS). For repellency tests of leaves, tea, and EO of *P. neochilus,* and others EOs, tubes with four levels of distance were used to count the *Drosophila melanogaster* at each level. The insecticidal effect was evaluated by the mortality of these flies in these tubes. The higher percentage of *P. neochilus* volatile compounds was at 1 p.m (GC-MS/headspace) and the composition of EO (hydrodistillation, GC-MS) showed the Caryophyllene (13.54%) and 1-Octen-3-ol (10.74%) as majority. *P. neochilus* leaves and tea did not show repellency or insecticide, just the EOs: *Plectranthus neochilus*> *Lavandula angustifolia*> *Foeniculum vulgare*. *Citrus paradisi* showed only repellency. This study showed that *P. neochilus* EO is a strong bioinsecticide candidate.

Keywords: GC-MS; Toxicity; Boldo gambá; *Lavandula angustifolia; Foeniculum vulgare; Citrus paradisi*.

Graphical abstract



1. Introduction

Plectranthus neochilus is a fragrant plant, whose leaves and other green parts give off a characteristic aroma and this species is used for several purposes. It is known as “boldo gambá” in Brazil, is originally from Africa, and its tea is used worldwide for stomach and liver problems (COUTO, 2006; DUARTE; LOPES, 2007; LAMBRECHTS, 2020). It is a plant of easy territorial adaptation and resistant to the climate (COUTO, 2006). It is used in form of tea (medicinal medicine), essential oil (EO) (AGUIAR et al., 2018b) and phytoremediation (research) (RAMBORGER et al., 2017b, 2021), for gardening (POOLEY, 1992), and green roof (MORAU; LIBELLE; GARDE, 2012b).

Studies performed with the EO of *P. neochilus* in Africa, Brazil and, Portugal identified different terpenoid constituents (monoterpenes and sesquiterpenes). Besides, this EO had antimicrobial activity (AGUIAR et al., 2018b; CREVELIN et al., 2015), activity against *Schistosoma mansoni* (CAIXETA et al., 2011), inhibition of tomato fly oviposition (*Bemisia tabaci* type B) (BALDIN et al., 2013; FANELA et al., 2016), as well as in the prevention of lipid oxidation (MOTA et al., 2014). The area that has been gaining prominence is the use of green pesticides or bioinsecticides, replacing dangerous synthetic pesticides. Therefore, many EOs and their bioactive compounds have been studied all over the world to verify repellent, insecticide, fumigant or antifeeding activities (MOSSA, 2016; REGNAULT-ROGER; VINCENT; ARNASON, 2012; SAROJ et al., 2019).

For these purposes, different experimental models are used, such as *Aedes aegypti* and *Culex quinquefasciatus* (SOONWERA; PHASOMKUSOLSIL, 2017), *Bemisia tabaci* and, *Trialeurodes ricini* (HUSSEIN; SALEM; SOLIMAN, 2017), *Drosophila suzukii* (REHER et al., 2019), *Stephanitis pyrioides* (JOSEPH, 2020), *Tribolium castaneum* (TABAN; SAHARKHIZ; HOOSHMANDI, 2017), *Lasioderma serricorne* and, *Liposcelis bostrychophila* (CAO et al., 2018). The experimental model *Drosophila melanogaster* is one of the most used in olfactory research, due to one of the main tasks of a fly, which is to locate and evaluate a substrate for feeding and oviposition (DEKKER et al., 2006; STENSMYR et al., 2003). And, for this odor to be detected, *D. melanogaster* use about 1,200 olfactory sensory neurons located in their antennae, and about 120 of these olfactory sensory neurons are in the upper palps, which is the second olfactory organ (SHANBHAG; MÜLLER; STEINBRECHT, 1999).

The present study aims to analyze the volatile compounds of the leaves of *P. neochilus* according to the time of harvest and to verify the repellent and insecticidal effect of the leaf,

tea, and essential oil of this plant. Besides, the repellent and insecticidal effect of the EO of *P. neochilus* was compared with other EOs with these activities (*Citrus paradisi, Foeniculum vulgare* and *Lavandula angustifolia*).

2. Materials and methods

2.1 Plant material and harvesting hour

The specimens of P. neochilus used in this study were grown in soil (open field) of the Federal University of Pampa (Uruguaiana – RS, Brazil), with light/dark cycle according to sunlight, rain, and tap water for periods of scarcity of rain. *The plant* was identified under the registration number: 108/2016 and stored in the herbarium of this university.

Leaves of *P. neochilus* (2 g) were harvested and placed in headspace vial (5 mL) at 8 a.m., 1 p.m., and 6 p.m. These samples were stored (-20ºC) until the chromatographic analysis, by GC-MS/headspace, to identify the volatile compounds (RAMBORGER et al., 2021). The analyzes were performed in triplicate.

2.2 Extraction of *P. neochilus* EO

After choosing the hour of harvest, leaves of *P. neochilus* were harvested and stored (-20ºC) to extract its EO. The hydrodistillation extraction procedure was adapted of Aguiar et al. (2018) methodology. For this, the 400 g of frozen leaves were smashed by compression and submitted to hydrodistillation (Clevenger) for 5 hours. The EO was collected in an Eppendorf (2 mL) and stored at - 20ºC to separate the excess water by freezing. This hydrodistillation procedure with new leaves on different days was repeated until an adequate volume was reached for further analyzes (chemical composition with GC-MS, repellent, and insecticide effects). Therefore, at 1 p.m., the harvests took place in May, June, July, August, September, October, November, and December. The other EOs used in this study were purchased (Via Aroma and WNF by Porto Alegre-RS, Brazil).

2.3 Chromatographic analysis

Chemical analysis of leaves and EO of *P. neochilus* was performed according Ramborger et al. (2021) with a gas chromatograph coupled to a mass spectrometer GC-MS (Shimadzu model QP-2010Plus, Shimadzu Corporation, Kyoto, Japan). The GC-MS equipment contained: an RTX-5MS capillary column (30 m x 0.25 mm i.d x 0.25 µm film thickness) comprising a stationary phase of 5% diphenyl and 95% dimethylpolysiloxane. The carrier gas used was

helium with a flow rate of 0.95 mL/min. The oven temperature programming was as follows: the initial oven temperature was holding at 50ºC for 5 min, and then increased to 280ºC at a rate of 10ºC/min and finally held for 30 min. Ion source and transfer line temperatures were 280ºC. The identification of compounds was performed by comparing the mass spectrum and the data from the NIST library that are available on the instrument. The percentage of the relative area of each compound was used for quantification purposes (similarity ≥ 85%).

For the volatile compounds analysis, we used flasks containing 2 g of leaves and the headspace procedure. The flasks were incubated for 15 minutes at 80ºC, and the injection was performed in CT spitless mode at an injector temperature of 250ºC. For the chemical composition of EO, 2 µL of oil was injected in CT spitless mode at an injector temperature of 250ºC. The equipment, conditions of separation in the chromatographic column, identification, and percentage of each compound used were performed at the same form for both analyses.

2.5 *Drosophila melanogaster* stock

The fly species used was *D. melanogaster* of the wild type obtained from the National Species Stock Center (Bowling Green, USA). The flies were bred on a standard cornmeal diet with yeast granules as the protein source at constant temperature and humidity (22 ± 1ºC; 60% relative humidity), under a cycle of 12 hours light/12 hours dark (SOARES et al., 2017).

2.6 Preparation and toxicity analyze of *P. neochilus* tea for *D. melanogaster*

The way to define the concentration of the tea to be used in the repellency and insecticide tests was that which obtained the highest number of dead flies (toxicity), by ingestion. For this purpose, the method of continuous liquid feeding was used (SOARES et al., 2017). Teas were prepared by decoction of the fresh leaves (harvested at 1 p.m.) in distilled water for 10 minutes. Then, teas were filtered, and sucrose (1%) was added in all concentrations (24, 48, and 72 mg/mL). These concentrations represented the treatment, and the control received only distilled water with sucrose (1%). Flies (n = 15) from both groups received 300 µL of this feed every 24h for five days. After the fifth day, dead flies were counted to define the most toxic concentration.

2.7 Repellent and insecticidal activity

The tubes used to perform the repellency and insecticide tests are showed in Fig. 1. This apparatus was based on the work of Chu et al. (2010) with changes in the material and assembly.

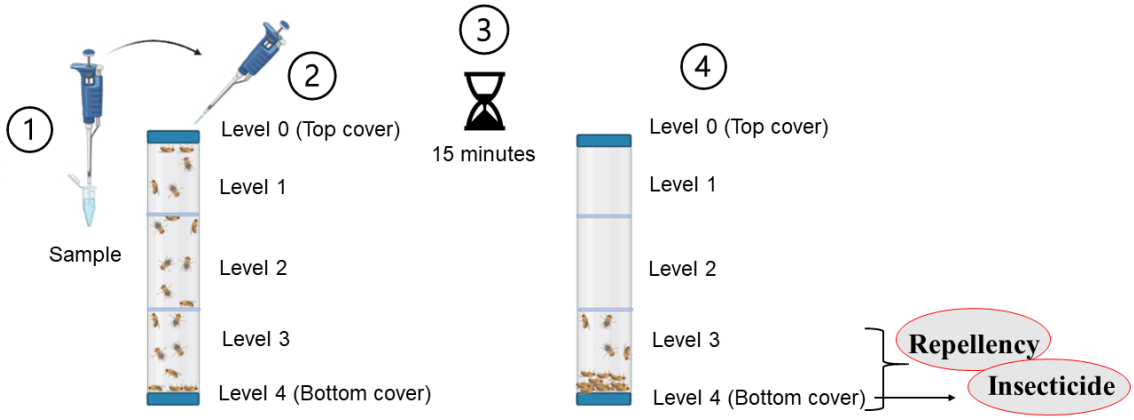


Fig. 1 Scheme drawing used to count flies at each level and dead flies. Level 0 (top cover), the position where the samples were added (100 µL). Degree of distance: level 0 < 1 < 2 < 3 < 4. Level 3 and 4, longest distance position (repellency). Level 4 (bottom cover), position for counting dead flies (insecticide). Source: Author, adapted by Biorender.

Each tube was developed by fixing tree tubes of falcon (total of 50 cm), which represented the distance levels for the repellency test. The ends (top and bottom) were closed with covers affixed with a paper inside (Whatman filter paper). Only on top cover paper, 100 µL (volume that soaked all the paper) of each sample was added (control or treatment - Table 1), because *D. melanogaster* performs negative geotaxis when confined. Therefore, the top cover (level 0) represented the flies' absence of distance concerning the samples added there (without repellency or toxicity). The falcon tubes and the bottom cover represented different levels of distance (1, 2, 3, and 4) for the repellency tests. In the bottom cover (level 4), there was only dry paper and, it represented the furthest position and served to the dead flies count (insecticidal effect).

For the analysis of the repellent and/or insecticidal activity of the samples, 20 cold-anesthetized adult flies (4 days) were used in the tube (Fig. 1). After all the cold-anesthetized flies recovered, it was waited 15 minutes to start the analyzes. After that, the tubes were shaken ten times at the same time for the flies started from the same position (bottom cover, level 4). Every 15 minutes, the number of flies was counted at each level of distance (top cover, levels 1, 2, 3, and 4) and died flies. The tests were performed in triplicate according to the following groups (Table 1):

Table 1. Samples used in repellency and toxicity tests.

Group of *P. neochilus* samples		
Sample (100 µL)	**Negative control (NC) (100 µL)**	**Positive control (PC) (100 µL)**
Crushed leaves of *P. neochilus* (2 g)	NC_{DW}: distilled water	PC_{SR}: "Super Repelex" repellent (Brazil) Composition: N, N-dietil-meta-toluamide (DEET), alcohol, isopropyl myristate, and fragrance
Tea of *P. neochilus* (72 mg/mL)		
P. neochilus EO		
Group of different kinds of EOs samples		
Lavandula angustifolia EO	NC_{MO}: Mineral oil (Farmax®, Divinópolis, Brazil)	
Foeniculum vulgare EO		
Citrus paradisi EO		
P. neochilus EO		

NC_{DW}: Negative Control Distilled Water; PC_{SR}: Positive Control Super Repelex; NC_{MO}: Negative Control Mineral Oil.

2.8 Statistical analysis

The differences in volatile compounds percentage, at different hours of the day, were verified with one-way ANOVA and Tukey post-test. It was analyzed the toxicity of tea in *D. melanogaster* with two-way ANOVA with Tukey post-test. The repellency and toxicity (insecticidal effect) with one-way ANOVA with Bonferroni post-test. Values of $p \leq 0.05$ were considered significant, and they were employed using Graph Pad Prism®.

3 Results and discussion

3.1 Volatile compounds percentage according to harvest time (GC-MS/headspace)

The harvest of plant material generally is performed according to the season, the interval of days (COSTA et al., 2020; DA SILVA JÚNIOR et al., 2019; DE ALENCAR FILHO et al., 2017; KAVOOSI; ROWSHAN, 2013; SELLEM et al., 2020), and/or, the collection site for the extraction of EOs (BEN FARHAT; SOTOMAYOR; JORDÁN, 2019; COSTA et al., 2020; FALLAH et al., 2020; GIOVANELLI et al., 2017; LEONTARITOU et al., 2020; SOILHI et al., 2019). Hardly, studies assess the best harvest time for EOs. Therefore, the present work

verified it to find the best period of the day with an increase in the percentage of volatile compounds. Fig. 2 shows the 17 compound percentages identified at each hour (8 a.m., 1 p.m. and, 6 p.m.). These compounds, shown in Fig. 2, are arranged according to the proximity of their relative percentages.

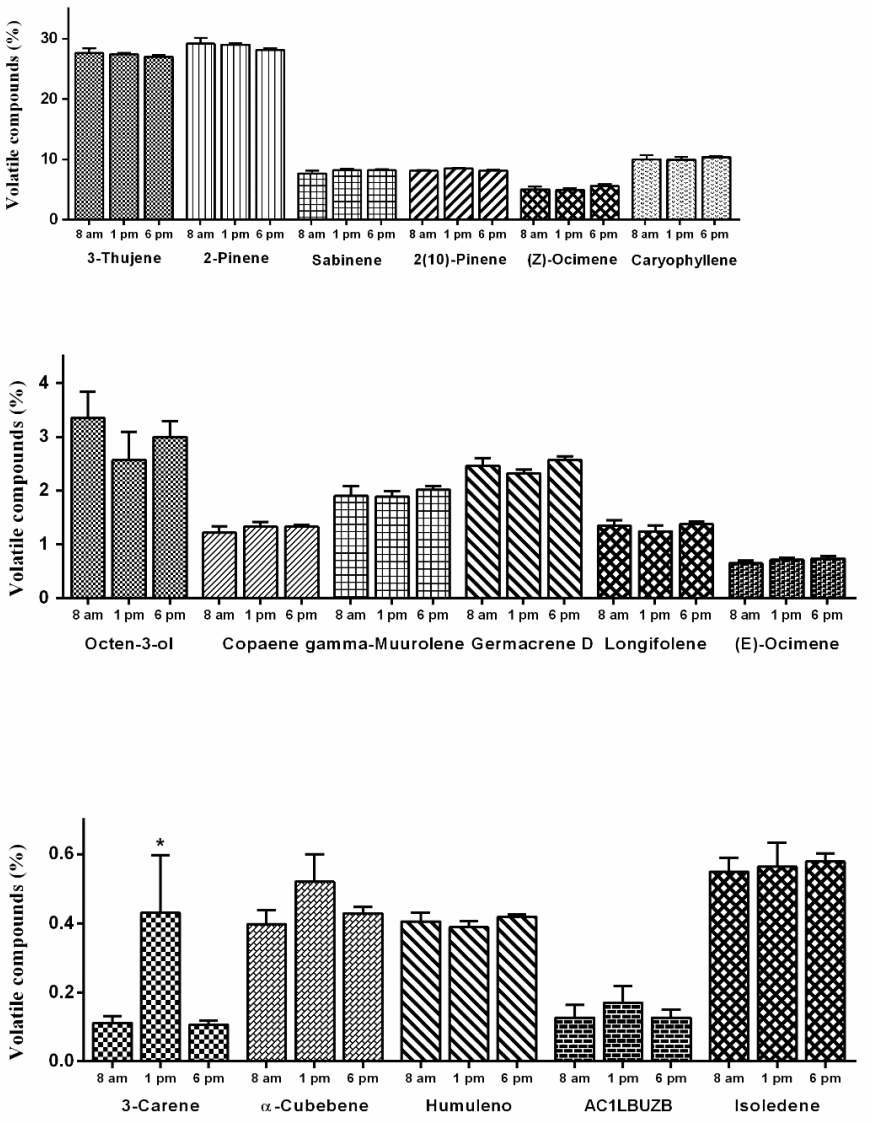


Fig. 2 Relative percentage of volatile compounds identified in *P. neochilus* leaves at 8 a.m., 1 p.m. and 6 p.m. by GC-MS/headspace. *Significant difference concerning different times.

Concerning the identified compounds, each one had a percentage value of peak area that varied during the hour of the day. However, at 1 p.m., the number of compounds that increased was superior (Copaene, 3-Carene, alpha-Cubebene, Bicyclo [5.2.0] nonane, 2-methylene-4,8,8-

trimethyl-4-vinyl- and AC1LBUZB). Only the compound 3-Carene had an increasingly significant at 1 p.m. These small variations that occurred in the period of 1 p.m. may have been due to the climatic conditions that changed mainly from this hour. Although the compounds with the highest percentages (alpha-Thujene, alpha-Pinene, Sabinene, beta-Pinene, cis-beta-Ocimene, and Caryophyllene) did not suffer many changes, the variation of the other compounds was considered to get up a EO activity greater. This choice was on the results of Crevelin et al. (2015) based, where the EO of *P. neochilus* had more effect on bacteria than just the use of its majors' compounds or the mixture of these major ones.

This small oscillation in the percentage of volatile compounds may have been influenced by abiotic factors. Therefore, we evaluated the data stored at the National Institute of Meteorology (INMET, 2020) in the days of the experiment. According to the data collected, solar radiation may have contributed to this data because, on the first day, there was a significant increase in radiation from 8 a.m. until 1 p.m. (05/15 - 8 a.m.: 0 Kj/m^2, 1 p.m.: 321.695 Kj/m^2 and 6 p.m.: 1101.776 Kj/m^2; 05/16 – 8 a.m.: 0.000 Kj/m^2, 1 p.m.: 1130.274 Kj/m^2 and 6 p.m. 1328.79 Kj/m^2). Another factor that registered oscillation at 1 p.m. on the second day was the atmospheric pressure (05/15 - 8 a.m.: 1002.5 hPa, 1 p.m.: 1003.9 hPa and 6 p.m.: 1004.0 hPa; 05/16 – 8 a.m.: 1010,6 hPa, 1 p.m.: 1013.2 hPa and 6 p.m.: 1011.2 hPa). The wind speed may also have influenced the second day at 1 p.m. (05/15 – 8 a.m.: 1.50 km/h, 1 p.m.: 2.60 km/h and 6 p.m.: 2.90 km/h; 05/16 – 8 a.m.: 1.40 km/h, 1 p.m.: 4.30 km/h and 6 p.m.: 3.20 km/h). Regarding the temperature, there were no drastic changes that could have culminated in this variation because, at 8 a.m. and 1 p.m., the average temperature increased by 1ºC, and, at 6 p.m., which obtained the highest temperature, there were no more variations in the percentages of the compounds (05/15 – 8 a.m.: 17.60ºC, 1 p.m.: 18.60ºC and 6 p.m.: 22.90ºC; 05/16 – 8 a.m.: 13.60°C, 1 p.m. 14.80°C and 6 p.m. 17.50°C). The data related to the dew point were also not significant (05/15 – 8 a.m.: 16.60ºC, 1 p.m.: 16.70ºC and 6 p.m.: 17.00ºC; 05/16 – 8 a.m.: 8.50ºC, 1 p.m.: 6.20ºC and 6 p.m.: 5.80ºC). We suggest that some changes in hormones or other factors in the plant itself may have led to this low change in volatile compounds.

3.2 Chemical composition of *P. neochilus* EO (GC-MS)

For the analysis of the constituents of the EO by GC-MS, it was performed a dilution (10 times) with ethyl acetate to acquire the best chromatographic peaks. The analysis allowed us to identify and quantify (relative percentage of peak area) 36 compounds (Table 2).

Table 2: Chemical composition of *P. neochilus* EO by GC-MS.

Name	Rt[a]	%[b]
2-Hexenal	5.56	0.46
(alpha-Thujene) Bicyclo [3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	6.88	4.58
(beta-Ocimene)1,3,6-Octariene, 3,7-dimethyl-, (E)-	7.02	4.51
(Sabinene) Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	7.16	0.45
3-Carene	7.23	0.42
beta-Phellandrene	7.71	2.45
(beta-Pinene) Bicyclo [3.1.1]heptane,6,6-dimethyl-2-methylene-, (1S)-	7.77	2.16
(Octenol) 1-Octen-3-ol	8.20	10.73
(beta-cis-Ocimene) 1,3,6-Octariene, 3,7-dimethyl-, (Z)-	8.99	2.60
gamma-Terpinene (1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-;)	9.19	1.76
((+)-3-Carene,4-isopopenyl-)Bicyclo[4.1.0]hept-3-ene, 3,7,7-trimethyl-4-(1-methylethenyl)-	9.65	0.59
(Linalool) 1,6-Octadien-3-ol, 3,7-dimethyl-	9.95	0.94
Thujone	10.18	0.25
(Terpinen-4-ol) 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	11.29	4.36
(alpha-Terpineol) 3-Cyclohexene-1-methanol, α,α4-trimethyl-	11.53	2.24
Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl, acetate	12.70	0.73

(alpha-Terpineol acetate) 3-Cyclohexene-1-methanol, α,α4-trimethyl- 1-acetate	13.69	5.49
Copaene	14.05	2.69
Caryophyllene	14.85	13.54
(cis-Caryophyllene) Bicyclo [7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-	14.93	1.63
alpha-Caryophyllene	15.16	2.33
Bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl-	15.22	0.84
(gamma-Cadinene) Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1α,4aβ,8aα)-	15.56	6.03
(alpha-Funebrene) Di-epi-α-cedrene	15.76	1.53
(Cyclosativene)1,2,4-Metheno-1H-indene, octahydro-1,7a-dimethyl-5-(1-methylethyl)-, [1S-(1.alpha.,2.alpha.,3a.beta.,4.alpha.,5.alpha.,7a.beta.,8S)	15.84	6.78
Epiglobulol	16.19	1.67
(Nerolidol) 1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-	16.35	2.47
Cariofileno oxide	16.86	4.82
tau-Cadinol	17.42	4.68
(Amboryl) (-)-Isolongifolene, acetate	18.52	0.95
2-Pentadecanone. 6.10.14-trimethyl	19.34	0.61
Phytol	23.35	0.46
Tricyclo[20.8.0.0(7.16)triacontane. 1(22).7(16)diepoxy	24.05	1.05

3,7,11,15 tetramethyl -2-hexaden-1-ol	24.47	1.11
Nonacosane	29.19	1.17
Triacontane	33.09	0.92

[a] Rt - Retention time.

[b] the relative percentage of each chromatographic peak identified regarding the total of compounds identified by the GC-MS NIST library.

The major compounds found in the *P. neochilus* EO were Caryophyllene (13.54%) and 1-Octen-3-ol (10.74%), and the minor ones were Cyclosativene (6.78%), gamma-Cadinene (6.03%), and alpha-Terpineol acetate (5.49%). Most of the compounds identified agree with other studies that also used the EO of *P. neochilus* leaves. The differences found in these studies were regarding the majority compounds identified in the different locations (Africa, Brazil and, Portugal) and the relative percentage (AGUIAR et al., 2018b; BALDIN et al., 2013; CAIXETA et al., 2011; CREVELIN et al., 2015; LAWAL; HUTCHINGS; OYEDEJI, 2010; MOTA et al., 2014; ROSAL et al., 2011). This factor reflects on the genetic, climatic, geographic factors, and stage of development of the plant. Therefore, it promotes different extraction compounds in the same plant species (PILATTI et al., 2019).

3.3 Repellent and insecticidal activity on *D. melanogaster*

The results regarding the repellent or insecticidal activities of the samples from the *P. neochilus* group are shown in Fig. 3 (a, b and, c) and are correlated with Fig. 1 (section 2.7 of the methodology).

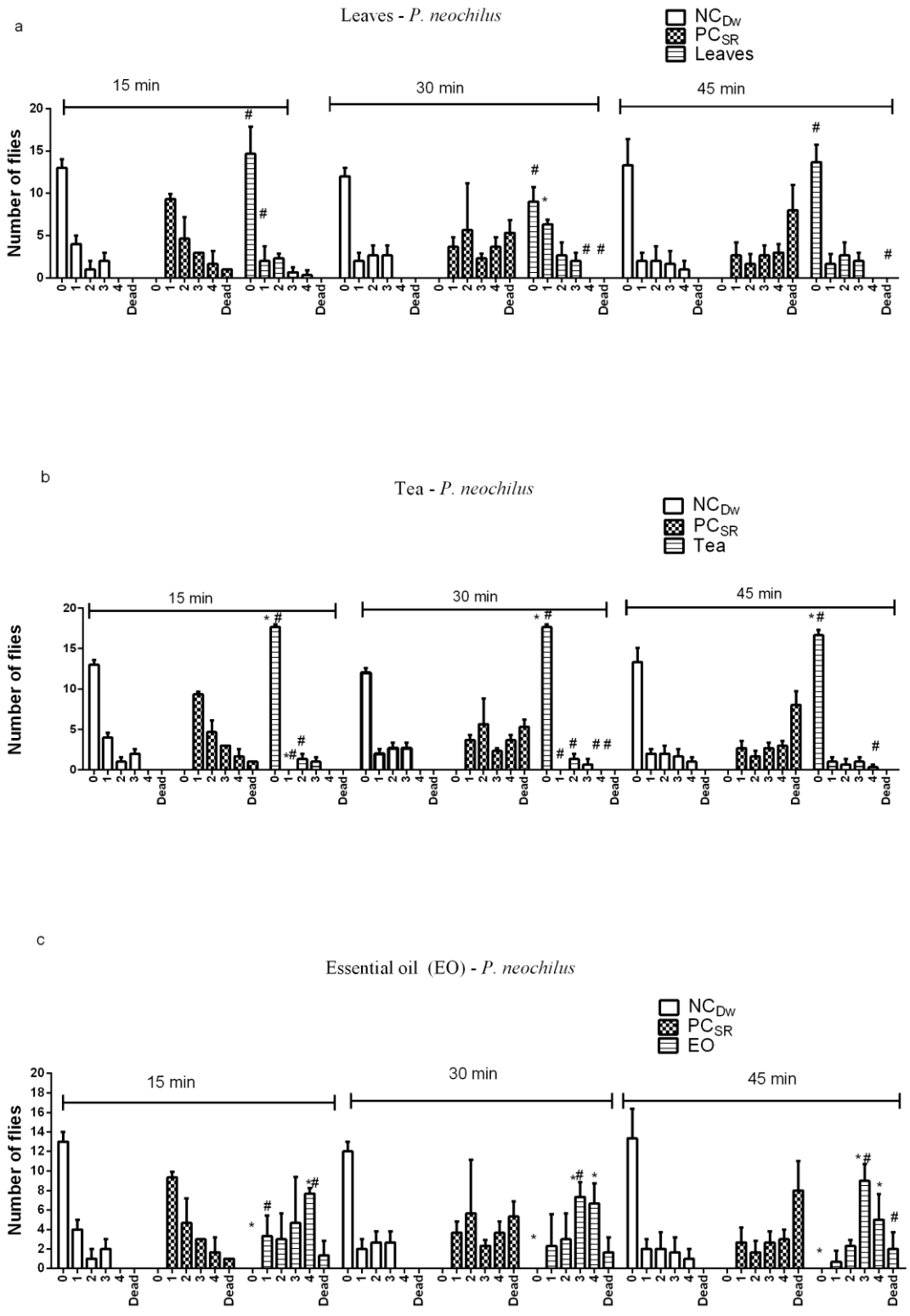


Fig. 3 Repellency and insecticide effect tests of *P. neochilus group*. a – Leaves; b - Tea; c - Essential oil. NC_{DW}, distilled water; PC_{SR}, commercial repellent. * Significant difference concerning NC_{DW}. # Significant difference concerning PC_{SR}.

Through the data in Fig. 3 (a, b, and c), after 30 minutes of exposure in the positive control (PC_{SR}), the flies already indicated a high permanence rate at different positions of distance (levels 1, 2, 3 and, 4). This behavior proved their repellent activity. Besides, some flies died in these 30 minutes of exposure to PC_{SR}, showing its insecticide effect (toxicity). After 45 minutes in Fig. 3 a, b, and c, most of the flies in PC_{SR} died and, none remained at level 0, where this PC_{SR} was added. For this reason, the significance of samples (leaves, tea and, EO of *P. neochilus*) was concerning PC_{SR}. In the negative control (NC_{DW}), the flies showed an opposite behavior because, throughout the exposure period (15, 30, and 45 min), they remained on level 0 (Fig. 3 a, b, and c). Besides, in the NC_{DW}, no flies died, and few of them were or remained at level 4. Thus, the same samples were also compared to NC_{DW} for assessing the absence of repellent or insecticidal activity.

About the sample of leaves (Fig. 3 a), the flies remained at the highest levels (level 0 and 1), and this result was significantly different concerning the PC_{SR}. No flies died, and the only significant difference from NC_{DW} was at level 1 at 30 minutes. But this is the closest level to level 0, the location where was added these leaves. Therefore, this sample did not show repellency or toxicity for *D. melanogaster*. The behavior of the tea sample (Fig. 3 b) was like that of the leaves since the flies remained mainly in level 0 throughout the exposure period. Besides, the number of flies on level 0 was higher in the tea sample (17.66, 17.66, and 16.66 flies) than in the NC_{WD} (13; 12; 13.33 flies). With these data, the tea also showed no repellency or toxicity, and in this case, it may be that the tea sample was attractive for *D. melanogaster*. According to research with *P. neochilus*, it is already known that the tea from its leaves contains flavonoids and phenolic acids, such as caffeic, ferulic, coumaric acids (RAMBORGER et al., 2017b) and rosmarinic acid (BRITO et al., 2018a). However, there are no studies that report the existence of any compound that causes repellency or that it is extremely toxic due to contact or volatility in the leaves or tea of *P. neochilus*. Through this study, it can be inferred that there are no such compounds. If they are present, they are in quantity low because the number of leaves used was enough to soak the filter paper in the tube, as well as the highest concentration of tea (72 mg/mL), which was considered toxic by ingestion (data not shown). In this case, the concentrations of 48 and 72 mg/mL of tea were toxic (mortality of 53 and 55%, respectively) for *D. melanogaster* when ingested continuously for five days. Thus, the concentration of 72 mg/mL was used because it has a higher concentration of compounds, although, in the tests with the tube, the flies inhaled the tea for 45 minutes. If there was tea ingestion (in the tea

present in the filter paper), this did not affect the analyzes of repellency and insecticide during the test.

The results of the *P. neochilus* EO sample (Fig. 3 c) showed that this sample had a high repellent and insecticidal effect (toxicity) like the behavior observed in the PC_{SR}. In the EO sample, the flies were at levels 1, 2, and mainly at levels 3 and 4 during the 45 minutes of exposure. In addition, most of the flies stayed at level 3 in the EO sample (7.33 and 9 flies) than in the PC_{SR} (2.33 and 2.66 flies), in the exposure time of 30 and 45 min. And, in the 45 minutes of exposure, there were flies' mortality too. From these results, the *P. neochilus* EO and different EOs acquired in the market were compared. Fig.5 shows the data obtained on the repellent and/or insecticide behavior of the different EOs and with the longest exposure time (90 minutes).

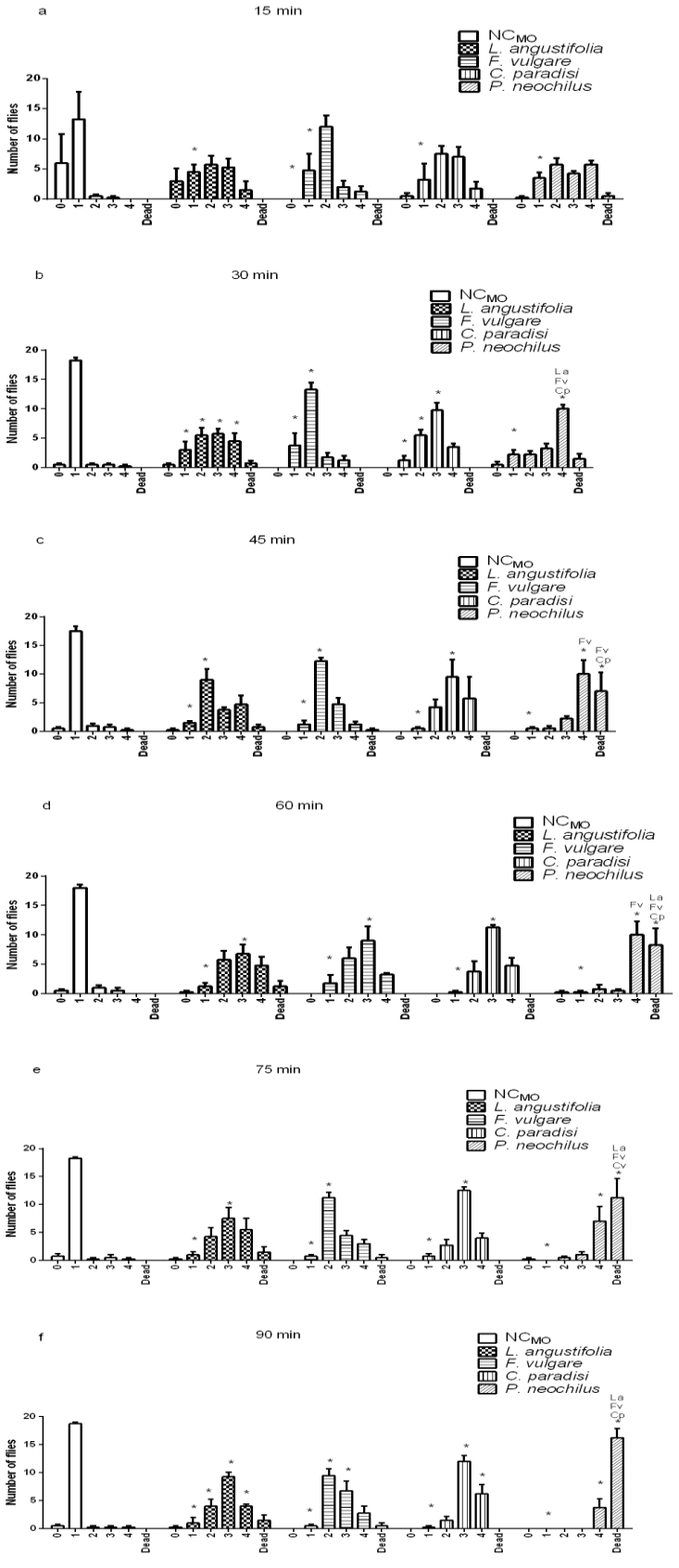


Fig. 4 Evaluation of the repellent and insecticide (toxic) activity of different EOs concerning the exposure time (minutes: a - 15 minutes; b – 30 minutes; c – 45 minutes; d – 60 minutes; e

– 75 minutes; f – 90 minutes). * Significant difference concerning the negative control (NC_{MO}). La, significant difference concerning *Lavandula angustifolia* (level 4 and dead); Fv, significant difference concerning *Foeniculum vulgare* (level 4 and dead); Cp, significant difference concerning *Citrus paradisi* (level 4 and dead).

The repellent effect of *P. neochilus* EO was observed again through the dispersion of *D. melanogaster* at levels 1, 2, 3, and 4 (Fig. 4 a -15 minutes). After 30 minutes, the flies were, mainly, at the most distant level (level 4) (Fig. 4 b). Its confirmed insecticide activity was through the gradual death of the flies and the mean of 16.25 value deaths (81.25%) in the 90 minutes of exposure (Fig. 4 f). When analyzing only the EO of *P. neochilus*, Baldin et al. (2013) verified its power to inhibit the oviposition of the whitefly (*Bemisia tabaci*, type B) in tomatoes. Therefore, the present study is following the literature, and the repellent and insecticide activities found can be attributed to the chemical composition of its EO (GC-MS). In the study of Baldin et al. (2013), they identified the Caryophyllene compound as the majority too. In other studies, this compound, as well as some of its isoforms, was highly toxic and, or a repellent, considered for other experimental models, including mosquitoes (CAO et al., 2018; GILLIJ; GLEISER; ZYGADLO, 2008; GUO et al., 2017; MA et al., 2020; NARARAK et al., 2019; PLATA-RUEDA et al., 2018). The second compound major (1-Octen-3-ol) is considered attractive at low concentrations. However, it becomes repellent at high concentrations in the experimental model *Cryptolestes ferrugineus* (MUSHOBOZY; PIERCE; BORDEN, 1993; PIERCE et al., 1991). Besides, Knaden et al. (2012) showed that 1-Octen-3-ol caused aversion in *D. melanogaster* and, Wallingford et al. (2018) showed that it decreased the oviposition of *Drosophila suzukii* in red raspberries. Therefore, these related data can be to our study, where there is a high prevalence of this compound (10.74%) by analyzing the GC-MS. Another compound that also causes aversion to flies is linalool (KNADEN et al., 2012). Although the detection percentage of the linalool (0.94%) was low compared to the total percentage area, it can be related to the repellent effect, as already reported by Yoon et al. (2011), Taban et al. (2017), Cao et al. (2018) and Kheloul et al. (2020). We suppose this because it is not known what is the actual concentration of this compound in the *P. neochilus* EO. The minority compounds, Cyclosativene, gamma-Cadinene, and alpha-Terpineol acetate, are generally in lower percentages in EOs and have other characteristics and activities. However, gamma-Cadinene showed acaricidal activity for *Psoroptes cuniculi* (GUO et al., 2017) and, alpha-Terpineol acetate caused repellency against *Tribolium castaneum* (WANG et al., 2019).

Fig. 4 (a, b, c, d, e, and f) also showed that the other EOs (*L. angustifolia*, *F. vulgare*, and *C. paradisi*) had a repellent effect because of the local of the flies in the most distant levels (levels: 2, 3, and 4, respectively) during the entire exposure period, differently of the negative control. Regarding the degree of toxicity (insecticide effect) of each EO in the 90 minutes of exposure time (Fig. 4 f), was observed the following sequence: *P. neochilus* (81.25%) > *L. angustifolia* (7.5%) > *F. vulgare* (2.5%) and *Citrus paradisi* (0%). Thus, the present study confirmed the repellent and, or insecticidal effect already reported for *L. angustifolia* (HAN et al., 2020; KHOSRAVI; SENDI, 2013; YAZDANI et al., 2013), *F. vulgare* (GUSMÃO et al., 2013; RANA; RANA, 2012; ZOUBIRI et al., 2014), as well as for *C. paradisi* (ABBAS et al., 2012; DUTRA et al., 2016). The results in Fig. 4 b also show that since the 30 minutes of exposure, the *P. neochilus* EO has already had a repellent effect superior to the other EOs. Besides, after 60 minutes (Fig. 4 d), *P. neochilus* also starts to have an insecticidal effect superior to other EOs.

4 Conclusions

Through this study, it was possible to establish the harvest time at 1 p.m. of *P. neochilus leaves*. For the first time, it was verified the repellence and insecticide effects in the leaves, tea, and EO of *P. neochilus*. However, both leaves and tea of the plant did not cause repellency or toxicity in the flies, only its EO. The repellency and the insecticide effect of *P. neochilus* EO were confirmed through a longer exposure time of the flies. These effects are due to the chemical composition of this EO, which presented Caryophyllene and 1-Octen-3-ol as compounds majority. In addition to these results, the *P. neochilus* EO showed to have a superior repellency and insecticide effect than the tested EOs (*L. angustifolia*, *F. vulgare,* and *C. paradisi*), making it a strong candidate for its use as a green pesticide.

Conflict of interest

The authors declare the absence of conflicts of interest.

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CAPÍTULO V

Low toxicity of *Plectranthus neochilus* tea in alternative experimental models

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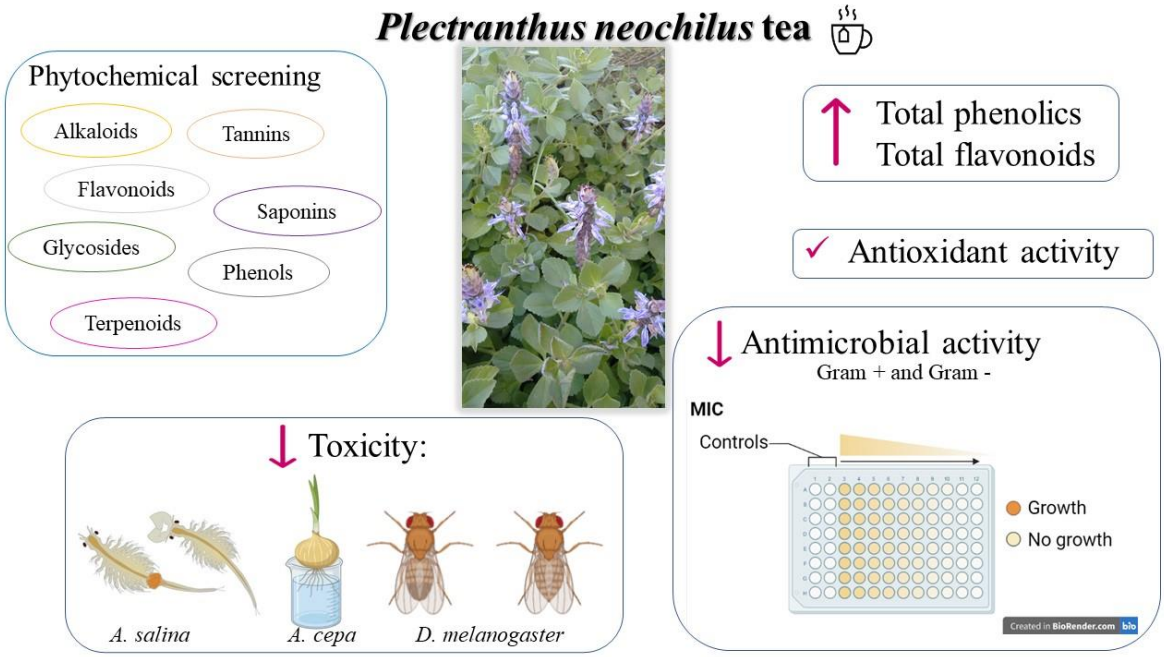
Abstract

Plectranthus neochilus tea is widely used in traditional medicine for stomach and liver problems. In this study, it was evaluated for the phytochemical constitution, antioxidant, antimicrobial, and toxicological activity. The tea was prepared by decoction followed by evaporation of the water and obtaining the dry extract to be resuspended with water in different concentrations. Phytochemical screening was carried out for alkaloids, flavonoids, tannins, glycosides, saponins, phenols, coumarins, and terpenoids. Antioxidant test was performed by DPPH. Folin Ciocalteu was used to detect the total phenolics and aluminum chloride complexation for the total flavonoids. In the toxicity bioassays, *Allium cepa*, gram positive and negative bacteria, *Artemia salina* and *Drosophila melanogaster* were used. It was possible to identify alkaloids, flavonoids, tannins, glycosides, saponins, phenols, and terpenoids. The antioxidant capacity reached 71.48% of DPPH inhibition, and the dosages of total phenolics and total flavonoids were 243.53 µgGAE/g and 103.16 mgRE/g, respectively. Tests with *A. cepa*, bacterial strains, *A. salina* and *D. melanogaster* showed that the tea was non-toxic in usual concentration of the traditional medicine. Thus, *P. neochilus* tea proved to be healthy, beneficial, and non-toxic up to a certain concentration according to alternative experimental models in vitro (*A. cepa*) and *in vivo* (*A. salina* and *D. melanogaster*). In the future, it can be used for the isolation of bioactive compounds intended for the pharmaceutical industry of herbal medicines.

Keywords: Boldo gambá; Screening phytochemical; Antioxidant; Polyphenols; Flavonoids.

Graphical abstract



1 Introduction

The medicinal plant extracts are a viable alternative for the population in disease treatments, symptoms, or health maintenance 1. It is an old practice since there are records of its use since the colonial Brazil period 2. Over the years, the use of plants for healing and treatment purposes has become more used by the population with more socioeconomic difficulties or with the impossibility of access to doctors and medicines 3. This ability that allows plants to help health is linked to their metabolism, specially the primary metabolites are essential for the survival of vegetables and are common to all plants. They are carbohydrates, lipids, and amino acids. While the so-called secondary ones, which are innumerable and are specific to each species, fall into one of the three main classes of molecules: terpenes, phenolic compounds, or alkaloids. Secondary metabolites are produced according to the needs of the plant, defense, and pollination. Therefore, plants can synthesize a wide range of secondary metabolites 4.

Plectranthus neochilus tea is used in the traditional medicine for stomach pains and liver problems 5. Also, there are regional reports of its use for respiratory infections 6, sedative and hypnotic purposes 7. This plant is known as the "boldo gambá" in Brazil and is found worldwide due to the ease of growth, adaptation, and propagation 5,8,9.

Many studies have investigated the composition and antioxidant actions of medicinal plant extracts. These studies also show that plant phytochemical constituents can be biologically active compounds which are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer 10,11. The antioxidant activity, for example, is verified after obtaining the plant extract (aqueous extract or by organic solvents), since organic extracts generally have antioxidant properties. Consequently, its antioxidant potential is analyzed through some tests, such as the iron-reducing antioxidant power of iron ion (FRAP), the capture power of the radical 2,2-diphenyl-1-picryl-hydrazil-hydrate (DPPH) test, or the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS). According to different reagents, the polyphenol content can be evaluated according to total phenolics and total flavonoids. In this case, it is used the aluminum chloride technique and Folin Ciocalteu, respectively 12,13. However, since some herbal teas can contain toxic substances 14, it is necessary to analyze their toxicity. It is already known about the traditional use of tea from *P. neochilus* leaves. But there are no references that relate to its security to ingesting it.

In this approach, the tea is evaluated in biochemical, pharmacological, or toxicological areas. This can be performed using alternative animal models due to the restrictions of animal

ethics committees. There are *in silico*, *in vitro, ex vivo*, or *in vivo* models for the pharmacological and toxicological studies of natural products 15. The *Allium cepa (onio)* bioassay (*in vitro*), which uses its roots grown to check the toxicity of solutions 16,17. This test uses different plant extract concentrations to examine chromosomal changes or meristematic cell divisions of the onion roots. Thus, it is possible to warn the population about a specific product consumption. Antimicrobial activity is also an *in vitro* test used, mainly in the search for new drugs effective against resistant microorganisms 18. There are the microcrustacean *Artemia salina*, considered an *in vivo* model and a target organism to detect bioactive compounds in plant extracts. This bioassay is an acute toxicity test (24–48 h), where a specific mortality number of the *A. salina* is verified, and the median lethal concentration (LC50) is calculated after 24 h. The toxicity in *A. salina*, can even be correlated with the cytotoxic activity against human tumors 19–21. Also, there is the *in vivo* model *Drosophila melanogaster*, known as the "fruit fly". *D. melanogaster* is widely used in toxicological studies due to its low cost, easy handling in the laboratory, high generation of genetically identical descendants, and short life span. This model can survive 10 - 14 days in generation time and 3 - 4 weeks in lifespan, depending on the diet. This alternative experimental model has been used in nutritional and toxicological sciences, due to its organ systems that operate in a similar form to mammalian organs 22–25. Therefore, the *D. melanogaster* is an ideal model in food toxicity research.

This study aimed to investigate the phytochemical, phenolic, and flavonoid detection, antioxidant, antimicrobial, and toxicity profile of *P. neochilus* tea.

2 Materials and methods

The summary of the experiments performed in this study is in Figure 1.

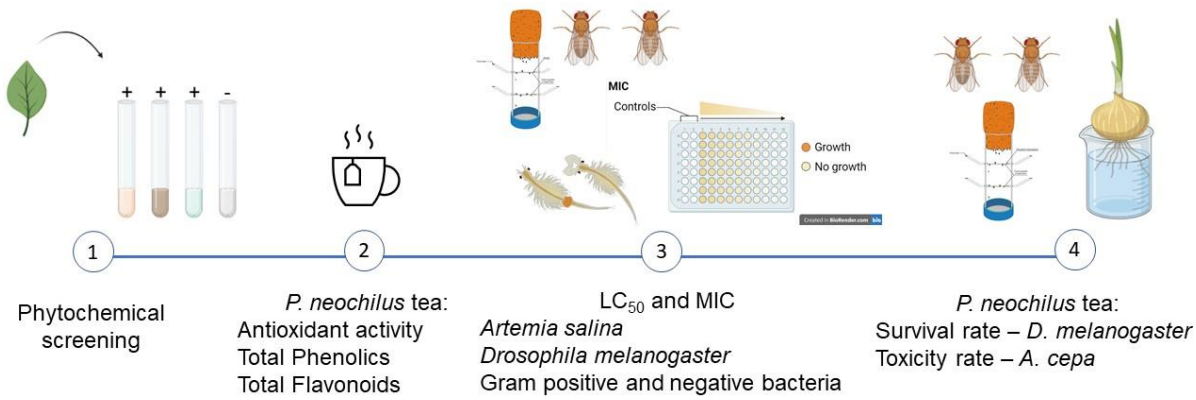


Figure 1. Experimental sequence design of the study.

2.1 Plant material, tea preparation, and extract

The plant *Plectranthus neochilus* was identified and stored in the herbarium of the Federal University of Pampa - Uruguaiana campus – RS. The number registration was 108/2016.

The tea was prepared by decoction of 1 g of fresh leaves in 100 mL of distilled water for 10 min. This value corresponds to the amount of plant material present in the tea bags (1 g of plant material per bag). After cooling, the solution was filtered with vacuum filter paper in the kitassato and Büchner funnel apparatus and evaporated in a vacuum rotary evaporator at 40ºC. For antioxidant activity, *A. cepa* bioassay, antimicrobial activity, and phenolic compounds, the dry extract was dissolved only in distilled water in different concentrations. The dry extract was dissolved in distilled water with sucrose 1% in the treatment concentrations for the tests with *D. melanogaster* and in distilled water with 0.9% NaCl for the *A. salina* tests.

For the microbiological analysis, the aqueous extract of the leaves of *P. neochilus* was used. The aqueous extract was prepared by decocting the dried leaves in distilled water for 10 minutes. After cooling, the solution was filtered and frozen in an ultrafreezer (-54°C) to be lyophilized. The obtained dry extract was weighed and diluted in ultrapure water at a concentration of 50 mg/mL to be used in antimicrobial analysis.

2.2 Phytochemical screening

The profile phytochemical of *P. neochilus* tea was estimated according to their presence or absence of the compounds. It was evaluated the alkaloids (Dragendorff or Mayer reagents), flavonoids - flavones and flavonols (AlCl3 reagent), hydrolysable tannins (FeCl3 or Pb(C2H3O2)2 reagents), glycosides (KOH reagent), saponins (mechanical agitation of the extract diluted in water distilled and added 1N HCl, presence of foam column of 1 cm for 10 minutes), phenols (FeCl3 reagent), coumarins (KOH reagent) and terpenoids (CHCl3 and H2SO4 reagent) according to 26.

2.3 Antioxidant potential

The quantification of the antioxidant activity was performed by DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capacity. This method was measured by the photometric method described by 27 and 28 with modifications in the reagents volume. 15 µL of the extract was added with 85 µL of distilled water and 50 µL of 0.3 mM DPPH solution in 96-well microplates. The sample was then incubated for 30 minutes, deprived of light, and subsequently read at 518 nm absorbance in the microplate reader. The results were expressed

in micrograms of ascorbic acid equivalents per milligram of extract and the percent of DPPH inhibition (%) was obtained through the formula:

PI%: = 100 – [(Abs sample – Abs blank) / Abs control] x 100

Where, PI% is the percentual of DPPH inhibition; Abs. sample is the absorbance of the mixture of the DPPH reagent with the extract; Abs. blank is the extract absorbance without the DPPH reagent; and Abs. control is the absorbance of the DPPH reagent.

Tea concentrations used were 1; 3; 6; 9; 12; 15; 18; 21 mg/mL.

2.4 Total phenolic compounds

The total phenolic compounds quantification used 29 methodology and adapted to 96-well microplates. 15 μL of the infusion was added to the microplate, with 37.5 μL of Folin-Ciocalteu solution 1 mol/L). After five minutes of reaction, 37.5 μL of sodium carbonate solution (20%) was added, and the volume was filled with distilled water to 300 μL. The solution was incubated for 30 minutes in a private light environment and then read at 750 nm. A blank control (water with tea) was used to discount the absorbance of the tea. Positive control (sodium carbonate, Folin-Ciocalteu, and water) was used to discount the blank solution. A calibration curve was constructed with 5 points between 1 and 38 μg/mg of gallic acid. The results were expressed in micrograms of gallic acid equivalent per mg of extract (μgGAE/mg). The tea concentrations were 1; 3; 6; 9; 12; 15; 18; 21 mg/mL.

2.5 Total flavonoid

Total flavonoid contents of *P. neochilus* extract were determined using the aluminum chloride method 30. Briefly, 99% ethanol (50 μL), 10% aluminum chloride (10 μL), 1 M sodium acetate (10 μL) and, 80 μL distilled water were added to 50 μL of *P. neochilus* extract 96-well microplates. This solution was incubated for 30 min at room temperature. Rutin was used as a standard for the calibration curve (2, 8, 16, 24, 32 e 40 mg/mL). Flavonoid contents were expressed as mg of rutin equivalent (RE) per g of the dry weight of the plant material (mgRE/g). The samples were read at 415 nm. The tea concentrations were 1; 3; 6; 9; 12; 15; 18; 21 mg/mL.

2.6 *Artemia salina* bioassay

The median lethal concentration (LC50) of *P. neochilus* tea was estimated with *A. salina*, according to Meyer et al. (1982). Cysts were induced to hatch in saline solution and aerating for 24 h. The nauplii were collected and transferred individually to a 96-well plate containing different tea concentrations or a control saline solution. Finally, the number of dead nauplii was analyzed after 24 h. Assays were performed in triplicate and n = 30 nauplii were used in each assay. The median lethal concentration was the required concentration to kill 50% of nauplii. It was considered LC50 < 1 mg/mL toxic.

2.7 *Drosophila melanogaster* tests

The fly species used was *D. melanogaster*, of the wild type, obtained from the National Species Stock Center, Bowling Green, OH, USA. The flies were bred on a standard diet of cornflour with yeast granules as the protein source at constant temperature and humidity (22 ±1°C and 60% relative humidity), under a 12h dark/12h light cycle.

For the toxicity of extract, the method of continuous liquid feeding was used according 31. Male and female flies (n = 20) with four age days were used and were divided into two groups: control - flies that received only distilled water with sucrose 1%; treatment - flies that received different concentrations of tea with sucrose 1%. In the first day all flies received the liquid feed of sucrose 1% solution for to put the flies in the same conditions.

D. melanogaster destined for the LC_{50} test received the continuous liquid feed every 24 hours during five days. The tea concentration used were 3; 6; 9; 12; 15; 18 and 21 mg/mL. The number of dead flies was counted after this exposure time.

D. melanogaster destined for the survival test received the continuous liquid feed every 24 hours for 31 days consecutively. The tea concentration used were 1; 2; 3; 4; 5 mg/mL. The number of dead flies was analyzed each day.

2.8 Antimicrobial assay (MIC)

Minimum inhibitory concentration (MIC) was performed using the broth microdilution technique 32. Six bacterial strains (gram positive and negative) standardized by the American Type Culture Collection were used (ATCC): *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonie* (ATCC 700603), *Acinetobacter baumannii* (ATCC BAA-747) e *Enterococcus faecalis* (ATCC 29212).

Broth microdilution used Mueller-Hinton broth in a 96U well plate in duplicate for each microorganism. A positive control was used, in which only the microorganism was inoculated, and a negative control, in which only Mueller-Hinton broth was added. For the inoculum, a suspension in 0.9% saline solution was prepared until reaching a turbidity equivalent to 0.5 on the McFarland scale of each microorganism (approximately 1.5 x 10^8 CFU/mL). The final volume of each microplate well was 0.1 mL and the plates were incubated at 35°C (+/- 2°C) for 18-24 hours. The gradual range of concentration of the aqueous extract was from 50 mg/mL. The reading of bacterial MIC was through visual verification with the aid of light contrast.

2.8 *Allium cepa* bioassay

The concentrations used in the *Allium cepa* roots (onion) were above the LC_{50} (*A. salina* and *D. melanogaster*), to further explore the toxicity of the tea. This methodology was according to 33. The tea concentrations were 6, 12, 24, 48, 96 mg/mL. The positive control (PC) was glyphosate 1%, and the negative control (NC) was distilled water. The root growth (cm) was established after 144 hours of exposure. The significant difference according to controls (PC and NC).

2.9 Statistical analysis

The data were expressed as means ± S.D. (n=3) for all testes. The DPPH data were expressed in percentage of DPPH inhibition (PI%). Total phenolic contents were expressed in micrograms of gallic acid equivalent per grams of dry extract (μgGAE/g). Total flavonoid contents were expressed as mg of rutin equivalent (RE) per gram of the dry extract (mgRE/g). The *A. salina* and *D. melanogaster* LC_{50} were analyzed by the linear and the nonlinear regression equation, respectively. For *D. melanogaster* survival analyzes, the mean and standard deviation of dead flies were performed every 24 h. Bacterial MIC was estimated by the concentration range visualized on the microplate. The *A. cepa bioassay* was analyzed with a One-Way ANOVA test followed by Bonferroni's and with $p < 0.05$ regarding negative control (NC) and positive control (PC).

3 Results and discussion

3.1 Phytochemical screening and biochemical tests of *P. neochilus* tea

In phytochemical screening, it was possible to observe that *P. neochilus* tea contains many secondary metabolites (Table 1). There were alkaloids, flavonoids, tannins, glycosides, saponins, phenols, and terpenoids present.

Table 1. Presence of secondary metabolites in *P. neochilus* tea. Present +. Absent -.

Phytochemical Compound	**Result**
Alkaloids	+
Flavonoids	+
Tannins	+
Glycosides	+
Saponins	+
Phenols	+
Coumarins	-
Terpenoids	+

Figure 2 shows the data of antioxidant capacity and quantification of total phenolic compounds and total flavonoids. The results showed good antioxidant activity, up to 71.48% inhibition of DPPH for 15 mg/mL concentration, and good results for total phenolic compounds (243.53 µgGAE/g) and total flavonoids (103.16 mgRE/g).

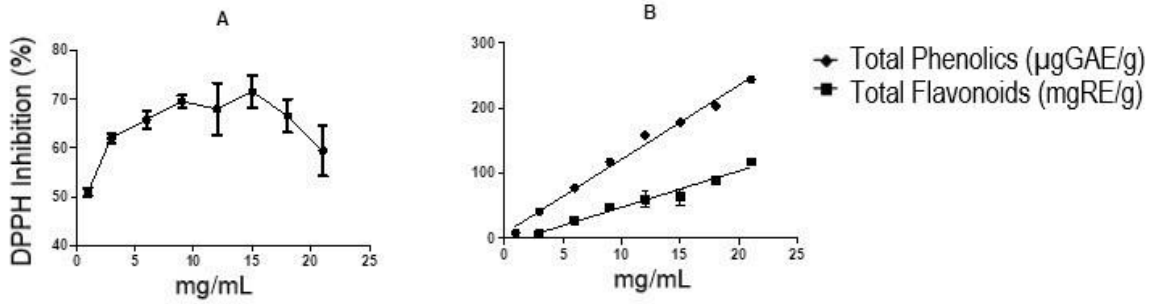


Figure 2. Graphics of P. neochilus tea: A - the antioxidant activity (DPPH inhibition); B - total phenolics (µgGAE/g) and total flavonoids (mgRE/g). Range concentration: 1 - 21 mg/mL.

The detection of the different secondary metabolites in this tea (Table 1 and Figure 2) contributes significantly to its medicinal use. Phytochemicals are reported to have several types of biological activities. It can be including antioxidant, anti-inflammatory, and cytotoxicity activities. Therefore, these compounds have beneficial effects for many diseases, such as cancer, diabetes, immune disease, inflammation, and neurodegenerative diseases 38.

In *P. neochilus* aqueous extracts, chlorogenic acid and rosmarinic acid have previously been isolated 35. Matias et al. (2019) identified other polyphenols (chlorogenic, caffeic and, rosmarinic acids), and the flavonoid glycoside, rutin. In 37, The three extracts of *P. neochilus* from the Cuban cultivars showed eight flavonoids, seven abietane diterpenes, and rosmarinic acid as the major constituents. Furthermore, 38 showed that rosmarinic acid (phenolic acid), common in *Plectranthus* species, including *P. neochilus*, is responsible for acting in the digestive and hangover process. Therefore, through these data, the consumption of *P. neochilus* tea in traditional medicine has been shown to bring benefits.

Figure 2 A showed that the percentage of inhibition of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) reached 71.48% in the concentration of 15 mg/mL of *P. neochilus* tea. 36 found values < 50% of the antioxidant capacity in their aqueous extracts of *P. neochilus* at a concentration of 100 μg/mL, but Brito et al. (2018b) showed high antioxidant capacity by the DPPH method.

Figures 2 B corroborate the antioxidant activity because there is a linear increase in phenolic compounds and total flavonoids. However, as tea concentration increased, the amount of these compounds increased too (phenolic and flavonoids).

3.3 *Artemia salina* and *Drosophila melanogaster* (LC_{50})

The LC_{50} concentration value obtained from *P. neochilus* tea in *A. salina* was 4.94 mg/mL and therefore was not considered toxic (below 1 mg/mL) 20. The value found in vivo model *D. melanogaster* obtained a similar LC_{50}, 6.93 mg/mL. Figure 3 presents these data.

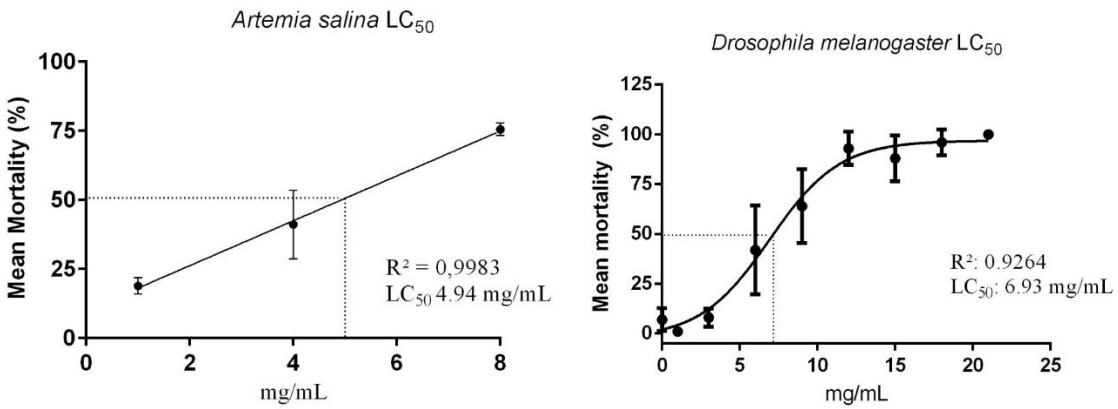


Figure 3. Median Lethal Concentration (LC_{50}) of *P. neochilus* tea in the experimental models *A. salina* (n = 30, linear regression) and *D. melanogaster* (n = 20, nonlinear regression). Data were collected after 24 hours for the bioassay with *A. salina* and after 5 days for *D. melanogaster*.

The results from the present study corroborate with 36-38. They showed that the decoctions of *P. neochilus* did not present toxicity to human cell lines. Even the study of 37 showed that the aqueous extracts do not affect cell viability at the maximum concentration tested. Thus, the LC_{50} values of the present study are in high concentrations, and, even so, they did not present toxicity in biological models, showing the low toxicity of *P. neochilus* tea.

The microbiological data obtained did not show antimicrobial activity because the MIC values for each microorganism were above 1 mg/mL 39 (Table 1).

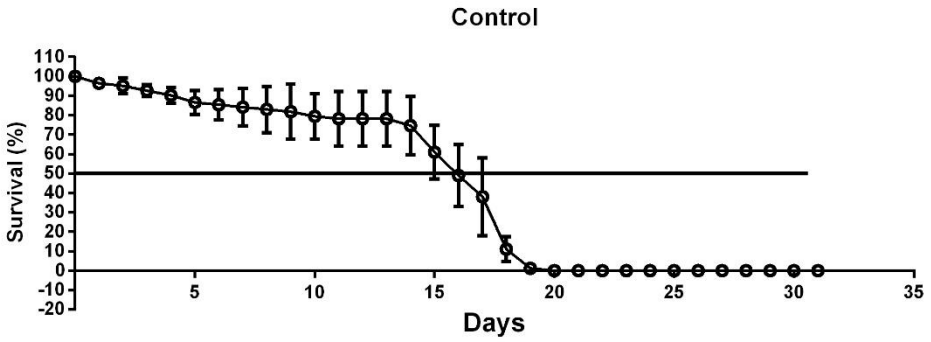
Table 1. Minimum inhibitory concentration (MIC) of *P. neochilus* tea in gram positive and negative bacteria.

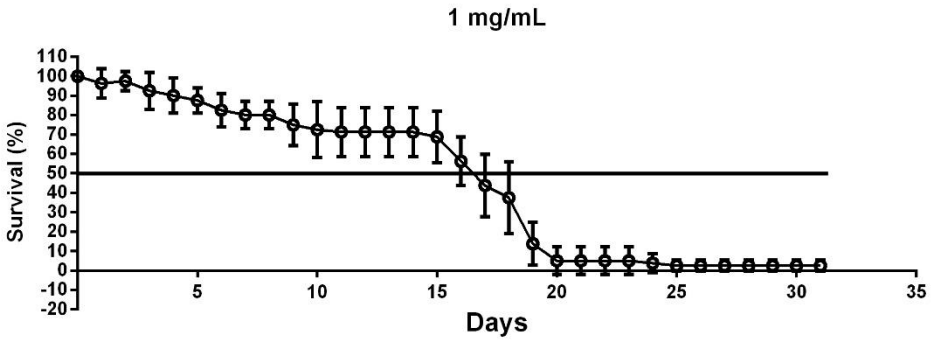
Microorganism	**MIC (mg/mL)**
Staphylococcus aureus (ATCC 29213)	6.25
Escherichia coli (ATCC 25922)	25
Pseudomonas aeruginosa (ATCC 27853)	12.5
Klebsiella pneumonie (ATCC 700603)	25
Acinetobacter baumannii (ATCC BAA-747)	12.5
Enterococcus faecalis (ATCC 29212)	3.12

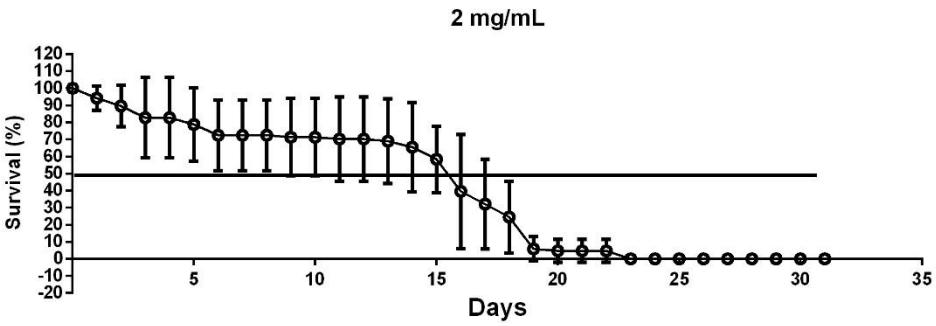
These values agree with (Matias et al. (2019); Pereira et al. (2015); Rodríguez-Ferreiro et al. (2020), who also did not obtain an antimicrobial effect with the aqueous extract of *P. neochilus*. Besides, Rodríguez-Ferreiro et al. (2020) did not obtain effect against the yeast *Candida albicans*, the fungus *Aspergillus fumigatus* and the parasites *Leishmania infantum*, *Leishmania amazonensis*, and *Trypanosoma cruzi* with the aqueous extract of *P. neochilus* leaves. Previously published studies have shown antimicrobial activity, or against other species, with the essential oil of the plant or other organic extracts, such as the acetone extract 36,40-45.

3.5 *Drosophila melanogaster* survival rate

Regarding the survival of the flies (figure 4) at concentrations that were not toxic, considering the LC_{50} (1 - 5 mg/mL), the flies survived for a more prolonged period (between 4 to 7 days) than the control group.


Control
Survival (%)
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
5
10
15
20
25
30
35
Days


1 mg/mL
Survival (%)
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
5
10
15
20
25
30
35
Days


2 mg/mL
Survival (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
5
10
15
20
25
30
35
Days

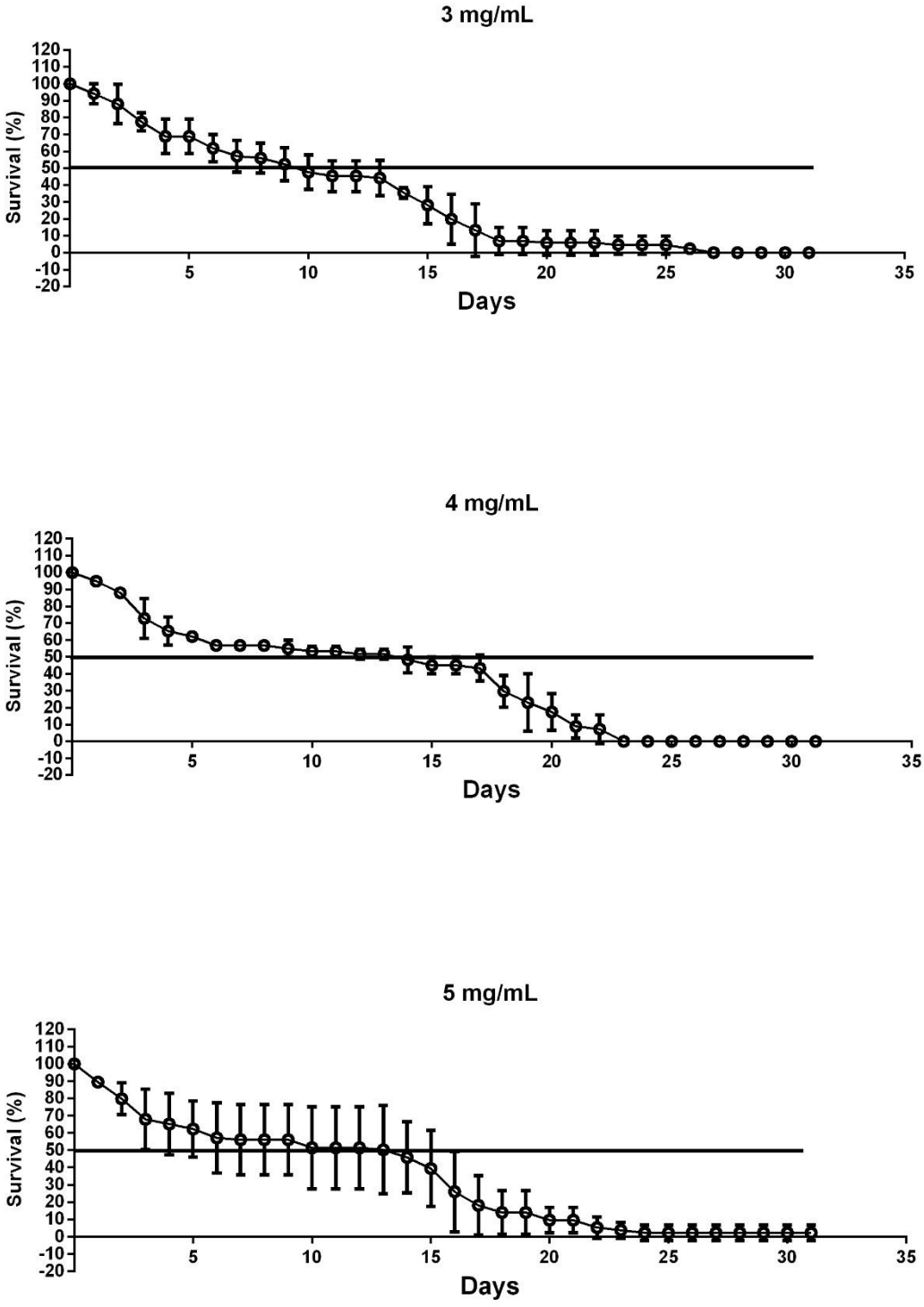


Figure 4. *Drosophila melanogaster* survival rate for 30 days. Control group: sucrose 1%. Treatment groups: 1, 2, 3, 4, and 5 mg/mL of *P. neochilus* tea with sucrose 1%.

Figure 4 shows that although flies that received the tea for 30 days survived a few more days (30, 23, 27, 23 and 30 days at concentrations of 1, 2, 3, 4 and 5 mg/mL, respectively) compared to the control (19 days), it does not mean that the tea has no toxicity. This can be verified by the 50% mortality of flies. At low dosages (1, 2 and 3 mg/mL) the tea shows similarities to the control group, as the mortality of 50% of the flies occurred on days (16, between 16 and 17 and between 15 and 16, for the control and treatments of 1mg/mL and 2mg/mL, respectively). The mortality of 50% of flies from the concentration of 3 mg/mL occurred earlier: day 9, between 11 and 12 and 10 for concentrations of 3 mg/mL, 4 mg/mL, and 5 mg/mL, respectively. These results corroborate the data in Figure 3, which shows the low toxicity of *P. neochilus* tea in biological models. However, as its concentration increases, it becomes toxic.

3.6 *Allium cepa* toxicity

The *A. cepa* bioassay was carried out with the onion roots to verify the toxicity of the different concentrations of *P. neochilus* tea. The data are shown in figure 5 and the inhibition of root growth represents the toxicity of the infusion.

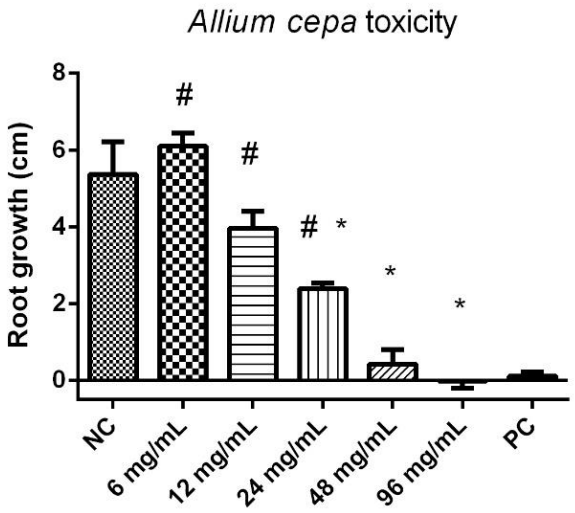


Figure 5. Bioassay of *P. neochilus* tea toxicity with the growth of onion roots (*A. cepa*) after 144 hours of exposure. * Significant difference ($p < 0.05$) concerning the negative control (NC - distilled water). # Significant difference ($p < 0.05$) concerning the positive control (PC - glyphosate 2%).

Through figure 5, the lowest concentration used (6 mg/mL) stimulates the growth of the roots because its root size grew a little more (6.1 cm) than in the control group (NC,5.3 cm). While the concentration increases, extension root decreases and becomes even toxic (48 mg/mL and 96 mg/mL with 0.4 and - 0.02 cm respectively). At the concentration of 96 mg/mL, tea becomes even more toxic than PC (0.1 cm). Through these results, *P. neochilus* tea showed to be beneficial and non-toxic up to a particular concentration.

4 Conclusions

This study showed that the ingestion of *P. neochilus* tea is healthy, beneficial, and no toxic to ingestion by biological alternative experimental models. However, its consumption in high concentrations is not recommended. Therefore, these results confirm as reported in the literature and by ethnobotany.

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CAPÍTULO VI

1 DISCUSSÃO

Na presente tese foram investigados os diferentes usos da planta *Plectranthus neochilus* na área ambiental pela fitorremediação, biológica e toxicológica pelo efeito do óleo essencial e do chá das folhas em modelos biológicos. Dessa forma, a tese foi composta por um artigo de revisão dos usos tradicionais e não tradicionais de *P. neochilus* e de três estudos experimentais, sendo um artigo e dois manuscritos.

De acordo com o primeiro estudo sobre a planta (capítulo II), mostramos que existem diversos trabalhos que abordam o uso de *P. neochilus*. Ela se desenvolve em diferentes ambientes e requer pouco manuseio. É uma planta perene, as vezes anual, prostrada e ereta. Geralmente muito ramificada e densa. A espécie não produz sementes e sua propagação vegetativa por meio de estacas possibilita a produção de mudas em menor tempo, maior uniformidade e padronização. Ela pode ser plantada em qualquer época do ano. Ademais, suas folhas e flores possuem um odor forte e sabor amargo. Geralmente, a *P. neochilus* é cultivada em ambiente domiciliar e suas folhas são ingeridas na forma de infusão (chá), para combater os problemas hepáticos e dores de estômago (COELHO et al., 2014; COUTO, 2006; DUARTE; LOPES, 2007; LORENZI; MATOS, 2002).

O estudo de Brito et al. (2018) mostrou que o ácido rosmarínico é o responsável por esse caráter medicinal curativo dos problemas hepáticos e gástricos. E que este composto está presente entre as outras espécies também denominadas de boldo, como o “boldo do chile” (*Peumus boldus*). Outros estudos também investigaram os compostos presentes em diferentes extratos e frações orgânicas de *P. neochilus* através de extrações com diferentes solventes orgânicos (etanol, metanol, hexano, acetato de etila). Muitos destes extratos mostraram a atividade antioxidante, anticolinesterásica, leishmanicida e citotóxica em algumas células e modelos experimentais (ANTINARELLI et al., 2015; ARCANJO et al., 2012; BORGES et al., 2016; RIJO et al., 2014; TEMPONE et al., 2008; VIANA, 2011). Muitas pesquisas têm investigado a composição do óleo essencial e seus efeitos (BANDEIRA et al., 2011; LEE; MIN, 2019; MOTA et al., 2010; ROSAL et al., 2011). Os achados mostraram que esse óleo essencial apresentou diferentes composições e rendimento do óleo de acordo com a localização e quantidade de material vegetal. Além disso, as atividades biológicas apresentadas foram: antibacteriana (CREVELIN et al., 2015), antifúngica (AGUIAR et al., 2018a), contra o

Schistossoma mansoni (CAIXETA et al., 2011), antioxidante (ROSAL et al., 2011) e na inibição da oviposição da mosca branca do tomate (*Bemisia tabaci*) (BALDIN et al., 2013).

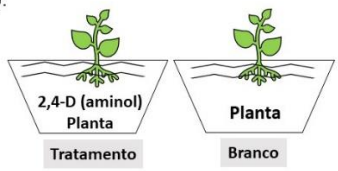
O cultivo de *P. neochilus* também pode ser usada para cobertura de solo (JAARSVELD; THOMAS, 2006; POOLEY, 1998), ou como purificadora do ar nos ambientes domésticos (POOLEY, 1998). A aplicabilidade desta planta também se destaca na área da tecnologia verde "*green technology*", uma vez que promoveu a fitorremediação do herbicida 2,4-D (RAMBORGER et al., 2017a, 2021) e, pode ser utilizada nos telhados verdes (MORAU; LIBELLE; GARDE, 2012).

Portanto, com esse artigo (capítulo II), foi possível ver as diferenças anatômicas, fisiológicas e de constituintes que diferenciam esse boldo (*P. neochilus*) de outras espécies também chamadas tradicionalmente de boldo, como o *Plectranthus barbatus, Peumus boldus* e *Vernonia condensata*. Também foi apresentado os diferentes compostos secundários extraídos das diferentes partes da planta em diferentes frações orgânicas. Por fim, foi mostrado as diferentes formas de cultivo não tradicionais dessa planta, apresentando seu caráter versátil para uso e cultivo.

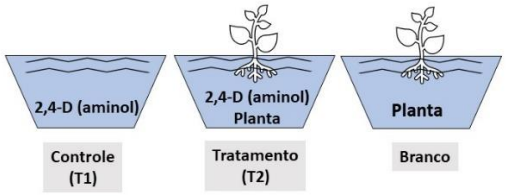
No capítulo III, correspondente a um estudo experimental, utilizamos a *P. neochilus* para realizar a fitorremediação do 2,4-D na água. Assim, investigarmos a eficiência da fitorremediação através da toxicidade do meio aquoso (resíduos) em modelos biológicos. Além disso, verificamos se havia alteração nos compostos voláteis das folhas e a possibilidade de utilizar seu óleo essencial para a atividade antimicrobiana e o chá para ingestão. Os testes foram realizados após a fitorremediação, ou seja, após a exposição da planta ao 2,4-D (na fórmula comercial aminol) na água. Os grupos experimentais e os dados encontrados no capítulo III estão expostos nas figuras 18 e 19.

Figura 18 - Grupos experimentais utilizados no capítulo III.

Grupo planta – alteração de compostos voláteis (GC-MS), do óleo essencial (MIC bacteriano) e toxicidade do chá (*D. melanogaster*):



Grupo resíduos aquosos: Degradação do 2,4-D (HPLC-DAD), toxicidade em *A. salina* e *A. cepa:*



Fonte: Autor.

De acordo com os resultados obtidos do tratamento do grupo planta (Figura 18), tanto os compostos voláteis quanto o óleo essencial não sofreram grandes alterações, pois a composição química permaneceu semelhante ao branco do grupo planta. Era esperado que ocorressem muitas alterações nesses compostos porque o 2,4-D leva a uma mudança no estado redox intracelular e extracelular devido a uma superprodução de espécies reativas de oxigênio (GROSSMANN, 2010) e isso o torna um agente estressante. Além disso, esse estresse do 2,4-D foi verificado no metabolismo dos compostos fenólicos de *P. neochilus* nos 30 dias de tratamento (RAMBORGER et al., 2017a). Porém, sugerimos que em relação ao metabolismo dos compostos voláteis esse período de 30 dias tenha sido suficiente para que a planta sofresse o estresse do herbicida e, se aclimatado, alcançando a homeostasia metabólica (TAIZ; ZEIGER, 2017; WANG; IRVING, 2011).

De acordo com os compostos voláteis majoritários identificados por cromatográfica a gás, houve aumento do trans-tujene apenas no branco do grupo planta após os 30 dias. Esse grupo branco da planta se refere a alguma alteração abiótica como luz ou temperatura no metabolismo vegetal. Portanto, supomos que a fitorremediação altera a biossíntese do trans-tujene. Assim, ele poderia estar envolvido nos processos de metabolização/degradação do 2,4-D, por ele não ter se alterado perante algum estresse abiótico no grupo tratamento da planta. Ou seja, poderia estar sendo usado para algum processo de metabolização do 2,4-D nesse processo de fitorremediação. Já o composto alfa-pineno apresentou diminuição apenas no grupo tratamento da planta após os 30 dias, evidenciando uma possível relação com o estresse

ocasionado pelo 2,4-D. Já o cis-sabinene obteve diferenças em ambos os grupos, então ele pode estar relacionado a algum estresse abiótico e não ao herbicida.

Em relação ao óleo essencial, apenas o composto 1-octen-3-ol, identificado por cromatografia a gás, obteve uma diminuição significativa (2,81%) no grupo de tratamento em relação ao grupo branco da planta. Essa alteração pode estar relacionada ao estresse causado pelo 2,4-D, porque 1-octen-3-ol ativa genes de defesa em plantas (KISHIMOTO et al., 2007). Já o composto (+)delta-cadinene (4,04%), apareceu apenas no grupo de tratamento da planta, porém estava em uma baixa porcentagem e pertence ao grupo cadinene, que estava presente nos grupos branco e tratamento da planta. Ao contrário do que foi verificado nesses dados, esperávamos que houvesse maiores variações nas quantidades dos compostos, uma vez que em nosso trabalho anterior (RAMBORGER et al., 2017a), o 2,4-D alterou significativamente a atividade antioxidante e de compostos fenólicos presentes nos chás da folha da planta. Portanto, o presente estudo mostrou que o 2,4-D afetou minimante o metabolismo secundário dos terpenos.

Ao averiguarmos a utilização do óleo essencial contra as bactérias gram positivas e negativas, observamos que não houve diferença significativa em relação a faixa de inibição de crescimento microbiano (técnica CIM, crescimento inibitório microbiano) nos grupos branco e tratamento da planta. Portanto, inferimos que o óleo essencial de *P. neochilus* pode ser utilizado para a fitorremediação e após essa técnica, ser extraído o óleo essencial de suas folhas para ser utilizado contra o crescimento de bactérias. Esse dado foi embasado na atividade desse óleo essencial ter atividade antimicrobiana em bactérias formadoras de cárie (CREVELIN et al., 2015). Inclusive, no estudo de Crevelin et al. (2015), o óleo essencial do *P. neochilus* apresentou maior capacidade antimicrobiana comparado apenas à mistura de padrões dos compostos majoritários detectados e quantificados no seu óleo.

Em relação à toxicidade do consumo do chá pelas moscas (*D. melanogaster*) após a fitorremediação, observamos que tanto o grupo branco quanto o grupo tratamento da planta foram tóxicos para este modelo experimental. E ainda, o chá do grupo tratamento foi mais tóxico do que o grupo branco pela mortalidade de 100% das moscas. Esse fato pode estar relacionado à exposição da planta ao agente tóxico (herbicida). Já é comprovado que muitas espécies vegetais crescem em locais contaminados ou adquirem esses compostos após contaminação ambiental. Portanto, sugerimos que o uso da planta após a fitorremediação para consumo em forma de chá não seja adequado.

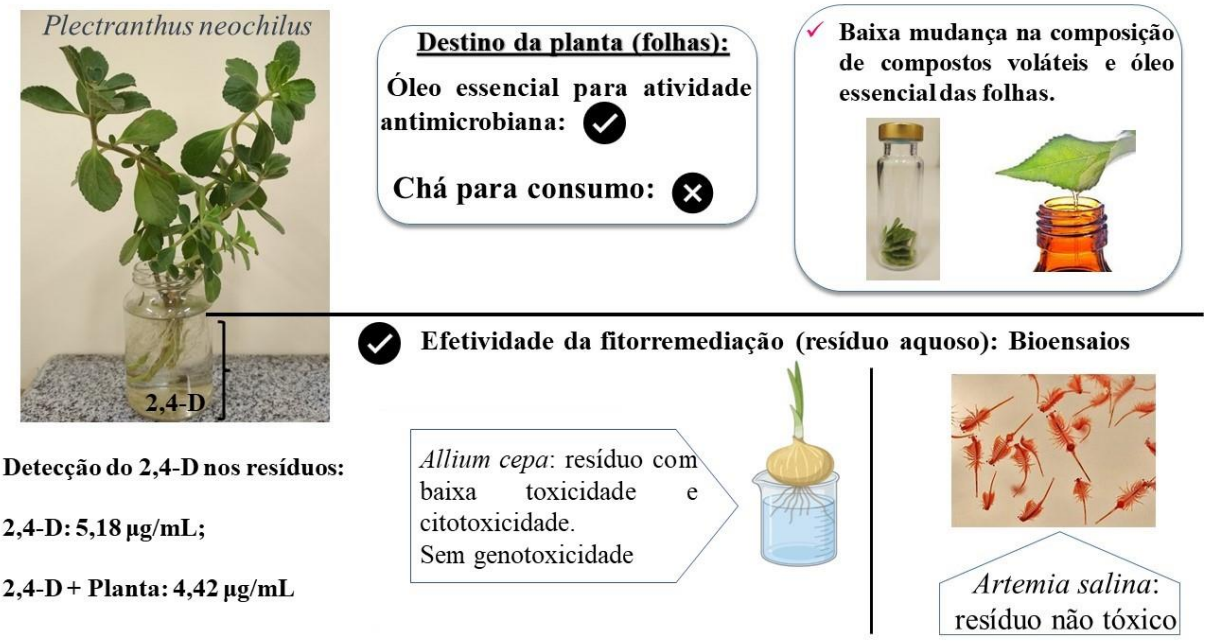
A fim de responder sobre a eficiência da fitorremediação, analisamos a toxicidade do meio aquoso após a fitorremediação (grupo resíduos, figura 16), com os modelos experimentais *A. salina* e *A. cepa*. Os resultados obtidos evidenciaram a efetividade da técnica de fitorremediação, pois o valor da dose letal mediana (DL_{50}) da *A. salina* foi de 5,6 mg/L, que é uma concentração maior do que a detectada de 2,4-D nos resíduos aquosos (5,18 mg/L e 4,42 mg/L em T1 e T2), respectivamente. Ou seja, a concentração de 2,4-D nos resíduos estava à baixo daquela que gera a DL_{50} em *A. salina*. Além do mais, ao utilizarmos os resíduos do controle e do tratamento (T1 e T2, figura 16), nenhum deles apresentou toxicidade no modelo de *A. salina*. Outros estudos também obtiveram dados de toxicidade do 2,4-D em organismos não alvo, que foi verificado através da concentração inibitória mediana (IC_{50}) urwurw. Já nos resultados pertinentes ao bioensaio com as raízes de *A. cepa*, houve toxicidade em T1 e T2. Ou seja, o resíduo aquoso afetou o crescimento das raízes de *A. cepa* em ambos os grupos. No entanto, T1 apresentou diferença significativa em relação aos grupos brancos (controles negativos: água do resíduo com a planta após 30 dias e água) e T2 teve uma diferença significativa em relação ao controle positivo (glifosato 15%). Nesse caso, há uma tendência de que T1 foi mais tóxico que T2. O que pode ter contribuído para essa toxicidade apresentada em T2, pode ter sido em razão das folhas e raízes mortas que ficaram nesse resíduo durante o período do tratamento. Podemos supor que o 2,4-D pode ter retornado ao meio aquoso quando as folhas e raízes morreram e permaneceram nesse meio durante todo o experimento. Porém, não foi possível verificar se o 2,4-D fica acumulado (fitoacumulação) na planta ou é degradado (fitodegradação). Aliado a isso, os valores de pH observados foram diferentes, sendo: 7,4 no BNC; 9,51 para T1; 8,4 em T2, o que pode ter contribuído para a degradação do 2,4-D no meio aquoso.

A toxicidade apresentada também foi observada nas análises microscópicas das células, pois houve diminuição no ciclo celular (citotoxicidade) maior em T1. Entretanto, não houve genotoxicidade nestes grupos, pois não houve aberrações cromossômicas significativas comparadas ao grupo controle positivo. Esses dados estão de acordo com Özkul et al. (2016), que obteve índice mitótico reduzido (diminuição no ciclo celular) com aumento da concentração de 2,4-D. Porém, nossos resultados diferem em relação ao achado no sangue periférico do peixe *Cnesterodon decemmaculatu*, exposto ao 2,4-D, onde Ruiz de Arcaute; Soloneski e Larramendy (2016) obtiveram um aumento na frequência de micronúcleos nos eritrócitos. E os autores Laborde; Larramendy e Soloneski (2020) observaram o potencial genotóxico do 2,4-D em células de ovário de hamster (CHOeK1). De acordo com Zuanazzi;

Ghisi; Oliveira (2020), a pesquisa sobre a toxicologia e mutagenicidade do 2,4-D avançou rapidamente e pode se concentrar na área da biologia molecular, como expressão gênica, avaliação da exposição em bioindicadores humanos ou outros vertebrados bioindicadores, além de estudos de degradação de pesticidas.

Portanto, no capítulo III (figura 19) foi possível concluir que: tanto os compostos voláteis quanto o óleo essencial apresentaram baixa alteração em suas composições na planta exposta ao 2,4-D; o óleo essencial de *P. neochilus* pode ser utilizado para atividade antimicrobiana, porém seu chá apresentou toxicidade; o meio aquoso com a planta diminuiu a concentração do 2,4-D e esse grupo de resíduos apresentou menor toxicidade nos modelos biológicos, comprovando a efetividade da fitorremediação.

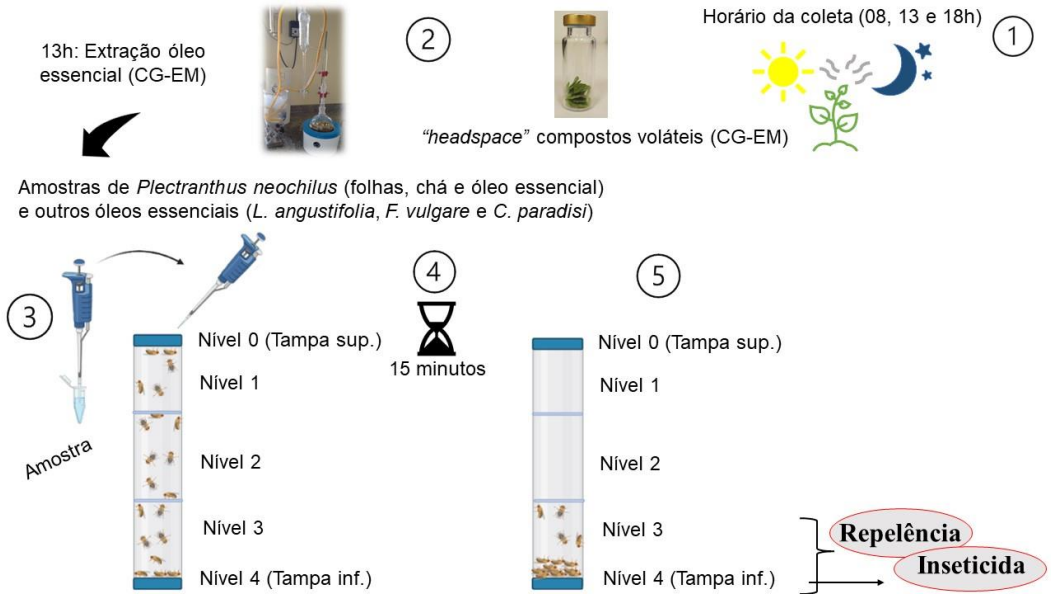
Figura 19 - Principais resultados obtidos no capítulo III.



Fonte: Autor.

No capítulo IV, investigamos se a folha, chá e óleo essencial (OE) de *P. neochilus* apresentavam atividades repelentes e inseticidas no modelo experimental *D. melanogaster* (mosca). O esquema geral da condução desse estudo está representado resumidamente na figura 20.

Figura 20 - Sequência experimental realizada no capítulo IV.



Fonte: Autor, adaptado de Biorender.com.

Primeiramente, realizamos as coletas das folhas de *P. neochilus* em diferentes horários (08, 13 e 18h) e analisamos a alteração dos compostos voláteis nesses períodos (figura 20, etapas 1 e 2). Assim, saberíamos qual seria o melhor horário para realizar as coletas. Com isso, observamos que o horário das 13 horas foi o que apresentou maior aumento nas porcentagens dos compostos voláteis (identificados por cromatografia a gás) e escolhido para as coletas das folhas para todas as amostras com o *P. neochilus*.

Os dados sobre o efeito repelente e inseticida de *P. neochilus*, observado pelo comportamento de distância das moscas nos níveis e morte delas (figura 20, etapas 3, 4 e 5 após 45 minutos), mostraram as folhas e, principalmente o chá, obtiveram efeito atrativo para as moscas, pois elas permaneceram apenas na parte superior (figura 20, nível 0 e 1). Apenas o OE da planta apresentou efeito repelente e inseticida. Baldin et al. (2013) obtiveram resultados semelhantes sobre a repelência, ao verificarem o potencial do óleo essencial de *P. neochilus* em inibir a oviposição da mosca branca (*Bemisia tabaci*, tipo B), em tomates. Ao analisarmos a composição química do OE através da cromatografia a gás acoplada ao espectrômetro de massas (CG-EM), observamos que dentre os compostos identificados e quantificados (%), o cariofileno (13.54%) e o 1-octen-3-ol (10.74%) foram os majoritários. No mesmo estudo de Baldin et al. (2013), também foi identificado o composto cariofileno como a majoritário. Além disso, outras pesquisas evidenciaram que este composto, assim como algumas de suas isoformas, foi altamente tóxico e, ou considerado repelente para outros modelos experimentais, incluindo mosquitos (CAO et al., 2018; GILLIJ; GLEISER; ZYGADLO, 2008; GUO et al., 2017; MA et al., 2020; NARARAK et al., 2019; PLATA-RUEDA et al., 2018). No modelo

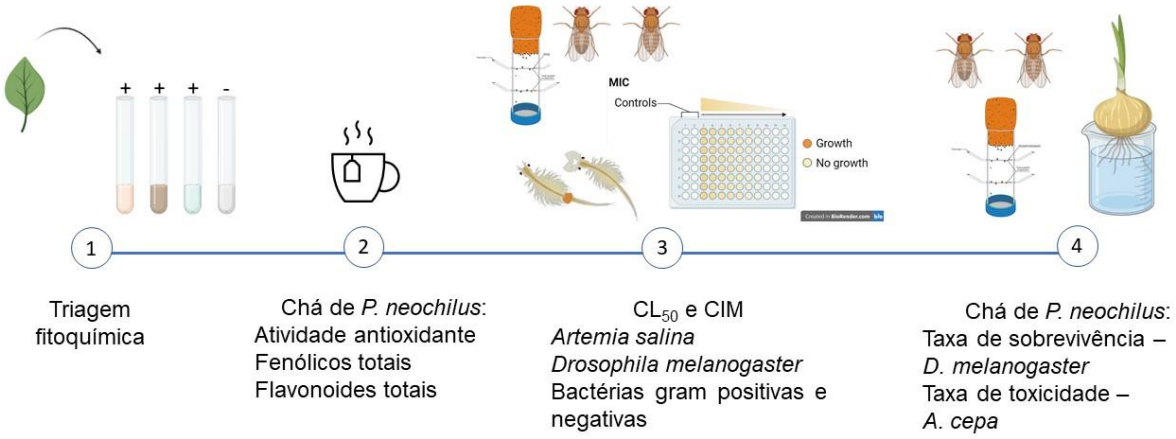
experimental *Cryptolestes ferrugineus*, o 1-octen-3-ol demonstrou ter atividade atraente em baixas concentrações e repelente em concentrações mais altas (MUSHOBOZY; PIERCE; BORDEN, 1993; PIERCE et al., 1991). Aliás, ele já mostrou causar aversão na espécie *Drosophila melanogaster* (KNADEN et al., 2012), e diminuiu a oviposição de *Drosophila suzukii* em framboesas vermelhas (WALLINGFORD; CHA; LOEB, 2018). Outro composto que também causa aversão às moscas é o linalol (KNADEN et al., 2012). No nosso estudo, sua porcentagem de área do pico foi baixa (0,94%). Entretanto, inferimos que ele pode estar relacionado ao efeito repelente, conforme já descrito por Cao et al. (2018), Kheloul et al. (2020), Taban; Saharkhiz; Hooshmandi (2017) e Yoon et al. (2011). Os outros compostos minoritários identificados nesse trabalho, Ciclosativeno, gama-cadineno e alfa-terpineol acetato, geralmente também estão em porcentagens mais baixas nos OEs, e possuem outras características e atividades. No entanto, o gama-cadineno mostrou atividade acaricida para *Psoroptes cuniculi* (GUO et al., 2017) e o alfa-terpineol acetato causou repelência contra *Tribolium castaneum* (WANG et al., 2019).

Através dos resultados obtidos do efeito repelente e inseticida do OE de *P. neochilus*, resolvemos comparar se esse efeito era equivalente a outros OEs disponíveis no mercado e que também apresentassem essas atividades. Portanto, utilizamos a mesma metodologia apresentada na figura 18 (etapas 3, 4 e 5) com *P. neochilus, Lavandula angustifólia, Foeniculum vulgare* e *Citrus paradisi* e aumentamos o tempo de exposição para 90 minutos. Os dados comprovaram que os efeitos repelente e inseticida de *P. neochilus* foram maiores e ocorreram mais rapidamente que os demais óleos. A sequência do efeito inseticida de cada OE após 90 minutos de exposição foi: *P. neochilus* (81,25%) > *L. angustifolia* (7,5%) > *F. vulgare* (2,5%) e *Citrus paradisi* (0%). Assim, o presente estudo confirmou o efeito repelente e/ou inseticida já relatado para *L. angustifolia* (HAN et al., 2020; KHOSRAVI; SENDI, 2013; YAZDANI et al., 2013), *F. vulgare* (GUSMÃO et al., 2013; RANA; RANA, 2012; ZOUBIRI et al., 2014), bem como para *C. paradisi* (ABBAS et al., 2012; DUTRA et al., 2016). Com esses dados, constatamos que mais testes com o OE de *P. neochilus* são importantes, como por exemplo: análises em outras espécies de insetos, tempo de permanência do óleo essencial no meio, entre outros. Assim poderemos verificar, futuramente, a possibilidade de seu uso como bioinseticida.

No capítulo V apresentamos o estudo sobre a toxicidade do chá de *P. neochilus* em diferentes modelos experimentais (*A. cepa, A. salina* e *D. melanogaster*). Realizamos também a análise fitoquímica das folhas da planta, bem como a composição fenólica, de flavonoides e

a capacidade antioxidante. O esquema metodológico desse capítulo está apresentado na figura 21.

Figura 21 - Sequência experimental realizada no capítulo V.



Fonte: Autor, adaptado de Biorender.com.

Os testes fitoquímicos permitiram identificar as seguintes classes de compostos: alcaloides, flavonoides, taninos, glicosídeos, saponinas, fenóis e terpenoides. A atividade antioxidante esteve à cima de 50% de inibição do radical DPPH em todas as concentrações testadas e atingiu 71,48% na concentração de 15 mg/mL. As dosagens dos compostos fenólicos totais e flavonoides totais obtiveram um aumento linear de acordo com o aumento da concentração do chá. Esses dados foram interessantes, uma vez que diversos fitoquímicos possuem muitas atividades biológicas (LEE; MIN, 2019). Em extratos aquosos de *P. neochilus*, os polifenóis ácido cafeico, clorogênico e rosmarínico foram identificados, bem como o flavonoide glicosídeo rutina (GROSSMANN, 2010; MATIAS et al., 2019; SHAREEF; SHAW, 2008). Inclusive, Brito et al. (2018) mostraram que o ácido rosmarínico é um composto em comum com outras espécies de *Plectranthus*, incluindo *P. neochilus*, e é responsável por atuar no processo digestivo e de ressaca.

Por fim, observamos que o chá se mostrou seguro para consumo nas quantidades usualmente utilizados em preparos caseiros, que em geral é de 1 g de chá para 100 mL de água (equivalente a 10 mg/mL). Esses resultados foram comprovados através dos valores de concentração letal mediana (CL_{50}) para *A. salina* que foi de 4,94 mg/mL. Essa concentração é à cima de 1 mg/mL, que é o valor considerado tóxico para esse modelo (MEYER et al., 1982). O valor de CL_{50} encontrado no modelo *D. melanogaster* foi de 6,93 mg/mL. Assim, nossos achados nesses modelos experimentais estão de acordo com (BRITO et al., 2018c; MATIAS et al., 2019; RODRÍGUEZ-FERREIRO et al., 2022), onde as decocções de *P. neochilus* também

não apresentaram toxicidade linhagens celulares. Portanto, pode ser observado que nossos valores de CL_{50} se encontram em altas concentrações e, mesmo assim, não apresentaram toxicidade no modelo biológico *in vivo*, evidenciando a baixa toxicidade do chá *P. neochilus*.

Através desses dados, investigamos a sobrevivência da *D. melanogaster* que também receberam alimentação líquida contínua, porém, nas concentrações não tóxicas para elas, de acordo com a CL_{50} obtida (1 - 5 mg/mL), por até 30 dias. Observamos então que no grupo controle (sacarose 1% em água destilada) as moscas sobreviveram por até 19 dias. Nos grupos de tratamento (sacarose 1% diluída nas diferentes concentrações do chá), as moscas sobreviveram por até 30, 23, 27, 23 e 30 dias nas concentrações de 1, 2, 3, 4 e 5 mg/mL, respectivamente. Porém, ao verificar a mortalidade de 50% das moscas durante esse período, observamos que em baixas dosagens (1 e 2 mg/mL), as menores concentrações do chá (1 e 2 mg/mL) apresentaram mortalidade semelhante ao grupo controle. Nesse caso, a mortalidade de 50% das moscas ocorreu nos dias 16, entre 16-17 e, entre 15 -16 nos grupos controle e tratamentos de 1 mg/mL e 2 mg/mL, respectivamente. A mortalidade de 50% das moscas das demais concentrações do chá (3 mg/mL 4 mg/mL e 5 mg/mL, respectivamente) ocorreram mais cedo (dia 9, entre 11-12 e no dia 10, respectivamente). Esses resultados corroboram com os achados da baixa toxicidade do chá de *P. neochilus* em baixas concentrações nos modelos biológicos. À medida que sua concentração aumenta, o chá se torna tóxico.

A fim de investigar a toxicidade do chá em concentrações ainda mais altas, utilizamos o modelo A. cepa para verificar o crescimento das raízes de A. cepa em concentrações superiores daquelas até então utilizadas (6 mg/mL - 96 mg/mL). Assim, novamente foi observado que com o aumento da concentração do chá, as raízes não cresceram normalmente, ocorrendo parada de crescimento nas maiores concentrações (48 mg/mL e 96 mg/mL). Portando, a partir de 24 mg/mL o chá se tornou tóxico para as raízes, entretanto essa concentração é considerada à cima do que normalmente se utiliza na medicina tradicional. Por meio desses resultados foi possível verificar que o chá de *P. neochilus* apresenta compostos secundários que permitem ele ter uma boa atividade antioxidante. Porém, com o aumento da concentração desse chá, ele se torna tóxico para os modelos experimentais complementares utilizados.

2 CONCLUSÕES tópicos

Com este estudo foi possível verificar a alta versatilidade de uso da planta *P. neochilus* e uma diversidade de atividades e efeitos biológicos. Os principais achados do estudo foram:

- Capacidade de utilizar o óleo essencial da planta após a técnica de fitorremediação para atividade antibacteriana. E, comprovação da menor toxicidade do resíduo aquoso utilizando os modelos *Artemia salina* e *Allium cepa*.
- O óleo essencial da planta comprovou ter efeito repelente e inseticida no modelo *D. melanogaster*.
- Seu chá se mostrou atóxico nas concentrações nos modelos *D. melanogaster*, *A. salina* e *A. cepa.*

3 PERSPECTIVAS FUTURAS

- Otimizar o tempo e condições para *P. neochilus* na fitorremediação do 2,4-D com uma técnica semelhante às "*phytobeds*".
- Realizar testes da fitorremediação com a amostra real (resíduo de rios, lagos ou barragens próximas a aplicação do 2,4-D) e com outros pesticidas altamente utilizados no RS.
- Verificar diferentes condições do meio aquoso, como pH, temperatura e luminosidade, que levam à autodegradação do 2,4-D no período de 30 dias.
- Testar diferentes modelos experimentais (mosquitos e larvas, por exemplo) para os testes de repelência e efeito inseticida do óleo essencial.
- Comparar a atividade repelente e inseticida do óleo essencial de *P. neochilus* com outros óleos essenciais.
- Analisar e verificar quais são os compostos bioativos presentes no chá de *P. neochilus* que o tornam tóxico de acordo com a concentração.

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