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DOCTORADO EN CIENCIAS EN HORTICULTURA

Metabolómica y actividad biológica del aceite esencial de hojas de árboles hembra y macho de *Pimenta dioica* L. Merrill en diferente época del año

TESIS

Que como requisito parcial para obtener el grado de:

DOCTORA EN CIENCIAS EN HORTICULTURA

Presenta:

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Bajo la supervisión de: Dra. María del Rosario García Mateos

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APROBADA

METABOLÓMICA Y ACTIVIDAD BIOLÓGICA DEL ACEITE ESENCIAL DE HOJAS DE ÁRBOLES HEMBRA Y MACHO DE *Pimenta dioica* L. MERRILL EN DIFERENTE ÉPOCA DEL AÑO

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RESUMEN GENERAL

METABOLÓMICA Y ACTIVIDAD BIOLÓGICA DEL ACEITE ESENCIAL DE HOJAS DE ÁRBOLES HEMBRA Y MACHO DE *Pimenta dioica* L. MERRILL EN DIFERENTE ÉPOCA DEL AÑO[1]

Pimenta dioica L. Merrill, conocida como pimienta gorda o allspice, es una especie aromática de gran valor económico y biológico debido a la diversidad de metabolitos presentes en su aceite esencial y su potencial para aplicaciones en las industrias farmacéutica, cosmética, alimentaria y agrícola. Sin embargo, la mayoría de los estudios se han enfocado en los frutos, mientras que las hojas de árboles macho y hembra han sido escasamente caracterizadas y subutilizadas. En el presente estudio se abordaron de manera integral los aspectos moleculares, fitoquímicos y biológicos de *P. dioica* con el objetivo de caracterizar la variabilidad genética, identificar los metabolitos volátiles en hojas de ambos tipos de árboles y evaluar su citotoxicidad y su actividad fungistática. La caracterización molecular se realizó mediante análisis de agrupamiento con el método de varianza mínima de Ward, las distancias genéticas fueron calculadas con el coeficiente de similitud de Jaccard y un análisis de varianza molecular (AMOVA). Los extractos hexánicos se analizaron mediante cromatografía de gases acoplada a espectrometría de masas (GC–MS), se identificaron 42 metabolitos, 17 se reportaron por primera vez en el tejido foliar. Además, el aceite esencial obtenido por hidrodestilación y arrastre de vapor en tres épocas del año (junio, septiembre y enero) permitió detectar 126 compuestos volátiles, 27 exclusivos de árboles hembra y 28 de árboles macho. El análisis multivariado demostró diferencias metabólicas significativas asociadas al tipo de árbol, al método de extracción y a la época de muestreo, mientras que los bioensayos mostraron actividad fungistática frente a *Fusarium oxysporum* f. sp. *lycopersici* (FOL3), donde destacaron los aceites de árboles macho ($p \leq 0.001$). Estos resultados demostraron la alta variabilidad genética y química de *P. dioica.* Así como, su potencial agrobiotecnológico fuente de metabolitos bioactivos y de diferenciación sexual.

Palabras clave: allspice, pimienta gorda, citotoxicidad

[1] Tesis de Doctorado en Ciencias en Horticultura, Universidad Autónoma Chapingo
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GENERAL ABSTRACT

METABOLOMICS AND BIOLOGICAL ACTIVITY OF LEAF ESSENTIAL OIL FROM FEMALE AND MALE TREES OF *Pimenta dioica* L. Merrill AT DIFFERENT SEASONS[2]

Pimenta dioica L. Merrill, known as allspice or Jamaican pepper, is an aromatic species of high economic and biological value due to the diversity of metabolites in its essential oil and its potential applications in the pharmaceutical, cosmetic, food, and agricultural industries. However, most studies have focused on the fruits, while the leaves of male and female trees have been poorly characterized and remain underutilized. In this study, the molecular, phytochemical, and biological aspects of *P. dioica* were comprehensively addressed with the aim of characterizing its genetic variability, identifying volatile metabolites in leaves of both trees, and evaluating their cytotoxicity and fungistatic activity. Molecular characterization was performed through cluster analysis using Ward's minimum variance method. Genetic distances were calculated with the Jaccard similarity coefficient, and an analysis of molecular variance (AMOVA) was also conducted. Hexane extracts were analyzed by gas chromatography–mass spectrometry (GC–MS), resulting in the identification of 42 metabolites, 17 of which were reported for the first time in leaf tissue. Additionally, the essential oil obtained by hydrodistillation and steam distillation in three periods of the year (june, september, and january) allowed the identification of 126 volatile compounds, 27 exclusives to female trees and 28 to male trees. Multivariate analysis revealed significant metabolic differences associated with sex, extraction method, and sampling period. Bioassays showed fungistatic activity against *Fusarium oxysporum* f. sp. *lycopersici* (FOL3), with essential oils from male trees exhibiting the strongest inhibition ($p \leq 0.001$). These results demonstrated the high genetic and chemical variability of *P. dioica*, as well as its agrobiotechnological potential as a source of bioactive metabolites and for sexual differentiation.

Keywords: allspice, pimienta gorda, cytotoxicity

[2] Thesis of Doctor in Science in Horticulture, Universidad Autónoma Chapingo
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CAPÍTULO 1. INTRODUCCIÓN GENERAL

Pimenta dioica L. Merrill es una especie arbórea y perennifolia perteneciente a la familia Myrtaceae, originaria del Caribe y Centroamérica. En náhuatl se le conoce como xocoxóchitl (flor con olor a acedo), también se le denomina pimienta gorda por el tamaño de fruto y como pimienta de Jamaica debido a que es el principal país productor (Raj et al., 2023). En el idioma inglés se le conoce como *allspice* debido al conjunto de sabores y aroma que presenta el fruto, una combinación de nuez moscada, canela, clavo y pimienta negra (Krishnamoorthy y Rema, 2006). Sus hojas y frutos presentan compuestos orgánicos volátiles (COVs) en su mayoría de tipo terpenoide, que confieren sabor y aroma agradables. El aceite esencial obtenido de los frutos secos es el producto comercial de *P. dioica* de mayor importancia económica. Las técnicas convencionales para la obtención del aceite esencial son, principalmente, el arrastre de vapor y la hidrodestilación. Aunque existen otros métodos como la extracción por soxhlet y la extracción con fluidos supercríticos. Los estudios sobre su perfil químico reportaron aproximadamente 60 compuestos (Premachandran et al., 2024). Además del fruto, la hoja presenta una diversidad de compuestos aromáticos que pueden percibirse al comprimirse manualmente. En la zona del centro norte del estado de Veracruz, las hojas no presentan importancia económica relevante en comparación del fruto, ya que son utilizadas para fines culinarios y medicinales, en infusiones para el alivio de cólicos menstruales y dolores estomacales (Montalvo-Hernández et al., 2024).

Estudios recientes de *P. dioica* están relacionados principalmente con la composición del aceite esencial de los frutos y de las hojas; sin embargo, muy pocas investigaciones consideran su descripción botánica al considerarse una especie hermafrodita funcionalmente dioica, es decir, en su sistema de producción se encuentran árboles hembra, que son los únicos productores de fruto, y árboles macho, los cuales se eliminan por no ser productores de fruto, por ende, su hoja es desechada y subutilizada (Montalvo-López et al., 2021). A la fecha, solo hay dos estudios que involucran árboles hembra y macho. El estudio de Minott y Brown (2007) donde se analizó el aceite esencial obtenido de hojas secas de árboles hembra y macho ubicados en unidades de

producción del país Jamaica y el estudio realizado por Montalvo-López et al. (2021) que reporta el perfil de COVs identificados en hojas de diferente estado de desarrollo mediante la técnica SPME-GC-MS (Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry).

Actualmente, la metabolómica ha sido una herramienta en el estudio de las plantas para conocer la composición, la variación y la función de los metabolitos presentes. Este análisis puede ser de manera dirigida realizando mediciones de grupos específicos de compuestos químicos o de manera no dirigida que comprende el análisis de todos los analitos posibles en una muestra incluyendo aquellos no identificados (Emam et al., 2025). Los avances tecnológicos han convertido este campo en un proceso complejo que abarca desde la recolección y extracción de muestras hasta el análisis estadístico multivariado (Hao et al., 2025). De esta manera, el integrar la información de los metabolitos secundarios, específicamente los COVs, presentes en el aceite esencial de árboles hembra y árboles macho de *P. dioica*, y correlacionarlos con factores como época del año y el método de extracción mediante un análisis multivariado da lugar a un panorama de conocimiento y revalorización de esta especie que se encuentra en vías de desarrollo económico y cuyas hojas subutilizadas presentan alto potencial en la industria farmacéutica, cosmética, alimentaria y agronómica. Aunado a lo anterior, el cultivo de *P. dioica* podría consolidarse como una actividad rentable y con alto potencial para transitar hacia un sistema de producción competitivo, capaz de mejorar las condiciones de los productores y contribuir al desarrollo regional. Su fortalecimiento impulsaría además procesos agroindustriales que detonen el crecimiento económico del productor y hagan de esta cadena productiva una alternativa atractiva para las regiones productoras.

El objetivo general de esta investigación fue determinar el perfil y rendimiento del aceite esencial de hojas de árboles hembra y macho de *P. dioica* L. Merrill localizados en el estado de Veracruz, obtenidos por diferentes métodos de extracción y en diferente época del año, así como evaluar la actividad biológica y, por primera vez, caracterizar molecularmente la variabilidad genética.

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CAPÍTULO 2. REVISIÓN DE LITERATURA

2.1. *Pimenta dioica* L. Merrill

2.1.1. Descripción botánica

Pimenta dioica L. Merrill es una especie que pertenece a la familia Myrtaceae y se distribuye en México, Centroamérica, el Caribe y las Antillas. Es un árbol que puede llegar a medir hasta 30 m de altura, sus hojas son simples, opuestas y con abundantes puntos glandulosos donde se encuentra el aceite esencial (Figura 1) (Martínez et al., 2013).

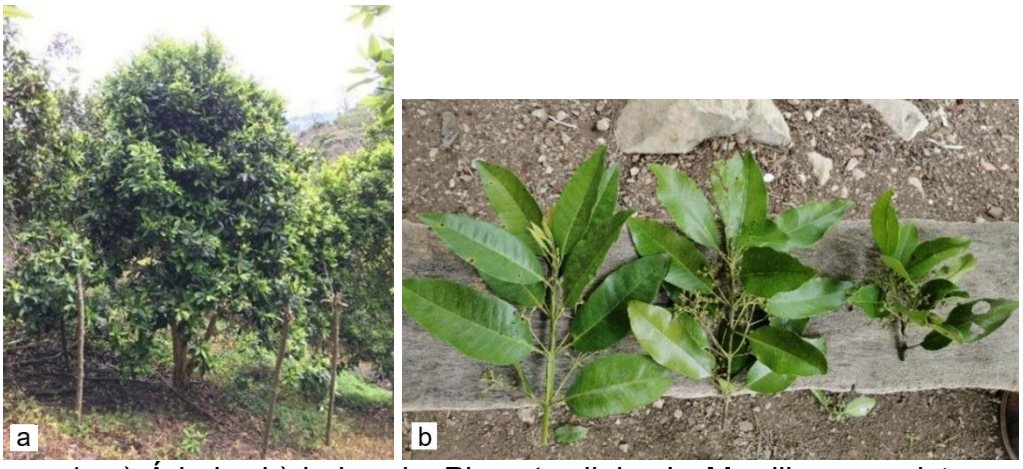

Figura 1. a) Árbol y b) hojas de *Pimenta dioica* L. Merrill en un sistema de producción en la comunidad de Tilzapota, municipio de Atzalan, Veracruz, México. Fotografías propias.

Sus flores se encuentran distribuidas en panículas, de color crema, con cinco pétalos; se describen como hermafroditas funcionalmente dioicas, es decir, los árboles poseen flores perfectas, con todos los verticilos; sin embargo, estas flores se diferencian funcionalmente en dos tipos de árboles: los árboles macho cuya flor tiene estambres de mayor longitud que sobresalen sobre el pistilo y que el número es el doble (aproximadamente 100) en comparación a la flores de árboles hembra cuyos estambres son en menor número (aproximadamente 50) y el pistilo sobresale de los estambres (Figura 2). El árbol hembra es considerado económicamente más importante ya que es el único tipo de árbol que produce frutos, de lo cual deriva su nombre, mientras que el árbol macho solo funge como polinizador. Sin embargo, observaciones en campo indican que los árboles hembra pueden producir fruto aun cuando reciben polen de otros árboles hembra. Hasta el momento no se ha publicado

un protocolo para la diferenciación de estos árboles la cual ocurre después de los cinco años de crecimiento y desarrollo (Montalvo-López et al., 2021). Es importante mencionar que, durante este tiempo, el productor realiza una inversión económica para el mantenimiento del sistema de producción y que, tras la diferenciación de árboles, muchos optan por la eliminación de los árboles macho ya que no proporcionan la parte económica de comercialización, el fruto.

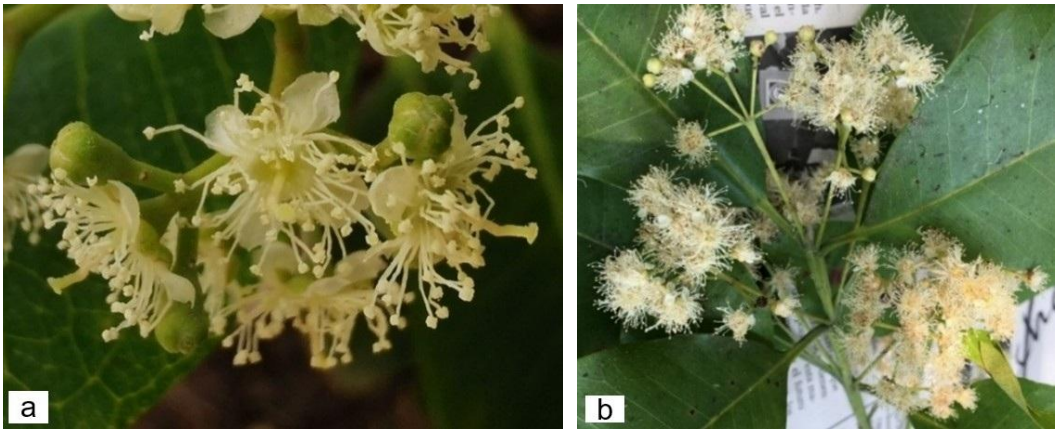

Figura 2. Flores de a) árboles hembra y b) árboles macho de *Pimenta dioica* L. Merrill. Fotografías propias.

2.1.2. Panorama nacional de producción de *Pimenta dioica*

En México los principales estados productores son Veracruz, Puebla y Tabasco con aportación del 97 % de la producción nacional (Morales-German et al., 2025). Esta especie se cultiva, en su mayoría, como policultivo, en asociación con maíz, plátano, café y algunas especies maderables como el cedro. El fruto es la parte económicamente más importante, es cosechado en estado verde inmaduro antes que llegue a su estado de madurez fisiológica, debido a que el fruto se vuelve dulce y es un alimento ideal para las aves (Montalvo-Hernández et al., 2024).

El rendimiento de pimienta gorda nacional reportado fluctúa entre 1.1 y 1.8 t ha^{-1} de fruto fresco, es la parte que comercializa el productor. Los rendimientos de esta especie han crecido, lo cual se ve reflejado en el valor de su producción ya que en el año 2011 la producción de fruto fue de 3452 t y en el año 2023 fue de 9800 t lo cual indica un incremento de 183.9 %

(Morales-German et al., 2025). Lo anterior se debe a la incorporación de tecnologías que ayudan a un desarrollo del sistema de producción adecuado, como fertilización, podas específicas, aplicación de fungicidas y herbicidas; así como, la siembra de injertos, ya que estos aseguran la presencia de árboles hembra productoras de fruto, aunque su costo puede oscilar entre 250 - 300 pesos (Montalvo-Hernández et al., 2024). No obstante, es importante destacar que se esperaría que el esqueje de las plantas injertadas proviniera de árboles sobresalientes, con características de interés comercial, tales como tolerancia a plagas y enfermedades, así como frutos y hojas de mayor tamaño, con aroma y sabor intensos característicos de la pimienta gorda para que el sistema de producción sea eficiente, rentable y genéticamente uniforme.

2.1.3. Diferencias entre pimienta gorda (*Pimenta dioica* L. Merrill) y pimienta negra (*Piper nigrum* L.)

Como se ha mencionado anteriormente, *P. dioica* es una especie cuyos frutos secos son usados principalmente como condimento, también es conocida como pimienta gorda, debido al tamaño que puede alcanzar su fruto, aproximadamente un cm de diámetro (Premachandran et al., 2025). Sin embargo, la mayoría de las veces la pimienta gorda es confundida con la pimienta negra (Figura 3), siendo que la primera presenta un fruto más grande de casi un centímetro de diámetro, pertenece a la familia Myrtaceae, es una especie arbórea, perennifolia y sus frutos deben ser cosechados en un estado fisiológicamente inmaduro, pero con su máximo tamaño. Por otro lado, la pimenta negra (*Piper nigrum* L.) pertenece a la familia Piperaceae, además de ser una planta herbácea trepadora cuyo fruto apenas alcanza 0.5 cm, es rugoso y presenta un sabor picante debido a la presencia de piperina (Wimalarathna et al., 2024).

Figura 3. Frutos a) verdes y secos de b) *Pimenta dioica* L. Merrill y c) Piper *nigrum* L. Elaboración propia.

2.1.4. Importancia económica

El fruto es la parte vegetal económicamente más importante de *P. dioica*. Es una baya verde que puede llegar a medir 1 cm de diámetro, en la etapa de madurez es de color púrpura e interior blando (Figura 4). Para su comercialización, los frutos se cosechan en estado verde inmaduro, momento en que han alcanzado su máximo tamaño; después son deshidratados para ser comercializados para la realización de moles, conservas, como sazonador de carnes, preparación de licores y en la elaboración de postres (Pennington y Sarukhán, 2005). El aceite esencial se usa en la industria de la perfumería, en la fabricación de velas, productos cosméticos y de limpieza. Se han realizado estudios que demuestran la capacidad del aceite esencial como nematicida, acaricida e insecticida (Contreras, 2018; El-Nashar et al., 2023).

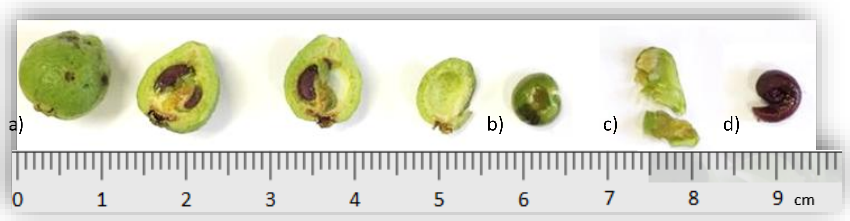

Figura 4. Disección del fruto inmaduro de *Pimenta dioica* L. Merrill: a) fruto; b) semilla; c) cubiertas seminales, y d) embrión. Elaboración propia.

2.1.5. Propiedades medicinales

Los frutos y hojas de *P. dioica* se han utilizado para el tratamiento de artrosis, dolor de estómago, dolor dental, neuralgia, gastritis, mialgia, flatulencia y candidiasis, debido a sus propiedades analgésicas, carminativas, digestivas, antimicrobianas, antiinflamatorias y antihipertensivas, resultado de la diversidad de metabolitos que presentan sus hojas y frutos, tales como compuestos fenólicos, taninos, flavonoides, entre otros (Premachandran et al., 2025). Las semillas de *P. dioica* se emplean en Haití y Cuba mezcladas con ron y azúcar para aliviar el dolor abdominal. Además, esta especie también se ha utilizado como especia y agente sazonador de alimentos (El-Nashar et al., 2023).

2.2. Aceite esencial

El aceite esencial es una mezcla de metabolitos hidrofóbicos de bajo peso molecular, lo que explica su volatilidad, además presenta olor y características distintivas. Cada uno de los compuestos del aceite esencial es diferente debido a la estructura química que presentan, como es la presencia de los grupos funcionales. Esta diversidad de compuestos también se debe al número y posición de las insaturaciones, estructura cíclica o acíclica (Mohammed et al., 2024). Los aceites esenciales pueden emplearse en diferentes ámbitos como la cosmética, la farmacéutica, la aromaterapia y la industria de alimentos, entre otros (Elhawary et al., 2024).

Las plantas vasculares, tanto gimnospermas y angiospermas, incluyendo plantas mono- y dicotiledóneas, presentan tejidos reservorios de aceite esencial como tallos, raíces, hojas, flores, frutos, semillas, principalmente y son resultado de una variedad de rutas biosintéticas. Algunas de las familias botánicas que presentan un mayor número de especies con aceite esencial son Myrtaceae, Apiaceae, Rosaceae, Pinaceae, Umbelliferaceae, Lamiaceae, Poaceae, Cupressaceae, Rutaceae, Asteraceae, entre otras (Mohammed et al., 2024). No obstante, los compuestos del aceite esencial pueden presentar cambios en su composición debido a la temperatura ambiental en la época de obtienen, así como a la humedad, al tipo de suelo y a la ubicación geográfica, entre otros (Ali et al., 2025).

2.2.1. Principales métodos de extracción de aceite esencial

El aceite esencial se ha definido como una mezcla compleja de metabolitos obtenidos mediante métodos de hidrodestilación o arrastre con vapor, o en su caso, mediante procesos mecánicos sin la aplicación de calor, como en la obtención del aceite esencial del epicarpio de especies del género *Citrus*. De esta manera se obtiene la fracción volátil de una planta (El-Kharraf et al., 2022).

Algunos de los métodos para la extracción de los compuestos del aceite esencial es la extracción con CO_2 supercrítico, la extracción asistida por microondas, la extracción *headspace* o la microextracción en fase sólida (SPME, por sus siglas en inglés) la cual utiliza una fibra adsorbente cubierta de sílice. Entre los métodos tradicionales empleados para la extracción de aceite esencial se encuentran la hidrodestilación y el arrastre de vapor (El-Kharraf et al., 2022). La hidrodestilación emplea agua y calor para extraer los compuestos de las plantas. Este método involucra el contacto del tejido vegetal inmerso en agua que, cuando alcanza la temperatura de ebullición, facilita la permeación osmótica del aceite esencial, rompiendo los depósitos de aceite esencial y permitiendo su liberación. El vapor generado es enfriado y por tanto condensado para obtener el aceite esencial y el hidrolato. En cuanto al método de arrastre con vapor, el vapor es generado por separado y pasa a través del material vegetal, es decir, el agua no está en contacto con el tejido vegetal. El vapor generado, al igual que en hidrodestilación, pasa por un serpentín donde se enfría y condensa. El método de arrastre con vapor es idóneo para aquellos tejidos que son sensibles a altas temperaturas ya que reduce el riesgo de degradación de algunos metabolitos por alta temperatura (Perović et al., 2024).

2.2.2. Componentes del aceite esencial

La mezcla de metabolitos que conforman el aceite esencial es compleja debido a la presencia de un gran número de terpeno sintasas y enzimas ciclasas que llevan a cabo la biosíntesis de diferentes compuestos, principalmente de los tipos mono- y sesquiterpénicos, tanto acíclicos como alicíclicos. Su estructura química consta de dos o tres unidades de C5 razón

por la cual también son llamados isoprenoides. Hasta el momento se han identificado cerca de 50000, de los cuales, 90 % son del tipo monoterpeno. Los terpenoides pueden clasificarse en dos principales grupos: los oxigenados y los no oxigenados (Mohammed et al., 2024). El principal precursor de los monoterpenos es el geranilpirofosfato. En relación con los sesquiterpenos, el precursor directo es el farnesilpirofosfato (Ali et al., 2025). En esta mezcla de compuestos también se observa la presencia de fenilpropanoides, estos se sintetizan por la vía del ácido shikímico (Mohammed et al., 2024). Los terpenoides y los fenilpropanoides son los dos grupos principales de metabolitos que conforman el aceite esencial. Sin embargo, compuestos del tipo oxilipinas, así como nitrogenados, sulfurados y ésteres, también pueden encontrarse en el perfil de un aceite esencial (Ali et al., 2025).

2.2.3. Actividad biológica del aceite esencial

Los aceites esenciales han sido objeto de estudio desde su uso en la medicina tradicional y en la cocina. Los avances científicos y tecnológicos han permitido esclarecer y confirmar diversas actividades biológicas asociadas a estos compuestos. La revisión de varias investigaciones realizada por Mohammed et al. (2024) indicaron algunas de estas actividades y su modo de acción: citotoxicidad y actividad anticancerígena (los compuestos β-cariofileno y α-humuleno pueden inducir la detención del ciclo celular e inhibir la angiogénesis); antibacteriana (los compuestos alteran la membrana citoplasmática bacteriana y generan la coagulación del contenido celular de las bacterias); antifúngica (alteran la membrana celular e inhiben la polimerización de elementos estructurales como glucanos y quitina, lo cual conduce a la muerte celular o dificulta la esporulación y la germinación); antiviral (interfieren en diversas etapas del ciclo de vida viral, incluyendo la entrada, la replicación y la liberación, inhibiendo así la propagación del virus); así como actividad antiinflamatoria (tienen capacidad de eliminar las especies reactivas de oxígeno (ROS) y evitar su formación por parte de las células inflamatorias, reduciendo así el estrés oxidativo y la inflamación), entre otras.

2.2.4. Aceite esencial de *Pimenta dioica*

Como se mencionó anteriormente, el aceite esencial de los frutos de *P. dioica* es el producto más importante en términos económicos. Se extrae de los

frutos secos obteniendo un rendimiento de 3 a 4.5 %. Aproximadamente se han reportado 60 compuestos, entre ellos fenólicos, monoterpenos, sesquiterpenos y oxilipinas. Los compuestos mayoritarios identificados son eugenol, metileugenol, β-cariofileno, α-cariofileno, terpinen-4-ol y eucaliptol (Premachandran et al., 2024). Las hojas también se han empleado para la extracción de aceite esencial con un rendimiento de 0.3 a 3%, estas presentan un perfil similar de metabolitos que los frutos, destacando el eugenol como compuesto mayoritario. Diversos estudios se han realizado en los que se caracterizó la composición del aceite esencial evaluando diferentes técnicas de extracción como destilación por arrastre de vapor, extracción con fluidos supercríticos e hidrodestilación, siendo la última la más común (Perović et al., 2024). En algunos de estos estudios indicaron la actividad acaricida, nematicida y antimicrobiana del aceite esencial (Contreras, 2018). Youssef et al. (2021) reportaron la actividad citotóxica en líneas celulares de cáncer de mama (MCF-7), hígado (HepG-2) y cérvix (HeLa-2) mediante el ensayo MTT que consiste en una prueba colorimétrica que mide la viabilidad celular al cuantificar la actividad metabólica mitocondrial. El mecanismo bioquímico del ensayo MTT implica enzimas oxidoreductasas celulares dependientes de NAD(P)H, que convierten el tetrazolio amarillo MTT [3-(4,5-dimetiltiazolil-2)-2,5-difeniltetrazolio bromuro] en (E,Z)-5-(4,5-dimetiltiazol-2-il)-1,3-difenilformazán (formazán), un compuesto insoluble. El formazán formado puede disolverse con dimetilsulfóxido (DMSO) para generar un color púrpura con una absorción característica a 540 nm. La intensidad del color púrpura es directamente proporcional al número de células e indica la viabilidad celular (Bahuguna et al., 2017).

A pesar de que se han identificado los metabolitos volátiles presentes en el aceite esencial de *P. dioica*, aún se desconoce cómo varía su composición y abundancia a lo largo del ciclo anual de producción, particularmente en función de la estacionalidad de las hojas empleadas para la extracción. Este aspecto resulta especialmente relevante en regiones donde el tejido foliar permanece subutilizado. Rao et al. (2012) evaluaron el aceite esencial de las hojas en dos épocas del año (primavera y verano) por HPTLC (High Performance Thin Layer Chromatography) y reportaron que en verano hubo un mayor contenido de eugeno; sin embargo, no se consigna información

sobre el porcentaje real de los compuestos. Así mismo, Youssef et al. (2021) identificaron 89 metabolitos en el aceite esencial de *P. dioica* y *P. racemosa* en cuatro épocas del año de plantas cultivas en Egipto mediante GC-MS, eugenol fue el constituyente mayoritario en otoño (88.71 %) y en verano (88.41 %).

A pesar de que *P. dioica* es una especie vegetal que tiene relevancia biológica y económica, existen pocos estudios que consideren su descripción botánica como hermafrodita funcionalmente dioica. Investigaciones anteriores han aportado información sobre los COVs extraídos de árboles hembra y macho mediante diferentes técnicas, como la hidrodestilación y la microextracción en fase sólida acoplada a cromatografía de gases-espectrometría de masas (SPME-GC-MS) (Minott y Brown, 2017; Montalvo-López et al., 2021). Sin embargo, al margen de ese progreso científico técnico, resulta de gran interés profundizar en el estudio de la composición de los COVs empleando métodos de extracción convencionales como Soxhlet, la hidrodestilación y el arrastre de vapor, ya que las variaciones químicas derivadas de estas técnicas, así como su posible influencia en la actividad biológica, no se han explorado con suficiente detalle.

En este contexto, el presente estudio consistió en el análisis del tejido foliar de árboles hembra y macho de *P. dioica* con el propósito de generar información relevante para el aprovechamiento de un recurso tradicionalmente subutilizado. Los resultados de esta investigación contribuyen al aprovechamiento integral de sus hojas como fuente alternativa de compuestos bioactivos con aplicación en medicina aprovechando su diversa actividad biológica. Asimismo, la evaluación de su actividad biológica y la caracterización molecular contribuyen a establecer bases científicas para un desarrollo tecnológico orientado a la diversificación de productos derivados con potencial aplicación en las industrias alimentaria, farmacéutica, cosmética, agro biotecnológica y de saborizantes. Este enfoque genera conocimiento necesario para garantizar la calidad, la autenticidad y la trazabilidad del aceite esencial de *P. dioica*, además de aportar información sobre la variación en su composición en diferentes épocas del año.

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CAPITULO 3. Molecular Characterization, Identification of the Volatile Organic Compounds by GC–MS, and Assessment of the Cytotoxic Activity of Leaves of *Pimenta dioica* L. Merrill Trees from Mexico

metabolites

MDPI

Article

Molecular Characterization, Identification of the Volatile Organic Compounds by GC–MS, and Assessment of the Cytotoxic Activity of Leaves of *Pimenta dioica* L. Merrill Trees from Mexico[3]

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Abstract

Background: *Pimenta dioica* is a medicinal plant rich in various natural compounds, giving it significant potential for applications in the pharmaceutical, cosmetic, food, and agricultural industries. However, little is known about the metabolites present in the leaves of female and male trees, as well as their toxicity and genetic variability. Therefore, in this study, molecular characterization was conducted, the volatile compounds in the leaves of female and male trees were identified, and their cytotoxicity was assessed. **Methods**: For molecular characterization, a clustering analysis was performed using Ward's minimum variance method; genetic distances were determined using Jaccard's coefficient (similarity) and an analysis of molecular variance. Hexane extracts were obtained using the Soxhlet method and analyzed by gas chromatography coupled to mass spectrometry (GC–MS). The cytotoxicity of the extracts was evaluated by a bioassay with *Artemia salina*. **Results**: Forty-two metabolites were identified in leaf extracts from female and male trees, of which 17 are reported for the first time in this tissue. The female tree exhibited a distinct metabolite profile compared to the male tree and was slightly more toxic than the male tree. However, both were considered to be moderately toxic (282.00 and 222.87 µg/mL, respectively). **Conclusions**: *Pimenta dioica* has a high potential for various uses, primarily for anthropocentric purposes due to its composition of specific metabolites and moderate toxicity. The sampled trees showed a high molecular genetic variability among individuals.

Keywords: allspice; cytotoxicity; dioecism; GC–MS; Soxhlet

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1. Introduction

Pimenta dioica L. Merrill (Myrtaceae) is known as allspice, as the dried fruit flavor results from a mixture of clove, nutmeg, cinnamon, and black pepper flavors. It is an evergreen tree species, native to Mexico and Central America, growing up to 20 m tall. The leaves and fruits contain vesicles of essential oil [1]. In Mexico, the plant has been used ancestrally, primarily by the Totonaca culture, for medicinal purposes. Infusions of the leaves are used to relieve stomach pains and colic, as well as symptoms of nausea, dysentery, diarrhea, and rheumatic pain [2]. The leaf has a greater use in the preparation of

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various traditional Mexican dishes [3]. Mexico is the second-largest producer and exporter of *P. dioica* fruits, after Jamaica, with a planted area of 3448.70 ha and an annual production of 10,265.34 t [2,4]. The primary producing states in Mexico are Veracruz, Tabasco, Chiapas, and Puebla [5]. Despite its importance in Mexico, this crop requires further technological improvements for commercialization of the fruit, such as better harvesting techniques and appropriate soil nutrition management [5]. Because this species reaches a great height, canopy management is necessary to facilitate harvesting. The underutilized leaf tissue could increase the potential and commercial demand for this species as a source of aroma- related metabolites, instead of being used as a source of economically and medically important metabolites.

In some places, *pimienta gorda* or *xocoxóchitl* (as it is called in Mexico) is confused with black pepper (*Piper nigrum*, Piperaceae); the latter is a herbaceous climbing plant, while *P. dioica* is an evergreen tree. The fruits of both species contain some similar volatile compounds like α-thujene, α-copaene, eugenol, α-pinene, β-caryophyllene, methyl eugenol, sabinene, β-pinene, caryophyllene oxide, eucalyptol, and limonene [6]. However, the fruits from both species are morphologically different: the *P. dioica* fruits are larger, measuring up to 1 cm in diameter, while the *P. nigrum* fruits are small (up to 5 mm) and rough. As for aroma, black pepper is more pungent due to the presence of piperine, while the *P. dioica* fruits are sweeter. Although black pepper is more popular in the culinary arts, the fruits and leaves of *Pimenta dioica* also contribute aromas and flavors to foods, as well as metabolites with medicinal properties.

As its scientific name indicates, *P. dioica* has been described as a functionally dioecious hermaphrodite species [3,7], because its trees show perfect flowers; however, they are functionally different. Female trees are the only fruit producers with flowers with viable pistils and short stamens, while male trees are only pollinators, with flowers with double the amount of stamens and shorter, non-viable pistils. To identify trees, they must grow and develop for five years, and the first fruiting provides sex information [3]. Currently, the trees are differentiated, and both types are present in a 1:1 ratio; to ensure good pollination, only two male trees are left for every 10 female trees [8].

The fruit and leaves of *P. dioica* produce metabolites that make up the essential oil. In addition to the essential oil, the plant produces other metabolites such as flavonoids, phenylpropanoids, tannins, saponins, and fatty acids [9]. For this reason, it exhibits various medicinal properties, including anti-inflammatory, antidiabetic, antiseptic, antitumoral, antifungal, antioxidant, antimicrobial, anti-ulcer, and nematicidal properties [9,10]. The rich metabolite content favors the potential use of *P. dioica* in the cosmetic, food, and agricultural industries. Most studies of *P. dioica* have focused on identifying metabolites in the essential oils of its fruits and leaves. Only two reports on male and female trees provide information on volatile organic compound (VOC) composition derived by hydro-distillation and analyzed by GC–MS [11] and by SPME–GC–MS (Solid-Phase Micro-Extraction–Gas Chromatography–Mass Spectrometry) techniques [3]. In the first study conducted by Minott and Brown [11] on cultivated species in Jamaica, a similar profile of volatile components was reported in both female and male tree leaves, although their relative abundances differed. In contrast, Montalvo-López et al. [3] reported only the aroma constituents of leaves from both types of trees. Therefore, there is a growing interest in identifying additional volatile compounds that have not yet been characterized. Also, molecular studies have not been conducted to determine the genetic variability of plants for their conservation and genetic improvement. Hence, the aim of this study was to perform molecular characterization and identification of the volatile compounds of the hexane extract from leaves of female and male trees. Furthermore, the cytotoxicity of the components was tested using the BSLT (Brine Shrimp Lethality Test) method to obtain preliminary, rapid,

and cost-effective information on their potential antitumor and antimicrobial activities, among others [12].

2. Materials and Methods

2.1. Plant Material Sampling

Fresh leaves were randomly sampled from the terminal branches of three female and three male *Pimenta dioica* trees from a commercial production unit located in the town of Tilzapota, Atzalan, Mexico (97° 53′10′′ W, 19° 53′10′′ N; 633 masl), in June 2023. Leaves were collected according to the four cardinal points to cover the canopy. All 18-year-old trees were subjected to uniform irrigation and nutrient management. The taxonomic identification of the species was conducted at the National Herbarium (MEXU) of the Institute of Biology at UNAM, Mexico, and the specimen was assigned the number 1531279. The tissue was dried in an oven at 45 °C until it reached a constant weight.

2.2. Molecular Characterization

2.2.1. DNA Extraction

Fresh leaves from the three male and three female trees were disinfected with ethanol at 70% and stored at −80 °C. The CTAB-based extraction protocol, as modified by Silva et al. [13], was used for DNA extraction. CTAB 2% (Tris-HCl 10 mM at pH 8; $Na_2EDTA\text{-}2H_2O$ 20 mM at pH 8.0; NaCl 1.4 M) was used as an extraction buffer. The plant tissue was macerated in liquid nitrogen; then, 1 mL of 2% CTAB was added to microtubes at 80 °C. The mixture was incubated in a thermoblock (Thomas Scientific®, Henry Troemmer, LLC., USA) at 85 °C for 90 min. Subsequently, the sample was centrifuged at 11,500 rpm for 10 min, and the supernatant was mixed with 500 µL of chloroform–isoamyl alcohol (24:1) in a vortex until a homogeneous mixture was obtained. Again, it was centrifuged at 11,500 rpm for 10 min. The upper aqueous phase was collected, and 900 µL of absolute ethanol, previously cooled to -20 °C, was added and mixed gently by inversion. The mixture was centrifuged at 11,500 rpm for 20 min, and the supernatant was decanted, ensuring that the pellet that formed at the bottom of the microtube was not lost. This was washed with 600 µL of ethanol (70% at −20 °C) and centrifuged for 10 min at 11,500 rpm. The pellet was allowed to dry for at least 30 min. Finally, it was resuspended in molecular-biology-grade water (50 µL). Once the DNA was obtained from the samples, the quality was evaluated by electrophoresis (integrity) and spectrophotometry (quantity and quality).

2.2.2. DNA Quantification and Quality Assessment

DNA samples were quantified using a spectrophotometer NanoDrop (Thermo Fisher Scientific, DNA Factor: 50, China). For equipment calibration, 4 µL of TE buffers were used. From each sample, 4 µL of DNA dissolved in molecular-biology-grade water were taken, and absorbance was measured at a wavelength of 260 nm. Based on the concentrations obtained, each sample was diluted to a final concentration of 10 µL/mL using molecular-biology-grade water. DNA quality was evaluated through PCR amplification of a 330 bp fragment corresponding to the endogenous 16S gene (Figure 1). PCR reactions had a final volume of 25 µL. To each reaction, 2.5 µL of DNA (10 ng/µL), 1 µL of each primer (16S1: TGAGAATGGATAAGAGGCTC and 16S2: TGTTGTTCCCCTCCCAAGGG), 10 µL dNTPs, 2.5 µL PCR buffer, 1.5 µL $MgCl_2$, 0.3 µL Taq DNA polymerase, and 6.2 µL of molecular-biology-grade water were added. DNA amplification was performed in a thermal cycler (Veriti Applied Biosystems®, Singapore) using the following conditions: initial 1 min step at 94 °C, then 35 cycles of 30 s denaturation at 94 °C, 1 min alignment at 55 °C, 1 min of extension at 72 °C, and a final extension at 72 °C for 6 s.

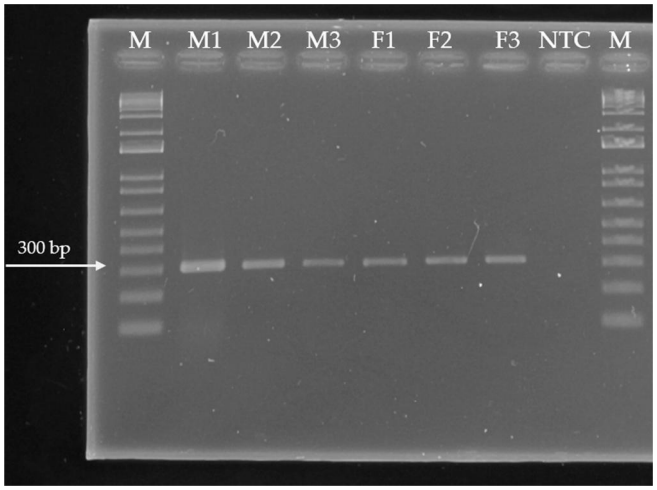

Figure 1. Electropherogram depicting PCR amplification products of the 16S gene (330 bp) from six DNA samples extracted from *Pimenta dioica* L. Merrill leaves. Ladder size: 100 bp. Order of paths on the gel: 1 M—size marker, 2—Male 1 accession, 3—Male 2 accession, 4—Male 3 accession, 5— Female 1 accession, 6—Female 2 accession, 7—Female 3 accession, 8—No-template control lane, 9 M—size marker.

2.2.3. ISSR PCR Reaction

Ten ISSR-type markers were selected due to their proven effectiveness in the molecular characterization of other species within the Myrtaceae family [14,15]. PCR reaction was performed to amplify loci based on the 10 ISSR markers presented in Table 1. PCR reactions for each marker had a final volume of 25 µL. To each reaction, 1 µL of DNA (10 ng/µL), 3 µL of primer, 10 µL dNTPs, 2.5 µL PCR buffer, 1.5 µL $MgCl_2$, 0.3 µL Taq DNA polymerase, and 5.2 µL of molecular-biology-grade water were added. DNA amplification was performed in a thermal cycler (Veriti Applied Biosystems®, Singapore) using the following conditions: initial 1 min step at 93 °C, then 40 cycles of 20 s denaturation at 94 °C, 1 min alignment according to each ISSR marker (Table 1), 20 s of extension at 72 °C, and a final extension at 72 °C for 6 s.

Table 1. Sequence of 10 ISSR primers and their respective annealing temperatures used for the molecular characterization of *Pimenta dioica* L. Merrill accessions.

Primer	Sequence 5′–3′	Annealing Temperature
ISSR 1	$(CA)_8$AAGG	60 °C
ISSR 2	$(CA)_8$AAGCT	62 °C
ISSR 3	$(GA)_8$CTC	58 °C
ISSR 4	$(AG)_8$CTC	58 °C
ISSR 5	$(AC)_8$CTA	56 °C
ISSR 6	$(AC)_8$CTG	58 °C
ISSR 7	$(AG)_8$CTG	58 °C
ISSR 8	$(AC)_8$CTT	56 °C
ISSR 9	$(AG)_8$C	52 °C
ISSR 10	$(GA)_8$ T	50 °C

2.2.4. Electrophoresis

PCR products were separated on a 2% agarose gel in 1X TAE buffer at 100 V for 40 min. Fragment sizes were determined using the 100 bp DNA Step Ladder (DNA Ladder Thermo Fisher Scientific, Lithuania). After separation, the gel was visualized under a UV transilluminator and photographed with a gel documentation system (UVP Digidoc-It® Imaging System, Upland, CA, USA). The resulting gels were analyzed by quantifying

the number of polymorphic bands generated by the amplification for each primer. The presence of a band was scored as 1 and its absence as 0, thereby constructing a basic binary data matrix.

2.3. Extraction Procedure

The extracts were obtained with analytical-grade hexane (J.T. Baker, USA) in a Soxhlet apparatus at 70 °C for 7 h from 100 g of dried leaves from each tree to extract lipophilic components, some of which are responsible for the aroma of *P. dioica* leaves. In a round-bottom flask, 250 mL of hexane was added. The solvent was removed under vacuum using a Büchi R-210 rotary evaporator to obtain the crude extract. Each extract was flushed with nitrogen gas to preserve it and stored at −20 °C until analysis.

2.4. Chromatography–Mass Spectrometer Analysis

Extracts were analyzed using a gas chromatograph (Agilent Technologies 7890B, China) coupled to an autosampler (7683B) and a mass selective detector (5977A). Chromatographic separation was performed on an HP5-MS column (30 m × 0.25 mm × 0.25 µm). For each hexane extract, 5 mg was re-dissolved in 1 mL of hexane. The injection volume was 1 µL. Samples were injected in the splitless mode at 280 °C. The oven conditions were as follows: an initial temperature of 60 °C for 1 min, then increased at a rate of 6 °C/min to 270 °C, and maintained for 1 min. The column flow rate was 1 mL/min of helium. The transfer line was maintained at 300 °C throughout the analysis. The quadrupole and ionization source temperatures were 150 °C and 230 °C, respectively. Data acquisition was performed in electron impact mode over a range of m/z 50–m/z 550 in SCAN mode. The identification of compounds was carried out by analyzing the data generated by the GC–MS system (Total Ion Chromatograms), recorded using the Mass Hunter Workstation software version B.08.00 (Agilent Technologies, Inc., USA). The resulting data were processed with the Automated Mass Spectral Deconvolution and Identification System software (AMDIS v 2.71) to isolate each compound's mass spectrum from the background. Compounds were identified by comparing their mass spectra with those in the NIST 2011 library, and some were also compared with standard compounds: p-xylene ≥ 99% (134449), α-terpineol 90% (432628), eugenol ≥ 99% (E51791), and trans-caryophyllene ≥ 98% (C9653) (Sigma-Aldrich, Saint Louis, MO, USA). The relative abundance of each compound in the extracts was obtained by integrating the peak area using the MSD ChemStation Data Analysis Application F.01.01.2317 (Agilent Technologies Inc., USA). Data presented are mean values of three repetitions for each tree.

2.5. Evaluation of the Extract Toxicity in Artemia salina

The bioassay was performed using *Artemia salina* (Brine Shrimp Lethality Test, BSLT), where the percentage mortality in these crustaceans was evaluated after 24 h of exposure to the plant extract to determine the LC_{50} [12]. The nauplii hatched in saline water with NaCl (35 g/L) at 25 °C after 24 h. Ten nauplii and 5 mL of saline water were placed in 25 mL vials. Hexane extracts were evaluated at three concentrations (10, 100, and 1000 ppm) and saline water was used as the control. From each extract, a stock solution was prepared by dissolving 20 mg of the extract in 2 mL of saline water supplemented with Tween 20 (0.1%); therefore, vials containing only saline water and vials containing saline water with Tween 20 (0.1%) were used as controls. Each test was performed with five replicates. The vials were kept under illumination at 25 °C for 24 h. After this time, the surviving crustaceans were counted. With the data obtained, a Probit analysis was performed to calculate the Mean Lethal Concentration (LC_{50}).

2.6. Statistical Analysis

For the molecular statistical analysis, the Jaccard coefficient (a measure of similarity) was used to determine the genetic distances between the *P. dioica* trees. A dendrogram was obtained using Ward's method (clustering analysis) of minimum variance. Based on Hotelling's T_2 pseudo-statistic [16] and pseudo-F [17], the cutoff height was determined using the SAS statistical program, ver. 9.4. To explore the hierarchical structure of genetic variation among accessions, an analysis of molecular variance (AMOVA) was applied using the Infogen® package ver. 2016. A *t*-test was performed to determine whether there were significant differences in the composition of the extracts. The Mean Lethal Concentration (LC_{50}) was calculated by the Probit method with RStudio ver. 2025.05.0+496.

3. Results

3.1. Molecular Characterization

The 10 primers were polymorphic (77.78%); ISSR-5 (16 bands) amplified the most bands at 61.11%, followed by ISSR-8 (12 bands) at 48.81%, ISSR-2 (10 bands) at 42.42%, and ISSR-3 (10 bands) at 60.26% amplification. Based on Hotelling's T_2 pseudo-statistic, the cut-off height in the dendrogram was a value of 0.53 semi-partial r^2, which gave rise to three groups: Group I with two individuals (M-1 and F-3), Group II with three individuals (M-2, M-3 and F-2), and Group III with one individual (F-1) (Figure 2).

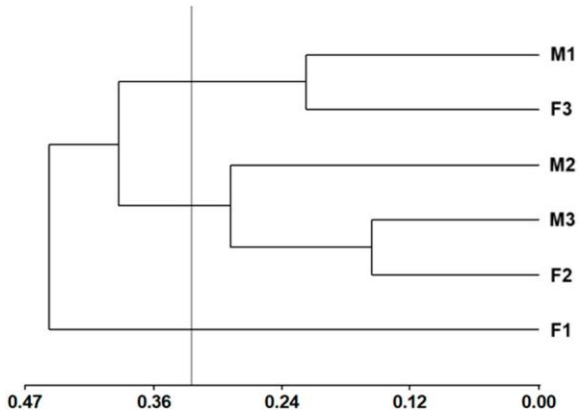

Figure 2. Dendrogram constructed by Ward's minimum variance method and Jaccard's genetic distance of three female trees (F-1, F-2, and F-3) and three male trees (M-1, M-2, and M-3) of *Pimenta dioica* L. Merrill.

The primers that showed the highest polymorphic information content (PIC) values were ISSR-9 (0.36), ISSR-1 (0.34), ISSR-5 (0.33), ISSR-8 (0.32), ISSR-10 (0.32), and ISSR-2 (0.30). These primers were considered to be the most useful for further studies on the molecular characterization of allspice. The hierarchical structure of genetic variability among trees was explored using an analysis of molecular variance (AMOVA), which showed that the molecular genetic variability among accessions was very high (100%) (Table 2).

Table 2. Molecular analysis of variance of female and male *Pimenta dioica* trees based on their ISSR molecular profiles.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	*p*-Value	Variance Components	%
Between accessions	5	97.67	19.53	0.9325	1.26	100.0
Within accessions	0	0.0	0.0	<0.0001	0	0
Total	5	97.67	19.53		1.26	100.0

3.2. Yield and Chemical Profile of Leaf Extracts by GC–MS

No statistically significant differences were found in the hexane extract yield between leaves from female trees (3.13%) and male trees (4.07%). A total of 42 compounds were identified in the two extracts (Table 3). Both extracts had 29 metabolites in common. The relative abundance of each compound changed according to the type of tree; however, 32 compounds were identified in the male tree leaf extract, while a higher number (39 compounds) were identified in the female tree. These compounds were not found in the male tree extract: 4-octen-3-one; 2-methylnonane; 5-ethyl-2-methyl-heptane; 3-methyl-undecane; dodecane; α-fenchene; β-cubebene; δ-guaiene; 3,7,11,15-tetramethyl-2-hexadecen-1-ol; and 1-docosene. On the other hand, compounds not identified in the female tree extract included 5-methyl-1-undecene, (+)-epi-bicyclosesquiphellandrene, and eugenol.

Table 3. Relative abundances (%) and metabolite profiles identified in hexane leaf extracts of male and female *Pimenta dioica* L. Merrill trees.

No.	RT	Compound	Male (%)	Female (%)
1	3.747	p-xylene †	0.77 ± 0.12	1.17 ± 0.23
2	3.819	Nonane	0.37 ± 0.59	0.27 ± 0.11
3	3.953	4-Octen-3-one	nd	0.07 ± 0.01
4	4.238	Cumene	0.13 ± 0.1	0.16 ± 0.03
5	4.773	Isocumene	0.03 ± 0.00	0.04 ± 0.01
6	4.901	2-Methylnonane	nd	0.04 ± 0.01
7	4.999	Artemisia ketone	0.06 ± 0.02	0.09 ± 0.7
8	5.117	β-Thujene	0.01 ± 0.01	0.03 ± 0.02 *
9	5.420	β-Myrcene	1.89 ± 0.74	1.26 ± 0.73
10	5.586	Decane	0.38 ± 0.18	0.55 ± 0.05
11	6.044	5-Ethyl-2-methyl-heptane	nd	0.04 ± 0.03
12	6.235	Eucalyptol	1.02 ± 0.26	0.77 ± 0.23
13	6.621	β-Ocimene	0.34 ± 0.06	0.35 ± 0.17
14	7.060	3-Methyl-decane	0.03 ± 0.02	0.05 ± 0.03
15	7.497	(+)-4-Carene	0.07 ± 0.01	0.08 ± 0.02
16	7.710	Undecane	0.10 ± 0.02	0.21 ± 0.24
17	9.289	3-Methyl-undecane	nd	0.05 ± 0.04
18	9.517	1-Terpinen-4-ol	0.22 ± 0.06	0.21 ± 0.09
19	9.856	α-Terpineol †	0.25 ± 0.19	0.12 ± 0.08
20	9.958	Dodecane	nd	0.15 ± 0.11
21	13.028	α-Fenchene	nd	0.02 ± 0.01
22	13.673	Eugenol †	17.66 ± 35.17	nd
23	13.879	Copaene	0.68 ± 0.83	0.73 ± 0.43
24	14.220	(-)-β-Elemene	0.47 ± 0.29	0.93 ± 0.37
25	14.548	Methyl eugenol	59.20 ± 34.67	68.93 ± 2.75
26	14.821	β-Caryophyllene †	6.50 ± 1.55	6.65 ± 1.11
27	15.014	β-Cubebene	nd	0.56 ± 0.23
28	15.525	α-Caryophyllene	0.84 ± 0.14	0.75 ± 0.16
29	16.088	Germacrene D	0.22 ± 0.07	0.19 ± 0.10
30	16.199	β-Selinene	0.20 ± 0.09	0.11 ± 0.04
31	16.390	α-Selinene	0.19 ± 0.08	0.13 ± 0.05
32	16.613	α-Farnesene	0.08 ± 0.04	0.11 ± 0.05
33	16.747	(+)-Epi-bicyclosesquiphellandrene	0.03 ± 0.00	nd
34	16.919	(+)-δ-Cadinene	0.16 ± 0.01	0.13 ± 0.06
35	18.129	Caryophyllene oxide	0.44 ± 0.21	0.22 ± 0.05
36	19.599	δ-Guaiene	nd	0.25 ± 0.12
37	20.905	5-Methyl-1-undecene	0.05 ± 0.01	nd
38	22.762	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	nd	0.12 ± 0.03

Table 3. *Cont.*

No.	RT	Compound	Male (%)	Female (%)
39	33.528	Vitamin E	3.53 ± 1.47	9.18 ± 3.23
40	34.942	Heptacosane	0.77 ± 0.54	0.69 ± 0.71
41	36.079	1-Docosene	nd	1.25 ± 1.25
42	36.496	Squalene	3.32 ± 0.60	3.31 ± 3.40

† Compared to a standard reference; nd: not detected. Values were compared using *t*-test. An asterisk (*) indicates a statistically significant difference ($p < 0.05$). Comparisons were conducted within rows. Data are presented as mean ± standard deviation.

According to the statistical *t*-test, β-thujene (8) was the only compound that statistically differed, with the female tree exhibiting a higher relative abundance in the leaf extract. The five main compounds in both extracts were methyl eugenol, β-caryophyllene, vitamin E, squalene, and β-myrcene (Figures 3 and 4).

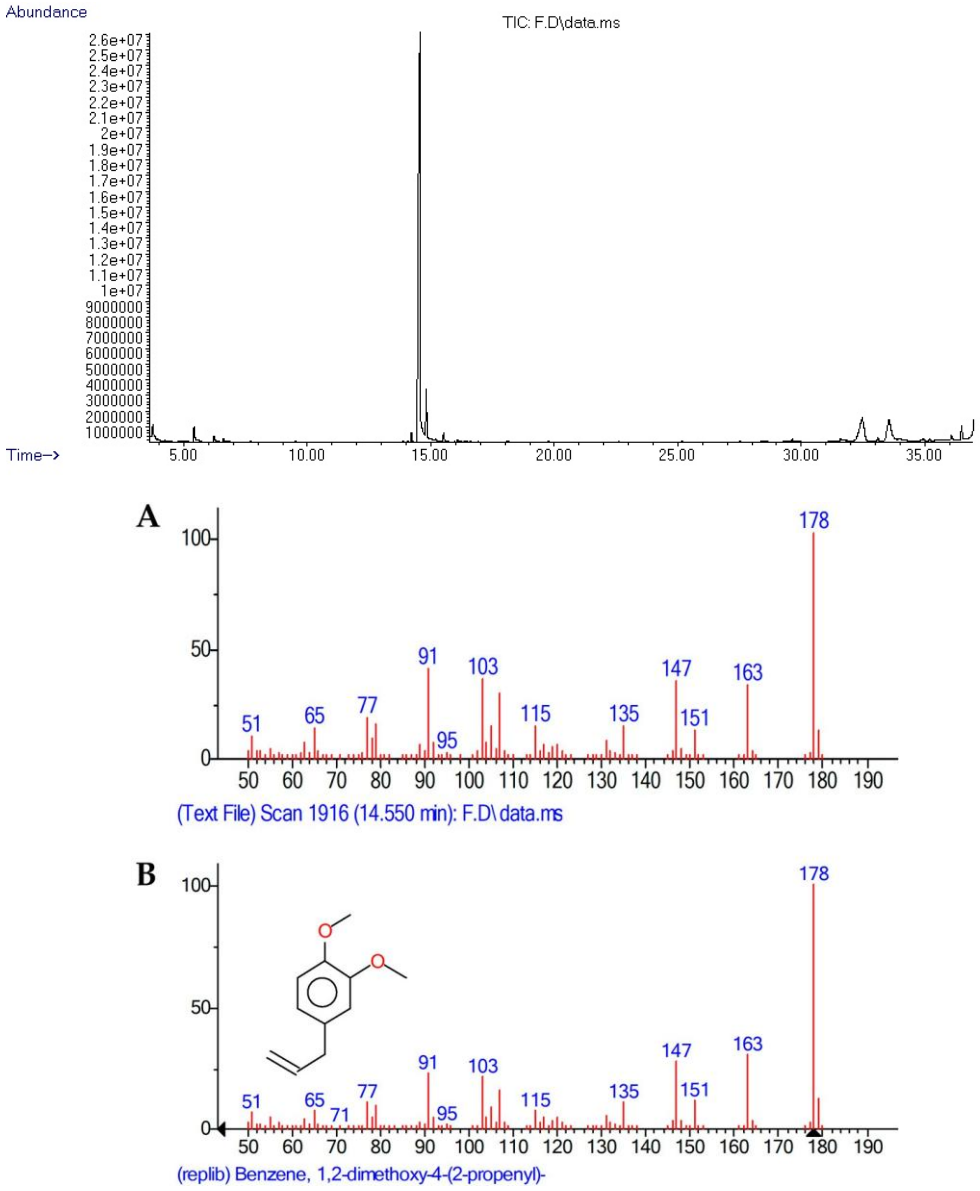

Figure 3. Chromatogram of the hexane leaf extract from a female *Pimenta dioica* tree and mass spectrum of the major peak: methyl eugenol. A: sample spectrum, B: NIST library spectrum.

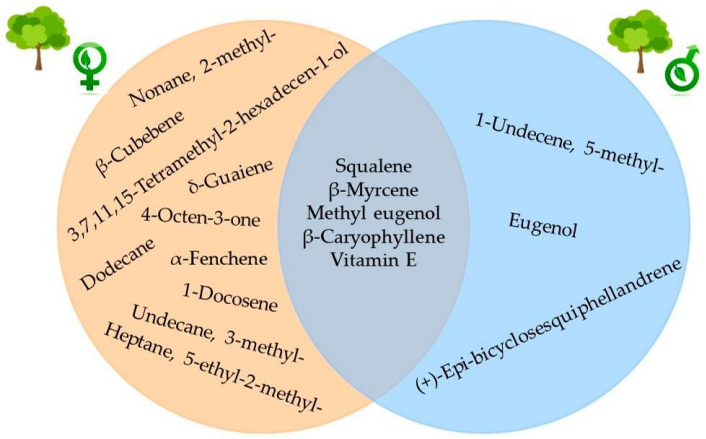

Figure 4. Compounds identified exclusively in the hexane leaf extract of female trees (orange) and male trees (blue) of *Pimenta dioica* L. Merrill, as well as the five major compounds present in both extracts.

3.3. Evaluation of the Extract Toxicity in Artemia salina

The cytotoxicity values for the male and female tree leaf extracts were determined to be 222.87 µg/mL and 282 µg/mL, respectively (Table 4). According to the criteria proposed by Betancurt et al. [18], an extract with an LC_{50} greater than 1000 µg/mL is considered to be non-toxic. An LC_{50} in the range of 100–1000 µg/mL indicates moderate toxicity, while an extract with an LC_{50} of 10–100 µg/mL is classified as very toxic. Finally, an extract with an LC_{50} between 0 and 10 µg/mL is regarded as highly toxic. According to the results obtained, leaf extracts from both female and male trees were found to be moderately toxic.

Table 4. Significant parameters of the probit model for the determination of LC_{50} of the hexane leaf extracts from female and male *Pimenta dioica* trees.

Leaf	b	*p*-Value	e	*p*-Value
Male	−1.2653	1.355×10^{-10}	222.872	6.550×10^{-12}
Female	−1.5747	2.536×10^{-6}	282.000	1.191×10^{-6}

4. Discussion

Genetic variability between female and male trees of *Pimenta dioica* has not been reported. The dendrogram revealed the formation of three groups: Group I comprised two individuals (M-1 and F-3) and Group II comprised three individuals (M-2, M-3, and F-2), with each group likely sharing the same parent. Group III contained only one individual (F-1) (Figure 1). This explains the high genetic variability detected, possibly due to the different origins of the parent trees, which provide a diverse genetic load in the descendants (individuals of the three groups). Nemara et al. [19] reported that polymorphism levels (PIC) lower than 0.25 are slightly informative, a marker with a PIC between 0.25 and 0.5 is considered moderately informative, and a PIC higher than 0.5 indicates that it is highly informative. According to Nemara et al. [19], the primers that showed the highest PIC values were ISSR-9 (0.36), ISSR-1 (0.34), ISSR-5 (0.33), ISSR-8 (0.32), ISSR-10 (0.32), and ISSR-2 (0.30). These primers could be used in further studies for the molecular characterization of this species. In the dendrogram, no grouping was observed for female and male trees, possibly due to the ISSR molecular marker used, as this marker is widely applied for genetic characterization but does not provide sufficient specificity to be employed as a sex-linked marker. The trees showed 100% variability within the samples according to the analysis of molecular variance (AMOVA); this result allowed us to infer that the six sampled trees had different progenitors, even though they were collected from the same site. Likewise,

Raichand et al. [20] noted that other climatic factors, such as temperature and rainfall, may also contribute to the variability that influences plant diversity. For example, diversity is greater when rainfall is between 1000 and 1500 mm per year or greater than 1500 mm, particularly in tropical forests. In the present study, the sampling site has a mean annual rainfall of 1500 to 2500 mm and a warm humid climate, a factor that could partly explain the high variability of the analyzed trees. Raichand et al. [20] conducted a phylogenetic analysis of *P. dioica* trees from different localities in Tanzania and other species of the family Myrtaceae, explaining that the observed genetic diversity and significant genetic variations are attributed to factors such as geographic isolation, founder effects, or natural selection. Likewise, these factors could also explain the results in the present study.

Currently, no varieties of *P. dioica* have been reported in Mexico. The trees sampled in production units are wild, and few have been propagated through grafting using tree scions, which favors desirable characteristics such as large fruit, a very aromatic flavor, and disease tolerance. Therefore, these wild trees exhibit significant genetic variability, which is essential for a genetic improvement program. This allows for the selection of cultivars tailored to the needs of producers and the market, as well as the development of new cultivars with characteristics suitable for various soil and climatic conditions. Accordingly, the significant genetic variability observed explains the differences in the relative abundance and metabolite profiles in the extracts analyzed from leaves of female and male trees, as well as their relationship with the edaphoclimatic characteristics of the place of origin [20].

The studies conducted on the leaves and fruits of *P. dioica* have focused exclusively on the composition of volatile compounds and their biological activity; however, only two studies have reported the composition of volatile compounds in both female and male trees [3,11]. In contrast, other studies have documented the yield and metabolite profile of leaf extracts from the same species. According to a survey by Pino et al. [21] the yield of the hexane extract from the leaves of trees in Havana, Cuba, was reported to be 5.67%. Similarly, the yield obtained in the leaves of trees of the same species grown in India was 6.21% [22], which is higher than the yields in female trees (3.13%) and male trees (4.07%) in the present study. On the other hand, Pino et al. [21] identified seven metabolites (eugenol, thymol, carvacrol, menthol, carvone, T-cadinol, and α-cadinol) in the hexane extract profile, whereas we identified 42 compounds (Table 3). In fact, eugenol was the only common compound in both studies. These differences may be attributed to edaphoclimatic factors or genetic variability.

This study identified 42 metabolites (lipophilic) in the hexane extract in both female and male trees. In the male tree extract, 32 metabolites were found; only three compounds (5-methyl-1-undecene, (+)-epi-bicyclosesquiphellandrene, and eugenol) were not present in the female tree extract. On the other hand, 39 metabolites were identified in the female tree leaf extract, of which ten compounds (4-octen-3-one; 2-methylnonane; 5-ethyl-2-methyl-heptane; 3-methyl-undecane; dodecane; α-fenchene; β-cubebene; δ-guaiene; 3,7,11,15-tetramethyl-2-hexadecen-1-ol; and 1-docosene) were not identified in the male tree extract (Table 3). Therefore, it is important to note that plant sex is a determining factor in the synthesis of certain metabolites that partly account for their biological activity). The differences in most metabolites identified in the profiles of female and male tree leaves in studies conducted by Minott and Brown [11], Montalvo-López et al. [3], and the present study could be attributed to the extraction method employed. Minott and Brown [11] employed hydrodistillation, and Montalvo-López et al. [3] utilized the SPME–GC–MS technique; both techniques are specific to the identification of volatile compounds, unlike the Soxhlet extraction with hexane used in this study. Eugenol has been reported as the main compound in the leaves and fruits of *P. dioica* [2,11,21] and other aromatic species [23]; in the present study, it was only found in male trees, although methyl eugenol was the

primary compound in both types of trees, which coincides with the results of the study by Padovan et al. [24], where they analyzed the diversity of terpenoids among species of the Myrtaceae family, showing that in the case of *P. dioica*, chemotype 1 has eugenol as the primary compound and chemotype 2 has methyl eugenol. This suggests that studies with a larger number of trees should be conducted to confirm the presence of these metabolites as chemotypes within the species, thereby contributing to chemotaxonomy and highlighting their potential for commercial and medicinal use.

Of the hydrophobic compounds in Table 3, 17 new unidentified metabolites are reported from *P. dioica* leaves (4-octen-3one; isocumene; cumene; 2-methylnonane; decane; dodecane; 5-ethyl-2-methyl-heptane; 3-methyl-undecane; α-fenchene; 5-methyl-1-undecene; vitamin E; artemisia ketone; heptacosane; δ-guaiene; 1-docosene; 1-docosene; (+)-epi-bicyclosesquiphellandrene; 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol)). Likewise, biological activity has been reported for only a few of the 17 metabolites; however, it is essential to mention that artemisia ketone exhibits antibacterial activity [25], as well as heptacosane [26] and δ-guaiene [27]. 1-docosene possesses antioxidant and antibacterial properties [28]; (+)-epi-bicyclosesquiphellandrene possesses antimicrobial and antitumoral activities [29], and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol) exhibits anticancer, anti-inflammatory, antimicrobial, and antioxidant activities [30], which could partly justify the medicinal usage associated with *P. dioica*.

Most of the compounds identified in the present study have also been reported in the essential oils of leaves and fruit [9]. Due to their volatile nature, they are responsible for the characteristic odor of the species. Three major volatile compounds in both extracts in the present study had a higher relative abundance (methyl eugenol, β-caryophyllene, and β-myrcene). β-caryophyllene is a primary metabolite in *Piper nigrum* leaves, as well as linalool, β-elemene, and β-selinene; while in the fruit, α-pinene, β-pinene, limonene, myrcene, and germacrene D [31,32], all identified in the present study, have been reported. However, piperine is the metabolite responsible for the pungency of *P. nigrum*. The compounds related to its flavor and aroma include α-pinene, β-pinene, myrcene, limonene, and β-caryophyllene, among others [31], which were also identified in the present study. These profiles may explain the similarity in aromas and flavors with *P. dioica*, which is why it is commonly referred to as allspice.

Vitamin E and squalene, present in the hydrophobic extract, are part of the five primary metabolites. Vitamin E is a lipophilic compound located in the cellular lipid compartments and is therefore part of the cell membrane and lipoproteins. It stops the propagation of the chain reactions of lipid peroxidation, while, in the cell, it preserves the integrity of the membrane; therefore, it plays a crucial role in maintaining the stability of cells, such as erythrocytes and neurons in the central and peripheral nervous systems [33]. As for squalene, it is a triterpenoid with antitumoral, antibacterial, antioxidant, detoxifying, and immune-activating properties, thus demonstrating a high potential for use as an ingredient in functional foods or disease prevention [34]. Its presence partly justifies the medicinal properties attributed to the leaves of *P. dioica* since pre-Hispanic cultures.

The presence of some metabolites identified in the species justifies several of its medicinal (antifungal, antimicrobial, and nematicidal) properties [9,10]. The cytotoxicity test performed with the *Artemia salina* bioassay found that the extracts were moderately toxic (222.87 µg/mL in male tree leaves and 282 µg/mL in female tree leaves). Razak et al. [35] reported on the same species grown in India, with LC_{50} values of 102.80 and 461.52 µg/mL for the methanolic and aqueous leaf extracts, respectively. However, the differences are attributed to the polar nature of the extracts. Finally, the profile of metabolites present in plants may vary due to genetic characteristics of each species, as well as the age of the plant, the type of soil, climatic conditions, altitude, geographical location, temperature and

humidity fluctuations, and rainfall are factors that could explain some chemical differences between these individuals; the sex of the tree was apparently the main factor responsible for the variation in composition [36]. Likewise, the presence of discriminant compounds between each type of tree can be considered biomarkers for their use in different industries, based on the composition and biological activity of plant extracts. Studies related to the profile of metabolites in trees of varying ages, collection times, sexes, and stages of development are required.

Among the limitations of the present study is the lack of a metabolomic analysis using different techniques (NMR and HPLC, among others) aimed at identifying other types of metabolites (flavonoids, phenolic compounds) to justify certain medicinal, antioxidant, and nutraceutical properties of interest to both producers and consumers. Furthermore, the identification of metabolites involved in signaling pathways for their synthesis would contribute to improving the yield of compounds of interest and determining the optimal harvest time for applications in the agro-food, cosmetic, and pharmaceutical industries.

5. Conclusions

Genetic variability was found between female and male trees. In the dendrogram, no clusters were observed that differentiate tree type. Forty-two compounds, mostly volatile metabolites and others of low polarity, were identified in the hexane extract of female trees (39 metabolites) and male trees (32 metabolites), and three compounds not present in the leaf extract of female trees were identified in the leaf extract of male trees. On the other hand, in the female tree extract profile, ten compounds were not identified in the male tree extract, demonstrating a slight difference in their biosynthetic pathways. However, 29 metabolites were present in both types of trees. Methyl eugenol was the primary compound in both extracts. Seventeen compounds were identified and are reported for the first time in *P. dioica* leaves. The diversity of compounds identified highlights the high potential of *Pimenta dioica*, with moderate toxicity observed in the bioassay using *Artemia salina*, for medicinal purposes as well as in the cosmetic and food industries.

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CAPÍTULO 4. A GC-MS-BASED METABOLOMIC APPROACH AND FUNGISTATIC ACTIVITY OF ESSENTIAL OIL FROM MALE AND FEMALE TREES OF *Pimenta dioica* IN THREE SEASONS OF THE YEAR[4]

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RESUMEN

Various aromatic plants have been studied phytochemically to determine their metabolic profiles; however, few studies have considered factors that could provide a more complete picture of their quantitative and qualitative chemical composition. *Pimenta dioica* L. Merrill is described as a functionally dioecious hermaphroditic species. Most research on this species has focused on fruit composition, while the underutilized leaves of male and female trees have been scarcely studied. In this study, the essential oil obtained from the leaves of female and male *P. dioica* trees was analyzed by hydro distillation and steam distillation at three times of the year (january, june, and september) to identify the profile of volatile compounds and their relationship with fungistatic activity against *Fusarium oxysporum* f. sp. *lycopersici* race 3 (FOL3). Using GC-MS data, multivariate analysis identified metabolites correlated with the factors mentioned above. A total of 126 metabolites were identified, with differential distribution across all samples. The steam distillation method yielded 118 metabolites, while hydrodistillation yielded 75; 67 metabolites were common to both methods. There were 27 metabolites exclusive to female trees and 28 to male trees. Tukey's post hoc analysis showed a significantly higher yield in june (p = 0.0057) compared to january and september. Most oils exhibited fungistatic activity against FOL3. Only the tree type factor was significant ($p \leq$ 0.001); oils from male trees exhibited greater fungistatic activity. Seventeen metabolites were identified that correlated significantly with fungal growth inhibition, including spathulenol and 12-oxabicyclo [9.1.0] dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-, which were present in male tree oil at all three sampling times.

Keywords: Dioecism, *Fusarium oxysporum,* pimienta gorda, allspice

[4] Sometido a: Sin someter
Estado: No enviado

4.1. Introduction

Pimenta dioica L. Merrill is an evergreen tree species in the Myrtaceae family, primarily distributed in Mesoamerica and the Caribbean islands. It has been described as a functionally dioecious hermaphroditic species, as its flowers occur in all whorls; however, the flowers differ between two types of trees: female trees, which are the only fruit producers, and male trees, which serve only as pollinators. This behavior is observed after five years of growth and development, when the first flowering and fruiting occur. Therefore, female trees are the most economically relevant, as they produce fruit marketed as a condiment and used for the extraction of essential oil, mainly for cosmetic and food purposes (Montalvo-López et al., 2021).

Most studies on *P. dioica* have focused on identifying the essential oils in fruits and leaves and on testing their biological properties, including anticancer, anti-inflammatory, and cytotoxic effects (El-Nashar et al., 2023). However, these studies do not specify whether the samples were derived from female or male trees; this variable is important because it could significantly influence the chemical composition of the essential oil. Only three studies by Minott and Brown (2017) and Montalvo-López et al. (2021; 2025) have reported this aspect; they analyzed volatile metabolites in leaves from trees grown in Jamaica and Mexico, respectively.

Among traditional methods for essential oil extraction, hydrodistillation and steam distillation are widely used but have not been systematically compared in profiling *P. dioica* essential oil. These methods could allow the identification of metabolites associated with tree type. The extraction profile may also vary with extraction method and sampling season. Furthermore, profile composition could influence fungistatic activity, specifically in this study against *Fusarium oxysporum* f. sp. *lycopersici* race 3. This fungus is an agriculturally significant pathogen responsible for vascular wilting and root rot (Aravena et al., 2021). It causes serious damage to more than 150 crop species, including bananas, tomatoes, melons, watermelons, and cotton (Rahman et al., 2021). Some studies have tested *P. dioica* essential oil against species of the *Fusarium* genus, such as *F. oxysporum* (Zabka et al., 2009), *F. verticillioides* (Zabka et

al., 2009; Achimón et al., 2021), *Fusarium* sp. (Everton et al., 2021), and *F. moniliforme* (Lam et al., 2024); however, to our knowledge, its effectiveness against *F. oxysporum* f. sp. *lycopersici* race 3 has not been reported.

In Mexico, production of *P. dioica* has increased due to improvements in the production system across both production and the value chain (Montalvo-Hernández et al., 2024). The *P. dioica* tree can reach up to 20 meters in height, which is why pruning is a key crop management practice. During this practice, a considerable amount of leaf tissue is discarded. Male trees are also pruned and, in many cases, even completely removed because they do not produce fruit. Their function in the production system is limited to pollination: one male tree for every ten female trees ensures adequate pollination. Consequently, removing male trees from the production system generates a large amount of leaves, which, according to previous studies, have high potential use (Montalvo-López et al., 2021; 2025). Thus, leaves are an underutilized resource within the *P. dioica* production system.

A metabolomic-based study may provide insights into the variation in profiles and concentrations of essential oil metabolites across tree types, extraction methods, and sampling seasons. Metabolomics is a fundamental tool for the comprehensive study of metabolites and the factors that influence their changes, such as sampling season, tissue type, and plant stress conditions. Metabolomics and multivariate analysis enable the exploration, modeling, and dimensional reduction of complex data to identify correlations and significant patterns, and to identify marker metabolites in specific samples to differentiate and enhance their commercial value (Elhawary et al., 2021).

In this study, a metabolomic approach was used to identify metabolites in the essential oils of female and male *P. dioica* leaves, obtained using two extraction methods at three periods of the year. Additionally, the fungistatic activity of these oils against *Fusarium oxysporum* f. sp. *lycopersici* race 3 was tested, and a multivariate analysis was performed to establish correlations between the experimental factors and the observed biological response. The application of metabolomics techniques to the data obtained in this study will improve the use of the leaf in agricultural, industrial, and biotechnological

applications. This work is the first comparative metabolomic approach study of female and male *P. dioica* trees at different times of the year, using two traditional methods of essential oil extraction.

4.2. Materials and methods

4.2.1. Plant material

Fresh leaves were collected from three female and three male trees in a production unit in the community of Tilzapota, Atzalan, Veracruz, Mexico (97°53′10″ W, 19°53′10″ N; 633 meters above sea level) in june (tree pruning season) and september (fruit harvest season) 2023 and january (tree pruning season) 2024. These trees were 17 years old. Leaves were sampled at the four cardinal points of the tree canopy. The collected leaves were dried in an oven at 45 °C until a constant weight was reached. Taxonomic certification of the species was performed at the MEXU Herbarium at Universidad Autonoma de México, with registration number 1245667.

4.2.2. Essential oil extraction

The essential oil from 100 g of dried, ground leaves from each of the three female and three male trees was extracted by hydrodistillation in a Clevenger-type apparatus and by steam distillation. For both methods, extraction was carried out for 3 hours using the same amount of plant material. The moisture residue in the essential oil was removed with anhydrous Na_2SO_4. The essential oil was flushed with nitrogen gas to preserve it and stored at -20 °C until analysis. The yield was calculated as a percentage (v/w) of the leaf dry weight.

4.2.3. Analysis by Gas Chromatography - Mass Spectrometry

The composition of the extracted essential oils was analyzed using an Agilent 7890B gas chromatograph coupled to a 5977A mass detector. An HP5-MS column (30 m x 0.25 mm x 0.25 µm) was used. Each 0.5 µL sample of essential oil was dissolved in 2 mL of hexane (HPLC grade). The injection volume was 1 µL in splitless mode at 280 °C. Helium (0.999%) was used as the carrier gas at a flow rate of 1 mL min^{-1}. The oven conditions were an initial temperature of 60°C for 1 min, followed by a ramp rate of 6°C/min to 270°C. The column flow was 1 mL min^{-1} of helium. The transfer line was maintained at 300 °C throughout the analysis. The quadrupole and ionization source temperatures

were 150 and 230 °C, respectively. Data acquisition was performed in electron impact (EI) mode in the *m/z* 50–550 range in SCAN mode.

The compounds were identified from GC–MS data (total ion chromatograms) recorded with the MassHunter Workstation software version B.08.00 (Agilent Technologies, Inc., USA). The resulting data were processed with the Automated Mass Spectral Deconvolution and Identification System (AMDIS v2.71) to isolate the mass spectra of each compound from the background. The compounds were identified by comparing their mass spectra with the NIST 2011 library, in some cases with standard patterns: α-terpineol 90% (432628), eugenol ≥ 99% (E51791), trans-caryophyllene ≥ 98% (C9653), β-pinene ≥ 99% (402753), β-myrcene 90% (M0382), eugenol methyl ether 99% (284424) (Sigma-Aldrich, Saint Louis, MO, USA). Each standard (5 µl) was dissolved in 1 mL of hexane for injection into the equipment. The relative abundance of each compound was determined by integrating peak areas using MSD ChemStation Data Analysis F.01.01.2317 (Agilent Technologies Inc., EUA).

4.2.4. Evaluation against *Fusarium oxysporum* f. sp. *lycopersici* race 3

The method described by Kim et al. (2016) was followed to test the fungistatic effect of the extracted essential oils. PDA medium (Difco PDA 213400, MD, USA) was prepared, and 20 mL was poured into each 90 mm Petri dish. After solidification, the fungus was sown at the center of the dish using agar discs (7 mm in diameter) impregnated with *Fusarium oxysporum* f. sp. *lycopersici* race 3 (Sombra-Argüelles et al., 2020). Each essential oil was tested at a concentration of 250 µg mL^{-1} on a 2 cm diameter circle of filter paper on the lid of the Petri dish. The Petri dishes were sealed with Parafilm to prevent the escape of volatile essential oil components. They were then incubated at 28 °C in the dark. All samples were evaluated at the same concentration to compare their intrinsic activity under identical experimental conditions. Fungal growth was evaluated from 24 to 168 h by measuring the growth radius every 24 h. Growth inhibition was calculated as the percentage of radial growth inhibition relative to the control (without essential oil).

4.2.5. Statistical analysis

To evaluate differences in essential oil yield, a three-way factorial analysis of variance (ANOVA) was conducted with extraction method (hydrodistillation and steam distillation), sampling period (June, September, and January), and tree type (female and male) as factors. When significant differences were detected (p<0.05), the Tukey HSD multiple-comparison test was used to identify groups that differed significantly. The analyses were performed in the RStudio statistical environment (version 2025.05.0+496).

The multivariate statistical analysis consisted of principal component analysis (PCA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA), performed using SIMCA v.18 software. The GC-MS data were previously normalized by total ion intensity (TIC). This procedure divided the intensity of each peak in a spectrum by the total ion intensity of that spectrum. In the present study, correlations among the relative abundances of the identified compounds were the primary parameters used to construct classification and clustering models. The validation of the OPLS-DA models was performed using the adjustment parameter (R^2X) and the prediction parameter (Q^2).

In the fungistatic activity bioassay, mycelial growth radius values were analyzed using a mixed linear model with repeated measures. In this model, measurement time (hours of growth) was treated as a random factor, and tree type, sampling season, and extraction method were fixed factors. An unstructured covariance structure was used to model the correlation between repeated measurements over time, yielding the best fit to the data set. The analysis was performed in the SAS Studio online environment (Version 3.82, Enterprise Edition) using the PROC MIXED procedure, with a significance level of $p \leq 0.05$ to determine statistically significant effects.

4.3. Results and discussion

4.3.1. Yield

The essential oil yield, depending on tree type, extraction method, and sampling period, ranged from 0.44% to 1.33%. A three-way factorial analysis of variance showed a significant effect of the sampling season on essential oil yield (p = 0.008). Tukey's post hoc analysis indicated that yield was

significantly higher in june (p = 0.0057), with no significant differences between september and january for either tree type or extraction method (hydrodistillation: male 1.13% and female 1.33%; steam distillation: male 1.22% and female 1.02%). Youssef et al. (2021) reported that the highest yield of essential oil from *P. dioica* leaves, obtained by hydrodistillation from trees grown in Egypt, was observed in July (1.46%) compared to spring, autumn, and winter. In contrast, studies conducted in Mexico by Pacheco-Hernández et al. (2024) reported yields of 1.9-2.4% of essential oil from leaves, obtained by hydrodistillation, in samples collected in july and september of 2022, 2023, and 2024 in the state of Veracruz. Similarly, Lam-Gutiérrez et al. (2024) analyzed the essential oil obtained by steam distillation from fresh leaves in the state of Chiapas, reporting a yield of 0.77%. These differences could be explained by seasonal, geographical, or climatic variations at the collection sites (Ben, 2025).

The yield of essential oil obtained by hydrodistillation from *P. dioica* leaves has been reported to range from 0.8 to 2.67% across different studies, with variations attributed to samples collected from trees grown in countries such as Cuba (Pino et al., 1997), Jamaica (Minott and Brown, 2007), Sri Lanka (Dharmadasa et al., 2015), India (Narayanankutty et al., 2021), Egypt (Youssef et al., 2021), and Mexico (Pacheco-Hernández et al., 2024; Lam-Gutiérrez et al., 2024). The above studies differed in sampling conditions, including trees up to 30 years old (Pino et al., 1997; Minott and Brown, 2007), backyard trees (Dharmadasa et al., 2015), and botanical gardens (Youssef et al., 2021).

4.3.2. Metabolite profile by extraction method

This study is the first to characterize the essential oil of *P. dioica* leaves from Mexico using two conventional extraction methods, hydrodistillation and steam distillation, to determine yields and metabolic profiles. A total of 126 metabolites were identified (Table 1). Each extraction method yielded a different number of metabolites: steam distillation extracted 118 metabolites, while hydrodistillation extracted 75 metabolites, and 67 metabolites were present in extracts obtained by both methods (Figure 1). In the study by Youssef et al. (2021), only 56 metabolites were identified in the essential oil extracted by hydrodistillation from *P. dioica* leaves grown in Egypt across four

seasons. As mentioned above, Pacheco-Hernández et al. (2024) analyzed and identified 18 compounds in the essential oil extracted by hydrodistillation from leaves in 2022, 2023, and 2024 in Veracruz, Mexico.

Table 1. Compounds identified in the essential oil from the leaves of *Pimenta dioica* trees. The symbols (▲; female) and (●; male) indicate metabolites identified exclusively in female and male trees, respectively.

#	Compound	RT		#	Compound	RT	
1	2-Hexenal, (E)-	3.695	●	**47**	Undecane, 3-methyl-	10.288	▲
2	3-Hexen-1-ol	3.760		**48**	Benzoic acid, ethyl ester	10.348	●
3	1-Hexanol	3.917	●	**49**	Terpinen-4-ol	10.545	
4	α-Thujene	5.009		**50**	p-Cymen-8-ol	10.709	●
5	α-Pinene	5.154		**51**	1-Dodecene	10.787	▲
6	Isocumene	5.515	▲	**52**	α-Terpineol*	10.847	
7	Benzene, 1-ethyl-3-methyl-	5.674	▲	**53**	Methyl salicylate	10.934	●
8	Benzene, 1-ethyl-2-methyl-	5.704	▲	**54**	Tridecane	10.972	●
9	β-Thujene	5.926		**55**	Estragole	11.006	
10	Bicyclo[4.1.0]heptane, 7-methylene-	5.993		**56**	2,6-Dimethyl-3,5,7-octatriene-2-ol, ,E,E-	11.200	
11	β-Pinene*	6.016		**57**	Undecane, 2,6-dimethyl-	11.293	
12	Cumene	6.051	▲	**58**	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	11.668	
13	β-Myrcene*	6.253		**59**	2-Butenoic acid, 3-hexenyl ester, (E,Z)-	11.799	●
14	Benzene, 1,2,4-trimethyl-	6.336		**60**	Cyclohexane, 2-propenyl-	11.885	▲
15	α-Phellandrene	6.573		**61**	β-Citral	11.964	
16	α-Terpinene	6.835		**62**	trans-Geraniol	12.250	
17	Isoterpinolene	6.837		**63**	Chavicol	12.280	
18	p-Cymene	7.008		**64**	2,2,4-Trimethyl-3-pentanone	12.414	▲
19	D-Limonene	7.106		**65**	α-Citral	12.634	
20	Eucalyptol	7.180		**66**	Anethole	13.005	
21	trans-β-Ocimene	7.256		**67**	Ethanone, 1-(2-hydroxy-5-methylphenyl)-	13.680	●
22	cis-β-Ocimene	7.502		**68**	Elixene	14.177	
23	Heptane, 4-ethyl-	7.697	▲	**69**	Eugenol*	14.652	
24	γ-Terpinene	7.772		**70**	α-Cubebene	15.057	
25	Decane, 2-methyl-	7.861	▲	**71**	Copaene	15.080	
26	Decane, 3-methyl-	8.004	▲	**72**	Geraniol acetate	15.105	▲
27	Terpinolene	8.453		**73**	1-Tridecene	15.250	
28	β-Linalool	8.682		**74**	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethenyl)-, [S-(Z,E)]-	15.301	
29	6-Methyl-3,5-heptadiene-2-one	8.796	●	**75**	(-)-β-Elemene	15.383	
30	Ethanone, 1-(2-methyl-1-cyclopenten-1-yl)-	8.822	●	**76**	Tetradecane	15.418	▲
31	trans-Decalin, 2-methyl-	8.989		**77**	Eugenol methyl ether*	15.653	
32	Phenylethyl Alcohol	9.021		**78**	cis-Isoeugenol	15.697	●
33	Octane, 3,4,5,6-tetramethyl-	9.062		**79**	Isopiperitenone	15.909	●
34	2-Cyclohexen-1-ol,1-methyl-4-(1-methylethyl)-, trans-	9.226		**80**	β-Caryophyllene*	16.022	
35	Decane, 3,7-dimethyl	9.294	▲	**81**	7-Methyl-3-methylene-7-octen-1-ol, propanoate (ester)	16.106	●
36	2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)-	9.367		**82**	Benzenemethanol, α,4-dimethyl-	16.150	●
37	α-Pyronene	9.366	●	**83**	β-Cubebene	16.235	
38	trans-4a-Methyl-decahydronaphthalene	9.370	▲	**84**	(E)-Isoeugenol	16.575	
39	1,3-Cyclohexadiene, 1,3,5,5-tetramethyl-	9.369		**85**	α-Caryophyllene	16.731	
40	1,3,8-p-Menthatriene	9.392	▲	**86**	(+)-Epi-bicyclosesquiphellandrene	16.947	▲
41	Cyclohexane, pentyl	9.501	▲	**87**	1-Dodecanol	16.989	▲

42	Heptane, 4-(1-methylethyl)-	9.922	▲	**88**	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	17.169	▲
43	Undecane, 5-methyl-	9.940	●	**89**	Germacrene D	17.290	
44	1,5,7-Octatrien-3-ol, 2,6-dimethyl-	9.997	●	**90**	β-Selinene	17.402	
45	Undecane, 4-methyl-	10.038		**91**	Isoeugenol methyl ether	17.504	
46	Undecane, 2-methyl-	10.135		**92**	α-Selinene	17.576	
93	α-Farnesene	17.708		**110**	α-Cadinol	20.436	
94	Phenol, 2,4-bis(1,1-dimethylethyl)-	17.795		**111**	(-)-δ-Cadinol	20.515	
95	γ-Cadinene	17.951		**112**	Juniper camphor	20.703	
96	1H-Indene, 5,6-dimethoxy-	17.969		**113**	Hexadecane	21.337	
97	(+)-δ-Cadinene	18.098		**114**	2-Propenal, 3-(3,4-dimethoxyphenyl)-	21.488	
98	2-Propanone,1-(4-hydroxy-3-methoxyphenyl)-	18.235	●	**115**	trans, trans-Farnesal	22.001	●
99	α-Calacorene	18.532	●	**116**	Coniferyl aldehyde, methyl ether	22.810	
100	D-nerolidol	18.833	●	**117**	E-15-Heptadecenal	23.002	▲
101	Elemicin	18.669		**118**	3-Octadecene, (E)-	23.005	●
102	trans-4-Methoxycinnamaldehyde	18.968	▲	**119**	Hexane, 3,3-dimethyl-	23.125	●
103	3-Hexen-1-ol, benzoate, (Z)-	18.999		**120**	Isopropyl Myristate	23.587	
104	Spathulenol	19.234	●	**121**	Benzeneacetic acid, 2-phenylethyl ester	25.204	▲
105	1-Hexadecene	19.320		**122**	Hexadecanoic acid, methyl ester	25.267	▲
106	Caryophyllene oxide	19.351		**123**	n-Hexadecanoic acid	25.863	●
107	Veridiflorol	19.520	●	**124**	1-Nonadecene	26.352	
108	12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-	19.857	●	**125**	Hexadecanoic acid,butyl ester	29.309	▲
109	Selina-6-en-4-ol	19.921		**126**	1-Docosene	29.419	●

*Compared to standard

Figure 1. Distribution of metabolites identified in the essential oil of *Pimenta dioica* leaves extracted by steam distillation (SD) and hydrodistillation (HD). Each number in parentheses corresponds to the metabolite reported in Table 1.

4.3.3. Metabolite profile by tree type

Of the 126 metabolites identified, each tree type showed a different number. Ninety-eight metabolites were detected in female trees, 99 in male trees, and 71 in both. Twenty-seven compounds were identified as exclusive to female trees, and 28 to male trees (Table 1). Among the metabolites extracted by steam distillation in june, aliphatic hydrocarbons (20) prevailed in female trees, whereas only 14 were identified in male trees (Figure 2). By chemical group, the percentage composition of monoterpenes and sesquiterpenes was higher

in male trees, and the percentage composition of phenylpropanoids was higher in female trees, regardless of the factor analyzed (Table 2).

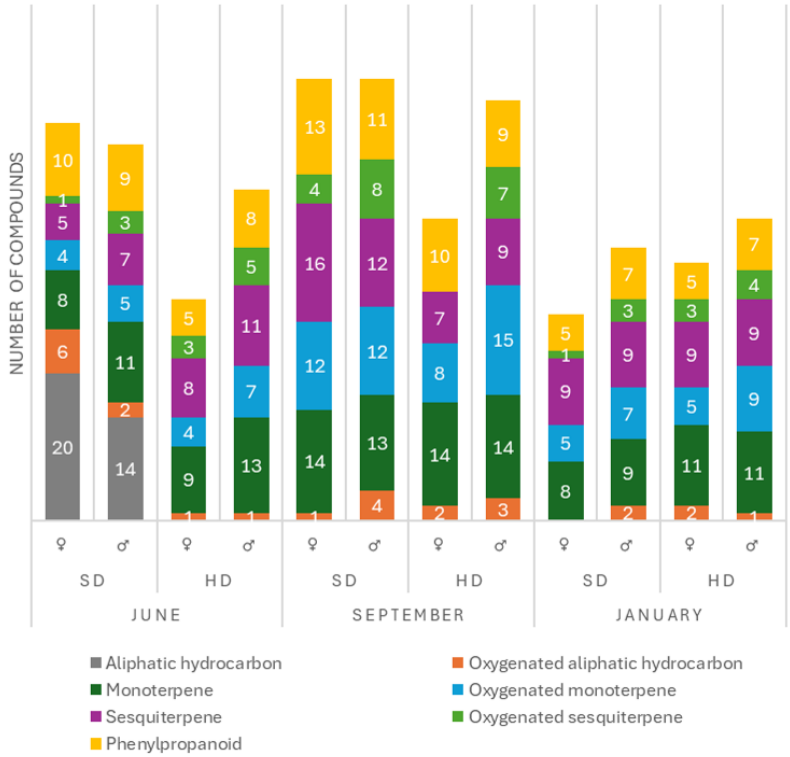


Figure 2. Number of metabolites identified in the essential oil of leaves from female (♀) and male (♂) *Pimenta dioica* trees obtained by steam distillation (SD) and hydrodistillation (HD) in june, september and january.

Table 2. Grouping by structural group and relative abundance (%) of metabolites in the essential oil of leaves from female (♀) and male (♂) *Pimenta dioica* trees obtained by steam distillation (SD) and hydrodistillation (HD) during june, september, and january.

Group	June				September				January			
	SD		HD		SD		HD		SD		HD	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Aliphatic hydrocarbon	4.45	1.08	-	-	-	-	-	-	-	-	-	-
Oxygenated aliphatic hydrocarbon	2.14	0.22	0.02	0.01	0.01	0.43	0.03	0.01	-	0.03	0.05	0.01
Monoterpene	1.53	5.56	6.51	9.91	10.26	13.24	9.58	16.07	4.11	3.67	6.34	8.16
Oxygenated monoterpene	4.45	5.89	3.14	5.32	5.1	9.9	4.89	9.31	3.23	4.97	4.82	7.67
Sesquiterpene	0.6	0.31	2.92	3.63	2.49	4.84	1.18	1.65	1.61	1.75	2.05	2.62
Oxygenated sesquiterpene	0.11	0.11	0.1	0.44	0.2	1.14	-	0.67	0.06	0.35	0.2	0.6
Phenylpropanoid	86.6	86.8	87.3	80.7	81.93	70.48	84.35	72.17	90.96	89.24	86.55	80.91
Total compounds	54	51	30	45	60	60	41	57	28	37	35	41
Yield (%)	1.02	1.22	1.33	1.13	1.06	0.63	1.04	1.02	0.68	0.79	0.92	0.44

Among the characteristic compounds of each tree type, 2-isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene stands out. It was identified exclusively in the essential oil from the female tree by steam distillation (Figure 3). Montalvo-López et al. (2021) also reported this metabolite, although they identified it by SPME-GC-MS. This metabolite could be considered a marker that distinguishes the female *P. dioica* tree from the male tree. Basaid et al. (2020) also reported this metabolite in the essential oil of *Senecio glaucus* subsp. *coronopifolius* (Asteraceae), where it exhibited antifungal, nematicidal, acaricidal, and repellent activity, highlighting its potential as a bioactive compound.

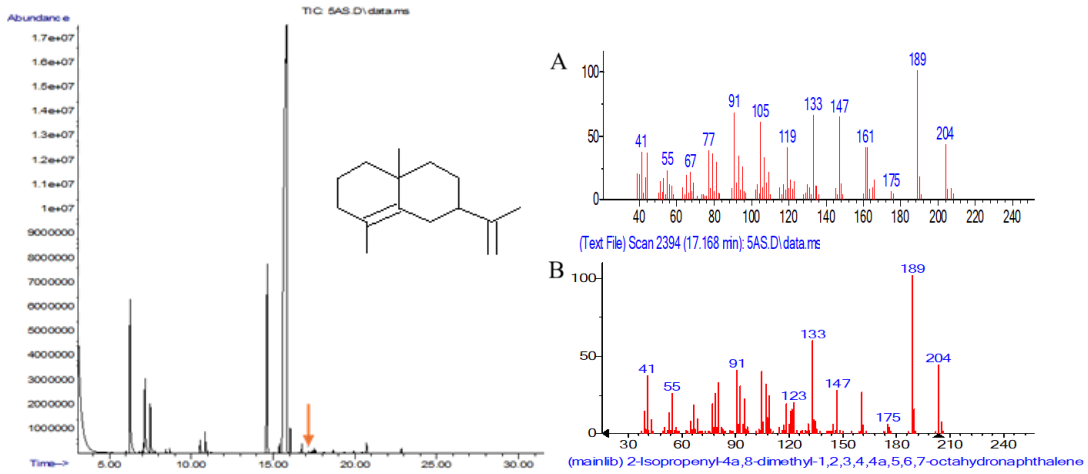


Figure 3. Chromatogram of essential oil from female leaves of *Pimenta dioica*. The orange arrow indicates the retention time (RT: 17.16) of the metabolite 2-isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene. Mass spectrum of the metabolite (A) and that of the NIST library (B).

4.3.4. Metabolite profile by sampling period

The June samples yielded the highest number of metabolites (87), followed by september (82) and january (50), regardless of extraction method. Likewise, the number and profile of metabolites varied across months (june 39; september 30; january 3) (Figure 4). There were 39 metabolites common to all three collection periods, 16 of which coincided with the major compounds reported by Minott and Brown (2007), Dharmadasa et al. (2015), Youssef et al. (2021), Lam-Gutiérrez et al. (2024), and Pacheco-Hernández et al. (2024) in the essential oil of *P. dioica* leaves. These metabolites included eugenol; eugenol methyl ether; α-terpineol; β-pinene; β-myrcene; α-terpinene; p-

cymene; D-limonene; eucalyptol; cis-β-ocimene; γ-terpinene; terpinolene; β-linalool; terpinen-4-ol; α-caryophyllene; and (+)-δ-cadinene.

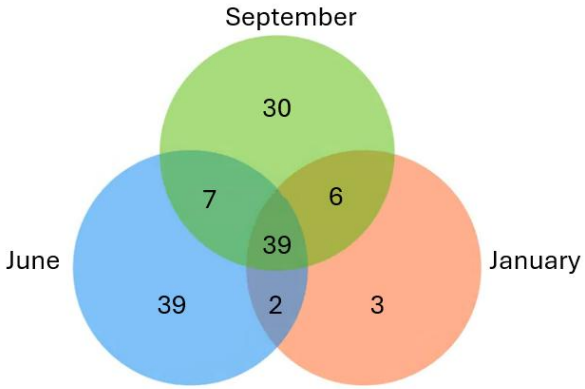


Figure 4. Distribution of the number of metabolites identified in the essential oil of *Pimenta dioica* leaves during three periods of the year.

More compounds were identified in leaves collected in June (52) and September (60) using steam distillation; in contrast, the January samples yielded the highest number (38) by hydrodistillation. As shown in Figure 2, metabolite groups, based on chemical structure, varied with extraction method, tree type, and sampling period. However, in September, the greatest chemical diversity was detected by both extraction methods and tree types, compared with june and january. Aliphatic hydrocarbons were identified only in the June samples obtained by steam distillation, while oxygenated aliphatic hydrocarbons were consistently in the minority. Of these, up to six metabolites were identified across the three factors, and their combined percentage contribution was less than 2% (Table 2).

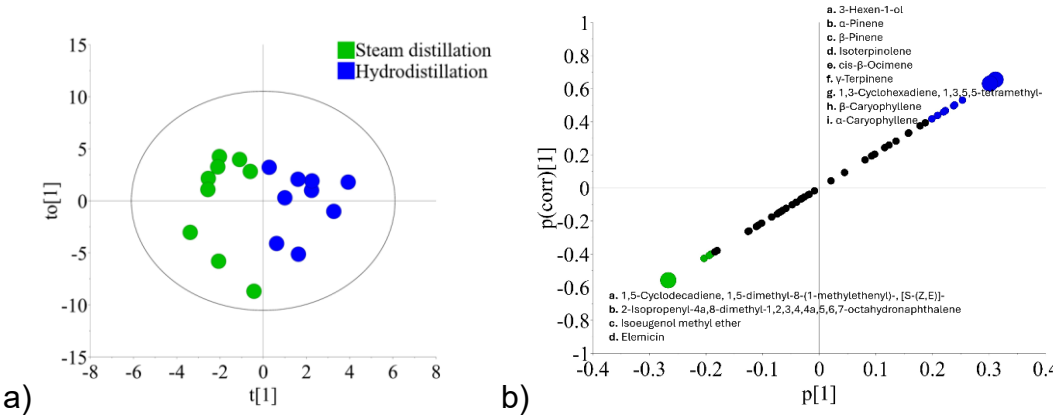
The percentage composition of phenylpropanoids was consistently higher across all factors analyzed (70.48 - 90.96%), followed by monoterpenes (6.98 - 25.38%) and, finally, sesquiterpenes (0.42 - 5.98%) (Table 2). In contrast, Elhawary et al. (2024) reported that oxygenated monoterpenes prevailed in essential oil from in *Citrus aurantium* leaves, regardless of extraction method (steam distillation or hydrodistillation). These results indicate that essential oil composition varies with factors such as extraction method, plant tissue, and seasonal variation. All or some of these factors could modify the composition

of the essential oil, even within the same plant species. The quality and quantity of the resulting essential oil depend on the appropriate selection and optimization of the extraction method (Elhawary et al., 2024).

4.3.5. Correlation of identified metabolites with the factors analyzed

To identify metabolites differentially correlated with each factor (extraction method, sampling period, and tree type) in the oil from *P. dioica* leaves, OPLS-DA models were constructed. Their validity was assessed using permutation tests ($Q^2 \geq 0.4$) and cross-validated analysis of variance (CV-ANOVA, $p \leq 0.05$).

Figure 5a shows the correlation of metabolites based only on the extraction method for the female tree ($Q^2 = 0.49$, $p = 0.05$). No significant correlation was found in the male tree model, indicating that the extraction method did not influence its composition. This suggests that the essential oil from male trees could be a resource with industrial potential. The compounds correlated with each extraction method for the female tree are shown in Figure 5b, where green dots correspond to metabolites correlated with steam distillation, blue dots to metabolites correlated with hydrodistillation, and black dots to metabolites not correlated with the extraction method.



a) b)

Figure 5. a) OPSL-DA scores plot and b) S-plot of the comparison of extraction methods in female *Pimenta dioica* trees.

The analysis validated the effect of tree type on variability in essential oil metabolites ($p = 2.53 \times 10^{-5}$ and $Q^2 = 0.64$). The results allowed metabolites correlated with male (green dots) and female (blue dots) tree types to be

grouped (Figure 6a). Metabolites that were not correlated with any tree type are shown as black dots.

Additionally, the S-plot of the model indicated that the 21 compounds correlated with the male tree (Figure 6b) were β-myrcene, α-terpinene, p-cymene; β-linalool; 2,4,6-octatriene, 2,6-dimethyl-, (E,Z); (-)-terpinen-4-ol; p-cymen-8-ol; α-terpineol; methyl salicylate; 2,6-octadien-1-ol, 3,7-dimethyl-, (Z)-; trans-geraniol; eugenol; α-cubebene; (E)-isoeugenol; β-selinene; (+)-δ-Cadinene; 3-hexen-1-ol,-benzoate, (Z)-; caryophyllene oxide; 12-oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-; and selina-6-en-4-ol. Six compounds were detected in correlation with the female tree: 1,3,8-p-menthatriene; (-)-β-elemene; eugenol methyl ether; 2-isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene; and isoeugenol methyl ether (Figure 6b).

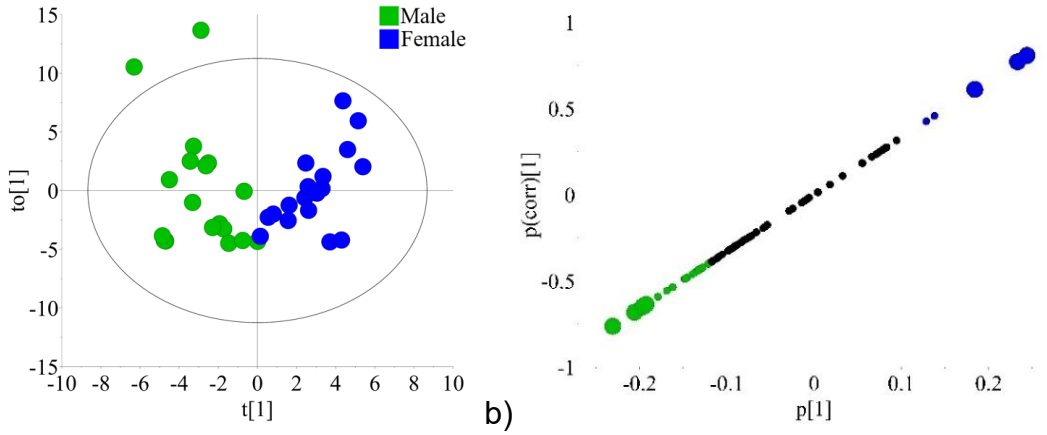


Figure 6. a) OPSL-DA scores plot and b) S-plot of the essential oil composition of female and male *Pimenta dioica* trees.

In the present study, eugenol was the major metabolite in male trees, whereas methyl eugenol was the major metabolite in female trees, regardless of extraction method or period. The structural difference between the two is due to the reaction catalyzed by the enzyme eugenol-O-methyltransferase (EOMT), which converts eugenol to methyl eugenol (Renu et al., 2014) after

eugenol biosynthesis via the shikimic acid pathway to synthesize phenylpropanoids. Eugenol has been reported as the major metabolite in species such as *Ocimum basilicum* (Lamiaceae), *Syzygium aromaticum* (Myrtaceae), *Cinnamomum verum* (Lauraceae), and *Laurus nobilis* (Lauraceae) (Smitha and Triphaty, 2016). On the other hand, methyl eugenol has been identified in species of other genera such as *Croton malambo* (Euphorbiaceae), *Cinnamomum chordatum* (Lauraceae), *Melaleuca* species (Myrtaceae), *Pepper racemosa* (Myrtaceae), *Piper divaricatum* (Piperaceae), and *Clusena anisata* (Rutaceae) (de Oliveira et al., 2024). Some of the properties reported for eugenol include antioxidant, analgesic, anticancer, bactericidal, and fungicidal activities (Ulanowska and Olas, 2021). Likewise, methyl eugenol has been reported to have antioxidant, anticarcinogenic, insecticidal, bactericidal, and fungicidal activity (Anwar et al., 2024). This could help explain the diverse biological activities attributed to *P. dioica.*

For the sampling period factor (june, september, and january), a SUS-plot scatter diagram was generated, showing loadings scaled as correlation coefficients (*p*(corr) [1]) from two independent OPLSDA models (Ivanović et al., 2021). For this, two OPLSDA analyses were performed for september and January (Q^2= 0.69; *p* = 0.07) (Figure 7a), and another for june and september (Q^2= 0.65; *p* = 0.0003) (Figure 7b), where the blue dots correspond to the september samples, the red dots to january, and the green dots to june. The SUS-plot (Figure 7c) from the two previous OPLSDA models showed that the most representative factor was september. The 12 correlated metabolites are shown in Figure 7c; 10 are monoterpenes and were found at greater relative abundance than in june and january, as shown in Table 2.

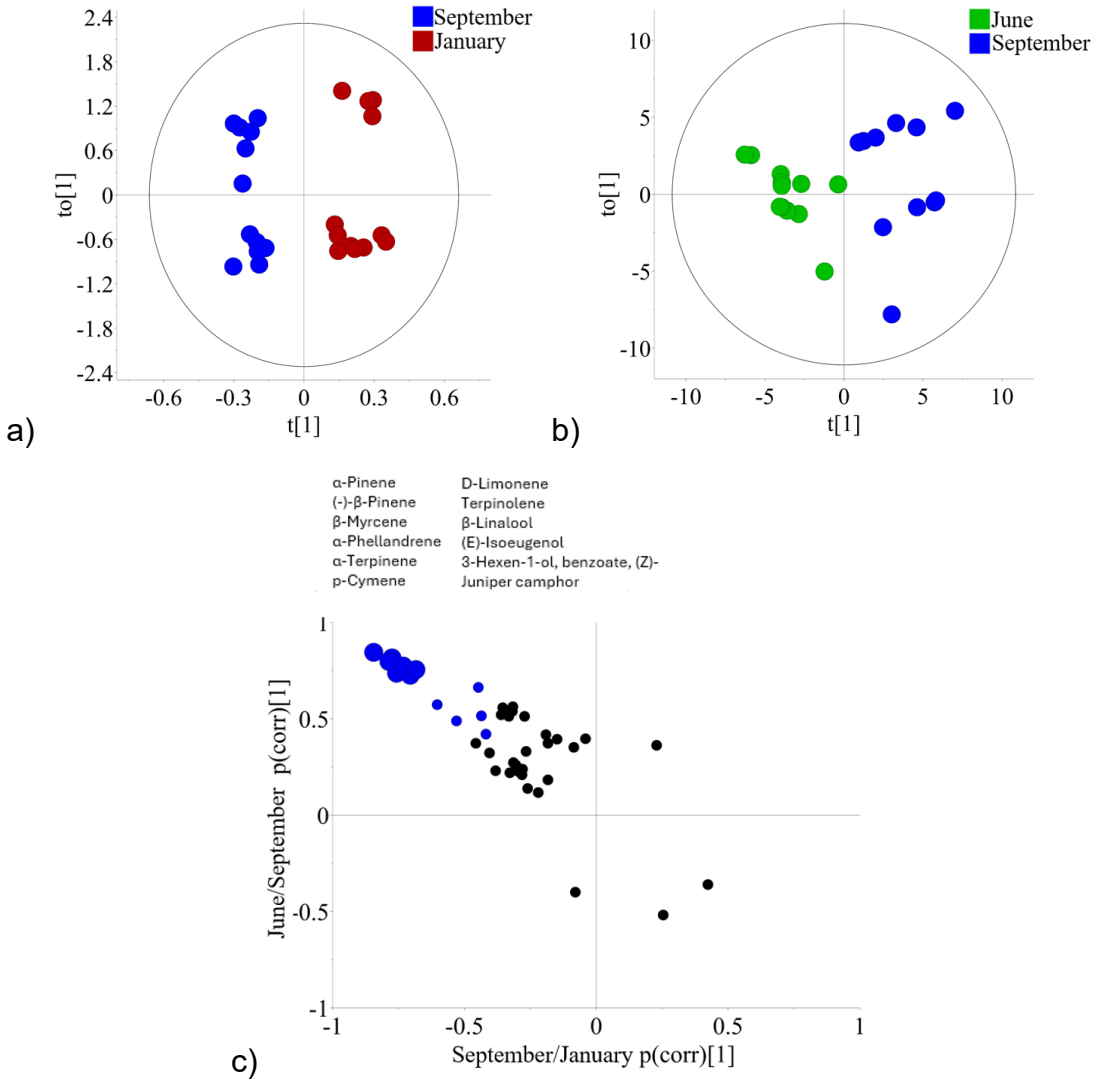


Figure 7. OPSLDA comparing the sampling periods of a) September and January, and b) June and September; c) SUS-plot correlating two OPLS-DA models matching the sampling period.

4.3.6. Evaluation of fungistatic activity against *Fusarium oxysporum* f. sp. *lycopersici* race 3

Significant differences ($p \leq 0.001$) were found in the fungistatic effect of the essential oil depending on tree type against *Fusarium oxysporum* f. sp. *lycopersici* race 3 (FOL3). The essential oil obtained from male trees showed a greater reduction in fungal mycelial growth compared with oil from female trees and the control (Figure 8), regardless of the extraction method. In specific treatments, particularly in january with both extraction methods and in september using hydrodistillation, essential oil from male trees exhibited a fungicidal effect compared with oil from female trees.

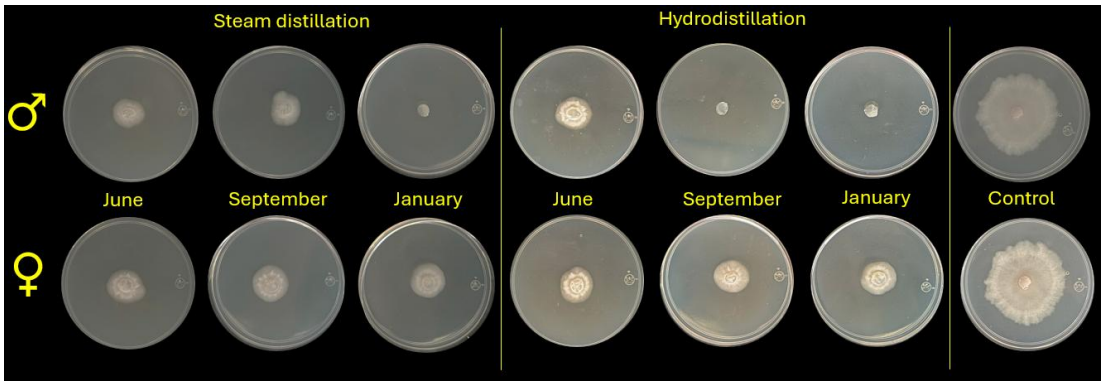


Figure 8. Representation of three replicates of fungicidal and fungistatic activity at 168 h of essential oil from male (♂) and female (♀) leaves of *Pimenta dioica* obtained by steam distillation and hydrodistillation in june, september and january against *Fusarium oxysporum* f. sp. *lycopersici* race 3.

Regarding fungistatic activity, growth dynamics (Figure 9) showed that male tree oil obtained by both extraction methods significantly delayed the onset of mycelial growth. In contrast, female tree treatments showed only moderate inhibition over the first 72 h.

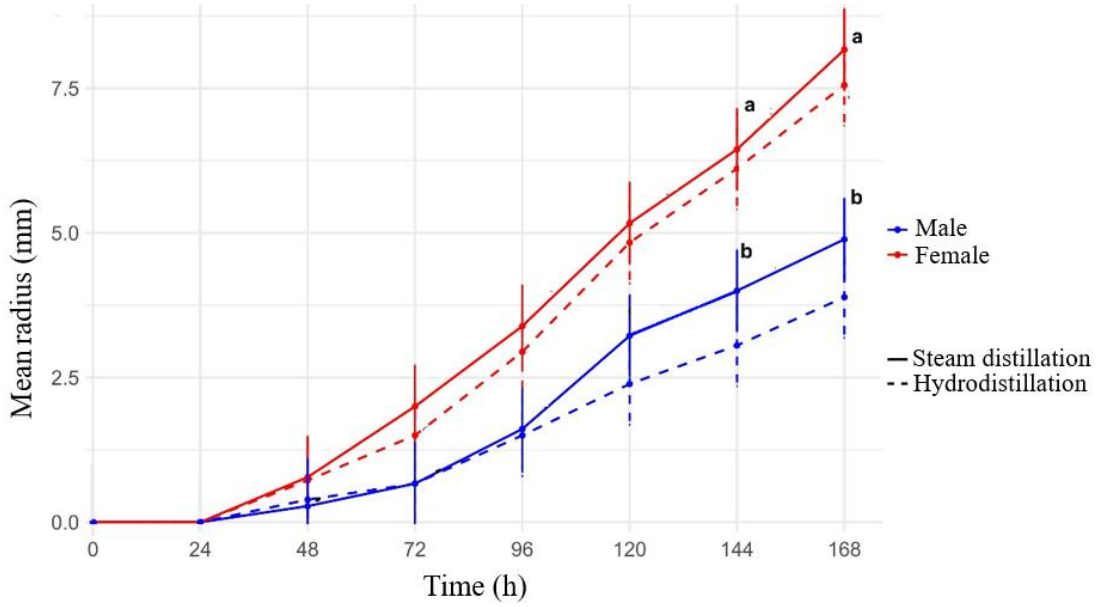


Figure 9. Growth dynamics of *Fusarium oxysporum* f. sp. *lycopersici* race 3 of essential oil from female and male *Pimenta dioica* leaves obtained by steam distillation and hydrodistillation. Different letters indicate significant differences (Tukey; $p \leq 0.05$).

The results revealed that only the tree type factor was significant ($p \leq 0.001$), indicating that oil from male trees consistently showed higher fungistatic

activity than that from female trees. Therefore, an OPLS model for male trees (Q^2 = 0.76; p = 0.03) was constructed, which clearly separated samples with low and high fungistatic activity at 168 h (Figure 10a). Samples with the highest inhibition were grouped in positive values in Figure 10a (orange and red dots). At the same time, those with the lowest activity were grouped in negative values (blue and green dots). This pattern indicated that differences in the chemical composition of the essential oil are strongly correlated with its fungistatic activity against FOL3. The VIPpred-plot identified 17 metabolites with the greatest contribution to the OPLS-DA model (Figure 10b), with VIP values > 1; therefore, they were considered significant and correlated with the increase in fungistatic activity (red bars, VIP > 1). Metabolites with VIP values below 1 were classified as low importance, as they did not contribute substantially to fungistatic activity (black bars).

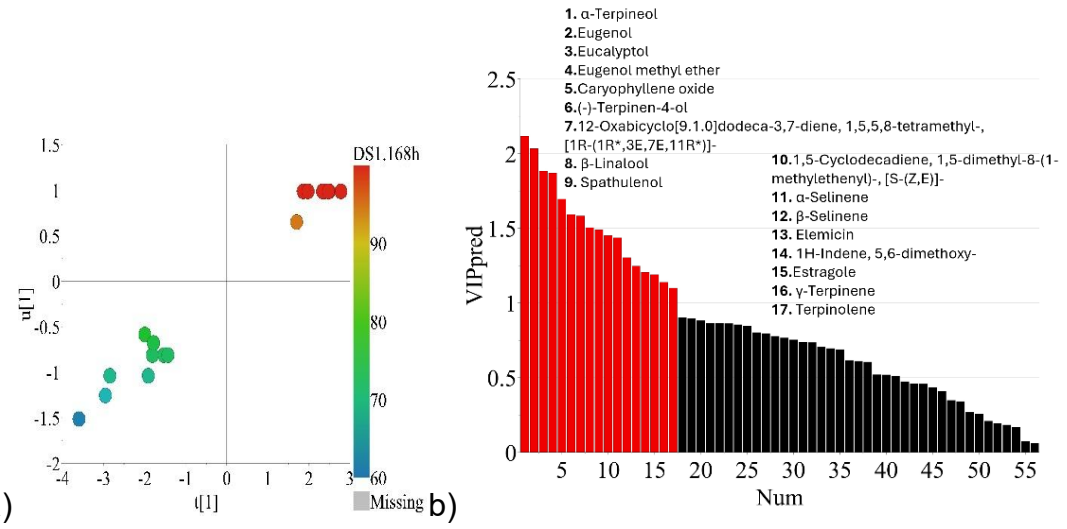


Figure 10. a) OPLS diagram and b) VIPpred-plot of essential oils obtained from male *Pimenta dioica* leaves by hydrodistillation and steam distillation in June, September and January. The VIP diagram showed 17 metabolites (in red) with VIP values > 1.

Of the 17 metabolites significantly correlated with fungistatic activity, the compounds spathulenol and 12-oxabicyclo [9.1.0] dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]- were identified exclusively in male trees and were obtained using both extraction method across in the three sampling periods analyzed. Da Silva et al. (2024) analyzed the essential oil of *Schinus molle* leaves, where spathulenol was the major compound. Both the essential oil and spathulenol exhibited bactericidal activity against *Xanthomonas citri*, a

disease of agricultural significance in citrus. On the other hand, Taha et al. (2023) reported that the essential oil of *Eucalyptus camaldulensis* and the metabolite spathulenol inhibited the growth of the fungi *Aspergillus flavus*, *Fusarium culmorum,* and *Aspergillus niger*. Furthermore, the metabolite 12-oxabicyclo [9.1.0] dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]- has been identified in the essential oil of *Calamintha nepeta* L. (Lamiaceae) (Zeynalova, 2018) and in the essential oil of *Picrasma quassioides* (Simaroubaceae) leaves, a medicinal plant used in China to treat cold and cough symptoms (Huang et al., 2013). Both compounds could serve as alternative sources of bioactive metabolites with potential applications in the formulation of natural products of agricultural, industrial, pharmaceutical, or cosmetic interest, thereby contributing to the comprehensive and sustainable use of the crop. This finding was particularly relevant for assessing the potential use of underutilized and discarded male tree leaf tissue in allspice production systems.

Other studies have reported that essential oil extracted from dried *P. dioica* leaves by hydrodistillation showed marked fungistatic activity against species of the genus *Fusarium,* with a minimum inhibitory concentration (MIC) of 450 μg mL^{-1}. The major compounds were eugenol, chavicol, and myrcene (Everton et al., 2021). Similarly, in the study conducted by Lam-Gutiérrez et al. (2024), the essential oil obtained from fresh leaves by steam distillation showed significant inhibition of 83.37% against *Fusarium moniliforme* at a concentration of 0.26 mg mL^{-1}, reaching total inhibition at 0.43 mg mL^{-1}. The major metabolites were eugenol (phenylpropanoid), β-caryophyllene, and β-pinene (terpenoids). These results support the antifungal potential of *P. dioica* essential oil, which is mainly attributed to its high content of phenylpropanoids and bioactive terpenes.

The same metabolites α-terpineol, terpinen-4-ol, and linalool identified in this study were also reported by Soliman et al. (2022), who noted that these metabolites caused the collapse of the mycelial hyphae of *F. oxysporum* f. sp. *lycopersici* treated with essential oils of *Mentha spicata* and *M. longifolia.* Li et

al. (2022) reported that linalool exhibits antifungal activity against the soil fungus *F. oxysporum* f. sp. *radicis-lycopersici*, both *in vitro* and *in vivo*, and that its action is associated with structural disruption of the cell membrane, redox dysfunction, and alterations in nutrient metabolism. Similarly, eugenol has shown potent antifungal activity through a multimodal mechanism: being lipophilic, it can penetrate the lipid bilayer of fungal membranes, alter their permeability, and cause ion leakage and loss of essential metabolites. Other studies indicate that this compound can bind to membrane proteins and enzymes, denaturing key proteins in fungal metabolism, such as those involved in respiration and cell synthesis, and interfering with mitochondrial oxidative phosphorylation, reducing ATP production. Finally, it induces oxidative stress, accelerating cell death (Giménez-Santamarina et al., 2025).

4.4. Conclusions

A total of 126 metabolites were identified in the essential oil of *P. dioica* leaves. In September, the highest essential oil yield was obtained, with no significant differences between tree type and extraction method. Steam distillation yielded 118 metabolites, while hydrodistillation yielded 75. A different number of metabolites was identified in each tree type: 98 compounds were identified in female trees, of which 27 were exclusive; 99 metabolites were detected in male trees, with 28 exclusives; and 71 metabolites were identified in both tree types. Most of the oils showed fungistatic activity against FOL3; however, significant differences were observed in the essential oils of male trees, where 17 compounds were significantly correlated with the inhibition of the growth of *Fusarium oxysporum* f. sp. *lycopersici* race 3, including spathulenol and 12-oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-, metabolites identified exclusively in male trees at the three sampling periods and which could be considered metabolites for the differentiation of male trees. This justifies the use of male tree leaf tissue as an alternative source of bioactive metabolites for the comprehensive and sustainable use of other crops affected by *F. oxysporum,* as well as for the development of natural products with agrobiotechnological potential.

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