



Universidade Federal de Uberlândia
Instituto de Biologia
Programa de Pós-graduação em Ecologia,
Conservação e Biodiversidade



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Relação entre estresse, alterações na parede celular, metabólitos e acúmulo de metais nas galhas induzidas em *Macairea radula* (Melastomataceae)

Uberlândia, agosto de 2024

Patrícia Dias Santos

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Tese apresentada à Universidade Federal de Uberlândia, como parte das exigências para obtenção do título de Doutora em Ecologia, Conservação e Biodiversidade.

Orientador: Prof. Dr. Denis Coelho de Oliveira

Coorientador: Prof. Dr. Diego Ismael Rocha

Uberlândia, agosto de 2024

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

S237r
2024 Santos, Patrícia Dias, 1988-
Relação entre estresse, alterações na parede celular, metabólitos e acúmulo de metais nas galhas induzidas em *Macairea radula* (Melastomataceae) [recurso eletrônico] / Patrícia Dias Santos. - 2024.

Orientador: Denis Coelho de Oliveira.
Coorientador: Diego Ismael Rocha.
Tese (Doutorado) - Universidade Federal de Uberlândia, Programa de Pós-graduação em Ecologia, Conservação e Biodiversidade.
Modo de acesso: Internet.
Disponível em: <http://doi.org/10.14393/ufu.te.2024.5028>
Inclui bibliografia.
Inclui ilustrações.

1. Ecologia. I. Oliveira, Denis Coelho de, 1981-, (Orient.). II. Rocha, Diego Ismael, 1986-, (Coorient.). III. Universidade Federal de Uberlândia. Programa de Pós-graduação em Ecologia, Conservação e Biodiversidade. IV. Título.

CDU: 574

André Carlos Francisco
Bibliotecário Documentalista - CRB-6/3408



UNIVERSIDADE FEDERAL DE UBERLÂNDIA

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Conservação e Biodiversidade

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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Ecologia, Conservação e Biodiversidade				
Defesa de:	Tese, número 94, PPGECEB				
Data:	vinte e oito de agosto de dois mil e vinte e quatro	Hora de início:	14:10	Hora de encerramento:	[17:55]
Matrícula do Discente:	12013ECR007				
Nome do Discente:	Patrícia Dias Santos				
Título do Trabalho:	Relação entre estresse, alterações na parede celular, metabólitos e acúmulo de metais nas galhas induzidas em <i>Macairea radula</i> (Melastomataceae)				
Área de concentração:	Ecologia e Biodiversidade				
Linha de pesquisa:	Ecologia de comunidades e biodiversidade				
Projeto de Pesquisa de vinculação:	Estrutura, fisiologia e química de galhas neotropicais				

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Documento assinado eletronicamente por **Bruno Garcia Ferreira, Usuário Externo**, em 02/09/2024, às 09:45, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



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Agradecimentos

Agradeço à Universidade Federal de Uberlândia (UFU) pelo espaço arborizado, pelo restaurante universitário, pela biblioteca, laboratórios, equipamentos emprestados e espaço da natação. Ao programa de Pós graduação em Ecologia, Conservação e Biodiversidade pelas atividades de campo, apoio com as disciplinas, participação em eventos científicos e atividades de extensão. À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) pelos 48 meses de bolsa de doutorado. Ao CNPq pelos 6 meses de bolsa de Apoio Técnico à Pesquisa. Ao Programa de Pesquisa Ecológica de Longa Duração (PELD/MPT) pelo apoio à pesquisa. Ao prof. Denis pela orientação, por ser aberto às colaborações, pelos incentivos, ajuda nas coletas em campo e oportunidades que tive para desenvolver esta tese. Ao meu coorientador prof. Diego Rocha pela colaboração com os experimentos de fitormônios, ensinamentos para uso de software e análise de dados, sugestões e dicas para apresentação de trabalhos. À secretária Juliana Pinheiro por toda atenção e gentileza. Ao grupo galhas, em especial à profa Rosy Isaias pelos poucos, mas valiosos momentos de convivência nos encontros científicos. À Profa. Elaine Carbonero pela colaboração com os experimentos de carboidratos e por todo apoio científico desde minha passagem pelo mestrado até o retorno ao doutorado, por todo acolhimento que recebi em Catalão. Aos meus colegas do Laboratório de Anatomia, Desenvolvimento Vegetal e Interações, Vitor, Phabliny e Pedro pela ajuda nos campos e por todo acolhimento e ensinamentos, à Uiara pelas dicas de anatomia e pelos seus trabalhos publicados que dão base para esta tese. Às alunas de IC pelas perguntas e alegria que trazem ao laboratório, Amanda, Bruna e Stephany. Ao laboratório de Fisiologia Vegetal pela colaboração e cafés da tarde, agradeço à Profa. Ana Silvia, Jéssica, Maria Eduarda, Amanda, Gisely e Denny pela convivência. Aos profs. Vinícius Kuster e Thiago Magalhães pela participação e considerações na minha banca de qualificação. À profa. Lubia Guedes pela colaboração e sugestões no capítulo de metabólitos secundários. Ao Igor Arriola pelos experimentos com os metais e suas boas perguntas nos momentos de socialização. Ao Núcleo de Pesquisa em Produtos Naturais (NuPPeN), pela colaboração com os experimentos fitoquímicos e análise de dados de LC-MS, especialmente ao Prof. Alberto Oliveira, Mário Martins e Tiara Costa, grata pela paciência que tiveram comigo, pela disponibilidade de tempo e material de laboratório. À Élide, Gisely, William e Maria pelos poucos, mas fortes momentos de lazer durante o doutorado. À Leticia Nobrega pela sua visita à Uberlândia, grata pelas conversas e cafés. À Fabi, Tábata, Selma e Hosana pelos nossos momentos na cozinha e sessão de filmes. Aos amigos de Oriximiná, Amauri Pereira e Maico Pimentel pelo apoio profissional. À Talita e Aninha, grata pelos incentivos e amizade. Aos meus pais, Ivana e Adenil, por todo incentivo. Gratidão a todos por encherem a minha vida com ciência, arte, cafés, livros e muito afeto.

1 Resumo

As plantas são acometidas por uma variedade de estresses bióticos e abióticos, os quais podem afetar seu crescimento, desenvolvimento, produtividade e distribuição geográfica. Para sobreviver às condições ambientais adversas, desenvolveram estratégias adaptativas como o acúmulo de metabólitos com funções protetoras, principalmente contra o ataque de herbívoros. Muitos desses organismos, especialmente os insetos, induzem estresse biótico nas plantas estabelecendo uma relação de parasitismo espécie-específica. Essa relação pode alterar o metabolismo vegetal e induzir o crescimento anômalo com hiperplasia e hipertrofia celular, levando à formação de órgãos neoformados, as galhas. Essas estruturas são controladas pela liberação de espécies reativas de oxigênio, enzimas, carboidratos, metabólitos secundários e fitohormônios; também podem acumular metais nos tecidos das plantas hospedeiras. É provável que a biossíntese dessas moléculas possa mudar ao longo do dia, seguindo um ciclo circadiano em resposta ao estresse biótico. Nesse sentido, esta tese tem como hipótese central que, quanto maior o estresse oxidativo nos tecidos das galhas maior será o acúmulo de metabólitos primários e secundários, mediados pela alta concentração de fitormônios e metais que mantém e protege a relação inseto-planta e, possivelmente, aumenta as chances de interação com outros organismos, como os inimigos naturais. A tese está dividida em cinco capítulos, uma introdução geral, conclusões finais e perspectiva. No primeiro capítulo mostro as alterações na composição de polissacarídeos da parede celular nos tecidos de reserva e nutritivo das galhas induzidas por *Palaeomystella oligophaga* (Lepidoptera) em *Macairea radula* (Melastomataceae) nos diferentes estágios de desenvolvimento do inseto galhador. No segundo capítulo apresento a variação de monossacarídeos para testar a hipótese de que em galhas com diferentes cores e na presença de inimigos naturais, a composição monossacarídica é mais abundante e diversificada em comparação com os tecidos não galhados em *M. radula*. No terceiro capítulo apresento a relação das enzimas antioxidantes, metabólitos primários e secundários encontrados nos tecidos galhados na presença ou ausência de inimigos naturais comparados com tecidos não galhados e suas implicações ecológicas. No quarto capítulo mostro a relação das poliaminas, fitohormônios e acúmulo de metais com a coloração das galhas, verdes e vermelhas na dissipação do estresse oxidativo. Por fim, no quinto capítulo, apresento uma síntese dos temas da tese com uma abordagem de ciclo circadiano para explicar como as plantas podem estar em sincronia com os insetos galhadores na produção de metabólitos, em especial, os carboidratos. Nesta tese, constatou-se a alteração na composição da parede celular nos compartimentos da galha de acordo com o estágio de desenvolvimento do inseto galhador, especialmente em relação aos homogalacturonanos e xiloglucanos, assim como a maior quantidade de glucose e galactose nas galhas, os quais dão suporte ao alto metabolismo e à disponibilidade de recursos ao herbívoro galhador; assim como a presença de ácidos fenólicos, flavonoides e taninos hidrolizáveis, principalmente no estágio larval onde também detectou-se maior atividade das enzimas CAT e PAL. Outras moléculas, como as poliaminas foram mais abundantes nas galhas vermelhas do que nas verdes. E, os níveis dos fitormônios ABA, GA3, IAA e ACC foram maiores nas galhas verdes do que nas vermelhas, enquanto SA e MeJA foram mais abundantes nas galhas vermelhas. Esses resultados foram associados com o maior acúmulo de Al, Fe, Pb e Cu nas galhas vermelhas do que nas verdes, o que pode estar relacionado com a sua translocação e biossíntese de compostos fenólicos quelantes para reduzir a toxicidade desses metais, transformando-os em formas menos tóxicas para as galhas para manter a coloração vermelha como forma de proteção ao inseto galhador e seus inimigos naturais.

Palavras-chave: carboidratos, fenólicos, enzimas, fitormônios, metais, inimigos naturais.

2 Abstract

Plants are affected by biotic and abiotic stresses, which can influence their growth, development, productivity and geographic distribution. For survive adverse environmental conditions, developed adaptive strategies such as metabolites accumulation with protective functions, mainly against herbivore attacks. Many of these organisms, especially insects, induce biotic stress in plants by species-specific parasitism relationship, which can alter plant metabolism and, to induce anomalous growth with hyperplasia and cellular hypertrophy, leading to gall formation. These structures are controlled by reactive oxygen species release, enzymes, carbohydrates, secondary compounds, phytohormones; and can also accumulate metals in the host plants tissues. It is probable that molecules biosynthesis may change throughout the day, following a circadian cycle in response to biotic stress. In this context, this thesis has as its central hypothesis that the greater the oxidative stress in gall tissues, the greater the primary and secondary metabolites accumulation, mediated by the high phytohormone concentration and metals to maintain and protect the insect-plant relationship and, possibly, increase the chances of interaction with other organisms, such as natural enemies. The thesis is divided into five chapters, a general introduction, final conclusions and perspective. In the first chapter, I show the changes in the cell wall polysaccharides compositions in the reserve and nutritive tissues of galls induced by *Palaeomystella oligophaga* (Lepidoptera) in *Macairea radula* (Melastomataceae) in the different development galling insect stages. In the second chapter, I present monosaccharides variation to test the hypothesis that in galls with different colors and in the natural enemies presence, the monosaccharide composition is more abundant and diverse compared to non-galled tissues in *M. radula*. In the third chapter, I present the antioxidant enzymes, primary and secondary metabolites relationship found in galled tissues in the presence or absence of natural enemies compared to non-galled tissues and their ecological implications. In the fourth chapter, I show the relationship of polyamines, phytohormones and metals accumulation with the galls coloration in the oxidative stress dissipation. Finally, in the fifth chapter, I present a thesis summary topics with a circadian cycle approach to explain how plants can be in synchrony with galling insects in the metabolites production, especially carbohydrates. In this thesis, the cell wall composition changes in the gall compartments in according to the the galling insect development stage, especially in relation to homogalacturonans and xyloglucans, as well as the greater amount of glucose and galactose in the galls, which support the high metabolism and availability of resources to the galling herbivore; and the phenolic acids, flavonoids and hydrolyzable tannins presence, mainly in the larval stage wherein greater activity of the enzymes CAT and PAL was also detected. Other molecules, such as polyamines, were more abundant in red galls than in green galls. And the levels of the hormones ABA, GA3, IAA and ACC were higher in green galls than in red galls, while SA and MeJA were more abundant in red galls. These results were associated with the Al, Fe, Pb and Cu accumulation in red galls than in green galls, which may be related to their translocation and biosynthesis of phenolic chelating compounds to reduce the toxicity of these metals, transforming them into less toxic forms for the galls to maintain the red coloration as a form of protection against the galling insect and its natural enemies.

Keywords: carbohydrates, phenolic compounds, enzymes, phytohormones, metals, circadian cycle.

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Lista de siglas

- NT: Nutritive tissue
- ST: Storage tissue
- VB: Vascular bundle
- LM19: Unesterified homogalacturonans (HGs)
- LM20: Methyl esterified (HGs)
- LM5: (1→4)- α -D-galactans
- LM6: (1→3)- α -L-arabinans
- LM21: Heteromannans
- LM15: Xyloglucans
- LM11: (1→3)- α -D- xylans
- LM1: Extensins
- LM2: CArabinogalactan-proteins (AGPs)
- GC-MS: Gas Chromatography-Mass Spectrometry
- Rha: Rhamnose
- Fuc: Fucose
- Ara: Arabinose
- Xyl: Xylose
- Man: Mannose
- Glc: Glucose
- Gal: Galactose
- NGL: Non-galled leaves
- NGS: Non-galled stems
- GGL: Green gall with gall-inducer in larval stage
- GGP: Green gall with gall-inducer in pupal stage
- GGNE: Green gall with natural enemy inside
- EGG: Empty green gall
- RGP: Red gall with gall-inducer in pupal stage
- RGNE: Red gall with natural enemy
- ERG: Red gall with natural enemy (RGNE) and empty red gall
- PCA: Principal Component Analysis
- EDTA: Ethylenediaminetetraacetic acid
- PMSF: Phenylmethylsulfonyl fluoride
- CAT: Catalase
- APX: Ascorbate peroxidase

- POX: Peroxidase
- SOD: Superoxide dismutase
- PAL: Phenylalanine ammonia lyase
- ROS: Reactive oxygen species
- MDA: Malondialdehyde
- O_2 : Superoxide radicals
- OH: Hydroxyl radicals
- HO_2 : Perhydroxyl radicals
- H_2O_2 : Hydrogen peroxide
- NBT: p-nitro-tetrazolium blue
- HCl: Hydrogen chloride
- NaOH: Sodium hydroxide
- TLC: Thin Layer Chromatography
- PTFE: Polytetrafluoroethylene
- NP/PEG: Reagent mixture: Natural products - polyethylene glycol reagent
- HPLC-ESI: High performance liquid chromatography coupled to mass spectrometry tandem
- MS/MS: Tandem mass spectrometry
- ABA: Absciscic acid
- GA3: Gibberellic acid
- IAA: Indole-3-acetic acid
- ACC: 1-aminocyclopropane-1-carboxylate
- SA: Salicylic acid
- MeJA: Methyl jasmonate

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3 Introdução geral

As plantas são acometidas por uma variedade de estresses bióticos e abióticos, os quais podem afetar seu crescimento, desenvolvimento, produtividade e distribuição geográfica (ECKARDT *et al.*, 2023; McNICHOL & RUSSO, 2023). Para sobreviver às condições ambientais adversas, desenvolveram diversas estratégias adaptativas, entre as quais está o acúmulo de metabólitos que desempenham funções protetoras (KUSTER *et al.*, 2020; NICOLAS-ESPINOSA *et al.*, 2023; RAMAKRISHNA & RAVISHANKAR, 2011), principalmente contra o ataque de herbívoros. Muitos desses organismos, especialmente os insetos, induzem estresse biótico nas plantas estabelecendo uma relação de parasitismo espécie-específica (ISAIAS *et al.*, 2013; MANI, 1992; OLIVEIRA *et al.*, 2016; SHORTHOUSE *et al.*, 2005). Essa relação pode alterar o metabolismo vegetal conforme a disponibilidade dos sítios reativos, da sincronia do ciclo de vida da planta com o inseto durante as mudanças sazonais (OLIVEIRA *et al.*, 2016; PFEFFER *et al.*, 2018; YUKAWA, 2000) e até mesmo ao longo do dia (HIRANO *et al.*, 2023; VENKAT & MUNEER, 2022), e induzir o crescimento anômalo com hiperplasia e hipertrofia celular, levando a formação de órgãos neoformados, as galhas (HARRIS & PITZSCHKE, 2020; MANI, 1964; SHORTHOUSE *et al.*, 2005). Essas estruturas podem se estabelecer em diferentes órgãos da planta como em raízes, caules, botões florais e, em particular, nas folhas (ISAIAS *et al.*, 2014; MANI, 1964, 1992), criando um ambiente que fornece alimento ao galhador, abrigo e proteção contra inimigos naturais (PRICE *et al.*, 1987; STONE & SCHONROGGE, 2003).

Os estímulos para a indução e o desenvolvimento destes órgãos podem ser desencadeados por compostos salivares dos insetos, oviposição e ação de fitohormônios (RAMAN, 2011; SHORTHOUSE & ROHFRITSCH, 1992) que são coordenados por ação da expressão de genes (SCHULTZ *et al.*, 2019; SCHULTZ & STONE, 2022). Entretanto, a estrutura e o metabolismo da galha depende do táxon e, consequentemente, do comportamento do inseto galhador (OLIVEIRA *et al.*, 2016) e também, de como os tecidos da hospedeira e do órgão galhado respondem ao estresse oxidativo (ISAIAS *et al.*, 2015). Assim, as características estruturais e metabólicas das galhas têm sido a base para entender como ocorre a formação e diversificação deste novo órgão vegetal, principalmente no grupo dos insetos (JULIÃO *et al.*, 2014; MAIA & SILVA, 2020; SILVA *et al.*, 2023). E, para explicar o significado adaptativo desta neoformação, existem três hipóteses principais: a hipótese nutricional, do inimigo e do microambiente (STONE & SCHONROGGE, 2003). A hipótese nutricional defende que os tecidos das galhas oferecem recursos em qualidade e quantidade de forma mais eficientes para os insetos galhadores do que seus ancestrais de vida

livre (BRONNER, 1992), enquanto que a hipótese do microambiente diz que as galhas protegem o inseto de condições abióticas desfavoráveis, como excesso de radiação ultravioleta, temperatura, que são os principais problemas para o desenvolvimento larval (STONE & SCHONROGGE, 2003). E, de acordo com a hipótese do inimigo, a galha protege os insetos galhadores de seus predadores e parasitoides (PRICE *et al.*, 1987; STONE & SCHONROGGE, 2003).

Os herbívoros indutores de galhas estão inclusos, em sua maioria, dentro das famílias Cynipidae, Psyllidae, Eriophyidae, Apionidae, Gelechioidea e, principalmente, da Cecidomyiidae (RAMAN, 2012). Esses insetos possuem hábitos alimentares distintos, e se agrupam em guildas que vão desde mastigadores, raspadores até sugadores (OLIVEIRA *et al.*, 2016). Essas diferentes atividades alimentares podem desencadear crescimento diferencial nos tecidos da hospedeira (ISAIAS *et al.*, 2011; JORGE *et al.*, 2018; OLIVEIRA & ISAIAS, 2010; OLIVEIRA *et al.*, 2016) relacionadas diretamente ao estabelecimento de um estresse biótico (ISAIAS *et al.*, 2015), o qual é qualificado e quantificado por meio da presença de espécies reativas de oxigênio (EROs), geradas no tecido vegetal em resposta a indução e estabelecimento da galha (ISAIAS & OLIVEIRA, 2012; OLIVEIRA & ISAIAS, 2010; OLIVEIRA *et al.*, 2010). As EROs são decorrentes da redução do oxigênio molecular (O_2), formando substâncias que não necessitam da entrada de energia para reação, tais como superóxido (O_2^-), peróxido de hidrogênio (H_2O_2), radical hidroxila (OH) e oxigênio simples ($^1O_2$) (RESENDE *et al.*, 2003). Estas moléculas desencadeiam estresse oxidativo no vegetal e podem danificar a parede celular, os lipídios membranares, e até o material genético (DEMIDCHIK, 2015; MERIGA *et al.*, 2004). No entanto, podem atuar como segundos mensageiros em muitos processos celulares, como na transdiferenciação e hipertrofia celular durante o crescimento e desenvolvimento dos órgãos neoformados (ISAIAS *et al.*, 2015; SUGIMOTO *et al.*, 2011; YAN *et al.*, 2007).

Durante o desenvolvimento dos tecidos galhados, existem padrões distintos de crescimento e alongamento celular (MAGALHÃES *et al.*, 2014). Estes padrões podem levar à formação de um compartimento externo de reserva e um interno, o tecido nutritivo, responsável direto pela nutrição do galhador (BRAGANÇA *et al.*, 2020; BRAGANCA, 2017; FERREIRA *et al.*, 2019). Em geral, as células do tecido nutritivo são menores e menos alongadas, com citoplasma denso em comparação com as células do tecido de reserva, que são compostas por células hipertrofiadas e vacuoladas (BRAGANÇA *et al.*, 2020; FERREIRA *et al.*, 2019, 2017, 2020; ISAIAS *et al.*, 2018; OLIVEIRA *et al.*, 2010). Essas diferenças notáveis devem estar associadas à parede celular durante o desenvolvimento da galha (MAGALHÃES *et al.*, 2014). As mudanças na composição da parede celular das galhas têm sido estudadas para entender o rearranjo de microfibrilas e o

grau de metilesterificação das pectinas em diferentes sistemas de plantas hospedeiras e insetos indutores de galhas (MAGALHÃES *et al.*, 2014; MARTINI *et al.*, 2019; OLIVEIRA *et al.*, 2014; SILVA *et al.*, 2021). Além disso, esses polissacarídeos podem sofrer hidrólises, e liberar oligo e monossacarídeos como Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xilose (Xyl), Manose (Man), Glicose (Glc) e Galactose (Gal). Esses carboidratos são componentes essenciais das paredes celulares e, podem regular a atividade fotossintética nas galhas, assim como induzir a biossíntese de compostos fenólicos, levando ao fenômeno avermelhado nessas estruturas (CONNOR *et al.*, 2012). As galhas vermelhas em *Macairea radula* podem acumular mais açúcares solúveis em água do que as verdes (NAVES *et al.*, 2021). Para isso, o inseto galhador estimula continuamente o metabolismo da planta hospedeira para produzir metabólitos primários, como proteínas, lipídios e carboidratos, disponíveis não apenas para a dieta do inseto (FERREIRA *et al.*, 2019), mas também para a biossíntese de compostos fenólicos e poliaminas, por exemplo; os quais são regulados por fitormônios.

Todas essas alterações metabólicas que se iniciam na parede celular estão diretamente relacionadas aos mecanismos de controle do estresse oxidativo nos sistemas galhador-planta hospedeira. Nas galhas de *Aspidosperma australe* e *A. spruceanum*, por exemplo, a acumulação de EROs é encontrada desde as células iniciais da galha até a senescência (OLIVEIRA *et al.*, 2010). De fato, o sucesso e estabelecimento da galha depende do controle da produção e eliminação dos radicais livres por diferentes vias, enzimáticas e não-enzimáticas (ISAIAS *et al.*, 2015). Uma das formas de dissipação do estresse oxidativo em plantas é através da produção de compostos fenólicos que podem doar um átomo de hidrogênio para neutralizar a ação dos EROs e evitar danos celulares (DUMANOVIC *et al.*, 2021; IRATO & SANTOVITO, 2021; LEE *et al.*, 2007; SACHDEV *et al.*, 2021). Além disso, são capazes de sequestrar metais, assim como ativar as enzimas antioxidantes como superóxido dismutase, catalase, ascorbato peroxidases e peroxidases (ANJUM *et al.*, 2016), as quais atuam sinergicamente com fitohormônios induzidos por herbívoros galhadores e, possivelmente, afetando a sobrevivência de espécies vegetais vizinhas (BARONIO & OLIVEIRA, 2019; SERKEDJIEVA, 2011).

A manipulação da maquinaria celular pelos insetos galhadores envolve não somente os sistemas enzimáticos e não-enzimáticos, mas principalmente, a mediação e regulação pelos fitormônios. Estes reguladores desempenham um papel crucial na indução, desenvolvimento e estabelecimento de galhas de insetos, atuando como mensageiros químicos primários (BODY, 2019). Estes mensageiros podem estimular tanto a produção de metabólitos primários, responsáveis pela manutenção da maquinaria celular e nutrição dos insetos galhadores, quanto a

de compostos secundários, que teriam papel relevante na manutenção da homeostase, reações redox, defesa da planta e interações com outros organismos (BARONIO & OLIVEIRA, 2019; FORMIGA *et al.*, 2009; ISAIAS *et al.*, 2015). Assim, todo o desenvolvimento da galha e interações com outros organismos pode ser governado pela ação fitohormônios, principalmente auxinas e citocininas. A ação da auxina (IAA - ácido indol-3-acético) tem sido relacionada como o alongamento, enquanto que a citocinina, com a divisão celular (SOSNOWSKI *et al.*, 2023). Outros fitormônios como o ácido abscísico, as giberelinas, os precursores do etileno (ACC), o ácido salicílico e o metil jasmonato, assim como as poliaminas, embora participem das respostas aos estresses abióticos e bióticos; ainda pouco se conhece sobre suas funções na interação entre insetos indutores de galhas e plantas hospedeiras. É conhecido que essas moléculas estão associadas ao acúmulo de metais e compostos fenólicos (CHEN *et al.*, 2018; TYAGI *et al.*, 2023).

Para estudar as relações metabólicas da interação parasítica inseto-planta, usamos como sistema modelo as galhas globoides induzidas por *Palaeomystella oligophaga* Becker & Adamski (Lepidoptera) em *Macaírea radula* (Bonpl.) DC. (Melastomataceae). Essas galhas são iniciadas nos ramos e se desenvolvem como uma estrutura ramificada no caule com muitas projeções semelhantes às folhas, com emergências e tricomas (REZENDE *et al.*, 2023). Esses órgãos apresentam um tecido nutritivo típico que acumula lipídios e proteínas (REZENDE *et al.*, 2019), e um tecido de armazenamento com acúmulo polissacarídeos, malondialdeído e compostos fenólicos (NAVES *et al.*, 2021). As paredes celulares dos tecidos de armazenamento e nutritivos mostram distribuição diferencial de pectinas e epítomos de hemiceluloses, evidenciando funcionalidades distintas do tecido (SANTOS *et al.*, 2024). Assim, o inseto galhador ao longo dos diferentes estágios de vida, modifica o órgão galhado que assume diferentes cores variando do verde ao vermelho (CARDOSO *et al.*, 2022). Parte dessa coloração pode ser explicada pelo acúmulo de metabólitos, tanto primários quanto secundários. Acreditamos que diversos monossacarídeos são liberados dentro da galha para a síntese de outras moléculas do metabolismo secundário para dissipar o estresse oxidativo. **Nesse sentido, esta tese tem como hipótese central que, quanto maior o estresse oxidativo nos tecidos das galhas maior será o acúmulo de metabólitos primários e secundários, mediados pela alta concentração de fitormônios e acúmulo de metais para mantêm e protege a interação inseto-planta, e possivelmente aumenta as chances de interação com outros organismos, como os inimigos naturais. Portanto, com base na contextualização acima, apresento a tese que está dividida em cinco capítulos.**

- No primeiro capítulo mostro as alterações na composição de polissacarídeos da parede celular nos tecidos de reserva (externo) e nutritivo (interno) das galhas induzidas por *Palaeomystella oligophaga* (Lepidoptera) em *Macairea radula* nos diferentes estágios de desenvolvimento do inseto galhador. Como hipótese acreditamos que, com alto estresse respiratório e biótico nos tecidos nutritivos, pectinas metilesterificadas e também hemiceluloses estejam amplamente distribuídas nas paredes celulares com inseto galhador na fase larval. E, no tecido de armazenamento, uma grande distribuição de pectinas não esterificadas devido ao grau de maturação e rigidez deste tecido.
- No segundo capítulo apresento a quantificação dos monossacarídeos para testar a hipótese de que em galhas com diferentes cores e na presença de inimigos naturais, a composição monossacarídica é mais abundante e diversificada em comparação com os tecidos não-galhados em *M. radula* (Melastomataceae).
- No terceiro capítulo apresento a relação das enzimas antioxidantes, metabólitos primários e secundários encontrados nos tecidos galhados na presença ou ausência de inimigos naturais comparados com tecidos não-galhados e suas implicações ecológicas para o sistema de estudo - *P. oligophaga* (Lepidoptera) em *M. radula* (Melastomataceae).
- No quarto capítulo mostro a relação das poliaminas (PAs), fitohormônios e metais com a coloração das galhas verdes e vermelhas. Esperávamos que as galhas vermelhas contivessem mais PAs, fitohormônios e acúmulo de metais associados à dissipação de estresse para manter e proteger as galhas em *M. radula* (Melastomataceae).
- Por fim, no quinto capítulo, apresento uma síntese dos temas da tese com uma abordagem de ciclo circadiano para explicar como as plantas podem estar em sincronia com os insetos galhadores na produção de metabólitos, em especial, os carboidratos que são biossintetizados, principalmente, pela manhã. Enquanto que as moléculas do metabolismo secundário, como os compostos fenólicos podem ser produzidas ou estarem disponíveis somente em horários sob alta incidência solar por volta do meio-dia. Talvez, a manutenção dos insetos galhadores se deva pela quantidade e qualidade de moléculas produzidas em horários distintos ao longo do dia.

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5 Chapter I - Cell wall epitope distribution in the functional compartments of galls induced by *Palaeomystella oligophaga* (Lepidoptera) in *Macairea radula* (Melastomataceae)

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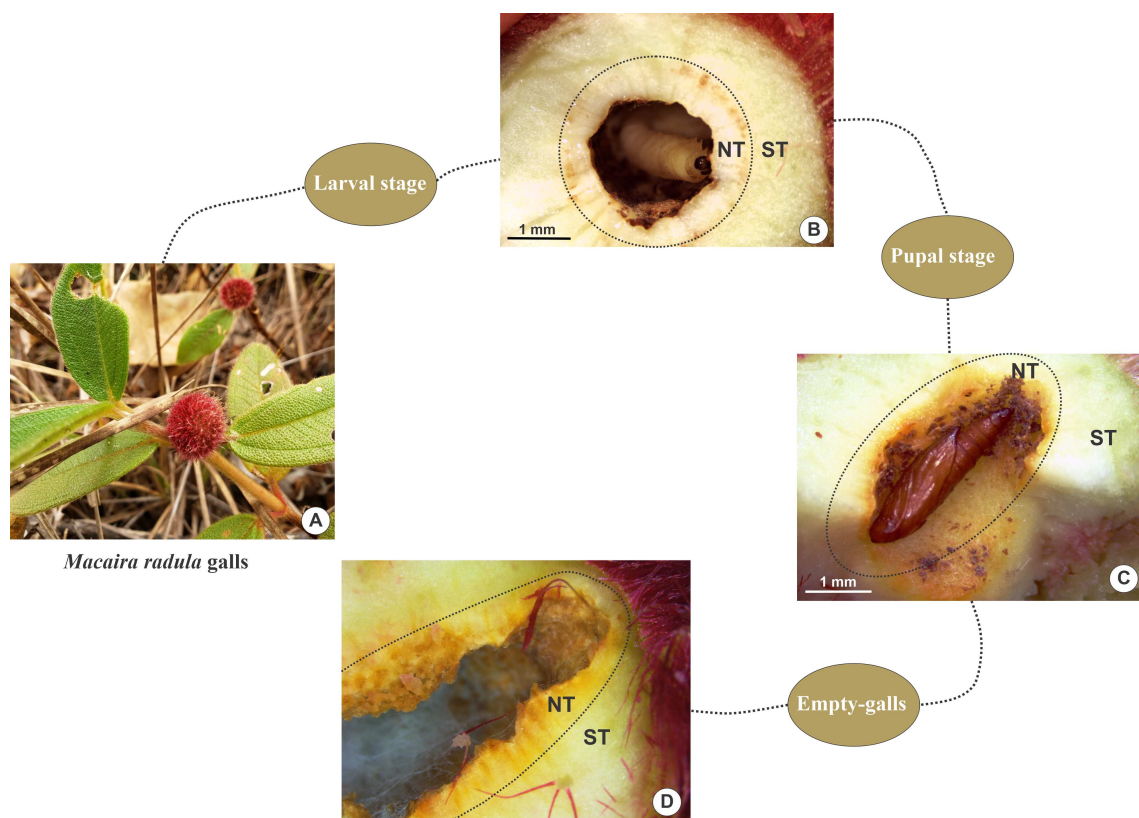
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Context

The parasitic interaction between the galling insect *Palaeomystella oligophaga* (Lepidoptera) and the host plant tissues of *Macairea radula* (Melastomataceae) leads to the formation of globoid galls. These galls have storage and typical nutritive tissues (outer and inner compartments, respectively), whose functions may be related to differential cell wall component distributions and to the stage of development of the gall inducer.

Cell wall epitope distribution in the functional compartments of galls induced by *Palaeomystella oligophaga* (Lepidoptera) in *Macairea radula* (Melastomataceae)

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Handling Editor:

James Camac

Received: 9 August 2023

Accepted: 1 December 2023

Published: 2 January 2024

Cite this:

Santos PD et al. (2024)
Australian Journal of Botany **72**, BT23064.
doi:10.1071/BT23064

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ABSTRACT

Context. The parasitic interaction between the galling insect *Palaeomystella oligophaga* (Lepidoptera) and the host plant tissues of *Macairea radula* (Melastomataceae) leads to the formation of globoid galls. These galls have storage and typical nutritive tissues (outer and inner compartments, respectively), whose functions may be related to differential cell wall component distributions and to the stage of development of the gall inducer. **Aims.** We evaluated whether the cell wall composition of the gall compartments (storage and nutritive tissues) changes according to the developmental stage of the gall inducer *P. oligophaga*. **Methods.** The galls occupied by the gall inducer in different stages of development (i.e. larval and pupal stages, as well as empty galls) were collected and submitted to histological examination, immunocytochemical analyses using monoclonal antibodies for pectins, hemicelluloses, and glycoproteins, and histochemical analyses using phloroglucinol for lignins. **Key results.** The histological results showed that nutritive tissues have smaller cells compared with storage ones when the galling insect is in the larval stage. Immunocytochemical analyses also showed the occurrence of both methyl-esterified and unesterified pectins, as well hemicelluloses in the nutritive tissue during the larval stage. The dynamic of some cell wall components changes when the gall inducer is in the pupal stage, or the galls are empty. **Conclusions.** The changes in the composition of the cell walls, especially regarding homogalacturonans and xyloglucans, support the high metabolism and resource availability in cell walls of nutritive tissue for the gall inducer. **Implications.** The cell wall composition may indicate important metabolic steps during gall formation and galling insect diet.

Keywords: gall-inducing insects, glycoproteins, hemicelluloses, immunocytochemistry, pectins, plant anatomy, polysaccharides, xyloglucans.

Introduction

Galls are new plant organs developed by specific interaction between the galling organisms and the host plant tissues (Mani 1992; Shorthouse et al. 2005). The development of these structures occurs by the reprogramming of plant cell growth induced by a phenotype manipulator, which impacts the host plant in different ecological ways (Oliveira et al. 2016; Harris and Pitzschke 2020). Insects are the main group of these phenotype manipulators that change the host plant's histological profile, leading to the formation of complex structures, i.e. the galls (Ferreira et al. 2017, 2019). During gall development, there are distinct patterns of cell growth and elongation that can lead to the formation of storage (outer) and nutritive (inner) tissue compartments (Bragança et al. 2017, 2020; Ferreira et al. 2017, 2019).

In general, the nutritive tissue cells are smaller and less elongated, and with dense cytoplasm compared to cells of storage tissue, which are composed of hypertrophied and vacuolated cells (Oliveira et al. 2010; Ferreira et al. 2017, 2019, 2020; Isaias et al. 2018; Bragança et al. 2020). These remarkable differences should be associated with cell wall

composition during gall development. Changes in the cell wall composition of galls have been studied in order to understand microfibril rearrangement and the degree of methyl-esterification of pectins in different host plant–galling insect systems, especially non-nutritive galls (de Oliveira *et al.* 2014; Magalhães *et al.* 2014; Martini *et al.* 2019; de Melo Silva *et al.* 2021). These studies have discussed, for example, the possible role of pectins, hemicelluloses, and cellulose microfibril arrangement during the main processes of gall formation, such as cell hypertrophy and tissue hyperplasia. However, the dynamics of the cell walls during the development of nutritive and complex bud gall, based on the galling insect stage of development, are poorly explored. In fact, the development of nutritive tissue in galls represent an adaptive significance to a wide range of insect taxa (Stone and Schönrogge 2003), and the cell wall composition and dynamics may have an important significance.

During the development of gall tissues the composition and structure of cell walls are continuously changed (de Oliveira *et al.* 2014; Magalhães *et al.* 2014; Costa *et al.* 2021) since the cell wall is not a static structure, but rather a dynamic one (Vaahtera *et al.* 2019; Du *et al.* 2022). Thus, the development of this new organ seems to involve a series of biosynthesis and degradation steps of cell wall components such as cellulose, hemicelluloses, pectins, proteins, and lipids (Motta *et al.* 2005; Oliveira *et al.* 2010). These components perform different functions such as mechanical strength, cell expansion, protection against the attack of microorganisms, storage, and as a source of signalling molecules (Knox 1992; Cassab 1998; Lamport *et al.* 2006; Xiao and Anderson 2013; Bellincampi *et al.* 2014). Pectins are non-cellulosic polysaccharides rich in galacturonic acid (GaLA) that can be assigned as homogalacturonan (HG), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), xylogalacturonan (XGA), and apiogalacturonan (AP) groups (Ridley *et al.* 2001; Willats *et al.* 2001a; Caffall and Mohnen 2009; Wolf *et al.* 2009; Albersheim *et al.* 2011). Homogalacturonans (HGs) are linear homopolymers containing galacturonic acid residues and each of their carboxyl groups can be methyl-esterified or not. Methyl esterification occurs when pectin methylesterases (PMEs) remove the methyl group under different stress conditions, with a change in HG functions such as elasticity and rigidity (Wolf and Greiner 2012). In fact, PME activities seem to be impaired by the increase in oxidative stress (Wu *et al.* 2018; Dabravolski and Isayenkov 2023) and the biotic stress induced by the gall inducer. Its impact on PME activities can be determined by detecting the variation in HG methyl-esterification (de Oliveira *et al.* 2014). In addition, other polysaccharides can occur, such as rhamnogalacturonans (RGI) composed of subunits of (1→2)- α -L rhamnosyl-(1→2)- α -D-galacturonyl disaccharides with variable side chains of arabinans and galactans (Wydra and Beri 2006; Atmodjo *et al.* 2013), showing different distributions and functions in galls (Silva *et al.* 2019). Another component of plant cell walls is hemicelluloses, a group of polysaccharides

(xyloglucans, xylans, and heteromannans), whose functions involve both carbohydrate reserves and cell expansion (Thompson 2005; Scheller and Ulvskov 2010; Albersheim *et al.* 2011). In addition, hemicelluloses have been detected on the cell wall of nutritive tissue of galls (inner compartment) and are discussed as an important resource available for galling insect diet (Bragança *et al.* 2020; Costa *et al.* 2021).

Palaeomystella oligophaga (Lepidoptera) stimulates host plant metabolism and reallocates resources for gall development, which provide protection and food resources to galling insect (Rezende *et al.* 2019, 2021). Within this structure, there are two tissue compartments, an inner compartment (= nutritive tissue) that accumulates proteins and lipids, adjacent to the larval chamber, and an outer compartment (= storage tissue) that mainly contains structural carbohydrates as a reserve, both of them depending on the stimulus of the galling insect in order to grow and develop (Rezende *et al.* 2019; Naves *et al.* 2021). Between these compartments, there are some cell layers with thick cell walls, probably with deposition of lignin (Rezende *et al.* 2019). Gall-inducing Lepidoptera feed voraciously during the larval stage, consequently contributing to the maintenance of the processes of hyperplasia and hypertrophy of their galls (Ferreira *et al.* 2019). The nutritive tissue is conspicuous during the larval stage and shows metabolic changes as the insect develops to the pupal stage, with degradation of lipids and proteins, as well as tissue disruption (Rezende *et al.* 2019). Thus, due to the high respiratory and biotic stress on nutritive tissues, we expect methyl-esterified pectins, and also hemicelluloses, to be widely distributed through these tissues when the galling insects are in the larval stage. In the storage tissue, we expect a large distribution of unesterified pectins to support tissue rigidity. When the larva of the *P. oligophaga* reaches the pupal stage or is killed by a natural enemy, the stimuli for gall development stop and nutritive tissue is completely degraded (Rezende *et al.* 2019). When the gall-inducer stimuli stop, this initiates the process of gall senescence, which can be associated with the formation of different carbohydrates in order to maintain the functional dynamics between the stiffness and porosity of the cell wall in the gall compartments (de Oliveira *et al.* 2014). To test these hypotheses, we asked the following question: how does the *P. oligophaga* life stage affect the functional dynamics of the cell wall in the two gall compartments?

Materials and methods

Histological and histochemical analyses

The galls induced by *Palaeomystella oligophaga* Becker and Adamski (Lepidoptera) (Becker and Adamski 2008) on *Macairea radula* Bonpl. (Melastomataceae) were collected in the Cerrado at Estação Ecológica do Panga (19°10'S, 48°24'W), Uberlândia municipality, Minas Gerais State, Brazil. For the histological analyses, the galls were divided into three life stages ($n = 10$

for each stage) (Fig. 1): galls with the inducer in the larval stage (GL), and galls with the inducer in the pupal stage (GP), as well as empty galls (EG) when the adult comes out. The samples were fixed in Karnovsky solution (8% paraformaldehyde and 25% glutaraldehyde in 0.2 M phosphate buffer, pH 7.3) (Karnovsky 1965), dehydrated in a graded ethanol series (70%, 80%, 85%, and 95%), and embedded in 2-hydroxyethyl methacrylate Histo-resin Leica® (Leica Instruments, Heidelberg, Germany). Transverse and longitudinal sections (5 µm) were obtained with a rotary microtome (YD315, ANCAP, Brazil). A set of sections were stained with 0.05% toluidine blue, pH 4.7 (O'Brien and McCully 1981). The histochemical tests for lignin were carried out in fresh samples hand cut in an acidified solution using 2% phloroglucinol (Johansen 1940). The slides for histological analysis were mounted with Entellan®, those for the histochemical test were mounted with water and both preparations were analysed with a Leica® DM50 light microscope coupled with an HD camera (DFC3000G).

Immunocytochemical analyses

A second set of gall sections with the three developmental stages of *P. oligophaga* ($n = 10$ for each stage) were submitted to immunocytochemical analysis. The samples were embedded in Histo-resin and sectioned with a rotary microtome. The samples selected for the labelling of

hemicellulose epitopes were immersed in 2 mM CaCl_2 buffer containing $10 \mu\text{g/mL}^{-1}$ pectate lyase (Sigma-Aldrich) and 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (Sigma-Aldrich), pH 10, for 2 h. After this step, all samples were incubated in blocking solution (3% powdered milk solution, Molico® in PBS) for 30 min to prevent crosslinking, followed by incubation with primary monoclonal antibodies (Table 1 – LM20, LM19, LM5, LM6, LM15, LM21, LM11, LM1, and LM2) at 1:5 dilution in PBS for 1 h and a half in the dark. The samples were washed three times with PBS for 5 min each time and incubated with the secondary anti-rat IgG FITC antibody (Sigma-Aldrich) in blocking solution (1:100) for 2 h in the dark. Samples without primary antibodies were used as a control group. The samples were washed in PBS, mounted in glycerin/distilled water (1:1 v/v), and examined with a Leica® DM4000 B fluorescence microscope coupled with a Leica® DFC3000 G camera for qualitative analyses.

Results

Histological features of galls

Galls on *M. radula* occupied by the galling insect *P. oligophaga* in the larval stage had a typical nutritive tissue (inner compartment) adjacent to the larval chamber, with smaller cells compared to the storage tissue (outer tissue compartment)

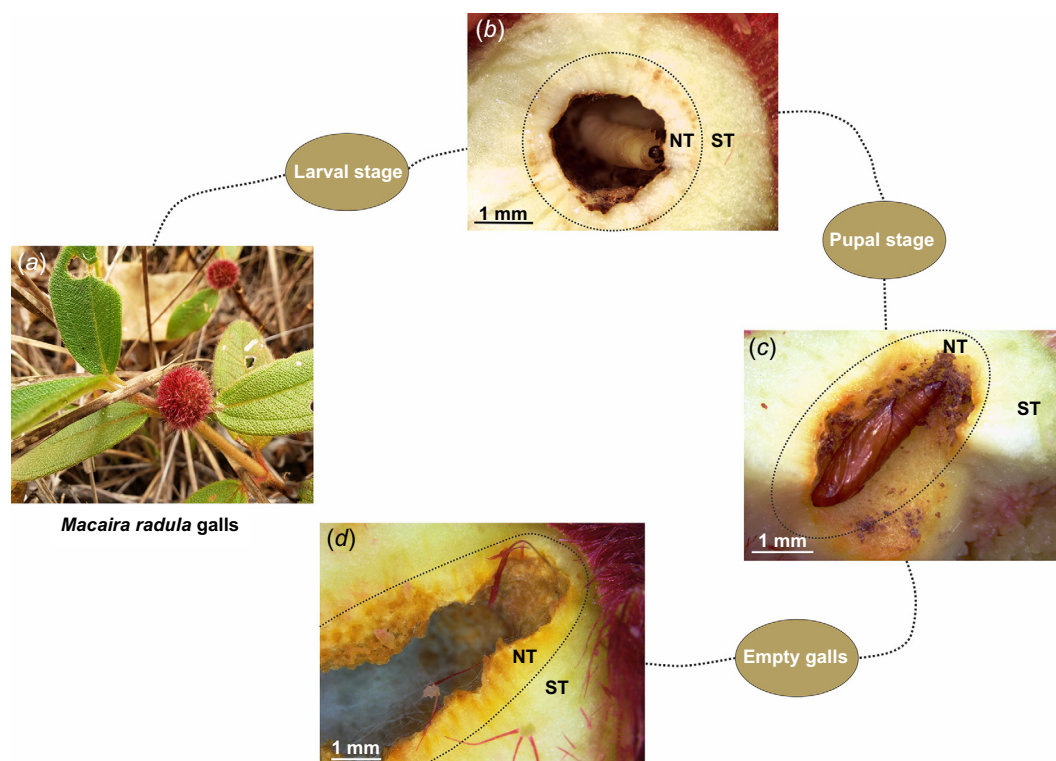


Fig. 1. Morphological traits of galls induced by *Palaeomystella oligophaga* in *Macairea radula*. (a) Globoid galls induced in the axillary buds; (b) galls with *P. oligophaga* in the larval stage; (c) galls with *P. oligophaga* in the pupal stage; (d) empty galls. NT, nutritive tissue; ST, storage tissue.

Table 1. Monoclonal antibodies and their epitopes used for labelling hemicellulose, protein, and pectin in cell walls of *Macairea radula* galls.

Monoclonal antibodies	Epitopes	References
LM19	Unesterified homogalacturonans (HGs)	Verhertbruggen et al. (2009)
LM20	Methyl esterified HGs	Verhertbruggen et al. (2009)
LM5	(1 → 4) β-D-galactans	Jones et al. (1997), Orfila and Knox (2000)
LM6	(1 → 5) -α-L-arabinans	Willats et al. (1998), Orfila and Knox (2000), Orfila et al. (2001), Verhertbruggen et al. (2009)
LM21	Heteromannans	Marcus et al. (2010)
LM15	Xyloglucans	Marcus et al. (2008)
LM11	(1 → 4)-β-D- xylans/ arabinoxylans	McCartney et al. (2005), Ruprecht et al. (2017)
LM1	Extensins	Smallwood et al. (1996)
LM2	Arabinogalactan-proteins (AGPs)	Smallwood et al. (1996), Yates et al. (1996)

(Fig. 2a, b). Both compartments were composed of parenchyma with thin and regularly thickened primary cell walls (Fig. 2b). A middle tissue layer, sclerenchyma, with thick and secondary cell walls, was observed between the inner and outer gall compartments; and vascular bundles were detected in both of these compartments (Fig. 2a, b). These tissues changed according to the life stage of the gall inducer, with the

number of lignified cell layers increasing in the sclerenchyma, when the galling insects were in the pupal stage (Fig. 2c, d). The inner compartment seemed to be degraded or partially consumed by the galling insect before the pupal stage (Fig. 2c), being more degraded when the gall was empty (Fig. 2e).

Distribution of epitopes on the cell walls of galls occupied by a galling insect in different stages of development

Larval stage

Unesterified HG epitopes recognised by LM19 were detected in the cell walls of the nutritive tissue near the larval chamber (Figs 3 and 4a), with no labelling in the cells of storage tissue. On the other hand, methyl-esterified HG epitopes recognised by LM20 were detected in cell walls of both nutritive and storage tissues, as well as in cells of the xylem and phloem of galls (Figs 3 and 4b). The epitopes of (1→4)-β-D-galactans and (1→5)-α-L-arabinans, respectively recognised by LM5 and LM6, were labelled in the cells of nutritive tissue (Figs 3 and 4c, d), storage tissue, and xylem and phloem (Fig. 3). Epitopes of heteromannans, recognised by LM21, and epitopes of (1 → 4)-β-D-xylans/ arabinoxylans, recognised by LM11, were detected only in the nutritive cell walls (Figs 3 and 4e, f). However, epitopes of xyloglucans, recognised by LM15, were detected in both tissue compartments (Figs 3 and 4g). Epitopes of extensins, recognised by LM1, and of arabinogalactan-protein glycans (AGPs), recognised by LM2, were detected only in the cell walls of the nutritive tissue (Figs 3 and 4h, i).

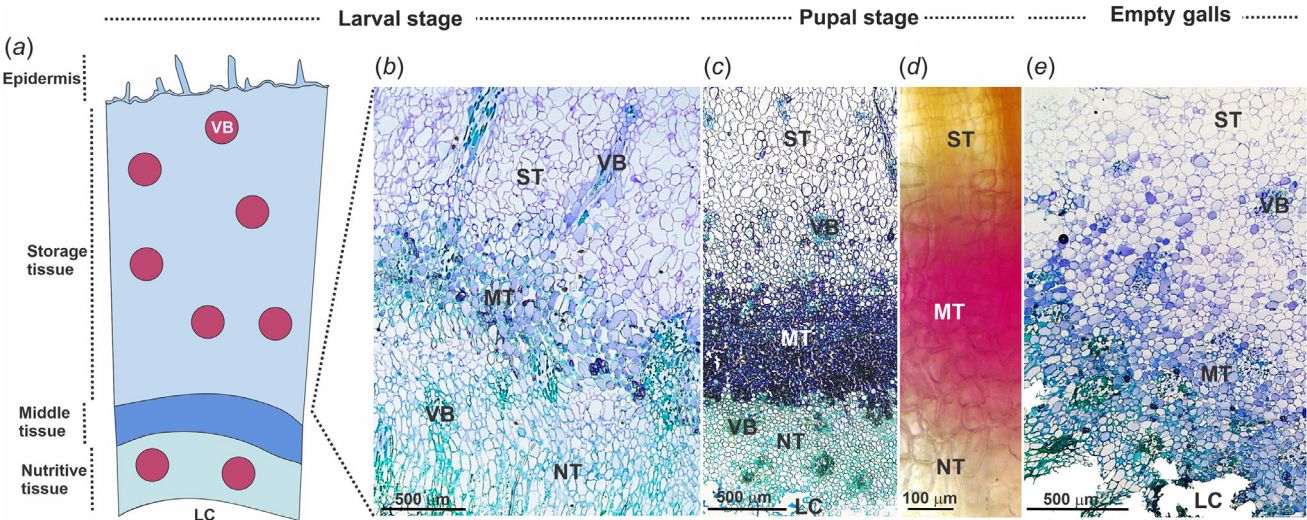


Fig. 2. Anatomical traits of galls induced by *Palaeomystella oligophaga* in *Macairea radula*. (a) Schematic illustration of galls in larval stage; (b) storage, middle and nutritive tissues of galls occupied by *P. oligophaga* in the larval stage; (c) storage, middle and nutritive tissues of galls occupied by *P. oligophaga* in the pupal stage; (d) positive result for lignin by the phloroglucinol test in the cell walls of the middle tissues of galls occupied by *P. oligophaga* in the pupal stage; (e) nutritive tissue consumed in an empty gall. NT, nutritive tissue; ST, storage tissue; MT, middle tissue; LC, larval chamber; VB, vascular bundle.

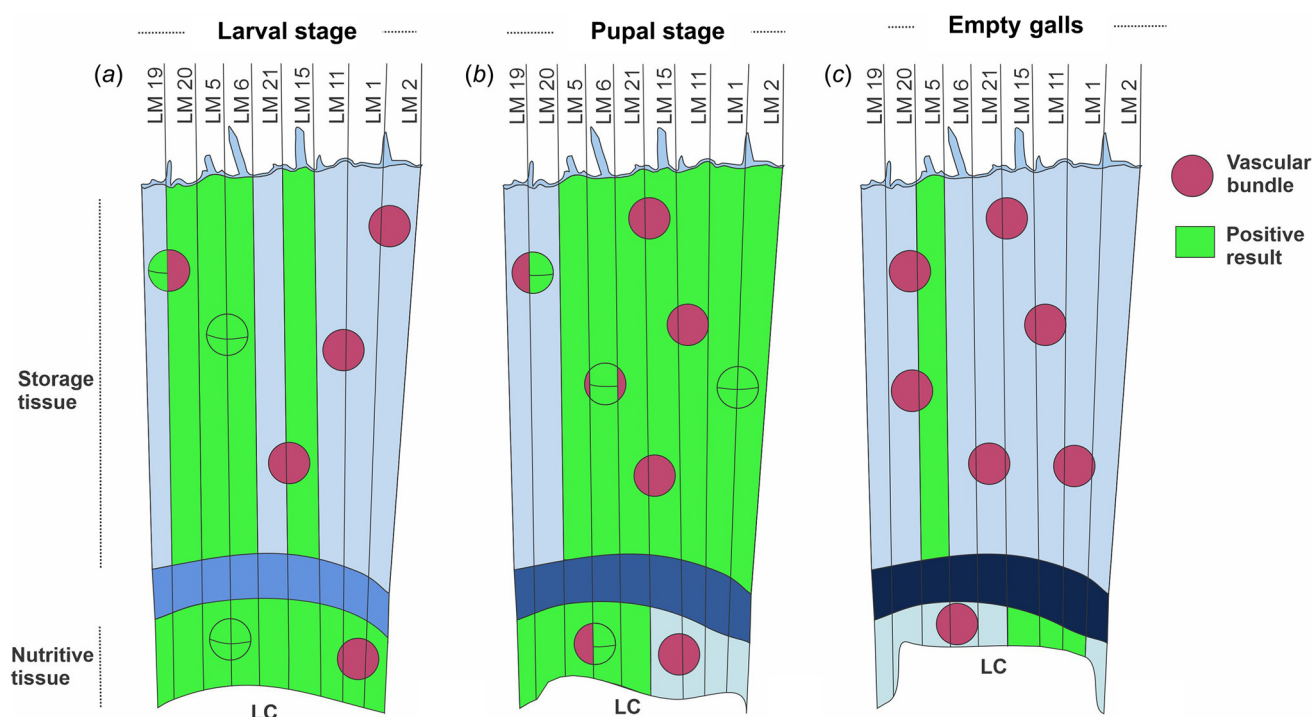


Fig. 3. Distribution of epitopes of pectins (LM19, LM20, LM5 and LM6), hemicelluloses (LM11, LM21 and LM15) and glycoproteins (LM1 and LM2) in the cell walls of nutritive tissue, storage tissue and vascular bundles of galls induced by *Palaeomystella oligophaga* on *Macairea radula*. (a) Galls with *P. oligophaga* in the larval stage; (b) galls with *P. oligophaga* in the pupal stage; (c) empty galls. Positive results are indicated by fluorescent green colour.

Pupal stage

Epitopes recognised by LM19 and LM20 were detected in the cell walls of nutritive tissues (Figs 3 and 5a, b) and only epitopes recognised by LM20 were detected in the xylem and phloem (Figs 3 and 5b). The cell walls of storage tissue were not labelled (Fig. 3). The epitopes for (1→4)-β-D-galactans and (1→5)-α-L-arabinans, respectively recognised by LM5 and LM6, were labelled in both tissue compartments (Figs 3 and 5c–e). The vascular bundles (xylem and phloem) were labelled only by the LM5 antibody (Figs 3 and 5c). Epitopes of heteromannans, recognised by LM21, were detected in the cell walls of both nutritive and storage tissues (Figs 3 and 5f), as also were the epitopes of xyloglucans, recognised by LM15 (Figs 3 and 5g). There was no labelling by the LM15 antibody in the vascular bundles (Fig. 3). Epitopes of arabinoxylans, recognised by LM11, were detected in storage tissue (Figs 3 and 5h). Epitopes of extensins, recognised by LM1, and of arabinogalactan-protein glycans, recognised by LM2, were detected in the cell walls of storage tissue (Figs 3 and 5i).

Empty galls

Epitopes recognised by LM20 and LM19 were not detected in the cell walls of nutritive and storage tissues or of the vascular bundles (Figs 3 and 6a–d). The epitopes of (1→4)-β-D-galactans, recognised by LM5, were labelled only in storage tissue (Figs 3 and 6a). Epitopes recognised by LM15

and LM11 were detected in the cell walls of nutritive tissues (Figs 3 and 6b, c), as also were epitopes of extensins recognised by LM1 (Figs 3 and 6d). There was no labelling for epitopes recognised by LM2.

Discussion

Anatomical and immunocytochemical analyses reveal that the cells of the outer and inner tissue compartments of the galls gradually lose their integrity after galling herbivore pupation. This loss of integrity is also evident at the level of cell wall components and is associated with the life stage of the gall inducer. The nutritive and storage tissue compartments of *P. oligophaga* galls have cells with different sizes and functions (Rezende *et al.* 2019), with different cell wall dynamics and composition. The developmental stage of the gall inducer seems to stimulate the cells of each tissue compartment in a different manner, consequently impacting the composition of unesterified and esterified HGs in the cell walls, as well as hemicellulose, galactan, and arabinan chains.

Galls occupied by the galling insect in the larval and pupal stages

HGs with different degrees of methyl-esterification were present in the *Macairea radula* galls occupied by larvae and

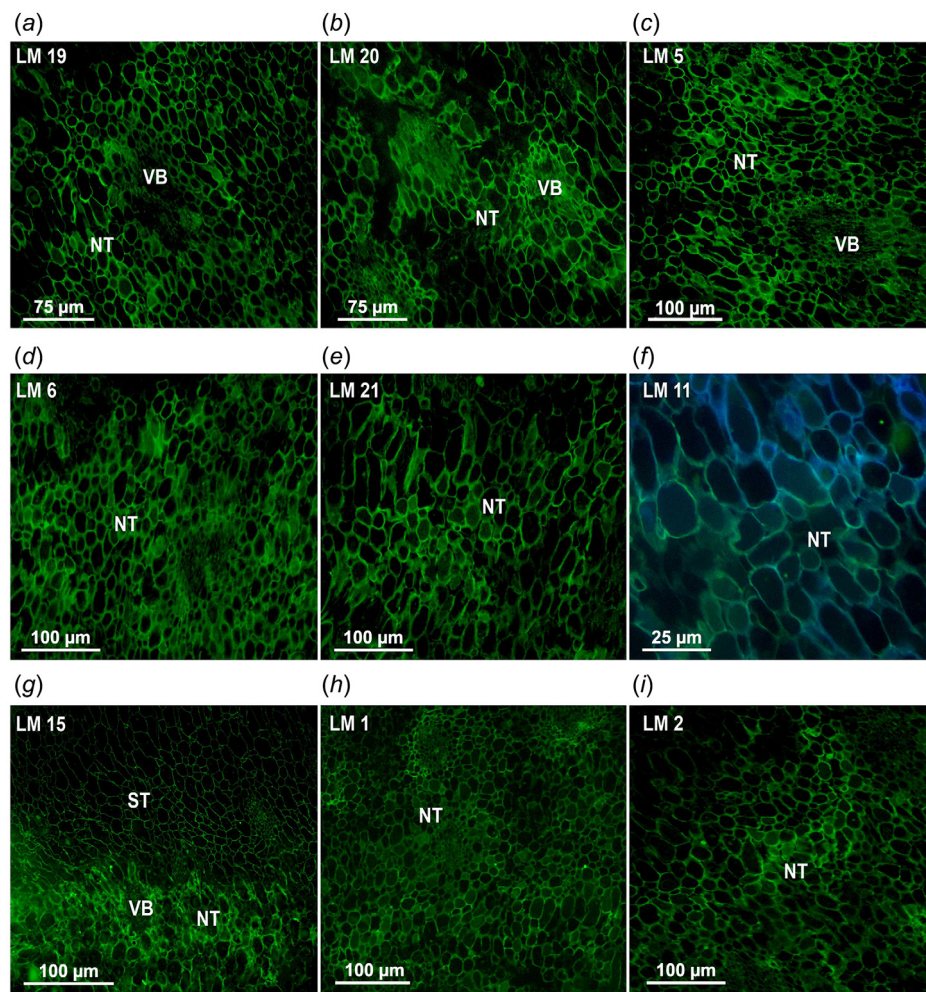


Fig. 4. Distribution of hemicellulose (LM11, LM21 and LM15), pectin (LM19, LM20, LM5 and LM6) and protein (LM1 and LM2) epitopes in the cell walls of galls on *Macairea radula* with *Palaeomystella oligophaga* in the larval stage. (a) Epitopes of unesterified homogalacturonans (HGs) labelled by LM19 in the cells of nutritive tissue; (b) epitopes of methyl-esterified HGs labelled by LM20 in the cells of nutritive tissue; (c) epitopes of (1→4)-β-D-galactans labelled by LM5 in the cells of nutritive tissue; (d) epitopes of (1→5)-α-L-arabinans labelled by LM6 in the cells of nutritive tissue; (e) epitopes of heteromannans labelled by LM21 in the cells of nutritive tissue; (f) epitopes of arabinoxylans labelled by LM11 in the cells of nutritive tissue; (g) epitopes of xyloglucans labelled by LM15 in the cells of nutritive and storage tissues; (h) epitopes of extensins labelled by LM1 in the cells of nutritive tissue; (i) epitopes of arabinogalactan-proteins labelled by LM2 in the cells of nutritive tissue. NT, nutritive tissue; ST, storage tissue; VB, vascular bundle.

pupae of the gall inducer, indicating a balance of flexibility and rigidity of the cell walls in the gall tissue compartments. Pectic polysaccharides, e.g. homogalacturonan epitopes, are biosynthesised in units of galacturonic acid and deposited in the cell walls in a highly methyl-esterified state (Caffall and Mohnen 2009; Albersheim *et al.* 2011), making the cell wall more malleable and able to expand (Pérez-Pérez *et al.* 2019; Reem *et al.* 2020). The degree of HG methyl-esterification depends on the activity of PME that remove the methyl groups, leading to the formation of un-methyl-esterified residues (Iribe and Buen 2013; Levesque-Tremblay *et al.* 2015). These

unesterified residues can form Ca^{2+} linkages and promote the formation of an ‘egg box’ structure leading to cell wall stiffening, which decreases the cell wall’s ability to expand and grow (Pelloux *et al.* 2007; Hongo *et al.* 2012). The dynamics of methyl-esterified and unesterified HGs in the cell walls of nutritive tissue may indicate juvenility (Ferreira *et al.* 2020) and stability (Willats *et al.* 2001b) required during the presence of the galling larva and pupa. The dual presence of methyl-esterified and unesterified HGs in the nutritive cells of *M. radula* galls was not affected by gall inducer pupation. We expected that, after the end of the galling herbivore feeding

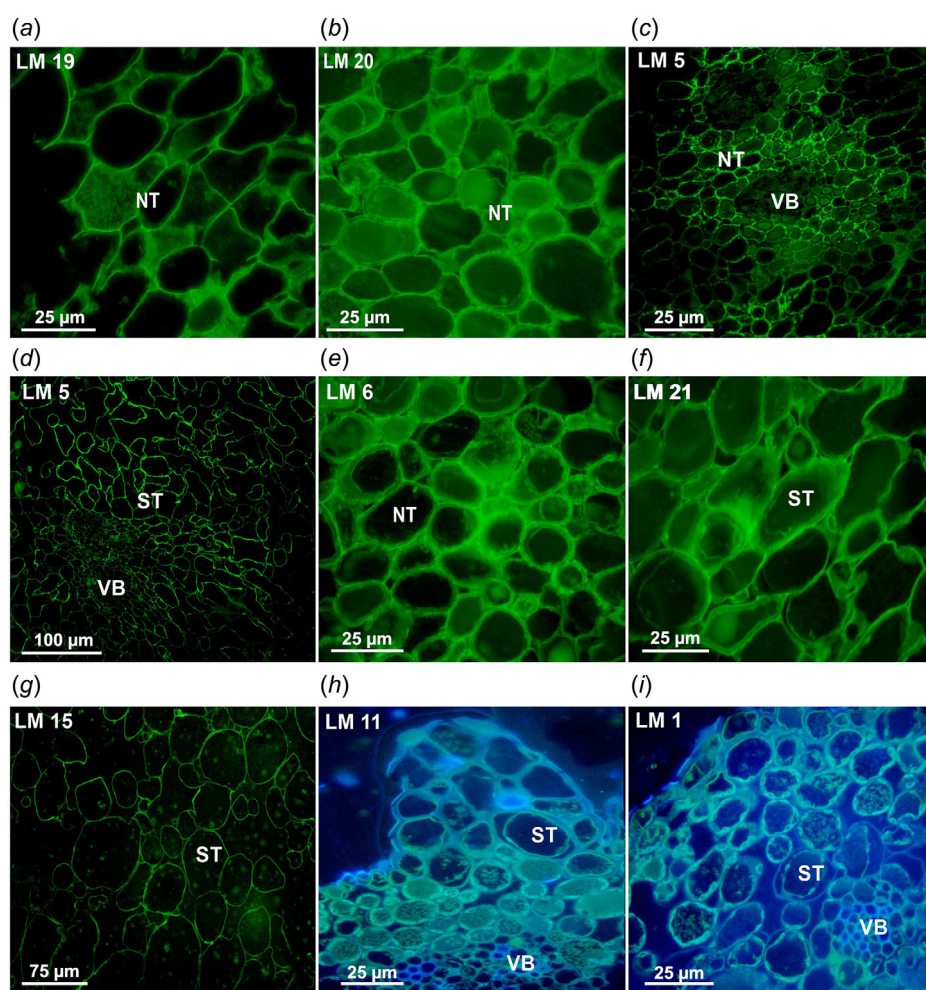


Fig. 5. Distribution of hemicellulose (LM11, LM21 and LM15), pectin (LM19, LM20, LM5 and LM6) and protein (LM1 and LM2) epitopes in the cell walls of galls on *Macaerea radula* with *Palaeomystella oligophaga* in the pupal stage. (a) Epitopes of unesterified homogalacturonans (HGs) labelled by LM19 in the cells of nutritive tissue; (b) epitopes of methyl-esterified HGs labelled by LM20 in the cells of nutritive tissue; (c) epitopes of (1→4)-β-D-galactans labelled by LM5 in the cells of nutritive tissue; (d) epitopes of (1→4)-β-D-galactans labelled by LM5 in the cells of storage tissue; (e) epitopes of (1→5)-α-L-arabinans labelled by LM6 in the cells of nutritive tissue; (f) epitopes of heteromannans labelled by LM21 in the cells of storage tissue; (g) epitopes of xyloglucans labelled by LM15 in the cells of nutritive and storage tissues; (h) epitopes of arabinoxylans labelled by LM11 in the cells of storage tissue; (i) epitopes of extensins labelled by LM1 in the cells of storage tissue. NT, nutritive tissue; ST, storage tissue; VB, vascular bundle.

period, that is, in the pupal stage, there would be an absence of methyl-esterified HGs and an increase in unesterified homogalacturonans. An alternative hypothesis for the similar distribution of HGs in cell walls of galls with larval and pupal stages is its bud origin. The *Macaerea* galls are induced on buds and develop like a stem-branch by shortening of internodes and, as a consequence, these galls remain attached and, sometimes, alive on the host plant for a long time once the gall inducer comes out (Rezende *et al.* 2023).

The (1→4)-β-D-galactan and (1→5)-α-L-arabinan epitopes were detected in all tissue compartments of the *M. radula* galls and seemed to be essential for their development. They were

recognised in both compartments and in the different regions of the cell wall in the gall occupied by the larval and pupal stages. In fact, galactan and arabinan epitopes can occur in different regions of the cell walls (Orfila and Knox 2000). These labelled carbohydrates are known as pectic rhamnogalacturonan-I (RGI), are highly heterogeneous, and are formed by a variable rhamnogalacturonan backbone and a variety of side chains composed of neutral carbohydrates. The (1→4)-β-D-galactan and (1→5)-α-L-arabinan epitopes may be related to the mechanical properties (extensibility and reinforcement) of the cell walls in the expansion and senescence of plant organs and galls (Caffall and Mohnen 2009;

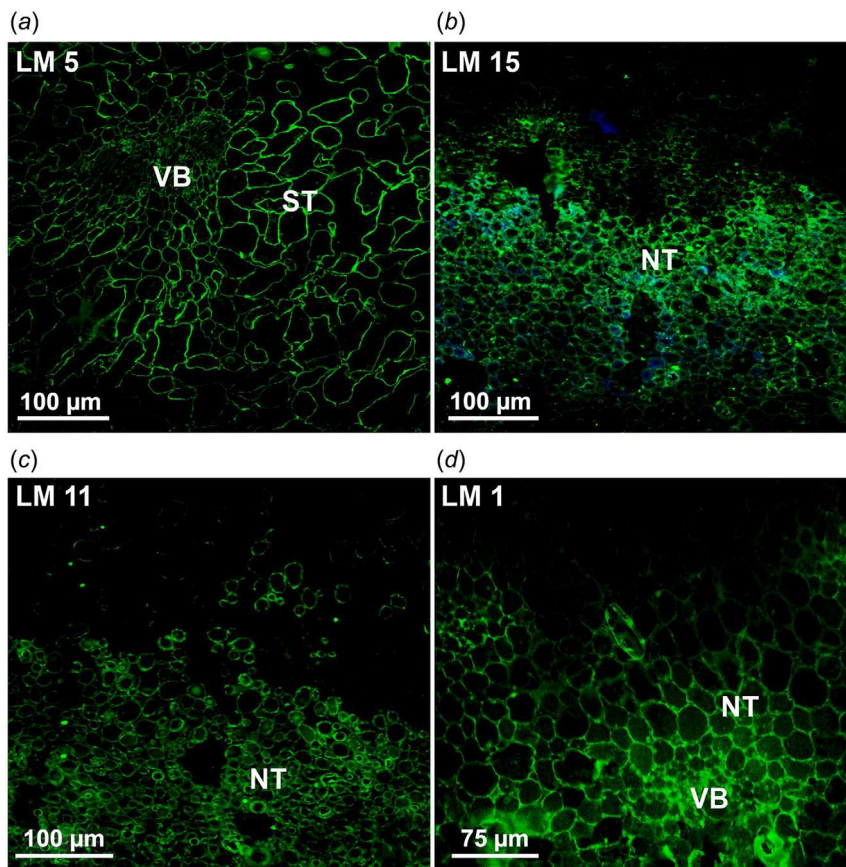


Fig. 6. Distribution of hemicellulose (LM11 and LM15), pectin (LM5) and protein (LM1) epitopes in the cell walls of empty galls induced on *Macairea radula* by *Palaeomystella oligophaga*. (a) Epitopes of (1→4)-β-D-galactans in storage tissue; (b) epitopes of xyloglucans labelled by LM15 in the cells of nutritive tissue; (c) epitopes of arabinoxylans labelled by LM11 in the cells of nutritive tissue; (d) epitopes of extensins labelled by LM1 in the cells of nutritive tissue. NT, nutritive tissue; ST, storage tissue; VB, vascular bundle.

Magalhães *et al.* 2014; Ferreira *et al.* 2020; de Melo Silva *et al.* 2021). In the nutritive tissue compartment, the presence of (1→4)-β-D-galactan and (1→5)-α-L-arabinan epitopes, associated with HGs, reinforced the porosity of the cell walls for the transport of molecules that can be directed to gall inducer feeding (Zykwinska *et al.* 2005; Verherbruggen *et al.* 2009; Ferreira *et al.* 2020).

Arabinogalactan proteins (AGPs) have several roles in plants (Lin *et al.* 2022) and gall development (de Oliveira *et al.* 2014; Teixeira *et al.* 2018; Ferreira *et al.* 2020; de Melo Silva *et al.* 2021). In galls occupied by larvae and pupae on *M. radula*, the AGPs can be involved in cell adhesion (Sala *et al.* 2017), as reported for other galls (Ferreira *et al.* 2020). The detection of AGPs in plants may be associated with cell death prevention (Mastroberti and Araújo Mariath 2008), an important role in gall tissues due to exposure to high oxidative stress (Isaias *et al.* 2015). Extensins are responsible for protecting against stress and reinforcing the cell wall in mature organs (Cassab 1998; Sabba and Lulai 2005; Albersheim *et al.* 2011; de Melo Silva *et al.* 2021). In *M. radula*, the presence of extensins in the nutritive tissue of galls occupied by *P. oligophaga* in the larval stage also reinforces their protection against oxidative stress.

Epitopes of heteromannans, recognised by LM21, and epitopes of (1→4)-β-D-xylans, recognised by LM11, were detected only in the nutritive cell walls of galls occupied by

larvae and pupae. These epitopes can bind to phenolic acids and influence the reduction of reactive oxygen species caused by the increase of cell respiration from the galling insect (Chowdhury *et al.* 2014; Isaias *et al.* 2015). The presence of heteromannans is commonly related to carbohydrate storage in the cell wall of the endosperm of seeds (Buckeridge *et al.* 2000) and in the nutritive tissue of some galls (Costa *et al.* 2021; Nogueira *et al.* 2022). Thus, heteromannans may be associated with the nutrition of *P. oligophaga* larvae, supplying the voracious feeding activity of Lepidoptera (Ferreira *et al.* 2019). Heteromannans are also related to the formation of new cell walls (Voiniciuc 2022), which may support the conservation of meristematic characteristics in nutritive cells of *M. radula*, with intense processes of cell division, as reported for the apical meristem of plants (Voiniciuc 2022). The presence of heteromannans in the inner and outer tissue compartments of galls occupied by pupae can help maintain the architecture of pectins and cellulose as discussed for non-galled tissues (Voiniciuc *et al.* 2015).

Xyloglucans are involved in cell wall loosening at specific points of association with microfibrils, i.e. the 'biomechanical hotspots' (Park and Cosgrove 2015). In galls, the role of xyloglucans is also related to the storage and availability of carbohydrates in the nutritive tissue (Ferreira *et al.* 2020; Bragança *et al.* 2020; Costa *et al.* 2021). The presence of

xyloglucans in both gall tissue compartments of *M. radula* occupied by gall inducers in the larval stage contributes to cell wall stability and nutrient reserve in the nutritive tissue. In galls occupied by pupae, the presence of xyloglucans in the cell walls of the storage tissue compartment reinforces stability during the remobilisation processes. However, their absence in nutritive tissue can indicate the utilisation of nutritive reserves by the galling larva.

Cell wall disruption in empty galls

In empty galls, only arabinoxylan, extensin, and xyloglucan epitopes were observed in the nutritive tissue, and (1→4)-β-D-galactan epitopes in the storage tissue. During the development of the gall from the pupal stage to empty galls, there was a decrease in pectin content, showing a possible loss of the cell wall flexibility necessary to maintain the gall in the host organ for a long period. In this case, the (1→4)-β-D-galactan epitopes may be related to the maintenance of cell stability due to their interaction with the other domains of the cell wall (Moneo-Sánchez *et al.* 2020). The absence of AGPs in senescent galls confirms the disruption, loss of integrity, and separation (Majewska-Sawka and Nothnagel 2000; Lampion *et al.* 2006; Seifert and Roberts 2007) of nutritive and storage tissue cells. The presence of extensins contributes to the reinforcement and prevention of stress to the cell walls for the permanence of the gall in the host organ (Cassab 1998; Sabba and Lulai 2005; Albersheim *et al.* 2011; de Melo Silva *et al.* 2021). Interestingly, the nutritive cells of empty galls contain xyloglucans, which may be related to the maintenance of inner cell rigidity and food reserve for interaction with other trophic levels (Rezende *et al.* 2021).

Conclusions

The formation of galls in *Macairea radula* leads to changes in the composition of the cell walls, especially regarding HGs, rhamnogalacturonans and hemicelluloses related to different functions, such as rigidity, porosity and/or flexibility. In the current work, we observed that the anatomical features and cell wall components of the tissues of the two compartments change throughout the developmental stages of *P. oligophaga*. These changes are associated with the stage of gall inducer development and reflect on the dynamics of the pectic components of the cell wall mainly in the inner compartment, i.e. the nutritive tissue. The detection of HGs with methyl and non-methyl-esterified groups in the nutritive tissue of galls in the larval and pupal stages indicates that, despite the end of larval feeding behaviour, the biosynthesis of HGs and PME activities is maintained. The xyloglucans in the nutritive tissue of *M. radula* galls occupied by gall inducers in the larval stage seem to be important for the insect's diet. The absence of these components in the nutritive tissue of galls when the insects are in the pupal stage corroborates their utilisation as nutritive reserves by the galling larva.

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Data availability. The data that support this study will be shared on request to the corresponding author.

Conflicts of interest. The authors declare that they have no conflicts of interest.

Declaration of funding. The current work was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Finance Code 001, Fundação de Amparo à pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), all from Brazil.

Acknowledgements. The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Brazil – Finance Code 001 and Fundação de Amparo à pesquisa do Estado de Minas Gerais (FAPEMIG – RED-00253-16) for scholarships and financial support, and the LADEVI and LAFIVE research laboratories for methodological support. The authors also thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for a DCO fellowship.

Author contributions. PDS collected the data, developed the methodologies and designed the experiments; GPPB, VCK and DIR developed the methodologies, analysed the data and supported result interpretations; DCO coordinated the project and supervised all steps. All authors wrote, revised and edited the manuscript.

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Chapter II: Monosaccharide composition in *Macairea radula* (Melastomataceae) galls vary depending on the gall-inducing stage, natural enemies presence, and gall coloration

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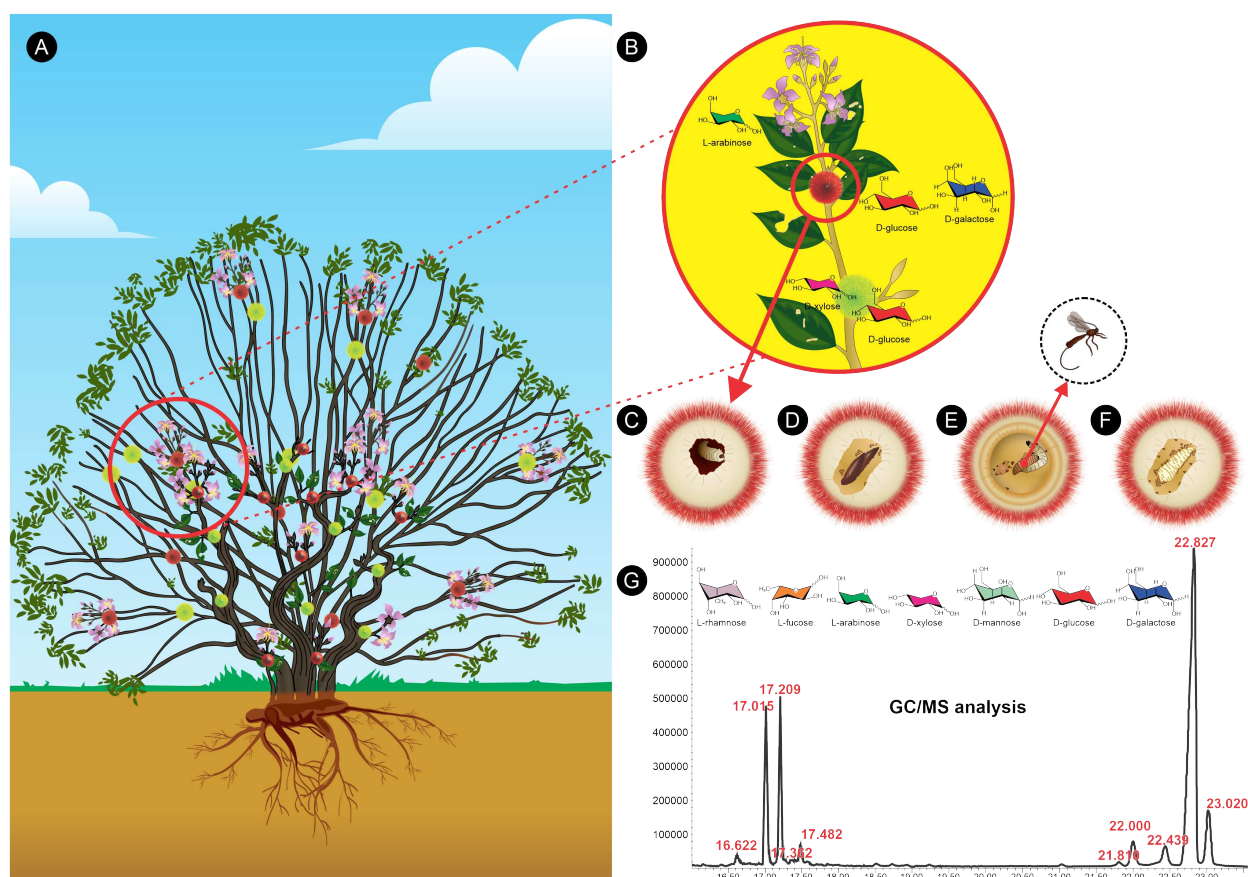
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A. *Macairea radula* (Melastomataceae); **B.** Stem galls of *Palaemystella oligophaga* Becker and Adamski (Lepdoptera); **C.** Gall in Larval Stage; **D.** Gall in Pupal Stage; **E.** Gall with Natural Enemy; **F.** Empty gall; **G.** Carbohydrates. The parasitic interaction causes physiological changes in the gall host plants, mainly in the composition of sugars. In this *Palaemystella oligophaga* (Lepidoptera) *M. radula* (Melastomataceae) system, we tested the hypothesis that the carbohydrates in galled tissues

17 with the presence of natural enemies are more abundant and diverse than in non-galled tissues. To
18 this end, the galls were subjected to acid hydrolysis in crude material and after ethanolic extraction
19 for analysis in Gas Chromatography-Mass Spectrometry (GC-MS).

20 2 Abstract

21 Galls are true plant organs induced by a huge diversity of organisms that can store different
22 kinds of substances. Among these substances, we can highlight the carbohydrates, essential components
23 of the plants produced by the photosynthesis processes. In the ecological interaction, carbohydrates
24 perform a variety of physiological functions such as providing energy for the cell growth, increasing
25 water-holding capacity, and as a food source for different organisms. Herein we evaluate the car-
26 bohydrate contents in galls induced by *Palaeomystella oligophaga* (Lepidoptera) on *Macairea radula*
27 (Melastomataceae) without and with natural enemy associated. We hypothesized that the composition
28 of carbohydrates in the galls, as well as galls with the presence of natural enemies are more abundant
29 and diverse than non-galled tissues. For that, we evaluate non-galled tissues (leaves and stems) and
30 galls (red and green) with the gall-inducing insect in the larval and pupal stages. The dry samples were
31 submitted to acid hydrolysis (crude samples) and to hydrolysis of ethanolic extract for analysis in gas
32 chromatography-mass spectrometry (GC-MS). Rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose
33 (Xyl), mannose (Man), glucose (Glc), and galactose (Gal) were detected in all groups of samples, with
34 a greater abundance of xylose and glucose in green galls with natural enemy followed by non-galled
35 tissues. In the hydrolyzed ethanolic extracts free monosaccharides and oligosaccharides with a greater
36 abundance of Glc were detected in the galled tissues. We conclude that there is variation in the com-
37 position of hydrolyzed polysaccharides composed mainly by Xyl, Glc, Gal, and Ara in the galls crude
38 extract. The abundances of Xyl and Ara were greater in non-galled organs, which may be associated
39 with the structural function of the leaves and stems. Unlike galls, which had a higher amount of glucose
40 and galactose; which can be conjugated with secondary metabolites, mainly flavonoids, responsible for
41 the protection and color of the galls.

42 **Keywords:** glucose, xylose, gall, carbohydrates, plant physiology.

3 Introduction

Galls are a very sophisticated parasitic interactions between host plant tissues and a biotic stressor, the gall-inducers, that belong to the most different groups of organisms such as bacteria, fungi, nematodes, mites, and insects (FERREIRA *et al.*, 2019; ISAIAS *et al.*, 2013; MANI, 1992; OLIVEIRA *et al.*, 2016; RAMAN, 2007; SHORTHOUSE *et al.*, 2005). These organisms can induce galls in different organs of the host plant, as vegetative as far as reproductive structures, but usually in leaves and branches (ISAIAS *et al.*, 2011; MANI, 1964). The gall induction and development lead to structural and physiological changes in the host tissues, increasing cell divisions and elongation and providing food and shelter for the gall-inducer (DREGER-JAUFFRET & SHORTHOUSE, 1992; OLIVEIRA *et al.*, 2016; RAMAN, 2007). Thus, the adaptive significance of galls to the gall-inducers depends on the reallocation and accumulation of different metabolic components to build the gall structure and provide food for the galling organism; among them, we can cite mono, oligo, and polysaccharides.

The carbohydrates are essential components of land plants associated with a variety of physiological functions, such as providing energy for the cell growth, increasing water-holding capacity, signaling molecules, and regulators of auxin biosynthesis (HARTMANN & TRUMBORE, 2016; LAST-DRAGER *et al.*, 2014; RASPOR *et al.*, 2021; SAIRANEN *et al.*, 2012). Furthermore, recent studies have also shown that carbohydrates play a key role in the plant defense responses to abiotic and biotic stress through the biosynthesis of secondary metabolites (CHUSTE *et al.*, 2020; DONG & BECKLES, 2019; JEANDET *et al.*, 2022; KANWAR & JHA, 2019; KEUNEN *et al.*, 2013; ROLLAND *et al.*, 2006), forming glycosylated compounds. These compounds can be relocated from other parts of the host plant to the gall, which functions as a true sink for photoassimilates (INBAR *et al.*, 1995; LARSON & WHITHAM, 1991; OLIVEIRA *et al.*, 2017), maintaining the growth and development of the neoformed gall, as well as the supply of resources for the gall-inducing and the associated community of natural enemies (REZENDE *et al.*, 2021).

Different monosaccharides such as rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc), and galactose (Gal) are essential components of cell walls and associated with other substances can determine cell wall functionalities (ALBERSHEIM *et al.*, 2011). The tissue hyperplasia and cell hypertrophy, as well as the determination of the gall functional compartments, are linked to changes in cell wall dynamics and composition during gall development (FERREIRA *et al.*, 2017; MARTINI *et al.*, 2019; SILVA *et al.*, 2021). In addition, carbohydrate accumulation may regulate the photosynthetic activity in galls and induce anthocyanin biosynthesis leading to the reddish phenomenon in galls (CONNOR *et al.*, 2012). These processes can correlate, for example, red galls can accumulate more water-soluble sugars than green ones (NAVES *et al.*, 2021). The gall-inducing insect stimulates continuously the host plant metabolism to produce primary metabolites such as proteins, lipids, and carbohydrates available not only for the insects diet (FERREIRA *et al.*, 2019) but also for

78 the associated fauna of natural enemies (REZENDE *et al.*, 2021).

79 Herein we used as a model system the globoid galls induced by *Palaeomystella oligophaga*
80 Becker and Adamski (Lepidoptera) on *Macairea radula* (Bonpl.) DC. (Melastomataceae) (BECKER
81 & ADAMSKI, 2008). These galls are induced on the buds and develop like a stem-branched gall with
82 a lot of leaf-like projections with emergences and trichomes (REZENDE *et al.*, 2023). The galls also
83 showed a typical nutritive tissue which accumulates lipids and proteins, and a storage tissue with starch
84 (REZENDE *et al.*, 2019). The cell walls of storage and nutritive tissues showed differential distribution
85 of pectins and hemicelluloses epitopes, evidencing distinct tissue functionalities (SANTOS *et al.*, 2024).
86 Despite globoid galls ranging from green to red with the formation of the projections, different groups
87 of natural enemies can be associated with the gall and gall-inducer (BECKER & ADAMSKI, 2008;
88 REZENDE *et al.*, 2021).

89 The modification of gall tissue associated with the presence of gall-inducer in different life
90 stages, natural enemies, and gall coloration can release the diverse of monosaccharides like rhamnose
91 (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc) and galactose (Gal).
92 These monosaccharides can be used for the synthesis of other molecules to ensure the carbon supply for
93 metabolic activities during the gall development. Monosaccharides constitute the most diverse class of
94 carbohydrates, a topic that has garnered the attention of various researchers due to its diversity and
95 ecological significance in biological systems (ALMEIDA *et al.*, 2015; AMICUCCI *et al.*, 2019; HARTMANN
96 & TRUMBORE, 2016; NESOVIC *et al.*, 2021; TIXIER *et al.*, 2019). In this study, we hypothesized that the
97 composition and quantity of carbohydrates within the galls of *M. radula*, especially those harboring
98 natural enemies and displaying red coloration, will be more abundant and diverse than non-galled
99 tissues.

100 4 Material and methods

101 4.1 Plant material

102 The galls induced on *Macairea radula* (Melastomataceae) were collected in an area of Cerrado
103 at the Estação Ecológica do Panga (19° 10' S, 48° 24' O), Uberlândia city, Minas Gerais State, Brazil.
104 We sampled and analyzed non-galled leaves (NGL) and non-galled stems (NGS), green galls with gall-
105 inducer in larval stage (GGL), green galls with gall-inducer in pupal stage (GGP), green galls with
106 natural enemies (GGNE), empty green galls (EGG), red galls with gall-inducer in pupal stage (RGP),
107 red galls with natural enemies (RGNE), and empty red galls (ERG). Samples of 10 individuals for each
108 treatment were dehydrated in an oven for carbohydrate analysis.

109 4.2 Samples preparation

110 The dry samples (5 mg) were hydrolyzed to detect the general carbohydrate composition.
111 Fractions of the dried samples were used to prepare an ethanolic extract for detection of mono-,
112 oligosaccharides, and secondary metabolites. Then, the ethanolic extracts were concentrated in a
113 rotary evaporator, weighed, and used to remove the low molar mass compounds. The residues left
114 over from the ethanolic extract were hydrolyzed with acid and subsequently injected into the GC-MS
115 chromatograph. The same procedure was repeated for all samples.

116 4.3 Gas chromatography coupled to mass spectrometry (GC-MS)

117 GC-MS analysis was performed with an Agilent 7820A gas chromatograph interfaced to an
118 Agilent 5975E quadrupole mass spectrometer, fitted with split/splitless capillary inlet system, an
119 Agilent G4513A autosampler, and a capillary DB225-MS column (30 m x 0.25 mm i.d.). Injections
120 of $1\mu/L$ were made in the splitless mode at injection temperature of $250^{\circ}C$ and detector at $280^{\circ}C$.
121 Helium was the carrier gas at a flow rate of $1\text{ ml}\cdot\text{min}^{-1}$. Electron impact (EI) analysis was performed
122 with the ionisation energy set at 70 eV.

123 4.4 Multivariate analysis

124 Multivariate analysis was performed with the PCA function ("FactoMineR" package) using
125 the matrix type correlation with data standardized. The chemical composition of the carbohydrates
126 was used as the variables. The graphs were created with the ggplot function and fviz_pca_biplot
127 ("ggplot2" package). All analyses were performed using R software version 4.3.1 (2023). And, the
128 data comparisons are described in the table at the end of this chapter.

129 5 Results

130 PCA analysis showed the separation of control groups (leaves, and stems) from the crude
131 extract of galls, with the presence of xylose and arabinose in non-galled organs and glucose and galactose
132 in the galls. On the other hand, in the ethanolic extracts, the non-galled stems and galls showed
133 similarities when compared with non-galled leaves. In the crude samples, the association of the first
134 two axes of the Principal Component Analysis (PCA) showed that monosaccharides explained 48.4% of
135 the data variation (Figure 10). The variation in data on axis 1 was explained positively by xylose and
136 negatively by glucose. The second axis of the PCA had a positive value for galactose and a negative
137 for glucose (Table 1) In the non-galled leaves (NGL), from ethanolic extract, the monosaccharides
138 Rha, Ara, Xyl, and Gal were significantly correlated with each first component, which explained 73.2%

of the total variance to PCA 1 (Figure 10). and Rha, Ara, Xyl, and Gal were positively correlated with the first axis of the principal component (Table 2). For the residual material (Figure 10), PC1 explained 47.7% of the total variance, with glucose (Glc) and galactose (Gal) with the highest positive correlation with the first axis of the component (Table 3).

Arabinose (Ara), Fucose (Fuc), Galactose (Gal), Glucose (Glc), Mannose (Man), Rhamnose (Rha), and Xylose (Xyl) were detected in all the crude extracts from all samples, with greater amounts of xylose in the leaves and stems (52.7%; 51.4%) and increase of glucose in galls (from 27.1% to 38.4%) (Table 4). Ara was detected in lower quantities in galls compared with non-galled tissues (Table 4). In galls, only those with the larval stage inside showed a greater amount of galactose (20.2%), see (Table 4). Red galls showed high quantities of Glc compared with green ones (Table 4). Also, the presence of natural enemies in both green and red galls seems to reduce the Glc quantity. In hydrolyzed ethanolic extracts free monosaccharides, predominantly composed of Glc, were detected with varying abundances ranging from 61.12% to 86.80% across different groups (Table 5). The leaves exhibited lower amounts, while the green galls containing gall-inducers in the pupal stage showed higher concentrations of Glc. In hydrolyzed ethanolic extracts Glc decreases in red galls compared with green ones. Man was detected in high quantities in galls compared with non-galled tissue (Table 5). We also detected a greater amount of mannose in red galls compared with green ones, especially red galls with natural enemies (Table 5). Xylose was detected in non-galled tissue and low quantities only in samples of red galls (Table 5). Ara was detected only in non-galled leaves and Fuc just in samples of galls. Rha was detected in high quantities in non-galled leaves compared with non-galled stems and galls. The composition of monosaccharides in the residue extracts, after acid hydrolysis, varied in the samples (Table 6). The quantity of Glc is bigger in non-galled leaves compared with non-galled stems and galls. However, the Glc quantities in gall are higher than in non-galled stems. Ara and Man were detected in high quantities in non-galled stem and gall samples compared with non-galled leaves. Fuc was detected only in non-galled tissues in similar quantities (Table 6).

6 Discussion

Globoid galls induced on *Macairea radula* by the *Palaeomystella oligophaga*, in both larval and pupal stages, along with the presence of natural enemies and distinctive patterns of coloration, exhibited monosaccharide compositions similar to the non-galled leaves and stems. In a PCA analysis, for instance, Rha, Ara, and Xyl were found to clustered non-galled tissues in the crude extracts, exhibiting higher quantities compared to the gall samples. Conversely, glucose levels were higher in galls than in non-galled samples, with the red galls displaying the highest values. In the ethanolic extracts, glucose appeared in greater quantities in the galls and stems compared with the non-galled

leaves. In addition, red galls showed low quantities of Glc compared with green ones, but high quantities of Xyl and Man. Manose also increased in all gall treatments compared with non-galled tissues (leaf and stem). Galls are a true sink of sugars (INBAR *et al.*, 1995; LARSON & WHITHAM, 1991; OLIVEIRA *et al.*, 2017), providing support for both the cellular processes within the gall and the feeding activities of gall-inducing insects (BRONNER, 1992; FERREIRA *et al.*, 2017; OLIVEIRA *et al.*, 2006, 2014). Also, the excess sugars can be utilized in the biosynthesis of secondary metabolites, such as anthocyanins, which contribute to the reddening of galls. The gall-inducing insect manipulates the host plant tissue for its own benefit (OLIVEIRA *et al.*, 2016) although the host plant can impose important constraints (ISAIAS *et al.*, 2014).

6.1 Monosaccharide functions in galls

The fragmentation of carbohydrates into monosaccharides can be used to regulate several functions in the different stages of the galls, mainly when gall-inducing is in the larval phase, the main trophic stage (BRONNER, 1992). In this study, it was found that there is diversity in the monosaccharide composition in the gall samples. The variation in PCA 1 was positively explained by xylose and glucose levels, and the second axis presented positive values for galactose and negative values for glucose. These sugar units have the possibility of branching due to the availability of many free hydroxyls (WANG & DEMCHENKO, 2019). Glucose has been identified as influencing several physiological processes in plants, including seed germination, root growth and development, floral transitioning, senescence, cell wall formation, and responses to biotic stress (ROLLAND *et al.*, 2006). Moreover, glucose is related to cell division, whereas sucrose supports cell expansion (BORISJUK *et al.*, 1998). Herein, the accumulation of glucose in galls is associated with cell division in the growth and developmental phase when the gall-inducer is in the larval stage. This accumulation is mediated by the auxin and cytokinins, phytohormones related to cell growth, elongation and divisions (ZORIC *et al.*, 2019). Glucose is produced in mesophyll cells, released to the phloem and distributed to the sink tissues where it is cleaved by different invertases to yield monomer hexoses (KOROLEVA *et al.*, 1998; LEMOINE *et al.*, 2013; WESCHKE *et al.*, 2003). These molecules can modulate metabolic and development processes, and stress responses in plants including source-sink carbon partitioning (MINCHIN & THORPE, 1996). Our data corroborate the galls as a sink of carbohydrates (INBAR *et al.*, 1995) and support what was found in the gall of *M. radula*, showing that galls accumulated more water-soluble sugars (NAVES *et al.*, 2021).

The red galls on *M. radula* accumulate more water-soluble polysaccharides than green galls (NAVES *et al.*, 2021) and herein we showed that crude extracts of red galls showed a high percentage of glucose compared with green ones. The reddish phenomenon in galls seems to be idiosyncratic and determined by the accumulation of anthocyanins (BOMFIM *et al.*, 2019). The accumulation of an-

thocyanins, and consequently the reddish color in galls, is discussed based on different ecological and physiological hypotheses as the defensive signaling against natural enemies (INBAR *et al.*, 2010), the senescing status of the galled tissues (WHITE, 2010), the high light exposure (BOMFIM *et al.*, 2019), the site of induction (CARDOSO *et al.*, 2022) and the fabrication noise hypothesis (CONNOR *et al.*, 2012). In general, this last hypothesis argues that cell divisions and consequently cytokinins accumulation increase the mobilizations of carbohydrates to the gall site and the production of flavonoids including anthocyanins. Glucose can also regulate secondary metabolite syntheses like glucosinolates and phenolic derivatives (SIDDIQUI *et al.*, 2020) and also induce the accumulation of anthocyanins (SALAMA *et al.*, 2023). However, the association between sugars, phytohormones, and the reddish phenomenon in gall is not mandatory and seems to be idiosyncratic. For example, the reddish color in galls induced on *M. radula* is also associated with the site of gall-inducing oviposition: if the bud site of induction is reddish, the gall has a high probability of being red (CARDOSO *et al.*, 2022).

The presence of natural enemies in both red and green galls results in a slight decrease in the quantity of glucose (Glc) in the crude extract. This decrease compared with galls with gall-inducing insects in the larval and pupal stages may be indicative of accelerated tissue degradation stimulated by natural enemies presence (REZENDE *et al.*, 2019). Other studies also showed, for example, the decrease in glucose during gall development caused by aphids (DONG *et al.*, 1997). The abundance of xylose decreased in galls compared with non-galled tissues. However, galls with natural enemies showed the greatest quantity of xylose compared with those with gall-inducing larvae and pupal stages. Xylose, as it is part of the branches of the chemical structure of xyloglucan, was also found in the cell wall of the nutritive and reserve tissues of *M. radula* galls (SANTOS *et al.*, 2024), and together with glucose, forms the hemicelluloses and contributes to the strengthening of the cell wall (SCHELLER & ULVSKOV, 2010). The accumulation of these monosaccharides has also been observed in the stem gall of *Linaria vulgaris* which xylose was higher in the larval stage of *Rhinusa pilosa* (ZORIC *et al.*, 2019).

6.2 Monosaccharide association with secondary metabolites

Carbohydrates may have similar functions to hormones as primary messengers in signal transduction (ROLLAND *et al.*, 2002; SMEEKENS, 2000), and thus coordinate the growth, development, and energy generation in plants (JEANDET *et al.*, 2022; LASTDRAGER *et al.*, 2014; WANG *et al.*, 2021). In this study, the main sugars are monosaccharides with five (xylose and arabinose) and six carbons (rhamnose, fucose, mannose, galactose, and glucose) which may function as osmoregulators, as substrates for respiration, or as precursors of secondary compounds biosynthesis to attract other organisms such as natural enemies, parasitoids and pollinators (HARTMANN & TRUMBORE, 2016; SWAIN, 1997).

The relationship between monosaccharides and secondary metabolites was observed in studies on *Populus deltoides*, where overexpression of UGPase resulted in an increase in leaf area and a decrease

240 in root biomass, as well as the amount of carbohydrates. However, it also altered the metabolism of
 241 secondary compounds, leading to the biosynthesis of phenolic compounds (PAYYAVULA *et al.*, 2013).
 242 The main secondary metabolites in galls are flavonoids and tannins (GUEDES *et al.*, 2024; HEMEG
 243 *et al.*, 2022; PAAVER *et al.*, 2010), which may have simpler or more complex chemical structures. The
 244 complexity of these molecules is associated with the conjugation with carbohydrates, commonly found
 245 in glycosidic forms, making them soluble in water; and also when linked to hydrolyzable and condensed
 246 tannins (LANDETE, 2011). These flavonoids molecules represent one of the largest groups of phenolic
 247 compounds and can be classified into several categories, such as anthocyanins, flavones and isoflavones
 248 (CHEN *et al.*, 2023). Proanthocyanidins, for example, are condensed tannins composed of catechin and
 249 epicatechin and their gallic acid esters can undergo acid hydrolysis in alcoholic solutions and release
 250 anthocyanidin molecules (YU *et al.*, 2023). While hydrolyzable tannins, such as ellagitannins, are
 251 associated with quinic acid and sugar units (JOURDES *et al.*, 2013; YOSHIDA *et al.*, 2010). But not
 252 all are associated to secondary metabolism, many participate in the maintenance of the cell wall and
 253 carbon allocation (HARMER *et al.*, 2000).

254 In plants, rhamnose and mannose are widely present as polymers in cell walls as well as part
 255 of the composition of several secondary metabolites (JIANG *et al.*, 2021). These monosaccharides are
 256 essential for synthesizing pectins and cell wall glycoproteins (MOHNEN, 2008), and compose homo-
 257 galacturone (HG), rhamnogalacturone I (RG-I) and rhamnogalacturonan II (CAFFALL & MOHNEN,
 258 2009). Both carbohydrates were immunolocalized in the cell wall of *M. radula* galls (SANTOS *et al.*,
 259 2024). They were also found in greater quantity in empty galls and galls with natural enemies in the
 260 ethanolic extract. Probably, the glycosylation of secondary metabolites can favor an increase in the
 261 hydrophilicity and stability of the compounds in the gall (LOUVEAU & OSBOURN, 2019). Generally,
 262 Rha forms O-glycosidic bonds with flavonoids and triterpenoid saponins; flavonol rhamnosides and
 263 quercetin 3-O-glucoside for example. These rhamnosides may play an important role in inhibiting
 264 auxin transport (YIN *et al.*, 2014) and, also, signaling the regulation of other plant hormone pathways
 265 (SMEEKENS, 2000) with a decline in chlorophyll content and photosynthetic activity in abundant sugar
 266 presence to contribute to growth and carbohydrate storage in the galls (KOCH, 1996; NAVES *et al.*,
 267 2021).

268 7 Conclusion

269 Despite there are no differences in the overall composition of monosaccharides in non-galled
270 tissues compared with galls, the quantity of carbohydrates within the galls of *M. radula*, especially
271 those harboring natural enemies and displaying red coloration, is greater than in non-galled tissues.
272 The xylose and arabinose content was higher in non-galled organs, which may be associated with the
273 structural function of leaves and stems. In contrast, galls had higher amounts of glucose and galactose,
274 which can be conjugated with secondary metabolites, mainly flavonoids responsible for the protection
275 and coloration of the galls. Besides that, they can be associated to the high primary metabolism in
276 galls for cell growth and development as well as supplying gall-inducing organism diet.

277 8 Acknowledgments

278 The authors thank the FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas
279 Gerais) for financial support and fellowship to PS Dias. We also thank CNPq – Conselho Na-
280 cional de Desenvolvimento Científico e Tecnológico for financial support and fellowship to AS Moreira
281 (309044/2021-9) and DC Oliveira (303691/2022-0). To the Laboratório de Bioquímica e Química
282 Orgânica (LaBiOrg) of Universidade Federal de Catalão, especially to Professor Elaine Carbonero for
283 the experimental analyses.

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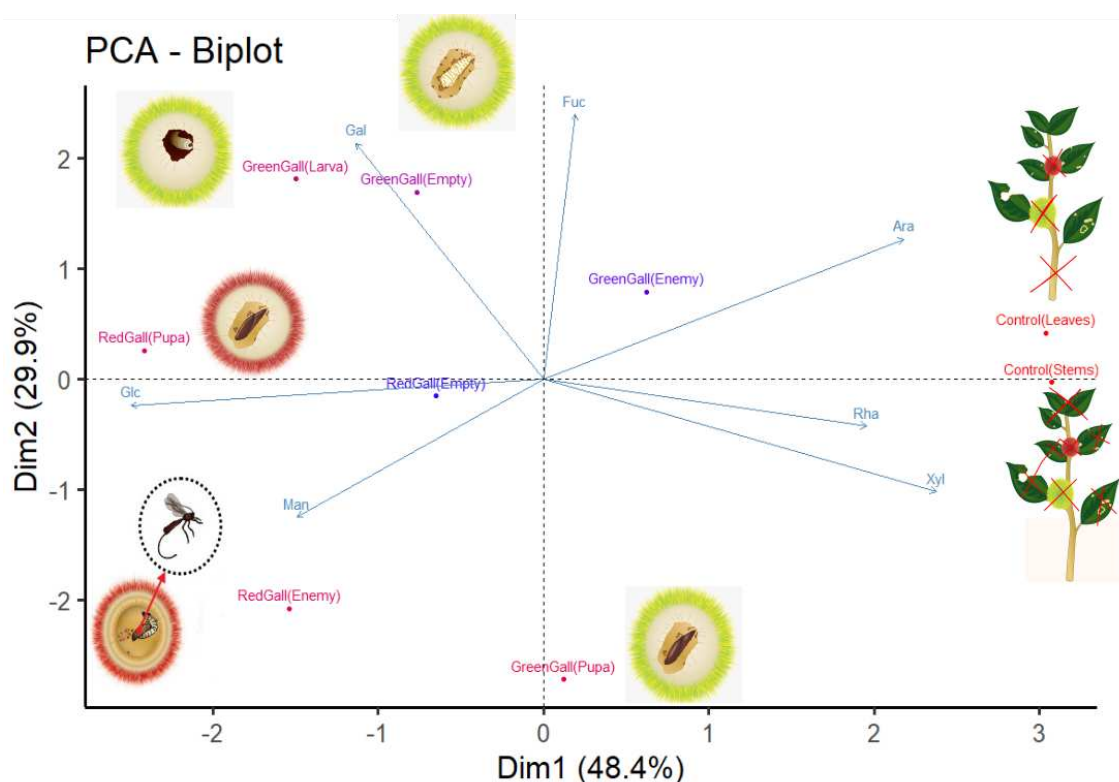


Figure 1: Biplot (PCA) of carbohydrates analysis performed in *M. radula* leaves, stems and galls. The data considered crude extracts from non-galled leaves and stems, green galls with gall-inducer in larval stage, green galls with gall-inducer in pupal stage, green galls with natural enemies, empty green galls, red galls with gall-inducer in pupal stage, red galls with natural enemies, and empty red galls. Rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc), and galactose (Gal) were detected in all groups of samples.

Table 1: Summary of principal component analysis (PCA) of crude samples. Values corresponding to the loadings of the parameters of the two PCA axes. Parameters that significantly correlated with each axis are shown in bold.

Variables	Parameters (%)	
	PCA 1	PCA 2
Rhamnose (Rha)	0.020	-0.013
Fucose (Fuc)	-0.007	0.067
Arabinose (Ara)	0.138	0.292
Xylose (Xyl)	0.698	-0.450
Mannose (Man)	-0.014	-0.022
Glucose (Glc)	-0.689	-0.528
Galactose (Gal)	-0.136	0.655
Explained Variance	48.4%	29.9%

Table 2: Summary of principal component analysis (PCA) of extract ethanolic. Values corresponding to the loadings of the parameters of the two PCA axes. Parameters that significantly correlated with each axis are shown in bold.

Variables	Parameters (%)	
	PCA 1	PCA 2
Rhamnose (Rha)	0.979	-0.066
Fucose (Fuc)	-0.333	-0.712
Arabinose (Ara)	0.987	0.118
Xylose (Xyl)	0.864	-0.099
Mannose (Man)	-0.753	-0.657
Glucose (Glc)	-0.895	0.443
Galactose (Gal)	0.916	0.265
Explained Variance	73.2%	19%

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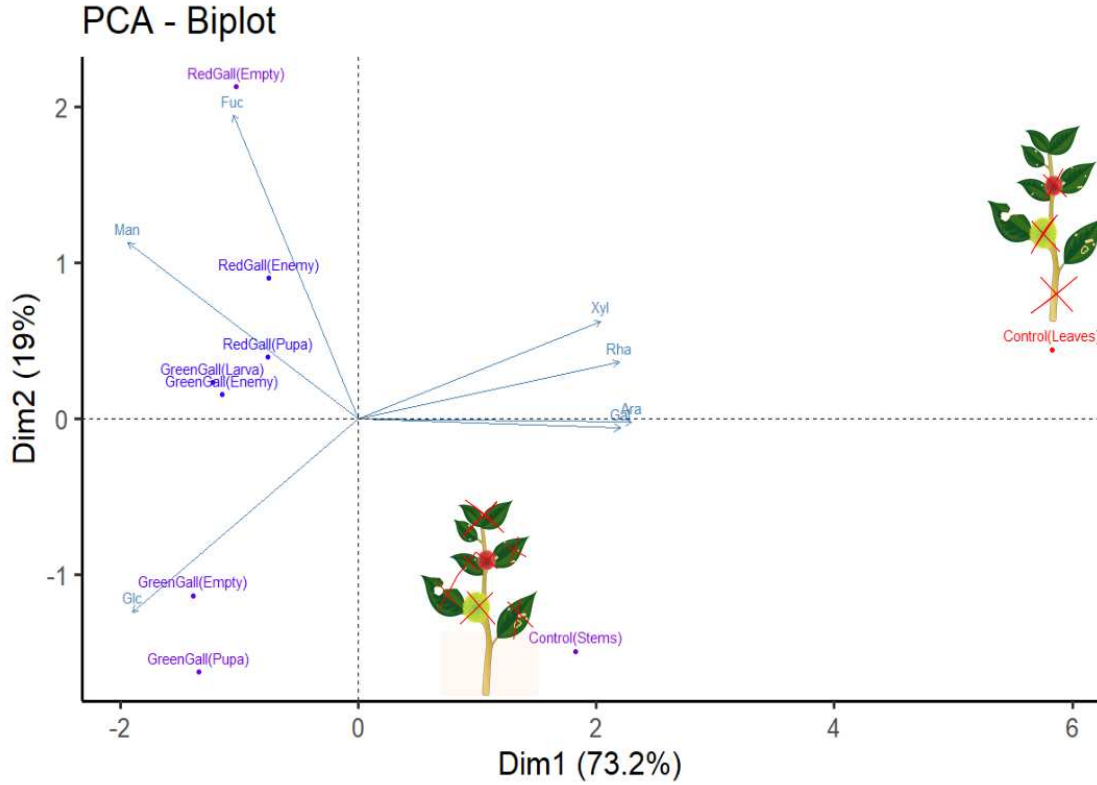


Figure 2: Biplot (PCA) of carbohydrates analysis performed in *M. radula* leaves, stems and galls. The data considered ethanolic extracts from non-galled leaves and stems, green galls with gall-inducer in larval stage, green galls with gall-inducer in pupal stage, green galls with natural enemies, empty green galls, red galls with gall-inducer in pupal stage, red galls with natural enemies, and empty red galls. Rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc), and galactose (Gal) were detected in all groups of samples.

Table 3: Summary of Principal Component Analysis (PCA) of residue material. Values corresponding to the loadings of the parameters of the two PCA axes. Parameters that significantly correlated with each axis are shown in bold.

Variables	Parameters (%)	
	PCA 1	PCA 2
Rhamnose (Rha)	0.383	0.027
Fucose (Fuc)	0.211	-0.731
Arabinose (Ara)	-0.568	0.701
Xylose (Xyl)	-0.983	-0.047
Mannose (Man)	0.586	0.740
Glucose (Glc)	0.929	-0.273
Galactose (Gal)	0.806	0.391
Explained Variance	47.7%	25.8%

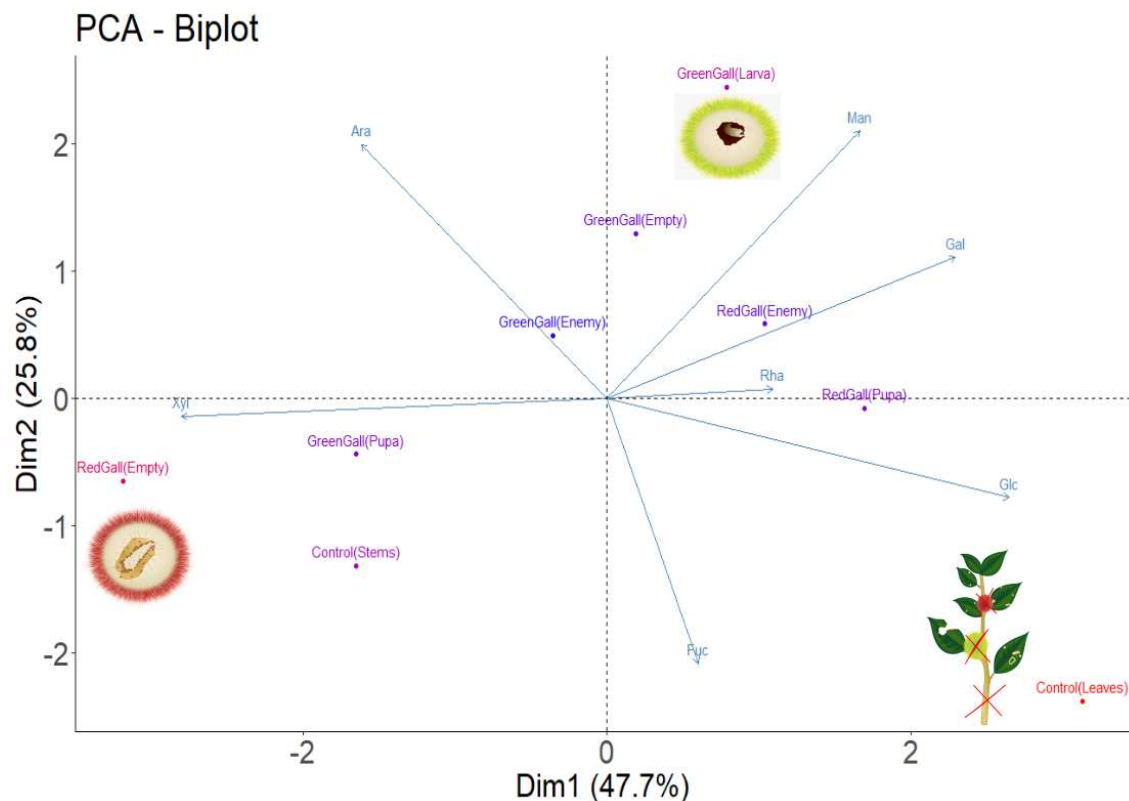


Figure 3: Biplot (PCA) of carbohydrates analysis performed in *M. radula* leaves, stems and galls. The data considered the residues from non-galled leaves and stems, green galls with gall-inducer in larval stage, green galls with gall-inducer in pupal stage, green galls with natural enemies, empty green galls, red galls with gall-inducer in pupal stage, red galls with natural enemies, and empty red galls. Rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc), and galactose (Gal) were detected in all groups of samples.

Table 4: Monosaccharides composition after acid hydrolysis of crude extracts

Plant material	Monosaccharides (%)							Sum(%)
	Rha	Fuc	Ara	Xyl	Man	Glc	Gal	
Non-galled leaves	2,8	0,7	10,1	52,7	1,6	19,5	13	100
Non-galled stem	3,2	0,7	10,2	51,4	2,0	19,4	13,1	100
Green galls in larval stage	2,4	0,9	7,1	33,6	2,1	33,7	20,2	100
Green galls in pupal stage	2,7	0,0	5,6	48,3	2,4	30,6	10,5	100
Green galls with natural enemies	2,6	1,0	8,8	43,4	2,2	27,1	14,9	100
Empty green galls	2,4	1,0	9,0	36,3	2,4	30,0	18,9	100
Red galls in pupal stage	2,1	0,6	4,9	33,9	2,0	41,1	15,2	100
Red galls with natural enemies	2,4	0,0	6,0	40,9	2,8	34,1	13,8	100
Empty red galls	3,1	1,0	6,7	35,5	2,4	38,4	12,8	100

Table 5: Monosaccharides composition after acid hydrolysis of ethanolic extract material.

Plant material	Monosaccharides (%)							Sum(%)
	Rha	Fuc	Ara	Xyl	Man	Glc	Gal	
Non-galled leaves	11,8	-	13,3	1,5	3,6	61,1	8,6	100
Non-galled stem	1,7	-	4,2	0,91	4,6	81,6	6,7	100
Green galls in larval stage	1,1	0,9	-	tr	14,6	81,9	1,36	100
Green galls in pupal stage	-	tr	-	-	13,20	86,80	-	100
Green galls with natural enemies	1,0	0,7	-	-	16,0	80,5	1,6	100
Empty green galls	tr	tr	-	tr	16,6	83,3	tr	100
Red galls in pupal stage	0,5	0,5	-	0,6	17,3	79,7	1,09	100
Red galls with natural enemies	0,9	0,7	-	0,6	18,5	77,6	1,50	100
Empty red galls	0,7	1,3	-	0,6	21,0	74,7	1,4	100

Table 6: Monosaccharide compositions after acid hydrolysis of the residue material

Plant material	Monosaccharides (%)							Sum(%)
	Rha	Fuc	Ara	Xyl	Man	Glc	Gal	
Non-galled leaves	4,6	0,7	6,7	29,7	1,2	42,0	14,7	100
Non-galled stem	1,4	0,7	10,3	62,8	1,0	10,8	12,7	100
Green galls in larval stage	3,4	tr	12,8	44,8	3,4	18,1	17,2	100
Green galls in pupal stage	3,9	tr	9,7	63,0	1,1	13,5	8,5	100
Green galls with natural enemies	3,2	tr	11,2	50,4	1,6	20,6	12,7	100
Empty green galls	3,5	tr	12,8	45,0	1,9	21,4	15,1	100
Red galls in pupal stage	2,9	tr	7,8	39,8	2,0	31,5	15,6	100
Red galls with natural enemies	4,6	tr	9,5	45,7	2,4	23,8	13,8	100
Empty red galls	3,8	tr	12,2	67,8	tr	10,0	6,1	100

Chapter III: Enzymatic and nonenzymatic defensive way against oxidative stress *in Macairea radula* gall system

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12 2 Abstract

13 Galls induction causes structural and physiological changes, such as an increase in the number
14 of cell divisions and hypertrophy. These new structures in host plants serve as a source of food and
15 shelter for the gall-inducing organism. The alterations in host cells and tissues involve the production of
16 reactive oxygen species (ROS) and can promote cellular damage. However, plants have both enzymatic
17 and non-enzymatic defense systems that include enzymes and phenolic compounds to scavenge ROS
18 and prevent cellular harm. Hence, the aim of this work was to evaluate the enzymes and molecules
19 related to oxidative stress in non-galled tissues (leaves) at different stages of *Macairea radula* galls
20 (Melastomataceae) in the presence or absence of natural enemies. Galled and non-galled tissues were
21 collected from *M. radula* with gall-inducer in larval and pupal stage, gall with natural enemy, empty
22 gall, and leaves in the Cerrado at Estação Ecológica do Panga (19° 10' S, 48° 24' O), Uberlândia city,
23 Minas Gerais State, Brazil. These samples were dried and submitted to extraction by maceration for
24 enzymatic assay and phytochemical analysis by HPLC-ESI-MS/MS. The enzymes ascorbate peroxidase
25 (APX), peroxidase (POX) and (SOD) were those that showed the greatest difference between leaves,
26 larval, pupal, natural enemy and empty gall stages. The catalase (CAT) and phenylalanine ammonia
27 lyase (PAL) enzymes showed greater amounts only in the larval stage. There was no difference in the
28 malondialdehyde (MDA), anion, peroxide and lignin levels among the groups tested. A total of 50
29 chemical compounds were annotated, including phenolic acids, flavonoids, tannins, carbohydrates, and
30 lipids. The activation of the primary enzymatic defense involved a greater amount of catalase in the
31 gall with gall-inducing in the larval phase, and a higher presence of PAL to protect and maintain the
32 galling insect in its initial stage. Among all the compounds detected, hydrolyzable tannins appeared
33 exclusively in the galls, indicating that their degradation products, including ellagic acid, play a role
34 in protecting and maintaining the gall on the host plant.

35 **Keywords:** plant defense, enzymes, secondary metabolites, hydrolyzable tannins, flavonoids.

3 Introduction

The gall induction causes structural and physiological changes in the host plant tissue leading to the formation of a new structure which serves as a source of food and shelter for the gall-inducer (DREGER-JAUFFRET & SHORTHOUSE, 1992; OLIVEIRA *et al.*, 2016; RAMAN, 2007). These modifications involve the production of reactive oxygen species (ROS), which can promote signals for growth and development as well as cause damage in the cells (DEMIDCHIK, 2015; GUEDES *et al.*, 2022; ISAIAS *et al.*, 2015; OLIVEIRA *et al.*, 2016). This biotic stress caused by gall-inducing organisms changes the host plant cells and tissues homeostasis leading to an increase in the accumulation ROS, such as superoxide radicals (O_2^-), hydroxyl radicals (OH^-), and hydroperoxyl radicals (HO_2) (ISAIAS *et al.*, 2015; MITTLER *et al.*, 2011). The production these molecules can result in the destruction of cell walls and cell organelles, membrane peroxidation, degradation of macromolecules, and consequently, cell death (SHARMA *et al.*, 2012). The lipid peroxidation of cellular membranes resulting in the production of malondialdehyde (MDA), which is widely used as a biomarker of oxidative stress in plants (DAVEY *et al.*, 2005).

Plants have enzymatic and nonenzymatic antioxidant defense, scavenging ROS and prevent cellular damage (IRATO & SANTOVITO, 2021a; LEE *et al.*, 2007; SACHDEV *et al.*, 2021). The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) (ANJUM *et al.*, 2016). The nonenzymatic defense system in plants includes the secondary metabolites that, like enzymes, can neutralize ROS (DUMANOVIC *et al.*, 2021). This nonenzymatic system includes mainly polyphenolic compounds such as phenolic acids, flavonoids, and tannins (RUDENKO *et al.*, 2023). The latter are one of the most studied classes of plant secondary metabolites in research related to chemical ecology (BARBEHENN & CONSTABEL, 2011; SALMINEN & KARONEN, 2011). In insect-plant interactions, these compounds benefit the inducer of the chinese horned gall aphid system (CHEN *et al.*, 2018). Thus, the tannins are classified into three groups based on their structures: hydrolyzable tannins, condensed tannins, and complex tannins, also function as defensive molecules by protecting plant tissues from herbivore attacks, primarily due to their astringent taste (CHANG *et al.*, 2019). Despite their antioxidant and defensive roles, phenolics in galls can also act as signaling molecules to gall growth and development as well attract natural enemies (DIVEKAR *et al.*, 2022).

The first steps of gall development involve insect recognition and initial gall induction (MANI, 1964). After that, the gall beginning to growth and developmental until the mature phase, during which the gall-inducers are in the main trophic stage, feeding on the nutritive tissue (in the case of nutritive galls). In the pupal stage, the gall-inducers stop feeding and prepare to emerge, entering in the senescence stage (BRONNER, 1992; FERREIRA *et al.*, 2019). When a natural enemy enters in the system (especially in the gall with the gall-inducer in the larval stage), the nutritive tissue is

71 disrupted, and the gall tissue may enter the senescent processes early (REZENDE *et al.*, 2019). We
72 can presume that enzymatic and nonenzymatic antioxidant defense systems play an important role in
73 maintaining tissue homeostasis during the stages of gall development. Herein, we use the globoid galls
74 induced by *Palaemystella oligophaga* Becker & Adamski (Lepidoptera) on buds of *Macairea radula*
75 D.C. (Melastomataceae) as model system (BECKER & ADAMSKI, 2008). These galls develop as stem-
76 like structures by shortening of internodes (REZENDE *et al.*, 2023), exhibit a wide range of colors
77 from green to red, and are attacked by various natural enemies despite the presence of projection
78 on the gall surface (REZENDE *et al.*, 2021). Inside the galls there are two tissues compartments: an
79 inner compartment (nutritive tissue) that accumulates proteins and lipids for insect diet; and an outer
80 compartment (storage tissue) containing starch and soluble carbohydrates for maintenance of the gall
81 structure as well secondary metabolites (KUSTER *et al.*, 2020; NAVES *et al.*, 2021; REZENDE *et al.*,
82 2019).

83 We argue that the development of this gall activates different enzymatic complexes for de-
84 fense against oxidative stress caused by gall-inducing insects and by the presence of natural enemies.
85 Additionally, these enzymatic defenses change during gall development and are supported by the nonen-
86 zymatic defense system, namely, the biosynthesis of various secondary metabolites. Thus, the objective
87 of the work was to evaluate the levels of antioxidant molecules and the chemical composition in the
88 non-galled tissue (leaves) and in the different stages of the galls of *M. radula* in the presence and
89 absence of natural enemies.

90 4 Material and methods

91 4.1 Galls material

92 Non-galled organs (leaves) and galls were collected from a population of *M. radula* in the
93 Brazilian Cerrado at Estação Ecológica do Panga (19° 10' S, 48° 24' O), Uberlândia city, Minas
94 Gerais State, Brazil. The galls were collected at different developmental stages, including galls with
95 the gall-inducer in larval and pupal stages inside, galls with natural enemies and, empty galls. The
96 mature non-galled leaves were also collected and use for comparative analysis. For the quantification
97 of enzymes was used the n=4 because there were few reagents. A portion of each sample was dried
98 for phytochemical analysis, and another portion was frozen in nitrogen and stored in an ultrafreezer
99 at -80 °C for antioxidant activity analysis.

100 4.2 Enzymatic assay

101 4.2.1 Determination of enzymes levels

102 The galled and non-galled tissue samples stored in an ultrafreezer at -80 °C were ground with
103 liquid nitrogen in a blender. Approximately 200 mg of each sample were weighed and homogenized in 1
104 mL of 50 mM potassium phosphate buffer (pH 6.8), containing 0.1 mM ethylenediaminetetraacetic acid
105 (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 3% polyvinylpyrrolidone (PVPP) (w/v).
106 The homogenate was kept in an ice bath for 3 h and then centrifuged at 12,000 g for 20 min at 4 °C.
107 The supernatant was used as an extract to carry out the enzymatic reactions for enzymes catalase
108 (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and nonspecific peroxidase (POX; EC
109 1.11.1.7). For superoxide dismutase (SOD; EC 1.15.1.1), the enzyme activities were expressed on a
110 protein basis, the concentration of which was determined by the Bradford method (1976).

111 4.2.2 Superoxide dismutase (SOD; EC 1.15.1.1) activity

112 Superoxide dismutase activity was determined as described by Del Longo et al. (1993).. A
113 reaction mixture was prepared with 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine,
114 75 M p-nitro-tetrazolium blue (NBT), 0.1 mM EDTA, and 2 μ /L riboflavin. For the reaction, 100
115 μ /L of galled and non-galled tissue extract was added to make a total volume of 1 mL. The reaction
116 was carried out at 25 °C under 15 W lamp lighting for 15 min. After this, the lighting was stopped
117 and SOD was evaluated by the enzyme's ability to inhibit the photoreduction of nitrotetrazolium blue
118 (NBT), measured in a spectrophotometer (Evolution 60, Thermo Fisher Scientific Inc., Massachusetts,
119 USA) at 560 nm, as described by Giannopolitis and Ries (1977). Control samples, containing the same
120 mixture but without the enzyme extract, were kept in the dark for 15 min, and their absorbances were
121 measured at 560 nm.

122 4.2.3 Catalase (CAT; EC 1.11.1.6) activity

123 Catalase activity was determined using the method of Cakmak and Marschner (1992). The
124 reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.8) and 20 mM H_2O_2 in a total
125 volume of 2 mL. The reaction mixture was prepared, consisting of 50 mM potassium phosphate buffer
126 (pH 6.8) and 20 mM H_2O_2 . For the reaction, 100 μ /L of each organ was added, performing 2 mL.
127 Activity was determined by consumption of H_2O_2 at 240 nm, for 3 min at 25 °C. The molar extinction
128 coefficient of 36 M-1 cm-1 (ANDERSON *et al.*, 1995) was used to determine CAT activity, which was
129 expressed as mmol min⁻¹ mg⁻¹ of protein.

130 4.2.4 Ascorbate peroxidase (APX; EC 1.11.1.11) activity

131 Ascorbate peroxidase activity was determined using the method of NAKANO & ASADA (1981).
132 A reaction mixture was prepared with 50 mM potassium phosphate buffer (pH 6.8), 1 mM H_2O_2 and
133 0.8 mM ascorbate. For the reaction, 50 μ/L of galled and non-galled tissue extracts was added to a
134 final volume of 2 mL. The reaction was measured by ascorbate-dependent oxidation of H_2O_2 at 290
135 nm, for 5 min at 25 °C. The molar extinction coefficient of 2.8 mM⁻¹ cm⁻¹ (Nakano and Asada, 1981)
136 was used to calculate APX activity, which was expressed as μ/mol min⁻¹ mg⁻¹ of protein.

137 4.2.5 Peroxidases (POX; EC 1.11.1.7) activity

138 Peroxidases activity was determined by the oxidation of pyrogallol, according to the method-
139 ology proposed by KAR *et al.* (2012). A reaction mixture was prepared, consisting of 25 mM potassium
140 phosphate buffer (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2 . For the reaction, 25 μ/L of galled
141 and non-galled tissue extract was added in a final volume of 2 mL. The reaction was determined by
142 the consumption of H_2O_2 at 420 nm, for 3 min at 25 °C. The molar extinction coefficient of mM⁻¹
143 cm⁻¹ CHANCE & MAEHLI (1955) was used to calculate POX activity, which was expressed as μ/mol
144 of purpurogaline produced min⁻¹mg⁻¹ of protein.

145 4.2.6 Phenylalanine ammonia lyase (PAL; EC 4.3.2.5) activity

146 Phenylalanine ammonia lyase activity was determined using the methodology proposed by
147 Data *et al.* 1984. For the reaction, 20 μ/L of 0.5 mol L⁻¹ tris-HCl buffer (pH 8.8), 140 μ/L of
148 ultrapure water, 200 μ/L of L-phenylalanine 0.1 mol L⁻¹ and 200 μ/L of crude enzyme extract was
149 mixed. The tubes with the reaction mixture were incubated in a water bath at 37 °C for 60 min,
150 using 50 μ/L of hydrochloric acid (5N HCl) to stop the reaction. Absorbance were measured using a
151 spectrophotometer set at 290 nm. One unit of PAL activity was defined as 1 μ/mol of trans-cinnamic
152 acid formed per minute under assay conditions.

153 4.2.7 Malondialdehyde assays

154 Samples of galled and non-galled organs (100 mg) were macerated in nitrogen liquid and
155 homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at
156 12,000 g for 15 min at 4 °C. A 0.5% (m/v) thiobarbituric acid solution (prepared in 20% TCA, m/v)
157 was added to 0.5 mL of the supernatant, totaling 1.5 mL. The reaction mixture was incubated in
158 a water bath at 95 °C for 30 min, followed by stopping the reaction in an ice bath. Samples were
159 centrifuged at 9000 g for 10 min, and the specific absorbance of the supernatant was determined at 532
160 nm (CAKMAK & HORST, 1991). Malondialdehyde concentration was calculated using the extinction

161 coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed in $\mu\text{g/Lmol kg}^{-1}$ fresh mass (HEATH & PACKER, 1968).

162 4.2.8 Lignin assays

163 The lignin determination was carried out according to the method described by BARBER &
164 RIDE (1988). The reaction was started with the addition of 1.5 mL of a solution of thioglycolic acid
165 (1:10) and 2N HCl. The tubes were sealed, gently shaken to hydrate the residue, and placed in a
166 water bath at 4°C for 4 hours. Then, the tubes were cooled in a bath of ice for a few minutes and
167 centrifuged at 12,000g for 10 minutes. The supernatant was discarded and the solid part washed with
168 1.5 mL of distilled water, followed by new centrifugation at 12,000g for 5 minutes. After discarding the
169 supernatant, the solid part was resuspended in 1.5 mL of 0.5N NaOH and left on a shaking table during
170 night, at room temperature. Subsequently, the mixture was centrifuged at 12,000g per 15 minutes,
171 and the supernatant transferred to a new tube, to which were added 200 μL of concentrated HCl. The
172 mixture was placed on ice (4°C) for 4 hours to precipitate thioglycolic acid bound to lignin. Then,
173 the mixture was centrifuged at 12,000g for 10 minutes, the supernatant discarded, and the solid part
174 (orange-brown colored) resuspended in 2 mL of 0.5N NaOH on a shaking table. The absorbance of the
175 solution was measured at 280 nm in the spectrophotometer, and the values calculated based on in the
176 lignin curve, with lignin expressed in grams percent (g%) of dry tissue.

177 4.3 Secondary metabolite extraction

178 The non-galled leaves, stems and galls in different developmental stages were dried in stove
179 (MedClave, model 3) at 35°C . The dry samples were extracted with ethanol. The extract was filtered
180 and the alcohol was removed by a rotary evaporator under reduced pressure (INDICAR MARCA,
181 MODELO Y PAIS). The extraction was performed on the EE (100 g) solubilized with 600 mL of
182 methanol/water (adicionar a proporção). The extraction by maceration extracts are shown in (Figure
183 4.3).

184 4.4 Phytochemical analysis

185 In order to qualitatively identify the presence of secondary metabolites in the galled and non-
186 galled samples, phytochemical prospecting was carried out with different chemical developers. The
187 samples were solubilized in methanol with a concentration of (1 mg mL^{-1}) and applied to commercial
188 plates of Thin Layer Chromatography (TLC). Two mobile phase systems were used to develop the
189 chromatographic plates. Mobile phase 1 composed of ethyl acetate:methanol:formic acid (10:1:1.1
190 v/v/v) was the most polar phase and mobile phase 2 composed of ethyl acetate:dichloromethane:formic
191 acid (5 :5:1.1 v/v/v) was the most nonpolar phase. These mobile phases were placed separately in the

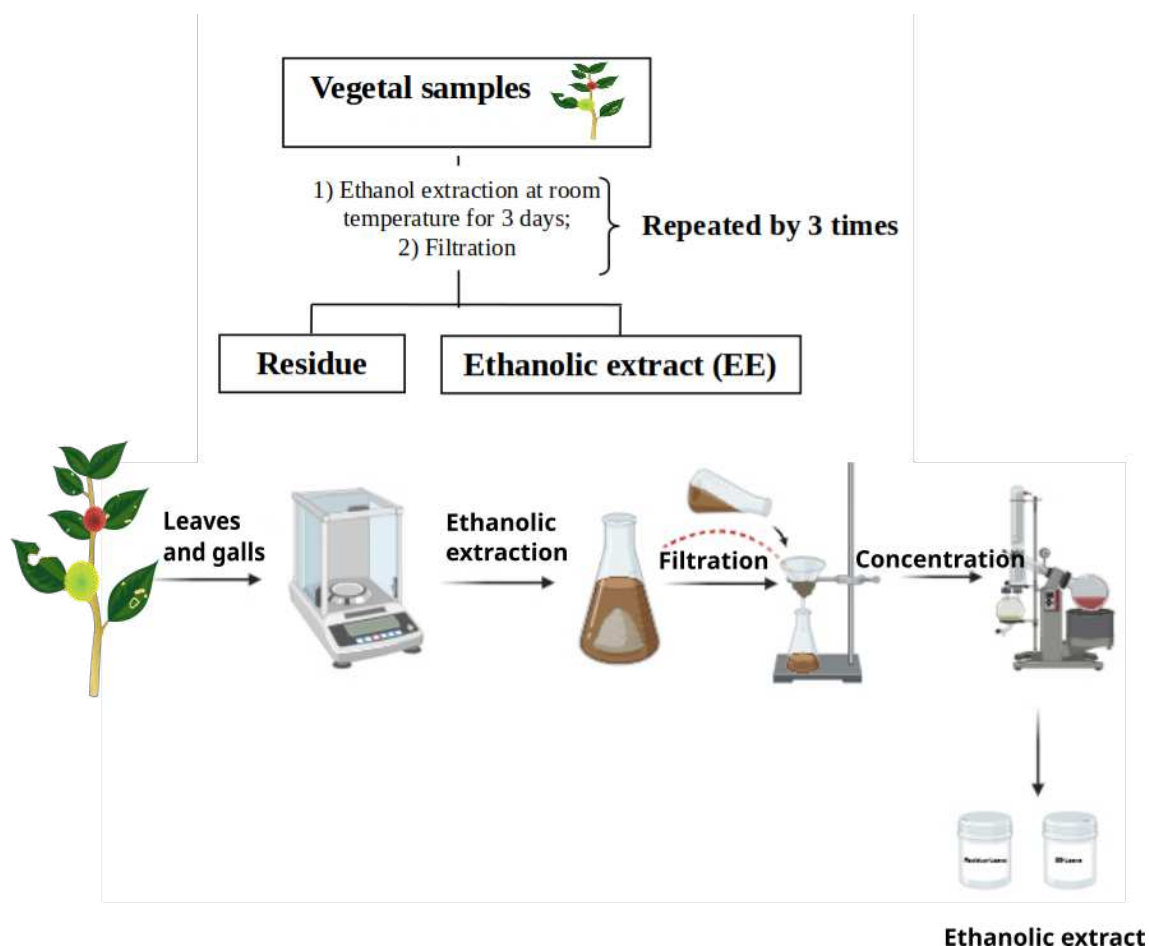


Figure 1: Ethanolic extraction schematic illustration from leaves and galls of *Macairea radula* (Melastomataceae).

vat where the chromatographic plates were made, and then they were developed with the following developers: Dragendorff and iodochloroplatinate, which indicate the presence of alkaloids, NP/PEG and $AlCl_3$, which indicate the presence of flavonoids, and the developers universal Anisaldehyde, Ceric Sulfate, Liebermann-Burchard, and vanillin sulfuric that indicate the presence of terpenes, steroids, flavonoids, phenylpropanoids, and proanthocyanidins respectively (CHAVES, 1977; WAGNER, 1996).

4.5 High performance liquid chromatography coupled to mass spectrometry tandem with electrospray ionization source (HPLC-ESI-MS/MS)

An aliquot of each sample was resuspended in methanol HPLC (5 mg mL^{-1}) and filtered through syringe filters with polytetrafluoroethylene (PTFE) membrane $0.45 \mu\text{m}$ membrane and injected into a liquid chromatograph (Agilent model Infinity 1260) coupled to an Agilent model 6520 B high-resolution Q-TOF mass spectrometer with electrospray ionization source, using an Agilent model Zorbax C18 column ($100.0 \times 3.0 \text{ mm}$, $2.7 \mu\text{L}$). The chromatographic parameters for analyzing the

205 samples were: mobile phase (A) composed of water acidified with formic acid (0.1% v v-1) and mobile
206 phase (B) methanol with the gradient starting at 10% B (0 min), 98% B (0 - 15 min) and 100% B
207 (15 - 17 min). The injection volume was 0.4 mL min⁻¹. The ionization parameters were nebulizer
208 pressure of 58 psi, drying gas at 8 L⁻¹) at a temperature of 220 °C and a power of 4.5 kVA was applied
209 to the capillary. Tandem mass spectrometry (MS/MS) analyses were carried out at different collision
210 energies (5, 10, 15, 20, 25 and 30 eV) in negative mode. The identification proposal was based on the
211 mass (m/z) at high resolution to calculate the error (ppm), comparing the fragmentation spectra with
212 literature articles and databases (Chempider, Pubchem, and Massbank).

213 4.6 Multivariate and Statistical Analysis

214 The data were analyzed using the one-way ANOVA followed by Tukey's multiple comparison
215 post test to CAT, SOD, PAL, Anion and Lignin considered statistically significant for $p < 0.05$. For
216 the enzymes APX, POX, MDA and peroxide, the Kruskal-Wallis test was performed. A multivariate
217 analysis was performed with PCA function ("FactoMineR" package) using the matrix type correlation
218 with data standardized. The hierarchical cluster was calculated with m/z ratio developed by the Ward
219 linkage method with Euclidean distance. The graphs were created with the ggplot function and fviz
220 pca biplot ("ggplot2" package). All analysis were performed using R software version 4.3.2 (2023).

221 5 Results

222 5.1 Enzymes Profiling

223 From the enzyme data obtained, we first performed a principal component analysis (PCA) to
224 gather an overview of the enzymatic profile (Figure 5.1). The graph showed the separation between
225 groups non-galled (leaves), with galls in the larval stage and galls with a natural enemy. The PCA
226 analysis of APX, POX, SOD, CAT, PAL, MDA, anion, peroxide and lignin variables in the leaves,
227 larval stage, pupal stage, natural enemy, empty gall groups resulted in 46,9% to PCA 1 and 31% to
228 PCA. Among the enzymes with the greatest contribution to component 1 were CAT and PAL (Table
229 1), which are associated with the larval stage .

230

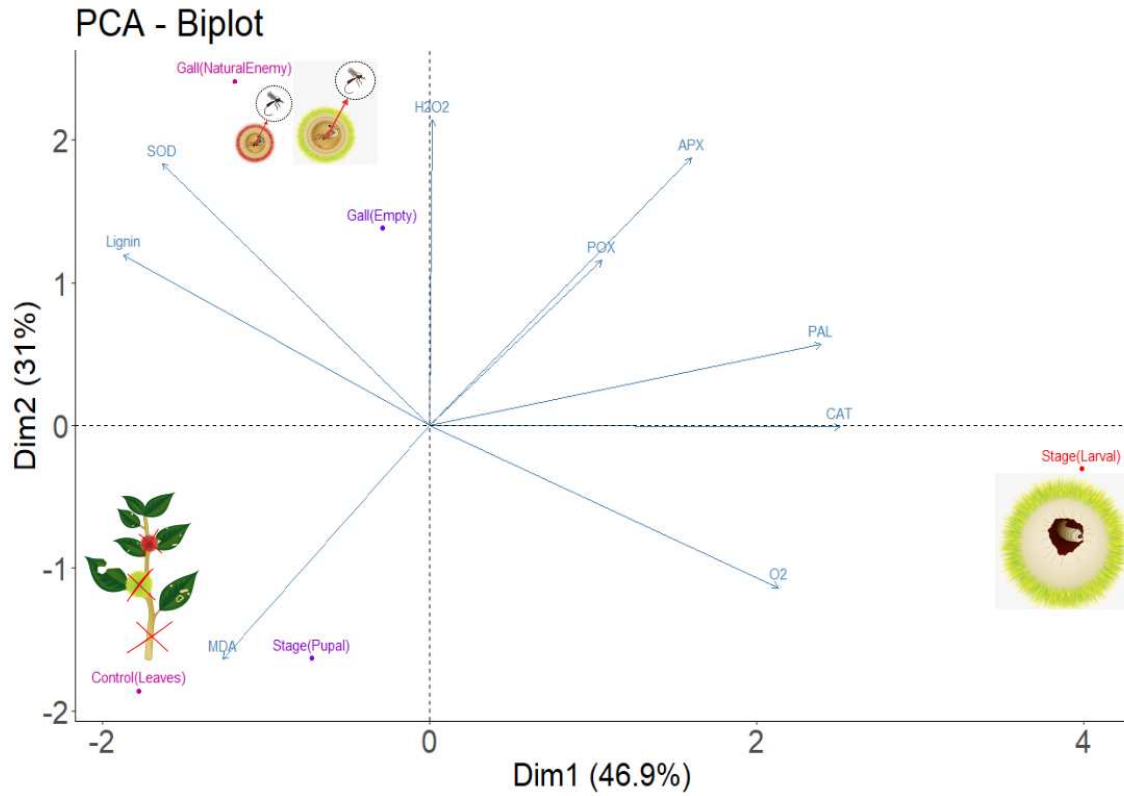


Figure 2: Biplot (PCA) from the analysis of APX, POX, SOD, CAT, PAL, MDA, Anion, Peroxide and Lignin in the groups A: leaves; B: larval stage; C: pupal stage; D: natural enemy; E: empty gall.

5.2 ROS level and lipid peroxidation

There was no significant difference between the groups regarding the levels of MDA (p-value= 0.168), anion (F-statistic: 0.984; p-value= 0.446), peroxide (p-value= 0.176) and lignin (F-statistic: 1.138; p-value: 0.376), with the exception of the group with the presence of natural enemies, which there was a greater amount of H_2O_2 . And, the MDA levels had a greater quantity in relation to the groups with galls (Figure 5.2).

5.3 Enzyme defense level

The enzyme superoxide (SOD) was that showed the greatest difference between the groups (A: leaves; B: larval stage; C: pupal stage; D: natural enemy; E: empty gall). The CAT and PAL the greatest difference was only in the larval stage. The Kruscal-Wallis test showed that there is that there is some difference between the groups on the APX variable (p-value: 0.003). The levels of POX (p-value= 0.032) and SOD (F-statistic= 24.32; p-value= 2.08e-06***) also showed significant differences. The post-hoc Tukey HSD showed that there are differences between D (natural enemy) and E (empty gall); D (natural enemy) and A (leaves) for POX and differences between groups C (pupal stage) and B (larval stage); D (natural enemy) and B (larval stage); D (natural enemy) and E (empty gall); D

Table 1: Summary of Principal Component Analysis (PCA).

Variables	Parameters (%)	
	PCA 1	PCA 2
PAL	0.929	0.022
CAT	0.974	0.004
Explained Variance	46,9%	-

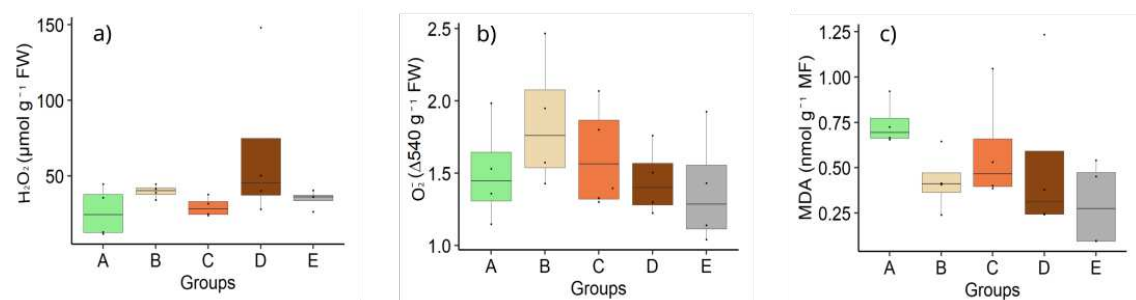


Figure 3: ROS level and lipid peroxidation evaluated in leaves and galls induced by *Palaeomystella oligophaga* on *Macairea radula*. (a) peroxide H_2O_2 ; (b) anion (O_2^-); (c) malondialdehyde (MDA) levels. A. leaves; B. larval stage; C. pupal stage; D. natural enemy; E. empty. One-way ANOVA and post-hoc Tukey HSD showed that there are no differences between the tested group ($p > 0.05$).

(natural enemy) and A (leaves); D (natural enemy) and C (pupal stage) for SOD. The post-hoc Tukey HSD showed that there are differences over group B (larval stage), but not between the other groups for CAT ($F = 12.26$; $p = 0.001$) and PAL (F -statistic: 74.42; p -value = $1.042e^*$) levels (Figure 5.3).

5.4 HPLC-ESI-MS/MS analysis and hierarchical cluster of annotated chemical compounds

A total of 50 chemical compounds from the leaves and galls of *M. radula* were annotated by HPLC-ESI-MS/MS in negative mode, including phenolic acids, flavonoids, tannins, carbohydrates, triterpene and lipids (Figure 5.4, 5.4 and Table 4). The results showed that the chemical composition of the leaves presented some flavonoids that were restricted to this group, including (epi)gallocatechin (m/z 305.0679), (epi)catechin (m/z 289.0724), myricetin-3-O-(2"-O-galloyl) glucoside (m/z 631.0955), myricetin-rhamnosyl hexoside (m/z 625.1427), myricetin-hexoside (m/z 479.0849), myricetin-acetyl-rhamnoside (m/z 625.1427), myricetin (m/z 317.0297) acetylquercitrin I and II (m/z 489.1048; m/z 489.1038), quercetin-acetylpentoside (m/z 475.0877) and the sesquiterpenoid glycoside roseoside (m/z 431.1937).

The flavonoids quercetin-3-galloyl hexoside (m/z 615.0995), myricetin-rhamnoside (m/z 463.08

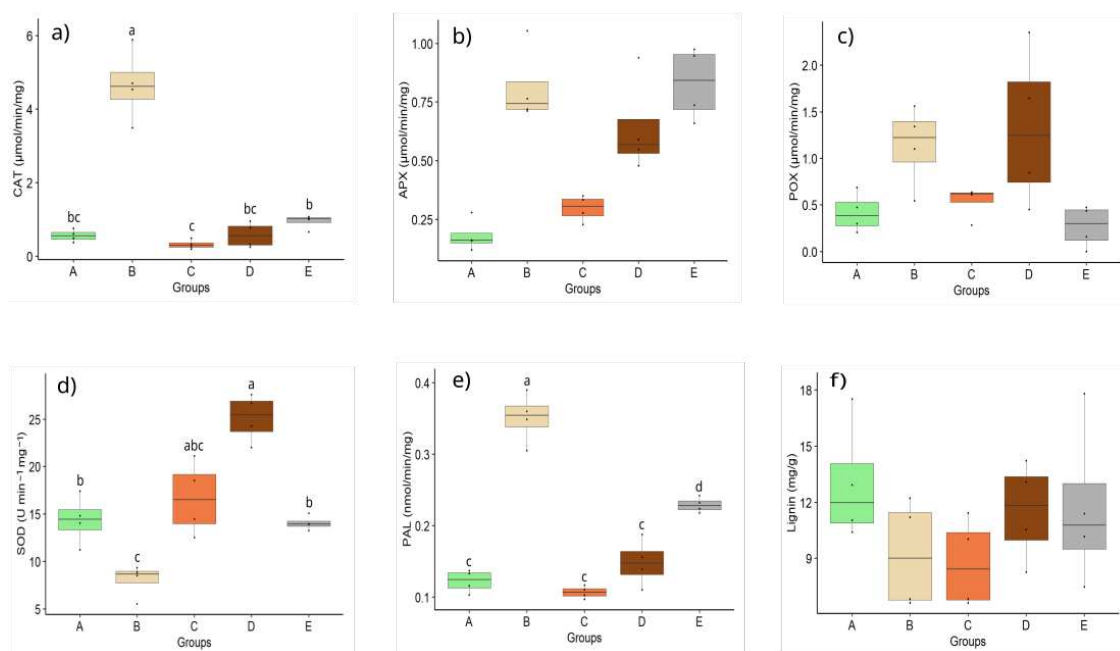


Figure 4: Enzyme quantification of leaves and galls induced by *Palaeomystella oligophaga* on *Macairea radula*. (a) CAT; (b) APX; (c) POX; (d) SOD; (e) PAL; (f) Lignin in galls induced by *P. oligophaga* on *M. radula*. A. leaves; B. larval stage; C. pupal stage; D. natural enemy; E. empty gall. One-way ANOVA and post-hoc Tukey HSD showed that there are differences between the tested group. But not among the groups evaluated for lignin ($p>0.05$).

92), quercetin-3-O-hexose-deoxyhexose (m/z 609.1476) and quercetin-3-O-pentoside I (m/z 433.0783) were found in both non-galled and galled tissues. Only in the galls were detected the hydrolyzable tannins including terflavin A (m/z 1085.0722) and valoneic acid dilactone (m/z 469.0059). Gallic acid ethyl ester (m/z 197.0461) were found only in the gall also. Among other compounds such as lipid such as linolenic acid (m/z 279.2335), palmitic acid (m/z 255.2328), oleic acid (m/z 281.2483) were found in all tissues, as also carbohydrates, including hexose (m/z 179.0568), others compounds such as quinic acid (m/z 191.0567), gallic acid (m/z 169.0142), pedunculagin-galloyl-HHDP-glucose (alnus-japonin A) (m/z 1415.1277), pedunculagin isomer I (m/z 783.0675), galloyl-HHDP-glucose isomer II (m/z 633.0733), digalloylglucose isomer I (m/z 483.0788), camellioferin A (m/z 1719.1498), ellagic acid (m/z 300.9994), quercetin-3-O-rhamnoside (m/z 447.0936); Quercetin (m/z 301.0354), Triterpene pentacyclic (m/z 487.3436) were detected in all tissues.

The hierarchical map showed the different compounds separated into lines by colors with different intensities, where the compounds Terflavin A and Valoneic acid dialactone were restricted to the galls (Figure 5.4). While the flavonoids Myricetin rhamnohexoside, Myricetin glucoside and two unknown compounds 1 and 2 were exclusively noted in the leaves. The tannins compounds, pedunculagin isomer I and II were detected in the galls and leaves. And, an unknown compound 3 was noted only for the green gall group with natural enemy. In addition to these findings, the map also showed a horizontal cluster where groups red gall without natural enemy (G1), green gall without

279 natural enemy (G2), red gall with natural enemy (G3) and green gall with natural enemy (G5) had
 280 more compounds in common. Also, a second cluster showing that the leaves have more compound
 281 similarities with the groups of green and red galls with the presence of natural enemies (G3 and G5).

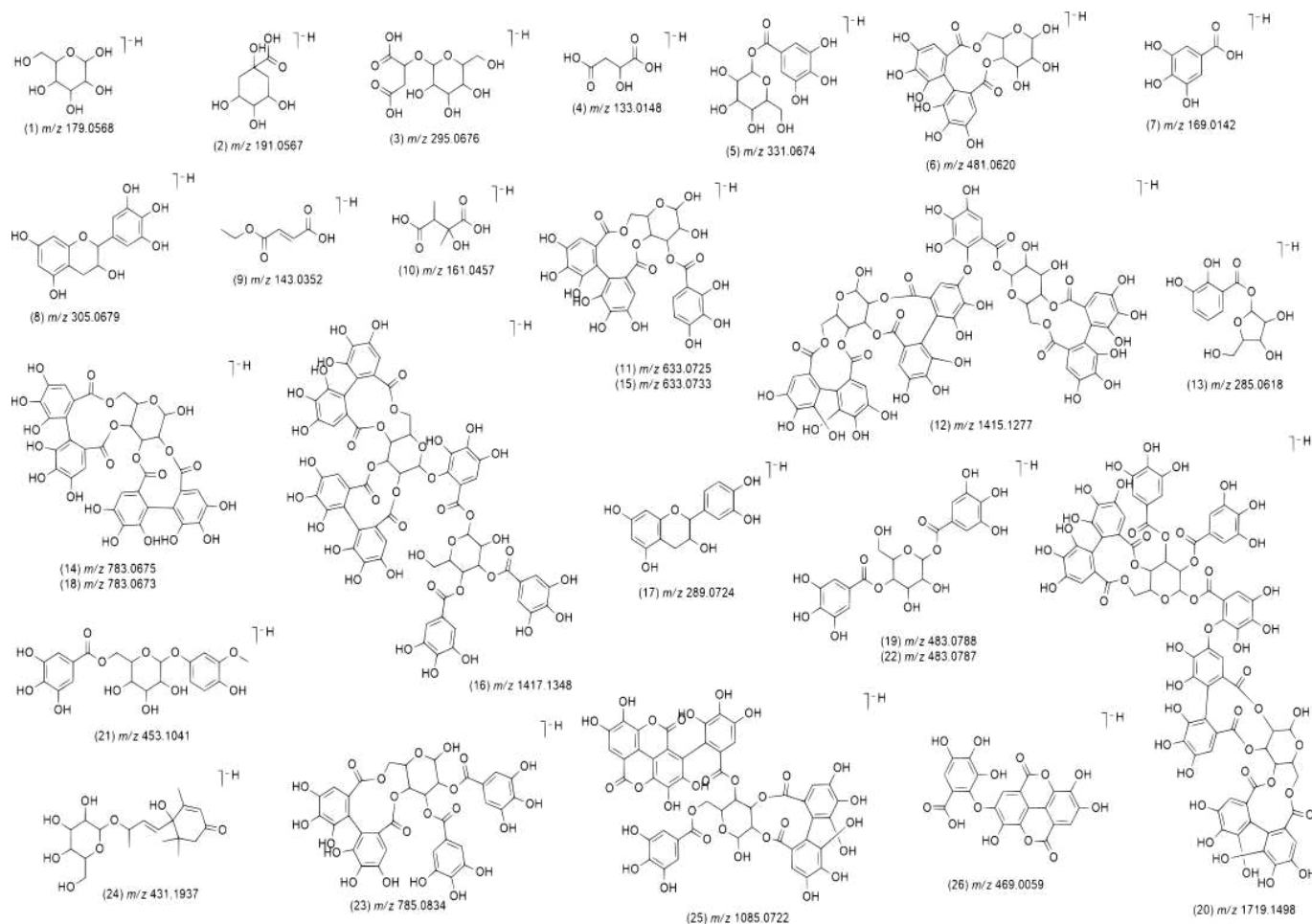


Figure 5: Structure of compounds annotated by HPLC-ESI-MS/MS in galls and leaves of *M. radula*. continue

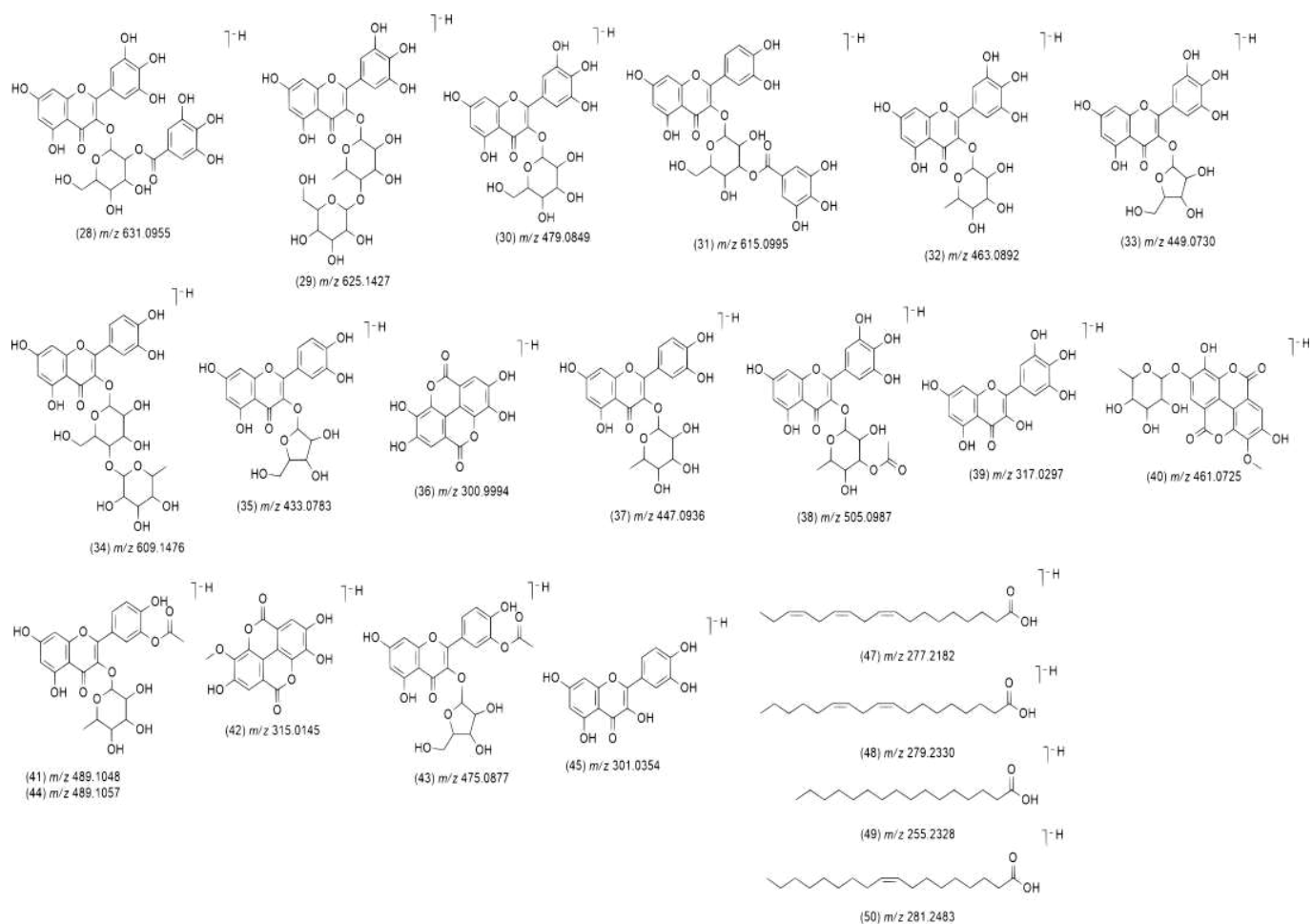


Figure 6: Structure of compounds annotated by HPLC-ESI-MS/MS in galls and leaves of *M. radula*. conclusion

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Table 2: Proposal for the identification of metabolites from Lepidoptera galls induced on branches of *M. radula* (Melastomataceae).

N ^o	Tentative identify	Parameters				Sample	Reference
		$T_{R(min)}$	$[M - H]^-$	Extract mass Error (ppm)	Fragment ions (m/z) MS/MS		
1	Hexose	1.11	179.0568	179.0561	3.90	5 eV: 143, 119, 101, 89, 71, 59	$C_6H_{12}O_6$ G1, G2, G3, G4, G5, G6, G7 and L.
2	Quinic acid	1.16	191.0567 295.0676	191.0561 295.0671	3.14 1.69	10 eV: 173, 129, 111 , 87, 85, 57 10 eV: 179, 133, 89 , 71	$C_7H_{12}O_6$ $C_{10}H_{16}O_{10}$ (LLORENT-MARTINEZ <i>et al.</i> , 2015) (SILVA <i>et al.</i> , 2019)
3	Malic acid-hexoside	1.16	295.0676	295.0671	1.69	10 eV: 179, 133, 89 , 71	$C_{10}H_{16}O_{10}$ (FRATERNALE <i>et al.</i> , 2015)
4	Malic acid	1.30	133.0148	133.0142	4.5	10 eV: 115, 89, 71	$C_4H_6O_5$ G1, G2, G3, G4, G5, G6, G7 and L.
5	Galloyl-glucose isomer	2.51	331.0674	331.0671	0.90	25 eV: 271, 211, 169 , 151, 125, 123, 89, 59	$C_{13}H_{16}O_{10}$ (SOBEH <i>et al.</i> , 2019)
6	HHDP-hexoside	2.51	481.0620	481.0624	-0.83	25 eV: 301 , 275	$C_{20}H_{18}O_{14}$ (MENA <i>et al.</i> , 2012)
7	Gallic acid	2.58	169.0142	169.0142	0.00	15 eV: 125	$C_7H_6O_5$ G1, G2, G3, G4, G5, G6, G7 and L.
8	(Epi)galocatechin	2.58	305.0679	305.0667	3.9	15 eV: 287, 261, 219, 197, 167, 139, 125	$C_{15}H_{14}O_7$ Leaves (SINOSAKI <i>et al.</i> , 2020)
9	4-Ethoxy-4-oxobut-2-enoic acid	2.98	143.0352	143.0350	1.40	10 eV: 99 , 84, 71, 55	$C_6H_8O_4$ (ESCOBAR-AVELLO <i>et al.</i> , 2019)
10	2,3-Dimethylmalate	2.98	161.0457	161.0455	1.24	5 eV: 143 , 99, 87, 71	$C_6H_{10}O_5$ G1, G2, G3, G4, G5, G6 and G7 Mass Bank ^a
-	Unknown	3.93	219.0520	-	-	10 eV: 157, 111 , 87, 67	G1, G2, G3, G4, G5, G6, G7 and L. Mass Bank ^b
11	Galloyl-HHDP-glucose isomer I	3.93	633.0725	633.0733	-1.26	30 eV: 301 , 275, 169	$C_{27}H_{22}O_{18}$ G1, G2, G3, G4, G5, G6, G7 and L. (MENA <i>et al.</i> , 2012), (PEREIRA <i>et al.</i> , 2017)
-	Unknown	4.13	1249.5857	-	-	30 eV: 1098, 933, 783, 633, 481, 301	G1, G2, G3, G4, G5, G6, G7 and L. -
12	Pedunculagin-galloyl-HHDP-glucose (<i>Atrusjaponin</i> A)	4.34	1415.1277	1415.1336	-4.12	-	$C_{61}H_{44}O_{40}$ G1, G2, G3, G4, G5, G6, G7 and L. (MENA <i>et al.</i> , 2012), (KHALIFA <i>et al.</i> , 2020)
13	Dihydroxybenzoic acid pentose	4.34	285.0618	285.0616	0.70	30 eV: 631, 481, 301 15 eV: 153, 152 , 108	$C_{12}H_{14}O_8$ G1, G2, G3, G4, G5, G6, G7 and L. Mass Bank ^c
14	Pedunculagin isomer I	4.41	783.0675	783.0686	-1.34	30 eV: 765, 745, 633, 481, 301 , 275	$C_{34}H_{24}O_{22}$ G1, G2, G3, G4, G5, G6, G7 and L. (BEELDERS <i>et al.</i> , 2014) (MENA <i>et al.</i> , 2012) (SINGH <i>et al.</i> , 2016)
15	Galloyl-HHDP-glucose	4.59	633.0733	633.0733	0.00	30 eV: 481, 301 , 275	$C_{27}H_{22}O_{18}$ G1, G2, G3, G4, G5, G6, G7 and L. (MENA <i>et al.</i> , 2012), (PEREIRA <i>et al.</i> , 2017)
16	Pedunculagin-trigalloyl-glucose	4.59	1417.1348 708.0700	1417.1348 708.0710	0.00 -1.41	- 25 eV: 633, 529, 481, 421, 301 , 191	G1, G2, G3, G4, G5, G6, G7 and L. Based on compound 2
17	(Epi)catechin	4.69	289.0724	289.0718	2.01	20 eV: 245, 221, 205, 203, 187, 175, 151 , 125, 109	$C_{15}H_{14}O_6$ L. (ESCOBAR-AVELLO <i>et al.</i> , 2019)
-	Unknown	4.71	1249.5862	-	-	30 eV: 1098, 933, 783, 633, 481, 301	G1, G2, G3, G4, G5, G6, G7 and L. -

^a<https://massbank.eu/MassBank/>
^b<https://massbank.eu/MassBank/>
^c<https://massbank.eu/MassBank/>

Table 3: Table continue

N ^o	Tentative identify	$T_{R(min)}$	$[M - H]^-$	Extract mass Error (ppm)	Parameters			Reference
					Fragment ions (m/z)	Molecular formula	Sample	
MS/MS								
18	Pedunculagin isomer II	4.76	783.0673	783.0686	-1.66	30 eV: 765, 745, 633, 481, 301 , 275	$C_{34}H_{24}O_{22}$	G1, G2, G3, G4, G5, G6, G7 and L. (MENA <i>et al.</i> , 2012) (SINGH <i>et al.</i> , 2016)
19	Digalloylglucose isomer I	4.87	483.0788	483.0780	1.65	30 eV: 331, 313, 271, 211, 169 , 124	$C_{20}H_{20}O_{14}$	G1, G2, G3, G4, G5, G6, G7 and L. (SINGH <i>et al.</i> , 2016)
20	Camellioferin A	4.94	1719.1498	1719.1555	-3.31	-	$C_{75}H_{52}O_{48}$	30 eV: 783, 765, 633, 481, 301 , 275, 169 PubChem proposito a partir do m/z 1415
21	3-Methoxy-4-hydroxyphenol-1-O-(6'-O-galloyl)-glucoside	5.14	453.1041	453.1038	0.66	30 eV: 313, 297, 267, 169 , 151, 139, 124	$C_{20}H_{22}O_{12}$	G1, G2, G3, G4, G5, G6, G7 and L. (SINGH <i>et al.</i> , 2016)
-	Unknown	5.14	417.1049	-	-	25 eV: 343, 285, 241, 152 , 109	-	-
22	Digalloylglucose isomer II	5.29	483.0787	483.0780	1.45	241, 152 , 109	-	G1, G2, G3, G4, G5, G6, G7 and L.
-	Unknown	5.34	1176.6049	-	-	30 eV: 1091, 783, 633, 481, 301	$C_{20}H_{20}O_{14}$	G1, G2, G3, G4, G5, G6, G7 and L. (SINGH <i>et al.</i> , 2016)
-	Unknown	5.34	1411.6071	-	-	30 eV: 1389, 633, 425, 264	-	G1, G2, G3, G4, G5, G6, G7 and L.
23	Digalloyl HHDP glucose	5.56	785.0834	785.0843	-1.15	30 eV: 633, 483, 301 , 275, 169	$C_{34}H_{26}O_{22}$	G1, G2, G3, G4, G5, G6, G7 and L. (SINGH <i>et al.</i> , 2016)
-	Unknown	5.69	1325.5897	-	-	30 eV: 935, 783, 633, 481, 301	-	G1, G2, G3, G4, G5, G6, G7 and L.
24	Roseoside (formate adduct)	5.71	431.1937	431.1923	3.24	20 eV: 385, 223, 205, 153	$C_{19}H_{30}O_2$ HCOOH	L. (SPINOLA & CASTILHO, 2016)
25	Terflavin A	5.87	1085.0722	1085.0749	-2.48	-	$C_{48}H_{30}O_{30}$	G1, G2, G3, G4, G5, G6, G7 (SINGH <i>et al.</i> , 2016)
26	Valoneic acid dilactone	5.91	469.0059	469.0049	2.13	15 eV: 451, 301 , 300, 191 30 eV: 301, 300	$C_{21}H_{10}O_{13}$	G1, G2, G3, G4, G5, G6, G7 (SILVA <i>et al.</i> , 2019)
27	Gallic acid ethyl ester	6.07	197.0461	197.0455	3.0	20 eV: 169, 125, 124 , 78	$C_9H_{10}O_5$	G1, G2, G3, G4, G5, G6, G7 (ESCOBAR-AVELLO <i>et al.</i> , 2019)
28	Myricetin-3-O-(2''-O-galloyl) glucoside	6.29	631.0955	631.0941	2.21	25 eV: 479 , 316	$C_9H_{24}O_{17}$	L. (NEGRI & TABACH, 2013)
-	Unknown	6.56	477.0691	-	-	30 eV: 314 , 298, 271	-	G1, G2, G3, G4, G5, G6, G7 and L.
29	Myricetin-rhamnohexoside	6.66	625.1427	625.1410	2.72	30 eV: 316	$C_{27}H_{30}O_{17}$	L. (BYSTROM <i>et al.</i> , 2008)
30	Myricetin-hexoside	6.72	479.0849	479.0831	3.75	30 eV: 317, 316	$C_{22}H_{22}O_{13}$	L. (PEREIRA <i>et al.</i> , 2017)
31	Quercetin-3-galloyl hexoside	6.87	615.0995	615.0992	0.49	30 eV: 463 , 301, 300	$C_{28}H_{24}O_{16}$	G1, G2, G3, G4, G5, G6, G7 and L. (LI & SEERAM, 2018)
32	Myricetin-rhamnoside	7.01	463.0892	463.0882	2.15	25 eV: 316 , 271	$C_{21}H_{20}O_{12}$	G1, G2, G3, G4, G5, G6, G7 and L. (PEREIRA <i>et al.</i> , 2017)
33	Myricetin-pentoside	7.01	449.0730	449.0730	0.00	25 eV: 316 , 271	$C_{20}H_{18}O_{12}$	G1, G2, G3, G4, G5, G6, G7 and L. (SALDANHA <i>et al.</i> , 2013)

Table 4: Table continue

No	Tentative identity	$T_{R(min)}$	$[M - H]^-$	Extract mass	Error (ppm)	Parameters		Molecular formula	Sample	Reference
						Fragment ions (m/z)	MS/MS			
34	Quercetin-3-O-hexose-deoxyhexose	7.21	609.1476	609.1461	2.46	30 eV: 301, 300 , 271		$C_{27}H_{30}O_{16}$	G1, G2, G3, G4, G5, G6, G7 and L.	(Li <i>et al.</i> , 2016)
-	Unknown	7.29	435.1302	-	-	30 eV: 271, 211, 169 , 151, 124		-	G1, G2, G3, G4, G5, G6, G7	-
35	Quercetin-3-O-pentose I	7.42	433.0783	433.0776	1.62	25 eV: 301, 300 , 271, 255		$C_{20}H_{17}O_{11}$	G1, G2, G3, G4, G5, G6, G7 and L.	(Li & SEERAM, 2018)
36	Ellagic acid	7.51	300.9994	300.9990	1.33	30 eV: 283, 257, 229, 201, 185, 173, 145, 141		$C_{14}H_5O_8$	G1, G2, G3, G4, G5, G6, G7 and L.	(Li & SEERAM, 2018)
37	Quercetin-3-O-rhamnoside	7.69	447.0936	447.0933	0.67	30 eV: 301, 300 , 271, 255, 179, 151		$C_{21}H_{20}O_{11}$	G1, G2, G3, G4, G5, G6, G7 and L.	(MENA <i>et al.</i> , 2012)
38	Myricetin-acetylfrhannoside	7.77	505.0987	505.0988	-0.20	25 eV: 316		$C_{23}H_{22}O_{13}$	L.	(Li & SEERAM, 2018) PubChem ^a
39	Myricetin	7.89	317.0297	317.0297	0.00	20 eV: 178, 151 , 137		$C_{15}H_{10}O_8$	L.	(PEREIRA <i>et al.</i> , 2017)
40	3'-O-Methyl-4-O-(rhamnopyranosyl) ellagic acid	7.97	461.0725	461.0725	0.00	20 eV: 315 , 300		$C_{21}H_{18}O_{12}$	G1, G2, G3, G4, G5, G6, G7 and L.	(SINGH <i>et al.</i> , 2016)
41	Acetylquercitrin I	8.12	489.1048	489.1038	2.04	20 eV: 301, 300		$C_{23}H_{22}O_{12}$	L.	PubChem
42	3-O-Methyl ellagic acid	8.22	315.0145	315.0146	-0.32	20 eV: 300		$C_{15}H_8O_8$	G1, G2, G3, G4, G5, G6, G7 and L.	(SINGH <i>et al.</i> , 2016)
43	Quercetin-acetylpenoside	8.24	475.0877	475.0882	1.05	25 eV: 300 , 271		$C_{22}H_{20}O_{12}$	L.	PubChem
44	Acetylquercitrin II	8.37	489.1057	489.1038	3.88	20 eV: 346, 301, 300 , 271, 209, 179		$C_{23}H_{22}O_{12}$	L.	PubChem
45	Quercetin	8.45	301.0354	301.0354	0.00	25 eV: 273, 229, 179, 151 , 121, 107		$C_{15}H_{10}O_7$	G1, G2, G3, G4, G5, G6, G7 and L.	(ABU-REIDAH <i>et al.</i> , 2015)
-	Unknown	10.78	429.1185	-	-	30 eV: 414, 399, 330, 315 , 287,		-	G1, G2, G3, G4, G5, G6, G7 and L.	-
-	Unknown	11.25	623.3557	-	-	30 eV: 555, 529, 487, 283		-	G1, G2, G3, G4, G5, G6, G7	-
46	Triterpene pentacyclic I	11.37	487.3436	487.3429	1.47	-		$C_{30}H_{48}O_5$	G1, G2, G3, G4, G5, G6, G7 and L.	(SUT <i>et al.</i> , 2018) Mass Bank ^b
-	Unknown	11.78	443.1338	-	-	30 eV: 428, 413, 359, 344, 329		G1, G2, G3, G4, 359, 344, 329	G5, G6, G7 and L.	-
-	Unknown	12.58	723.4134	-	-	20 eV: 677, 415, 397 , 279, 121		-	G1, G2, G3, G4, G5, G6, G7 and L.	-
-	Unknown	13.38	691.4238	-	-	25 eV: 265, 157, 105		-	G5	-
47	Linolenic acid		277.2182	277.2173	3.24	-		$C_{18}H_{30}O_2$	G1, G2, G3, G4, G5, G6, G7 and L.	Mass Bank
48	Linolenic acid	13.92	279.2335	279.2330	1.79	-		$C_{18}H_{32}O_2$	G1, G2, G3, G4, G5, G6, G7 and L.	Mass Bank
49	Palmitic acid	14.32	255.2328	255.2330	-0.78	-		$C_{16}H_{32}O_2$	G1, G2, G3, G4, G5, G6, G7 and L.	(GOMES <i>et al.</i> , 2020)
50	Oleic acid	14.57	281.2483	281.2486	-1.06	-		$C_{18}H_{34}O_2$	G1, G2, G3, G4, G5, G6, G7 and L.	(GOMES <i>et al.</i> , 2020)

^a<https://pubchem.ncbi.nlm.nih.gov/>^b<https://massbank.eu/MassBank/>

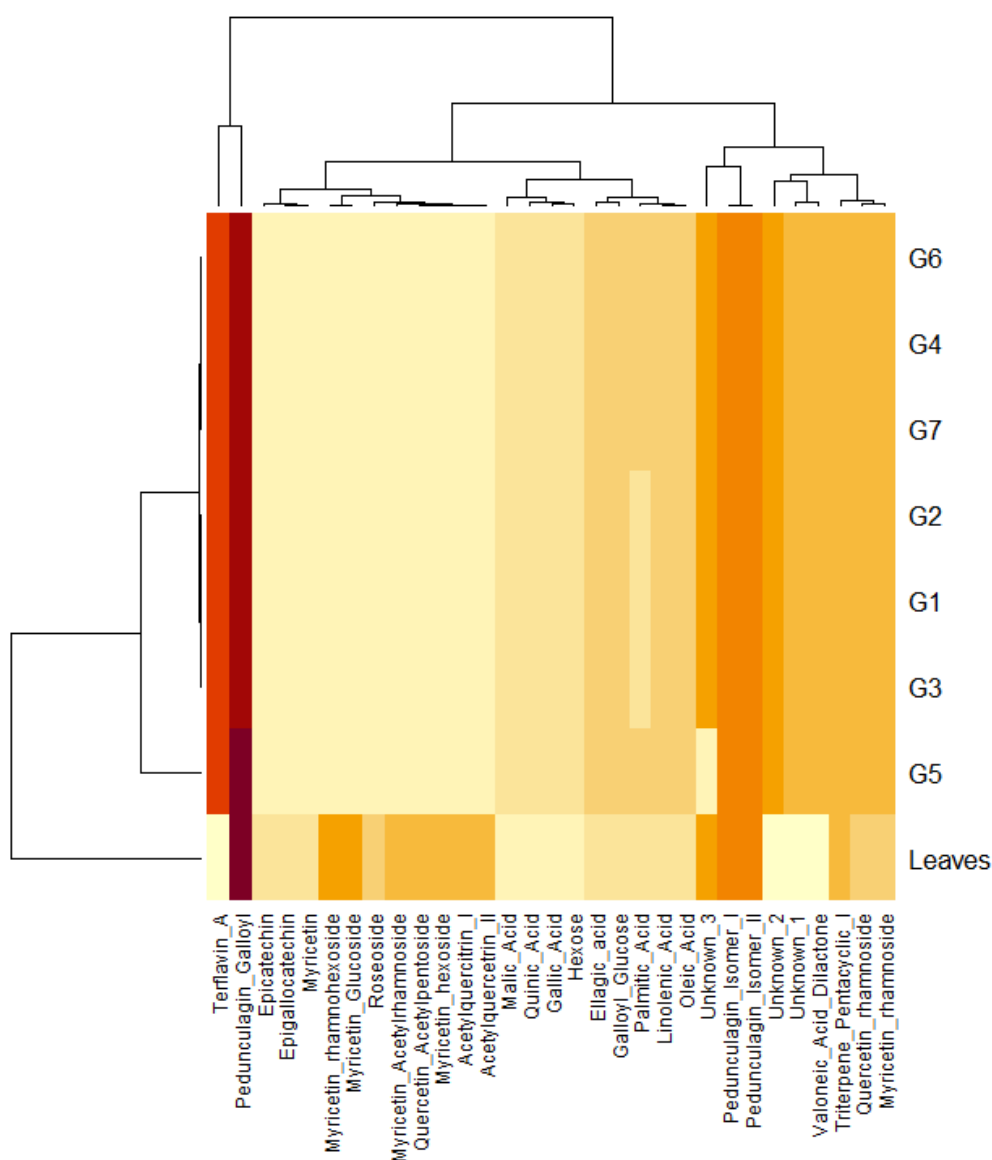


Figure 7: Hierarchical cluster heat map of the chemical compounds. The colors represent the intensities of the correlations, the darker colors have lower values and the weaker colors have higher values. The row indicates the groups separated into non-galled (leaves) and galled, and the column represents the chemical compounds

285 6 Discussion

286 The biotic stress caused by galling insects causes changes in the cell wall and membrane
287 of the host plant (MARTINI *et al.*, 2019; RAMAN, 2011; SILVA *et al.*, 2021). This process produce
288 oxidative stress signaling molecules and lipid oxidation products are one of the first physiological
289 responses of plant defense, being an indirect way to assess the level of oxidative stress in plants, and
290 insect galls (IHTISHAM *et al.*, 2023; NAVES *et al.*, 2021). In this study using the *Macairea radula* gall
291 system, the galling insect has a chewing habit that generates greater stimulation and stress in the
292 nutritive tissue (NAVES *et al.*, 2021; REZENDE *et al.*, 2019). This same stress can generate hydrogen
293 peroxide molecules, which can be converted by the enzymes into water and oxygen (CERNY *et al.*,
294 2018; HUBMANN *et al.*, 2022). Also, it can also be used as a substrate for the biosynthesis of phenolic
295 compounds such as lignin (FERRIZ-MARTINEZ *et al.*, 2023; WANG *et al.*, 2022). Thus, the system is
296 maintained and protected during gall development, whose these enzymes and sencondary metabolites
297 correspond to protective mechanisms non enzymatic and enzymatic defense system (HASANUZZAMAN
298 *et al.*, 2020; IRATO & SANTOVITO, 2021b; LEE *et al.*, 2007; MITTLER *et al.*, 2011). The superoxide
299 dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) correspond
300 to the enzymatic defense system in which the SOD starts the dectoxification process, and the CAT
301 converts peroxide HO_2 into HO_2 and O_2 , as well as APX and POX enzymes (TANG *et al.*, 2006). The
302 non-enzymatic antioxidants include molecules of secondary metabolism, such as phenolic compounds
303 which will be discussed in the next topic.

304 6.1 Oxidative stress during gall development and enemies attack

305 The ROS level and lipid peroxidation in gall induced on *M. radula* show that there is a
306 balance to control oxidative stress by the antioxidant systems since there was no significant difference
307 between the leaves and galls. However, a higher hydrogen peroxide content was observed in the group
308 of galls with the presence of natural enemies. These organisms are probably exerting some additional
309 metabolic activity in the system. And low amount of ROS and MDA can be explained by antioxidant
310 enzyme defenses and secondary metabolites found in the study. In open galls induced by *Obolodiplosis*
311 *robiniae* (Diptera) on *Robinia pseudoacacia* (Fabaceae), the ROS levels were also reduced, probably
312 through the ascorbate or glutathione peroxidase pathway (STASZAK *et al.*, 2023). In another system
313 of galls induced by *Trioza fletcheri* (Hemiptera) on *Terminalia arjuna* (Combretaceae), a greater
314 production of hydrogen peroxide was also observed in relation to non-infested leaves, showing that
315 these molecules indicate the first biochemical signs of protection not only to the galls, but also to
316 the host plant (KAR *et al.*, 2012). This protection can occur through different metabolic pathways
317 that might vary depending on the study system and environmental conditions. For example, anions

because they have a strong oxidant thermodynamic function, are not so reactive with most cellular components. In other words, they are a selective oxidant because they can be a substrate for SOD enzymes and, although they are not reactive with some cell structures, they can be with metals, such as iron and sulfur. When this complex of anion, enzyme and metals occurs in the lipid membrane, they can achieve greater chemical stability (SMETHURST & SHCHERBIK, 2021). In this work with *M. radula* galls, it is likely that the reduction in ROS levels is related to the greater quantity of other enzymes evaluated here, with the greater activity of catalase (CAT), superoxide dismutase (SOD) and phenylalanine ammonia-lyase (PAL), which will be addressed in the next subtopic.

6.2 The enzymatic defense pathway in galls

The enzymes catalase (CAT), superoxide dismutase (SOD) and phenylalanine ammonia-lyase (PAL) showed the greatest differences among all groups tested, especially the catalase (CAT) and phenylalanine ammonia-lyase (PAL), which had a larger quantity in the larval stage. These enzymes are considered biochemical markers of plant resistance and can be found in the saliva of aphids that cause galls and affect plant growth (GULSEN *et al.*, 2010; KUTSUKAKE *et al.*, 2019; NICHOLSON & PUTERKA, 2014). The greater amounts of CAT and PAL in the larval stage may be associated with the main trophic stage of the gall inducer, which, during stress, may lead to greater proliferation of peroxisomes. Catalase activity may be an adaptive response to avoid metabolic damage to galled tissues and reduce the amount of reactive oxygen species (MHAMDI *et al.*, 2010; SRIVALLI & VISHANATHAN, 2003). These enzymes and many others may be present in the saliva of inducer insects to aid in the disintegration of the cuticle and cell wall of the host plant during infestation and gall induction (DESNITSKIY *et al.*, 2023). The gall development may activate different metabolic pathways for defense and formation of new plant tissues with different shapes and colors, with activation of phenylalanine ammonia lyase (PAL), which is the key enzyme in the phenolic compound pathway in response to biotic stress caused by insects (AL-KHAYRI *et al.*, 2023; MARCHICA *et al.*, 2020).

6.3 The nonenzymatic defense pathway and chemical compounds in galls.

The chemical compounds such as phenolic acids, flavonoids, tannins, carbohydrates, and lipids found in the leaves and galls of *M. radula* can be associated with various biological functions, including defense responses to biotic and abiotic stress (SAMEC *et al.*, 2021), allelopathy (JORGE *et al.*, 2022), defense against herbivorous insects (SALMINEN & KARONEN, 2011), and pollinator attraction, for example (BARBERIS *et al.*, 2023). Flavonoids found in the leaves such as Myricetin-3-O-(2“-O-galloyl) glucoside, Myricetin-rhamno-hexoside, Myricetin-hexoside, Myricetin-acetylramnoside,

350 Myricetin, Acetylquercitrin I, Quercetin-acetylpentoside , Acetylquercitrin may be related to differ-
 351 ent metabolic pathways for host plant signalling and also oxidative defense. These compounds act
 352 as defenses by directly eliminating some reactive species, including hydroxyl, peroxy and superoxide
 353 radicals. In addition, they can bind to metals such as iron and copper, preventing the formation of
 354 ROS (DUMANOVIC *et al.*, 2021; JOMOVA *et al.*, 2019; KHOKHAR & OWUSU-APENTEN, 2003).

355 In galls induced on leaves of *Tibouchina pulchra* (Melastomataceae) by a lepidoptera gall-
 356 inducing despite of a lower quantity of carbohydrates detected, a greater quantity of soluble phenols,
 357 tannins, lignins, fibers, soluble carbohydrates and lipids was found in comparison with non-galled
 358 leaves, and no flavonoids were detected (MOTTA *et al.*, 2005). However, in this study we found dis-
 359 tinct results, with the presence of flavonoids with Quercetin-3-galloyl hexoside, Myricetin-rhamnoside,
 360 Quercetin-3-O-hexose-deoxyhexose, Quercetin-3-O-pentoside I detected in both non-galled and galled
 361 tissues.

362 Phenolic compounds have been commonly detected in several gall systems, especially in the
 363 outer tissues of the galls (KUSTER *et al.*, 2020). In a general way there is a increase of phenolic
 364 compounds on gall tissues, as occur in galls of *D. gallaeformans* on *M. albicans* and *M. ibaguensis*
 365 (Melastomataceae) compared to non-galled tissues (FERREIRA *et al.*, 2018) and, also on galls induced
 366 by *Leptocybe invasa* (Hemynoptera) on *Eucalyptus camaldulensis* (Myrtaceae) (ISAIAS *et al.*, 2018).
 367 Although phenolic compounds are associated to different biological function, especially defense against
 368 natural enemies (DIVEKAR *et al.*, 2022), they develop an important defensive role against oxidative
 369 stress, allowing the development of the gall structure (ISAIAS *et al.*, 2015). The different classes of
 370 flavonoids found here corroborate the idea of protection against oxidative stress in galls, as a nonen-
 371 zymatic via. As an example, phenolic compounds protecting the gall tissue against UV-B radiation
 372 (GUEDES *et al.*, 2024) and high light exposition (BOMFIM *et al.*, 2019). Herein, only in the galls of
 373 *M. radula* were detected the hydrolyzable tannins including Terflavin A and Valoneic acid dilactone.
 374 These compounds can be characterized by their solubility in water, having five to seven aromatic rings
 375 with the ability to precipitate some alkaloids and proteins, thus providing chemical defense against
 376 herbivores (HASLAM & CAI, 1994; OKUDA & HIDEYUKI, 2011). These condensed and hydrolyzable
 377 tannins have already been found in Cynipidae gall-wasp (ABRAHAMSON *et al.*, 2003).

378 The ecological function of these tannins were tested in studies involving, especially ellagitan-
 379 nins, have defensive effects against herbivores. The study of two generalist species (*Spodoptera exigua*)
 380 and one specialist (*Schinia florida*) showed that these compounds had effects on both groups, with
 381 a stronger effect on the generalist species (ANSTETT *et al.*, 2019). Othes studies involving parasitic
 382 relationship with leaf galls induced in tobacco leaves were evaluated for their chemical composition
 383 and compared with uninfected leaves, in which some acids, such as chlorogenic acid, were found in
 384 both tissues, but only caffeic acid was detected in greater quantities in leaf galls, as well as a coumarin

derivative, 7-O-methyl-6-O-(3-D-glucopyranosyl coumarin (7-methyl esculin) in the meristematic regions, locally influencing cell division of the host plant (VEREECKE *et al.*, 1997). Interestingly, phenolic compounds are also found in raw and heat-treated seeds. Ellagitannins, gallotannins, phenolic glycosides, hydroxybenzoic acid derivatives and ellagic acid derivatives were found in *Quercus rubra* seeds, with ellagitannins being predominant in raw seeds and ellagic acid derivatives in heat-treated seeds (ORACZ *et al.*, 2023). Also, galls induced in *Tamarix aphylla* (Tamaricaceae) were chemically evaluated and hydrolyzable tannins and low molecular weight phenolic compounds associated with tannins were detected and isolated (ORABI *et al.*, 2015).

The gallic acid ethyl found only in the gall can has its biosynthesis occurs through the shikimate biosynthetic route with the formation of shikimic acid, which is used in the formation of aromatic amino acids such as L-phenylalanine, which participates in the biosynthesis of gallic acid (BADHANI *et al.*, 2015). Among other compounds such as lipid such as Linolenic acid, Palmitic acid, Oleic acid were found in all tissues, as also carbohydrates, including hexose. These lipid compounds act as signaling agents, precursors of secondary metabolites and modulators of cell membranes, carbon and energy reserves. In addition, they have the function of an extracellular barrier to protect the gall structure (BOUCHNAK *et al.*, 2023). And, for the *M. radula* gall system it contributes to dissipating stress and keeping the galling insect on the host plant.

7 Conclusions

We concluded that the catalase (CAT) and phenylalanine ammonia-lyase (PAL) contributed to the greatest difference between leaves and galls in their different stages, especially in the larval stage, suggesting that they protect the initial stage of the gall for the maintenance of the galling insect. While, the superoxide dismutase (SOD) contributed to the greatest difference between leaves and galls with the presence of natural enemies, showing that perhaps these organisms are also altering the metabolism of the host plant and inducing antioxidant responses in the gall. And, among all the compounds detected, the hydrolyzable tannins were those that appeared exclusively in the galls, showing that they may have their degradation products, including ellagic acid that has an antioxidant function, and for the *M. radula* gall system it contributes to dissipating stress and keeping the galling insect on the host plant.

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Chapter IV: Reddish coloration in galls and its relationship with polyamines, phytohormones, and metals accumulation

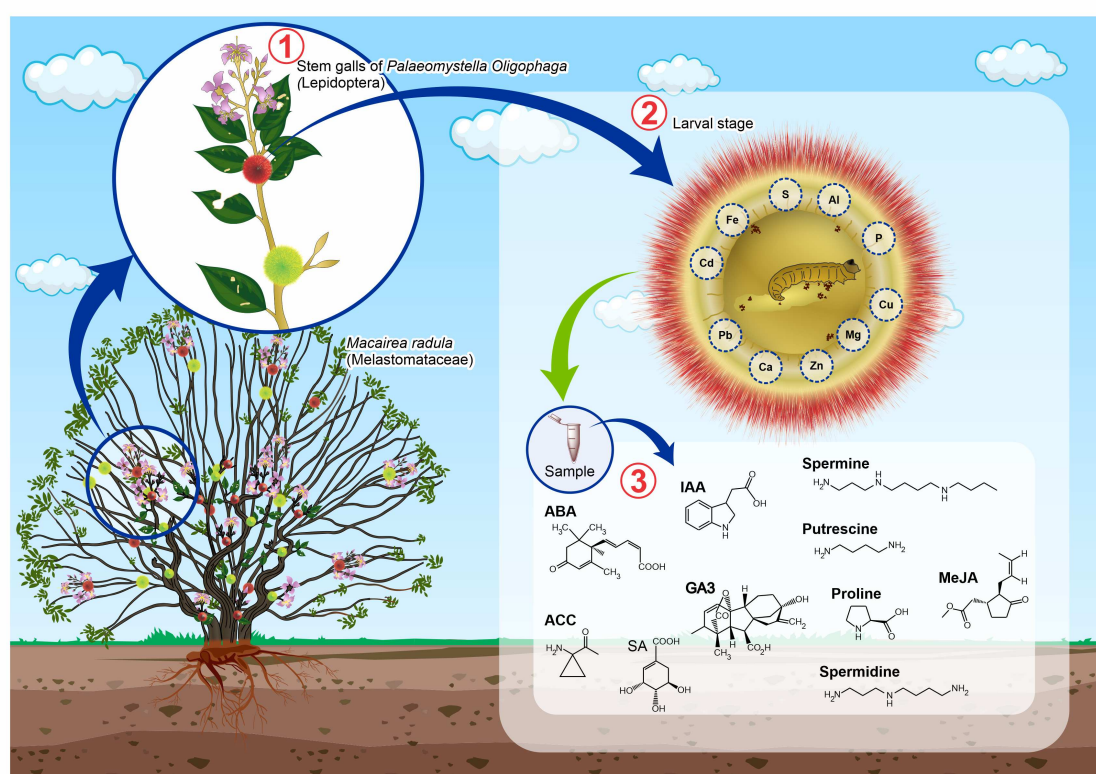
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Graphical abstract: Study system and polyamines, phytohormones and metals in the larval stage. 1) Green and red globoid galls induced by *Lepidoptera* on *Macairea radula* (Melastomataceae); 2) open gall in larval stage and metals accumulation; 3) polyamines phytohormones and structures.

13 2 Abstract

- 14 • The reddish phenomenon in galls is discussed based on distinct hypotheses, such as light stress
15 induced by high light exposure and anthocyanin accumulation for photoprotection. In addition,
16 other biomolecules, such as phytohormones and polyamines, associated with metal accumulation
17 may indicate promising ways to link these distinct hypotheses in response to abiotic and biotic
18 stresses. Until now, no study has evaluated the role of these molecules in galls based on their
19 coloration. Thus, we hypothesized that red galls would present more polyamines and phytohor-
20 mones than green galls and that an increase in metals would be associated with stress dissipative
21 molecules in galls induced by *P. oligophaga* (Lepidoptera) on *M. radula* (Melastomataceae).
- 22 • The galls were sampled randomly in two populations of cerrado vegetation located at Estação
23 Ecológica do Panga and Araguari municipality (waterfall Irmãs site) in the second infestation
24 period (July August) using liquid nitrogen, followed by storage in an ultrafreezer at -81°C .
25 Then, the galls were opened to select only those in the larval stage ($n = 3$) and kept inside the
26 gall for later analysis of phytohormones, polyamines and metals.
- 27 • The polyamines putrescine, spermine, and proline were more abundant in the red than green
28 galls, with the exception of spermidine. The levels of the phytohormones ABA, GA3, IAA, and
29 ACC were greater in green galls than in red galls, while SA and MeJA were more abundant in
30 red galls. These results were associated with more aluminum (Al), iron (Fe), lead (Pb), and
31 copper (Cu) in the red galls than in the green galls.
- 32 • We concluded that most of the polyamines evaluated presented high levels in red galls compared
33 to green galls, as did the phytohormones SA and MeJA, which may be related to the translocation
34 of metals and the biosynthesis of chelating phenolic compounds to reduce the toxicity caused by
35 the accumulation of aluminum (Al), iron (Fe), lead (Pb) and copper (Cu), transforming them
36 into less toxic forms for the galls and maintaining red coloration as a form of protection.

37 **Keywords:** coloration; red gall; biotic stress; primary metabolites, metallic ions.

3 Introduction

The induction and development of galls depend on the biochemical interactions between the gall-inducing organism and the host plant, leading to structural and physiological changes in gall formation (ISAIAS *et al.*, 2011; MANI, 1992; OLIVEIRA & ISAIAS, 2010; OLIVEIRA *et al.*, 2016; SHORTHOUSE *et al.*, 2005). The gall-inducing organism is a phenotype manipulator responsible for determining gall structure and metabolism, although the host plant tissue can impose constraints on gall development (ISAIAS *et al.*, 2014). The biotic stress generated by gall-inducing insects is controlled by several metabolic pathways in the host tissue, allowing gall development (ARRIOLA *et al.*, 2020; BEDETTI *et al.*, 2014; BODY *et al.*, 2019; NAVES *et al.*, 2021; SANTOS *et al.*, 2024; SARVAJEET & TUTEJA, 2010). As a result of this parasitic interaction, galls exhibit great morphological and physiological diversity (FERREIRA *et al.*, 2019; OLIVEIRA *et al.*, 2014; STONE & SCHONROGGE, 2003), including the accumulation of different pigments, especially anthocyanins, which are associated with red coloration in galls (LEV-YADUN, 2016).

The reddish phenomenon in galls is discussed based on distinct hypotheses, such as light stress induced by high-light exposure and anthocyanin accumulation for photoprotection (BOMFIM *et al.*, 2019); anthocyanin as a carbon sink due to the high accumulation of sugar promoted by cytokinin in galls (CONNOR *et al.*, 2012); the predisposition of host tissue to produce anthocyanins at the site of induction (CARDOSO *et al.*, 2022); and the defensive role of anthocyanins in galls (INBAR *et al.*, 2010). Although the reddish phenomenon in galls seems to be idiosyncratic, understanding the physiological pathways of the biotic stress induced by the galling organisms in plant tissue and the associated biomolecules may indicate promising ways to link these distinct hypotheses. Phytohormones and polyamines, for example, are important molecules involved in plant responses to abiotic and biotic stresses and are associated with the accumulation of metals and phenolic derivatives (CHEN *et al.*, 2019; TYAGI *et al.*, 2023). However, the functions of polyamines in the interaction between gall-inducing insects and host plants are poorly known (ASGHER *et al.*, 2018; KOT *et al.*, 2019; KULPA *et al.*, 1985; SRIVASTAVA, 1987), especially when they are associated with phytohormones, metal accumulation and the reddish phenomenon in galls.

Polyamines (PAs) are low-molecular-weight aliphatic nitrogenous bases containing one or more amino groups that are widely distributed in the cells of living organisms (LIU *et al.*, 2015; VUOSKU *et al.*, 2018). The main PAs found in plants are Putrescine (Put), Spermidine (Spd), and Spermine (Spm), which are involved in the regulation of diverse physiological processes, such as the control of oxidative stress (MUSTAFAVI *et al.*, 2018). PA cross-linking with phenolic derivatives is important not only for stress dissipation but also for plant morphogenesis (OLIVEIRA *et al.*, 2017). The induction and development of galls depend on the tunable control of oxidative stress (ISAIAS *et al.*, 2014) when the galling organism manipulates the host plant tissue (OLIVEIRA *et al.*, 2016). Once anthocyanin is

73 produced to relieve oxidative stress in plant tissue (DAVIES *et al.*, 2012), we believe that red galls can
74 accumulate more PAs and phytohormones than green galls.

75 Phytohormones, polyamines, and heavy metals can interfere with metabolism, development,
76 and possibly gall color (MALIK *et al.*, 2022; YADAV, 2010). Phytohormones such as Salicylic Acid
77 (SA) and Methyl Jasmonate (MeJa) are biosynthesized to regulate the defense mechanism of plants
78 (CHAMANDOOSTI, 2019; LI *et al.*, 2019), and their biosynthesis is directly associated with the metabolism
79 of PAs and secondary metabolites, especially those related to color such as chlorophylls, anthocyanins,
80 carotenoids, and betalains (TANAKA *et al.*, 2008; TYAGI *et al.*, 2023). The manipulation of the cellular
81 machinery by galling insects involves the release of secondary metabolites such as phytoalexins, phe-
82 nolic compounds, and phytohormones such as Indole-3-Acetic Acid (IAA), which regulate gall growth
83 (BEDETTI *et al.*, 2014). This process occurs because secondary metabolites, for example, flavonoids,
84 act synergistically with growth hormones (auxins) and metals to control reactive oxygen species dissi-
85 pation (ARRIOLA *et al.*, 2020; BUER *et al.*, 2010; MURPHY & PEER, 2000; PEER & MURPHY, 2007).
86 Some metals, such as Iron (Fe), Zinc (Zn), Copper (Cu) and Manganese (Mn) are essential for nutrition
87 at low levels, while others, such as Aluminum (Al), Cadmium (Cd), Lead (Pb), and Mercury (Hg),
88 have no biological function (ANGULO-BEJARANO *et al.*, 2021; BROWN *et al.*, 1987; LEI *et al.*, 2007).
89 However, the accumulation of metals can cause toxic effects on plants (ARIF *et al.*, 2016; SARWAR
90 *et al.*, 2017). To avoid the effects of toxicity, they can biosynthesize polyamines and phytohormones
91 that tend to bind to metals, forming complexes with antioxidant functions (GROPPIA & BENAVIDES,
92 2008; LIU *et al.*, 2015; WYBRANIEC *et al.*, 2013).

93 Herein, we evaluated the globoid galls induced by *Palaeomystella oligophaga* (Lepidoptera)
94 on *Macairea radula* (Melastomataceae). These galls can exhibit both green and red coloration (CAR-
95 DOSO *et al.*, 2022; NAVES *et al.*, 2021), however, until now, no study has evaluated the role of PAs,
96 phytohormones, and metals in galls based on their coloration. Thus, we expected that red galls would
97 contain more PAs and phytohormones associated with stress dissipation than green galls and that red
98 galls would contain more metals associated with dissipative stress molecules.

99 4 Material and Methods

100 4.1 Plant material

101 Galls induced by *Palaeomystella oligophaga* (Lepidoptera) on *Macairea radula* buds (Melas-
102 tomataceae) were sampled randomly from two populations of cerrado vegetation located at Estação
103 Ecológica do Panga and Araguari municipalities (waterfall at the Irmãs site). The green and red galls
104 were opened and considered for analysis only those with the larval stage of gall induction inside. The

galls were collected in the second infestation period (July August) using liquid nitrogen, followed by storage in an ultrafreezer at -81°C . Then, the galls were opened to select only those in the larval stage ($n = 3$). The larvae were kept inside the gall for later analysis of phytohormones, polyamines and metals.

4.2 Phytohormone and polyamine analysis by liquid chromatography–mass spectrometry (LC–MS)

Approximately 110 mg of fresh plant material (galls) was added to 1.5 ml Eppendorf tubes and macerated using glass or tungsten beads at 25 Hz/s for 3 minutes, considering three replicates. Then, 400 μL of extraction solution (methanol:isopropanol:acetic acid – 20:79:1) was added, and the control without plant material (white) was added. Each sample was vortexed 4 times for 20 s on ice and sonicated for 15 minutes. The samples were placed on ice for 45 minutes, sonicated (15 minutes), and centrifuged at $13,000 \times g$ for 10 minutes at 4°C . A total of 350 μL of the supernatant was pipetted into new 2.0 ml tubes (Eppendorf). Repeat steps 2 and 3 for the remaining pellet, and add the supernatants. Centrifuge again at $20,000 \times g$ for 5 minutes and transfer approximately 600 μL into new 2.0 ml tubes. The samples were passed through filters (13 mm, 0.2 μm PVDF syringe filter, Hexis brand, AG5190-5261ESTor HX0097-02623) to remove any particulate matter that could damage the LC–MS equipment. 300 μL of each vial was added to the triple quadrupole (QQQ) (FORCAT *et al.*, 2008).

4.3 Identification and quantification of phytohormones and polyamines

The phytohormone abscisic acid (ABA), gibberellic acid (GA3), indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC), salicylic acid (SA), methyl jasmonate (MeJA); polyamines putrescine (Put), spermidine (Spd), spermine (Spm) and proline data obtained by liquid chromatography coupled to mass spectrometry (LC–MS–QQQ) were identified and quantified by Skyline software (ADAMS *et al.*, 2020). Liquid chromatography coupled to mass spectrometry (LC–MS–QQQ) of the Nucleus of Biomolecules Analysis (UFV) was also used.

4.4 Metal quantification

The samples were dried, crushed, and sieved (approximately 0.200 g) and then digested in 8.75 ml of 2 mol/l nitric acid and 1.25 ml of hydrogen peroxide with the aid of a microwave (Ethos, Milestone) (?). After digestion, the volume was increased to approximately 18 ml with the addition of Milli-Q water. The final dilution of the sample in the digestion step was approximately 90 times. In the digestion stage, which was carried out every 22 samples, a vial containing only the reagents

used and a reference material (NIST 1515 - Apple Leaves) was also added. After each round with 24 bottles, the microwave was decontaminated. Then, the samples were diluted again in the following proportions for each round of analysis: 2x for trace elements copper (Cu), cadmium (Cd), lead (Pb), 50x for aluminum (Al), magnesium (Mn), iron (Fe), zinc (Zn) and 1000x for magnesium (Mg).

The concentrations of the chemical elements iron (Fe), copper (Cu), zinc (Zn), lead (Pb), cadmium (Cd), aluminum (Al) and magnesium (Mg) were analyzed using mass spectrometry with inductively coupled plasma–mass spectrometry (ICP-MS) (Agilent 7700, Agilent Technologies, Santa Clara, USA). The concentrations of calcium (Ca), phosphorus (P) and sulfur (S) were determined by total reflection X-ray fluorescence (TXRF). Quartz glass disks 30 mm in diameter were used as sample carriers and were covered with 10 μ/L of silicon solution in isopropanol (ServaTM, Heidelberg, Germany). The digested sample (450 μ/L) and 50 μ/L of 100 mg/L gallium solution were mixed for determination of the internal standard. These final solutions (10 μ/L) were applied to the siliconized disks, and the disks were dried at 60 °C for 15 min and analyzed in an S2 PICOFOX™ (Bruker Nano GmbH, Berlin, Germany).

4.5 Statistical analysis

Differences between means for treatments were tested using Students t test for polyamines, phytohormones and chemical element quantification. All the data were subjected to the Shapiro–Wilk normality test with a significance level of 0.05, which was performed using R software version 4.3.2 (2023).

5 Results

The compound putrescine was 60% larger in the red galls than in the green galls ($t = -7.812$; d.f. = 2.0027; p value = 0.01593). Additionally, spermine ($t = -4.8265$; d.f. = 2.0009; p value = 0.04031) and proline ($t = -1.0664$; d.f. = 2.0966; p value = 0.3935) were more abundant in red galls than in green galls, with the exception of spermidine, which was present at approximately 50% greater quantities in green galls than in red galls ($t = 2.3186$; d.f. = 3.9999; p value = 0.08126) (Figure 10). The levels of the phytohormones Absciscic acid (ABA), Gibberellic acid (GA3), Indole Acetic Acid (IAA), and Aminocyclopropanecarboxylic Acid (ACC) were greater in green galls than in red galls, while SA and MeJA were more abundant in red galls (Figure 10). There was 65% more ABA ($t = 7.11$; d.f. = 2.00; p value = 0.01) and 69% more ACC ($t = 20.865$; d.f. = 2.5756; p value = 0.0006075) in green galls than in red galls. Additionally, 15.8% and 10.6% ($t = 8.9908$, d.f. = 2.5071, p value = 0.005704; $t = 0.91878$; d.f. = 2.0077; p value = 0.4549) more GA3 and IAA, respectively, were detected in green galls than in red galls. 15.8% and 10.6% in green galls ($t = 8.9908$, d.f. = 2.5071,

168 p value = 0.005704; t = 0.91878; d.f. = 2.0077; p value = 0.4549) However, red galls have 96% SA (t
 169 = 17.199; d.f. = 2.1478; p value = 0.002439) compared with green galls and 25% more MeJA (t =
 170 -4.1218; d.f. = 3.8728; p value = 0.01559) than green galls (Figure 10).

171 Red galls accumulated more Aluminum (Al), Iron (Fe), Lead (Pb), and Copper (Cu) than
 172 green galls. On the other hand, green galls accumulate more Cadmium (Cd), Magnesium (Mg), Zinc
 173 (Zn), Calcium (Ca), Phosphorus (P), and Sulfur (S) than red galls. Specifically, red galls accumulate
 174 75% more Iron (Fe) (t = -10.586; d.f. = 2.1007; p value = 0.007416), 64.4% more Aluminum (Al) (t
 175 = -12.75; d.f. = 2.1731; p value = 0.004406), 52.9% more Lead (Pb) (t = -6.8959; d.f. = 3.2044; p
 176 value = 0.005036) and 19.4% more Copper (Cu) (t = -13.129; d.f. = 3.8705; p value = 0.0002367)
 177 than green galls. On the other hand, compared with red galls, green galls accumulated 66.9% more
 178 Cadmium (Cd) (t = 3.4325; d.f. = 2.3242; p value = 0.06078), 30.9% more Calcium (Ca) (t = 1.5979;
 179 d.f. = 2.0504; p value = 0.2481), 32.6% more Magnesium (Mg) (t = 1.2553; d.f. = 2.0983; p value =
 180 0.3311), approximately 26% more Phosphorus (P) (t = 0.84509; d.f. = 2.3832; p value = 0.4744) and
 181 Sulfur (S) (t = 0.95429; d.f. = 2.2454; p value = 0.4311) and 15% more Zinc (Zn) (Figure 10) and
 182 (Figure 10).

183 6 Discussion

184 As phenolic derivatives, anthocyanins accumulate in high amounts in galls induced by *Palaeomys-*
 185 *tella oligophaga* (Lepidoptera), leading to red coloration (CARDOSO *et al.*, 2022). These molecules
 186 can scavenge free radicals produced under stress in plants and are important for antioxidant activity
 187 (FALAHI *et al.*, 2018). Most of the polyamines evaluated in this study were more abundant in red
 188 galls than in green galls, which is indicative of the relationships between them and the phenolic deriva-
 189 tive anthocyanins (HUDEC *et al.*, 2006). Similar results were found, especially for the phytohormones
 190 associated with responses to stress, such as SA and MeJA. Some metals found in high quantities in
 191 red galls can lead to tissue toxicity and consequently are associated with an increase in PAs, SA, and
 192 MeJA (MENDOZA *et al.*, 2018; PENG *et al.*, 2021). Metals can also interact with phenolic derivatives,
 193 leading to the overexpression of red coloration (FEDENKO *et al.*, 2022).

194 Polyamines play protective roles in response to oxidative stress and metal toxicity, but their
 195 relationships with phytohormones and color gall are still not well understood. In plants, these nitroge-
 196 nous compounds are present in their free form, such as putrescine, spermidine, and spermine (CHEN
 197 *et al.*, 2019; GILL & TUTEJA, 2010). The higher percentages of putrescine, spermine and proline in
 198 red galls may be related to the translocation of metals and the biosynthesis of chelating compounds
 199 to reduce their toxicity, stabilize ROS, increase the efficiency of antioxidant enzymes and synthesize
 200 photosynthetic pigments (BALAL *et al.*, 2016; NAHAR *et al.*, 2016; RADY *et al.*, 2016). The greater

201 quantity of PAs in red galls, especially putrescine galls, may be also due to stress caused by the accu-
202 mulation of nonessential metals such as aluminum and lead (AMIST *et al.*, 2017). Polyamines reduce
203 cadmium (Cd) toxicity in *Inula crithmoides* (Asteraceae) (GHABRICHE *et al.*, 2017). These compounds
204 can protect cell membranes from oxidative damage through the formation of polyamines, phospholipids
205 and Fe_2^+ complexes (VELIKOVA *et al.*, 2000).

206 Galls induced by *Tetraneura ulmis* (Hemiptera) on elm tissues also showed high contents of
207 PAs in young and mature galls (KMIEC *et al.*, 2018). Additionally, studies carried out on potato tumors
208 have shown that PAs do not inhibit growth but increase growth by 30-50% (KULPA *et al.*, 1985). In
209 galls induced on oak leaves by Cynipidae species, the enzymes lysine decarboxylase (LDC), tyrosine
210 decarboxylase (TyDC), and ornithine decarboxylase (ODC) change the amount of polyamines (KOT
211 *et al.*, 2019). These molecules can help in the detoxification of metals, forming chelates to prevent the
212 excessive accumulation of nonessential metals in plant cells, transforming them into less toxic forms, for
213 example, through complexation with primary and secondary metabolism molecules, such as hormonal
214 regulators (RAHMAN *et al.*, 2023). Therefore, the increase in PAs in red galls seems to be a response
215 to the high level of metal accumulation and, consequently, increased capacity for ROS detoxification.
216 PAs play an important role in ROS metabolism and detoxification in plant tissues (ASIJA *et al.*, 2023).
217 Our results showed that red pigmentation can be associated to PAs accumulation in galls seems to
218 play an important role in stress dissipation.

219 Phytohormones play a crucial role in the induction, development, and establishment of insect
220 galls by acting as primary chemical messengers (BODY *et al.*, 2019). These hormonal messengers stim-
221 ulate both the production of primary metabolites and amino acids for the biosynthesis of polyamines,
222 which were observed in greater quantities in red galls, as well as the phytohormones Salicylic Acid (SA)
223 and Methyl Jasmonate (MeJa). These substances are involved in the protection and repair of damage
224 to plants caused by oxidative stress (TENG *et al.*, 2023; WAR *et al.*, 2011), suggesting that red galls
225 have more stressful tissue than green galls. On the other hand, green galls had higher concentrations
226 of ABA, GA3, IAA, and ACC than red galls. It is known that abscisic acid (ABA) is associated with
227 tolerance to stress caused by Lead (Pb) (PIOTROWSKA-NICZYPORUK *et al.*, 2020). Ethylene, auxin,
228 abscisic acid, jasmonic acid, and gibberellic acid are known to play important roles in regulating tol-
229 erance to aluminum (Al) toxicity in plants. These enzymes can activate phytochelatin biosynthesis,
230 stimulating the antioxidant system through the formation of ascorbate peroxidase, catalase, superox-
231 ide dismutase, and the biosynthesis of protective compounds such as proline and pigments involved in
232 secondary metabolism (PIOTROWSKA-NICZYPORUK *et al.*, 2020; TALAREK-KARWEL *et al.*, 2020) in
233 plants.

234 The growth and development of galls induced on Al-accumulator host species can be affected.
235 For example, Miconia host species accumulate more Aluminum (Al) in non-galled tissues than in galls;

236 however, the lower concentrations of these metals in galls are associated with the presence of phenolic
 237 compounds in the inner cell layers of galls (ARRIOLA *et al.*, 2020). Regarding the accumulation of
 238 Iron (Fe) in galls, it is believed that Fe_3^+ can be reduced to Fe_2^+ in the gall parenchyma, especially in
 239 the nutritive tissue (ARRIOLA *et al.*, 2020). Lead (Pb) does not play a role in biological systems and
 240 causes oxidative damage to plants, such as interference with enzymatic activities, stomatal closure due
 241 to the induction of abscisic acid (ALSOKARI & ALDESUQUY, 2011; ROELFSEMA & HEDRICH, 2005),
 242 and changes in photosynthetic structures such as the reduction of chlorophyll pigments (MUKHTAR
 243 *et al.*, 2010; ZULFIQAR *et al.*, 2019). The increase in Lead (Pb) in the red galls of *P. oligophaga* may
 244 be associated with an increase in oxidative stress and, consequently, an increase in the biosynthesis of
 245 phenolic derivatives, such as anthocyanins. Thus, there are changes in photosynthetic structures, such
 246 as a reduction in chlorophyll pigments (MUKHTAR *et al.*, 2010; ZULFIQAR *et al.*, 2019). Furthermore,
 247 metals such as Aluminum (Al) induce auxin biosynthesis and alter its transport (WANG *et al.*, 2019;
 248 YANG *et al.*, 2014). This phytohormone, in combination with other signaling molecules such as abscisic
 249 acid, regulates organic acids that may be involved in their tolerance (YANG *et al.*, 2011). Aluminum
 250 (Al) also favors the synthesis of ethylene (MASSOT *et al.*, 2002). In this research, we evaluated its
 251 precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), which is present at relatively high levels in
 252 green galls.

253 The greater amounts of Aluminum (Al), Iron (Fe), Lead (Pb), and Copper (Cu) found in red
 254 galls can be explained by the interaction of metallic ions with other stabilizing molecules, which leads
 255 to the formation of metallic pigment complexes in galls (MULYANINGSIH *et al.*, 2023). The available
 256 form of aluminum for plants is in its trivalent form (Al_3^+), which is highly toxic to cells, tissues,
 257 and organs (KOCHIAN *et al.*, 2015). When absorbed by cells, Al_3^+ indirectly induces oxidative stress,
 258 inhibition of antioxidant enzymes and lipid peroxidation (JONES *et al.*, 2006; RANJAN *et al.*, 2021;
 259 YAMAMOTO *et al.*, 2002).

260 7 Conclusion

261 Thus, we concluded that most of the polyamines evaluated presented high levels in red galls
 262 compared to green galls, as did the phytohormones Salicylic Acid (SA) and Methyl Jasmonate (MeJa),
 263 which may be related to the translocation of metals and the biosynthesis of chelating phenolic com-
 264 pounds to reduce the toxicity caused by the accumulation of Aluminum (Al), Iron (Fe), Lead (Pb) and
 265 Copper (Cu), transforming them into less toxic forms for the galls and maintaining red coloration as
 266 a form of protection.

267 8 Acknowledgements

268 We are grateful to Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG),
269 Laboratório de Anatomia, Desenvolvimento Vegetal e Interações (LADEVI-UFU), Laboratório de Fi-
270 siologia Vegetal (LAFIVE-UFU), Laboratório de Geoquímica Ambiental (UFOP), LEAQUAA (UFMG)
271 and Laboratório de Propagação e Micropropagação de Plantas (UFV).

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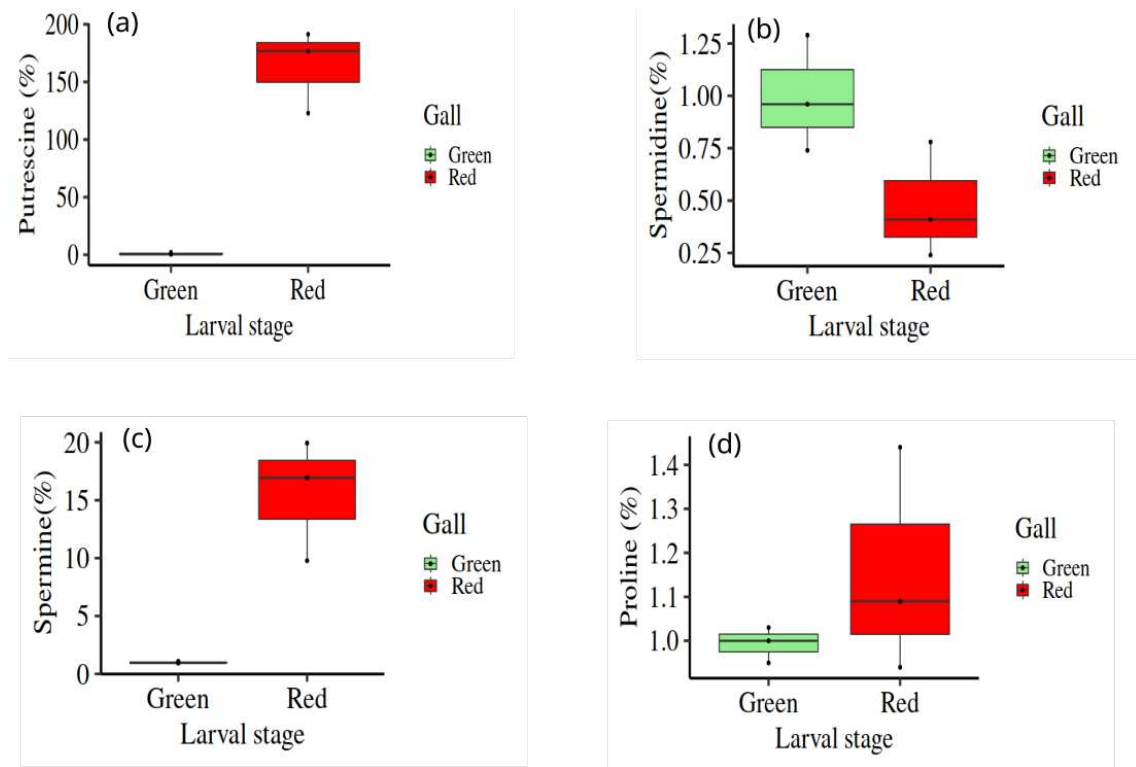


Figure 1: Polyamines quantification from red and green galls induced by *Paleomystella oligophaga* on *Macairea radula* in the larval stage. (a) Putrescine; (b) Spermidine; (c) Spermine; and (d) Proline. ($p > 0.05$).

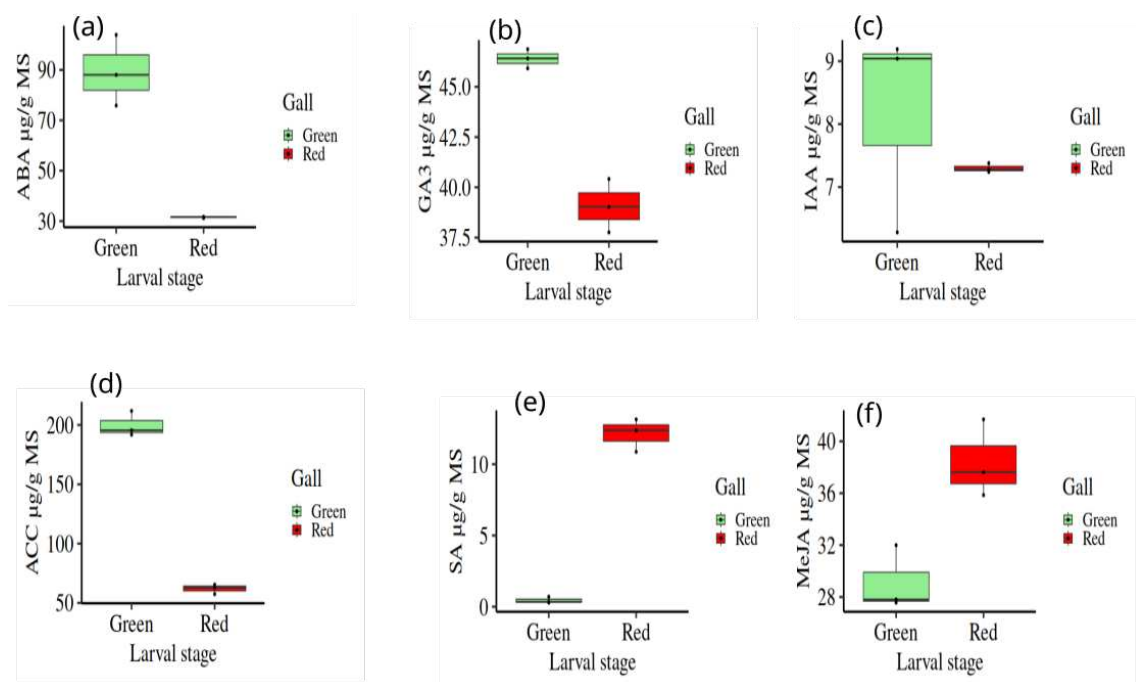


Figure 2: Phytohormone quantification from red and green galls induced by *Paleomystella oligophaga* on *Macairea radula* in the larval stage. (a) Absciscic acid (ABA); (b) Gibberellic acid (GA3); (c) Indole Acetic Acid (IAA); (d) Aminocyclopropanecarboxylic Acid (ACC); (e) Salicylic Acid (SA); and (f) Methyl Jasmonate (MeJA). ($p > 0.05$).

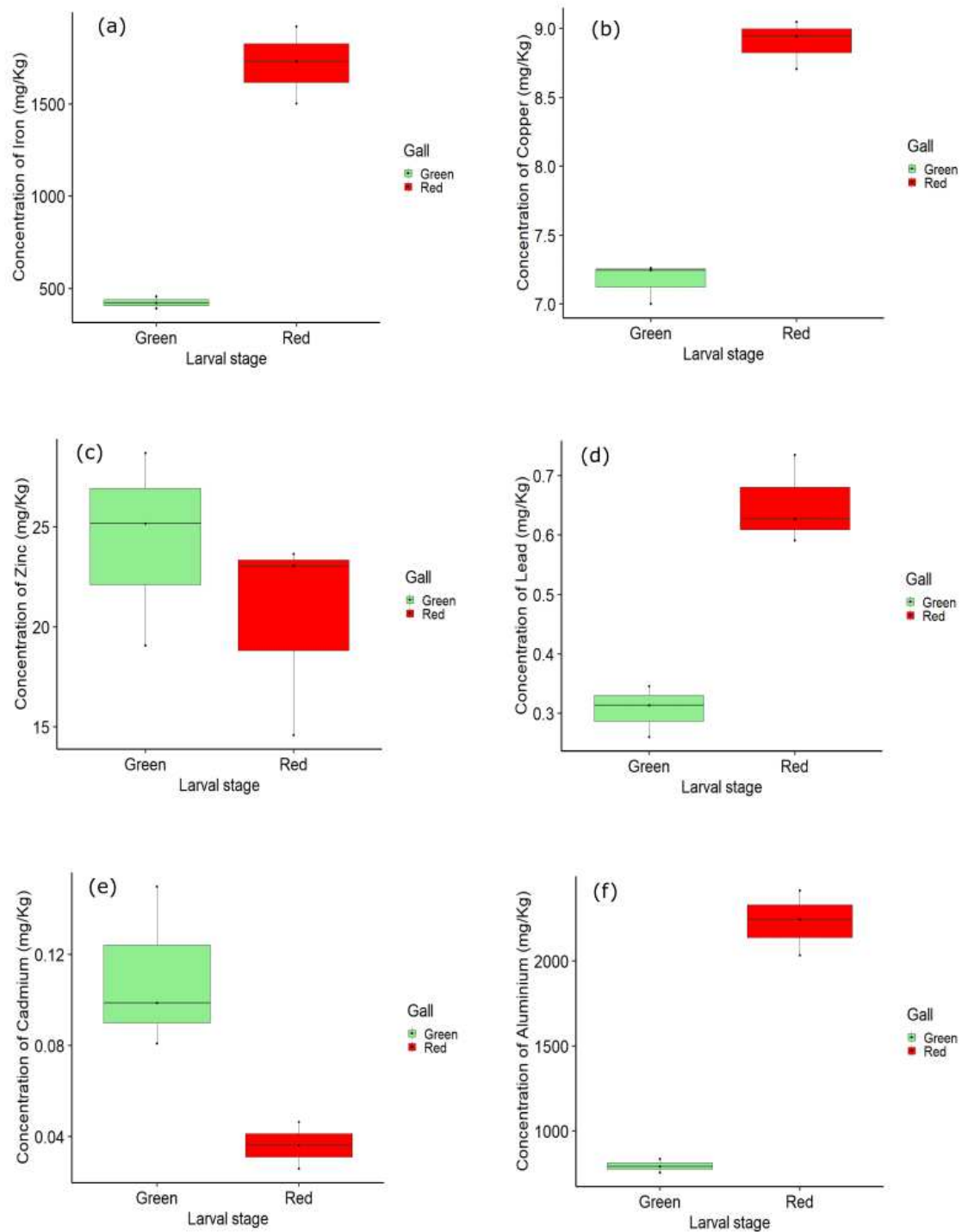


Figure 3: Metals quantification from galls induced by *P. oligophaga* on *M. radula* in the larval stage. (a) Iron (Fe); (b) Cooper (Cu); (c) Zinc (Zn); (d) Lead (Pb); (e) Cadmium (Cd); and (f) Aluminum (Al). ($p > 0.05$).

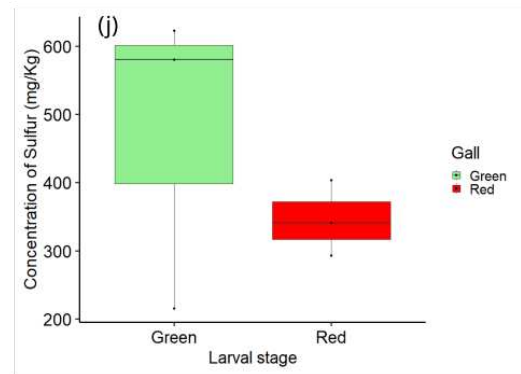
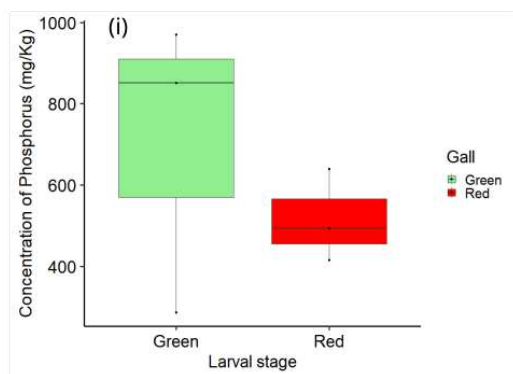
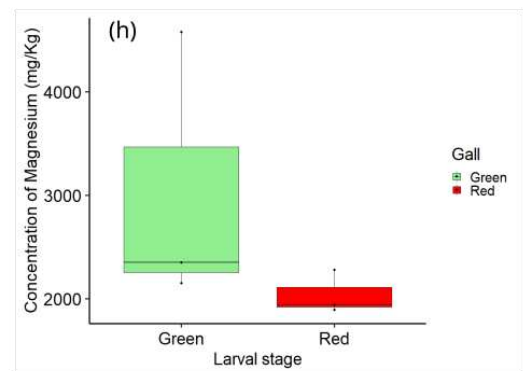
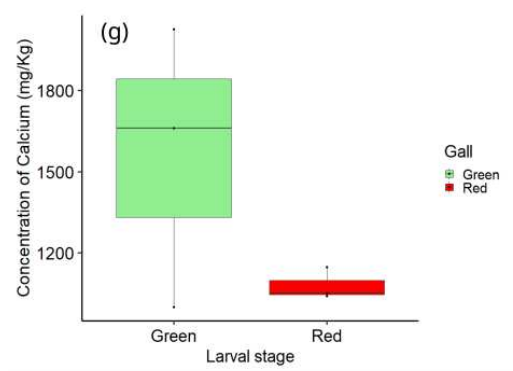


Figure 4: Chemical element quantification from galls induced by *P. oligophaga* on *M. radula* in the larval: (g) Calcium (Ca), (h) Magnesium (Mg), (i) Phosphorus (P), and (j) Sulfur (S) ($p > 0.05$).

1 Chapter V - Asking the biological clock: how the gall structure works? An integrative metabolic network approach

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2 Abstract

The formation of galls depends on biochemical and ecological interactions between the insect and host plant tissues. This relationship is controlled by biotic stress response generated from inducer insect, and mediated for the carbohydrates, secondary compounds, and phytohormones biosynthesis in the tissues of the host plant. Galls in growth and development are associated with primary metabolites whereas secondary molecules act in defense and ecological functions. These metabolites might change by circadian cycle and during the seasons in response to biotic stress. Thus, when inducer-insect cause the stress in the plant cause formation and dissipation of Reactive Oxygen Species (ROS) which might act as second messengers to signal a cascade of chemical events with several molecules biosynthesis. Phytohormones such as auxin, cytokinin, salicylic acid and ethylene are biosynthesized to cell division, growth and to regulate the defense mechanism of plant and preventing against attack of other insects. The manipulation of cellular machinery by inducer insect involves also the release of phenolic compounds, for example, flavonoids that act synergistically with auxins and linked with carbohydrates to control of ROS and maintain gall color and protection. This review chapter will address the circadian rhythm to understand the relationship between carbohydrates, phytohormones, secondary metabolites and metals present in galls for their maintenance on host plants.

Keywords: Reactive Oxygen Species (ROS), phytormones, phenolics compounds, metals, circadian clocks.

3 Introduction

Plants and insects can establish communication and create a new environment for survival (REDDY & GUERRERO, 2004; ZU *et al.*, 2023). This relationship can initiate with signals that plants express to attract the insects (QIAN *et al.*, 2024). These can be molecules in the air or other substances that are compatible with insect signals (PROFFIT *et al.*, 2020). It is very common to find different types of sugars in plants besides the volatile molecules, and these molecules might facilitate or prevent communication with the galling insect (BOUWMEESTER & SCHIESTL, 2019; JEANDET *et al.*, 2022; ZHOU & JANDER, 2022). When occurring the compatibility between the plant and the insect, a series of metabolic pathways are activated (KERCHEV *et al.*, 2012). This event depends on some factors such as the availability of reactive plant sites, the synchronization life cycles of host plants with galling insect, the distinct feeding behaviors of insect taxa (OLIVEIRA *et al.*, 2016; PFEFFER *et al.*, 2018; YUKAWA, 2000) and plant circadian cycle (HIRANO *et al.*, 2023; VENKAT & MUNEER, 2022).

This recognition is mediated by highly reactive molecules, Reactive Oxygen species (ROS), which represent the first signaling to produce mainly hydrogen peroxide to reinforce the membrane lignification (OLSON & VARNER, 2002). On the other hand, the ROS excess can cause cell damage and induce the membrane systems degradation of the plant cell (SHARMA *et al.*, 2012). The cell degradation can be reversed with the protective structures such as plastoglobules, which are lipoprotein particles that stay inside of the chloroplasts to repair metabolic imbalance (OLIVEIRA *et al.*, 2011). Some studies have been made with damage to the thylakoid systems to the maintenance of photosynthesis rates. In general, they show that there is an increase in the number and size of plastoglobules in galls (OLIVEIRA *et al.*, 2011). So, this high lipid content of the galls can represent the main energy source for the insect and biosynthesize molecules as primary and secondary metabolites and phytohormones to maintain the galling insect (KUSTER, 2020; NAVES *et al.*, 2021; SCHULTZ *et al.*, 2019).

All these chemical and molecular events result in galls formation. This new structure can be seen as ornaments of nature for presenting different shapes, sizes and colors (ISAIAS *et al.*, 2013). This diversity has been extensively studied, mainly regarding the process of growth, morphogenesis and differentiation, resulting in morphotypes generated by species-specific interaction between an inducing organism and a host plant (ISAIAS & OLIVEIRA, 2012). The gall can conserve patterns that are determined in plant meristems, manipulated by galling insects. In the studies of ROHFRITSCH (1992) galls development have four phases that are initiation or induction, growth and development, maturation, and dehiscence or senescence. Some insects are selected to induce galls in young tissues and, this process begins with the increase in the division and enlargement cellular (MAGALHAES *et al.*, 2014). In the initial phase there is a predominance of cellular hyperplasia and in the late phase cellular hypertrophy. During the maturation phase, the insect is in the last larval instar, which coincides with the highest food consumption. The last phase is senescence or dehiscence, when the insect completes

its cycle. To do this, nutrients need to be available. In general, inside the galls there are two compartments, one internal and one external. In the first there are more lipids, proteins (REZENDE *et al.*, 2019) and reducing sugars while in the external compartment there are phenolic compounds as a product of the accumulation of ROS (NAVES *et al.*, 2021). These compounds can be biosynthesized in synchrony with environmental changes and the host plant biological clock (MARCHEVA *et al.*, 2013).

Although the circadian clock regulates multiple metabolic pathways, the availability of both primary and secondary metabolites can influence the circadian rhythm and impact plant physiology through enzyme expression, phytohormone signaling, and regulation of metabolite levels in different plant organs (ATAMIAN & HARMER, 2016). Therefore, this review chapter will address the circadian rhythm to understand the relationship between carbohydrates, phytohormones, secondary metabolites and metals present in galls for their maintenance on host plants.

4 Does the relationship between phytohormone, carbohydrates, phenolic compounds and metals in galls depend on a circadian rhythm?

The primary and secondary plants metabolism change throughout the day - circadian cycle and during the seasons (BUCKLEY *et al.*, 2023; GREENHAM & MCCLUNG, 2015). Chemical reactions sequence occur cyclically in many organisms, especially in plants, which are regulated by an endogenous biological oscillator commonly called the biological clock (DODD *et al.*, 2005). This allows you to anticipate events, such as the presence or absence of light, water scarcity, changes in pH and nutrients in the soil (CERVELA-CARDONA *et al.*, 2021; HOTTA *et al.*, 2007). Before dawn, the leaves prepare to carry out photosynthesis, and for this, many signals are involved (KOYAMA & TAKEMOTO, 2014). It has long been believed that light would be its clockmaker, that is, a factor that could adjust the plants clock. But actually, there is evidence that it is small sugar molecules (trehalose-6-phosphate) that signal and regulate the metabolic rate (FRANK *et al.*, 2018). Thus, they are able to measure time using information coming from sugars, and not just light (BORDAGE, 2016). It is possible that the concentration of these molecules can adjust the metabolism to the rhythm of the sun, so that the plant is neither late nor early. Even without environmental stimuli, plants maintain biological rhythms (WEBB *et al.*, 2019).

This rhythm is maintained by the biological clock, which has a central oscillator with day-time or nighttime transcription factors for plant metabolism (DANTAS *et al.*, 2021). These receive input signals mediated by phytochromes (phyA and phyB) to regulate plant metabolic pathways. The transcription factors CIRCADIAN ASSOCIATED 1 (CCA1) and LATE Elongated HYPOCOTYLE

(LHY) that are expressed at dawn activate the expression of PRRs (PSEUDO-RESPONSE REGULATORS) (LAI *et al.*, 2012). While TOC 1 is expressed more at dusk and inactivates CCA1 and LHY (LI *et al.*, 2023). Thus, the biological clock is characterized by having periodic cycles (ALABADI *et al.*, 2001). Genes and proteins are regulated by these molecules, for example, the PRR7 protein gene is activated when the plant has a low level of carbohydrates (FRANK *et al.*, 2018). Otherwise, at high levels it can accelerate the plants biological clock, which is defined by a cyclical period of 24 hours, thus determining a circadian rhythm (BUCKLEY *et al.*, 2023). It has an endogenous origin, and was first observed in *Mimosa pudica* by Mairan in 1729, wherein plants were kept in the dark and even so the leaf movements were constant in the dark (GARDNER *et al.*, 2006). Until then, it was not known that the plants internal clock was maintained by a set of genes, proteins and carbohydrates. It was only centuries later that studies with *Arabidopsis* and agricultural cultivars showed the synchrony of events such as pollination and changes during the day and seasons (MARSHALL *et al.*, 2023). For example, *Arabidopsis* hypocotyls follow a circadian rhythm during elongation with a higher rate of growth at night (NIWA *et al.*, 2009). Other physiological events such as pollination are controlled by the biological clock, such as the release of volatile compounds in synchrony with pollinator activities (ZENG *et al.*, 2017). *Castanea sativa* orthologous genes LHY and TOC1 (chlorophyll binding protein regulator) are constantly expressed during winter (IBANEZ *et al.*, 2008). Clock regulation occurs through signals coming not from genes and proteins, but from other molecules, such as cyclic ribose adenosine diphosphate, which also signals various biotic and abiotic stress conditions (BLANCO *et al.*, 2020) and acts on plant defense mechanisms (BUTT *et al.*, 2020). How these molecules adjust the biological clock inside the cell is still a mystery. In addition to sugars as a clock regulator, metals such as calcium participate in the set of adjustment molecules, which is controlled by the expression of the TOC1 gene and integrate environmental signals into the oscillator (MARTI *et al.*, 2018).

Metals influence distinct circadian rhythm properties (HERMANS *et al.*, 2010; RAVET & PILON, 2013). In plant model *Arabidopsis* it has been shown that the increased concentration of calcium, iron, copper, magnesium, cadmium and zinc ions can cause changes in cell signaling and biological clock activity (CHEN *et al.*, 2013; MAISTRI *et al.*, 2011; PEREA-GARCIA *et al.*, 2016). In *Arabidopsis*, iron (Fe) and copper (Cu) deficiency, for example, increases the period of circadian clock components and alters gene expression (CHEN *et al.*, 2013; HONG *et al.*, 2013). Thus, an adequate supply of iron and copper could favor a rhythm synchronized with environmental signals to maintain plant metabolism adjusted for the adaptation of plants under stress (RAVET & PILON, 2013). On the other hand, the excess of other metals such as magnesium decreases the period of oscillation of some central oscillator genes and increase in the transcripts number when associated with illuminosity and temperature in excess (HERMANS *et al.*, 2010).

Under conditions of high solar incidence, plant metabolism is adjusted to avoid water loss and

keep protective metabolites active (KAPOOR *et al.*, 2020). In galls, in the initial phase of their development, before the biosynthesis of secondary metabolites, enzymatic pathways are active to deactivate IAA oxidases and increase the bioavailability of auxins (BEDETTI *et al.*, 2017). The action of auxin (IAA - indole-3-acetic acid) has been linked to an increase in the concentration of phenolic compounds in specific sites of cell growth, one of the most frequent responses to gall induction (FERREIRA *et al.*, 2018). Thus, this relationship between IAA and phenolic compounds in galls is related not simply to the process of growth and structural development, the increase in the concentration of phenolics in galls can contribute to the mechanisms to maintain oxidative homeostasis in host tissues, as occurs in induced galls by *Ditylenchus gallaeformans* (FERREIRA *et al.*, 2018). It remains to be seen how the concentration of IAA and other phytohormones vary throughout the development of the gall and how they influence the attack of natural enemies. Phytohormones play a regulating role in gall hypertrophy that suppresses the accumulation of gibberellins, auxin, and jasmonates during the first two stages of gall, while abscisic acid concentrations are suppressed throughout development (BEDETTI *et al.*, 2017; BODY *et al.*, 2019). There is evidence that the PRR7 protein participates in the signaling and regulation of abscisic acid (ABA) during seed germination (YANG *et al.*, 2021). And, regulates photoperiodic flowering (NAKAMICHI *et al.*, 2007; WANG *et al.*, 2024). However, for gall systems there are still no reports showing this association.

Many plant hormone signaling pathways are modulated by carbohydrates and circadian clock (CHEN *et al.*, 2023; S. *et al.*, 2006). When in low quantity, there is an increase in photosynthesis and, in its abundant presence, it promotes the growth and sugar storage, not only in the leaves, but in other organs, such as the galls (TAKAO *et al.*, 2006). And, the circadian clock may play an important role in the partition and allocation of these carbohydrates that can favor nutrients for the galling insect. In addition to molecules, secondary metabolites are in part regulated by the circadian clock in response to biotic and abiotic stress (PEREZ-LLORCA & MULLER, 2024). Phytochemical variations have been observed in response to environmental and circadian clock-related changes (LIEBELT *et al.*, 2019). Genes linked to the circadian clock turn on and off anthocyanin biosynthesis genes in relation to the time of day (NGUYEN & LEE, 2016). And, this class of secondary metabolites are accumulates in red galls induced by *Palaeomystella oligophaga* (Lepidoptera) on *Macairea radula* (Melastomataceae) (CARDOSO *et al.*, 2022). In addition to this function, they also contribute to reducing the amount of ROS and possibly protecting the integrity of the host plants biological clock under biotic stress condition and maintain the coloration in the galls (JIMENEZ *et al.*, 2021; PICCOLO *et al.*, 2022).

In addition to anthocyanins, other phenolic compounds present in different plant organs can exert functions as antioxidants and with allelopathic effects (GUEDES *et al.*, 2024; HU *et al.*, 2022). It is common to separate primary and secondary metabolites for teaching purposes, but it is difficult to find an integrative network between the two, with phytohormones and even with metals. The sec-

ondary metabolites are divided, in particular, the phenolic compounds that are commonly found in galls, into: phenolic acids, flavonoids, tannins, lignin; and also terpenoids, such as sterols, carotenoids and glycosides (BONCAN *et al.*, 2020; GUEDES LM, 2022; LATTANZIO, 2013; OLIVEIRA *et al.*, 2011; PAAVER *et al.*, 2010). These metabolites can vary in different gall systems depending on environmental conditions, seasonality, presence or absence of parasites and natural enemies (REZENDE *et al.*, 2023). All of these conditions can favor the biosynthetic pathways of phenolic compounds, which are biosynthesized via the shikimic acid and malonic acid routes, where the enzymes phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) participate (SALTVEIT, 2017). While terpenoids are biosynthesized through mevalonic acid (MVA) and the 2-C-methyl-D-erythriol-4-phosphate (MEP) pathway (MOVAHEDI *et al.*, 2022). Many of the compounds formed are generally linked with carbohydrates. However, secondary metabolites are the least studied regarding the circadian rhythm (PEREZ-LLORCA & MULLER, 2024).

5 Physiological aspects of gall formation

The formation of galls is known as a new plant organ that depends on biochemical interactions between the insect and the tissues of the host plant, inducing structural and physiological changes in response to the feeding activity of the galling insect (ISAIAS *et al.*, 2011; MANI, 1992; OLIVEIRA & ISAIAS, 2010; OLIVEIRA *et al.*, 2016; SHORTHOUSE *et al.*, 2005). This process results in hypertrophy, hyperplasia and transdifferentiation of plant cells and tissues host (BRAGANCA *et al.*, 2020; DREGER-JAUFFRET & SHORTHOUSE, 1992; OLIVEIRA *et al.*, 2016; RAMAN *et al.*, 2005; SUGIMOTO *et al.*, 2011). Thus, the gall may be related to changes controlled by responses of biotic stress generated by the galling insect and, especially, mediated by the relationship among phytohormones, carbohydrates, secondary compounds, and metals in the tissues of the host plant (ARRIOLA *et al.*, 2020; BRAGANCA *et al.*, 2020; NAVES *et al.*, 2021).

These changes in plant tissue are mediated by production hydrogen peroxide and oxygen superoxide known as (ROS - Reactive Oxygen Species) which reinforcement of the cell membrane with the deposition of polysaccharides (FORMIGA *et al.*, 2013). The presence of reactive oxygen species (ROS), that are originated from the plant tissue in response to the stimulus of the galling insect, cause oxidative stress in the plant and can damage everything from membrane lipids to genetic material (DEMIDCHIK, 2015; ISAIAS & OLIVEIRA, 2012; MERIGA *et al.*, 2004; OLIVEIRA & ISAIAS, 2010; OLIVEIRA *et al.*, 2010). However, theses changes in the intercellular environment can create favorable conditions for the activation of plant defense reaction and produce sencondary compounds such as phenolics. Additionally, biotic stress can be reversed by means of a highly reactive molecule, such as nitric oxide (NO). The molecule of NO participates in many physiological processes in plants, such as

germination and flowering (BELIGNI & LAMATTINA, 1999; HSU & KAO, 2004; MUR *et al.*, 2013). It is believed that NO can decrease the damage caused by ROS through its antioxidant activity, eliminating them directly, for example, using superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) (LASPINA *et al.*, 2005). The production of this molecule in plant tissues can follow two known pathways, nitrite-dependent (reducing) pathways and L-arginine-dependent (oxidative) pathways (GUPTA *et al.*, 2010). Therefore, the biosynthesis of NO goes beyond the activity of nitric oxide synthase (NOS), since it can be generated as the product of denitrification, nitrogen fixation, and/or respiration. In most cases, the production of NO in plant tissues has been associated with the accumulation of nitrogen dioxide (NO_2), which is mediated by light through carotenoids (COONEY *et al.*, 1994). Thus, after its production in response to biotic stress, (NO_2) can block the production of hydrogen peroxide (H_2O_2) induced by jasmonic acid and mediate the formation of plant organs induced by an increase in the concentration of phytohormones, such as auxins (KOLBERT *et al.*, 2008; OROZCO-CARDENAS & RYAN, 2002).

Phytohormones such as salicylic acid and ethylene are also biosynthesized to regulate the defense mechanism of plants (LI *et al.*, 2019). When they interact with insects changes occur in the cell wall and cause the release of ethylene (STOTZ *et al.*, 2000; WAR *et al.*, 2012). Because it is volatile, it spreads in the environment, preventing the attack of other insects (FARMER, 2001). It is believed that these signals mediated by NO and biosynthesis of volatile secondary metabolites may act in the process of growth and development of the gall structure, however, they can act as a signal attracting natural enemies (PARE & FARAG, 2008). The manipulation of cellular machinery by galling insects involves the release of secondary metabolites, such as phytoalexins, in particular, phenolic compounds that act as antioxidants, and phytohormones such as indole-3-acetic acid (IAA) that regulate the growth of galls (BEDETTI *et al.*, 2014). This process occurs because the secondary metabolites, for example, flavonoids act synergistically with growth hormones (auxins) in the control of reactive oxygen species (BUER *et al.*, 2010; MURPHY *et al.*, 2000; PEER & MURPHY, 2007).

In the tissues of *Piptadenia gonoacantha* (Mart.) MacBr. (Fabaceae) these molecules were found in structures of high division and cellular hypertrophy (BEDETTI *et al.*, 2014). It is suspected that these molecule biosynthesis processes can be mediated synergistically not only with phytohormones, but also with carbohydrates, favoring the flow of photoassimilates to galls as a defense strategy (OLIVEIRA *et al.*, 2017). Thus, phytohormones play a crucial role in the induction, development and establishment of insect galls acting as primary chemical messengers (BODY *et al.*, 2019). These chemical messengers can stimulate the production of carbohydrates and secondary compounds whose are responsible for the maintenance of cellular machinery, nutrition and protection of galling insects. Beside this, have a relevant role in maintaining homeostasis, redox reactions, plant defense and interactions with other organisms (BARONIO & OLIVEIRA, 2019; FORMIGA *et al.*, 2009; ISAIAS *et al.*, 2015). Finally, the entire development of the gall and interactions with other organisms can be governed by the action of

phytohormones, since there is variation in the concentration of these substances during the development of the gall and in specific compartments (BODY *et al.*, 2019).

6 Conclusion and Final Remarks

Gall host plants might achieve fitness gains by anticipating their metabolism at dawn to biosynthesize carbohydrates. And, also produce secondary metabolites, such as phenolic compounds, mainly anthocyanins at times with more light incidence to protect and maintain the color of reddish galls. Molecules of primary and secondary associated with metals metabolism accumulate under conditions of biotic and abiotic stress, and the circadian clock can integrate the biosynthesis cycles of compounds in favor of the survival of the galling insect. Not only does abiotic stress alter the circadian rhythm, but the quantity and quality these metabolites, even in the presence and quantity of calcium and other metals. As we saw in this review, it is possible to test these changes by measuring the amount of PRR transcripts that regulate part of the circadian rhythm. Because when the oscillator does not synchronize with environmental signals, the plants may have low performance, maybe producing fewer compounds, becoming more susceptible to pests and diseases. It is possible that secondary metabolites protect the circadian clock through their antioxidant functions and maintain the galls with their different shapes and colors on the host plants.

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8 Conclusão geral e perspectiva

- As análises anatômicas e imunocitoquímicas da planta hospedeira mostrou que a composição da parede celular dos compartimentos da galha muda de acordo com o estágio de desenvolvimento do inseto galhador, especialmente em relação aos homogalacturonanos e xiloglucanos, os quais dão suporte ao alto metabolismo e à disponibilidade de recursos nas paredes celulares do tecido nutritivo para o inseto galhador.
- Os experimentos químicos por cromatografia gasosa acoplada à espectrometria de massas mostrou a composição monossacarídica composta por ramnose, fucose, arabinose, xilose, manose, glucose e galactose nas galhas induzidas por *P. oligophaga* em *M. radula* com e sem os inimigos naturais. Existe variação na composição dos polissacarídeos hidrolisados, representados principalmente por xilose, glucose, galactose e arabinose no extrato bruto das galhas. E maior abundância de xilose e rabinose nos órgãos não galhados, o que pode estar associado à função estrutural das folhas e caules. Enquanto que nas galhas, houve maior quantidade de glicose e galactose; que podem ser conjugados com metabólitos secundários, principalmente flavonoides responsáveis pela proteção e coloração das galhas.
- Os ensaios enzimáticos mostraram que as enzimas a catalase (CAT) e a fenilalanina amônia-liase (PAL) contribuíram para a maior diferença entre folhas e galhas em seus diferentes estágios, especialmente no estágio larval, sugerindo que elas protegem o estágio inicial da galha para a manutenção do inseto galhador. Enquanto que a superóxido dismutase (SOD) contribuiu para a maior diferença entre folhas e galhas com a presença de inimigos naturais, mostrando que talvez esses organismos também estejam alterando o metabolismo da planta hospedeira e induzindo respostas antioxidantes na galha.
- Foram anotados cinquenta compostos químicos incluindo ácidos fenólicos, flavonoides, taninos, carboidratos e lipídios. E, dentre esses, os taninos hidrolisáveis foram aqueles que apareceram exclusivamente nas galhas, sugerindo que podem ter seus produtos de degradação, incluindo ácido elágico, contribuem para dissipar o estresse e manter o inseto galhador na planta hospedeira.
- Os experimentos das poliaminas, prolina e fitormônios, mostraram que a putrescina, espermina e prolina foram mais abundantes nas galhas vermelhas do que nas verdes, com exceção da espermidina. Os níveis dos fitormônios ABA, GA3, IAA e ACC foram maiores nas galhas verdes do que nas vermelhas, enquanto SA e MeJA foram mais abundantes nas galhas vermelhas. Esses resultados foram associados o maior acúmulo de alumínio (Al), ferro (Fe), chumbo (Pb) e cobre (Cu) nas galhas vermelhas do que nas verdes, o que pode estar relacionado com a sua translocação e biossíntese de compostos fenólicos quelantes para reduzir a toxicidade desses metais,

transformando-os em formas menos tóxicas para as galhas e mantendo a coloração vermelha como forma de proteção.

- **Perspectivas:**

Plantas hospedeiras de galhas podem obter ganhos de aptidão antecipando seu metabolismo ao amanhecer para biossintetizar carboidratos. E, também, produzir metabólitos secundários, como compostos fenólicos em horários distintos ao longo do dia, com maior incidência solar, por exemplo, para proteger e manter a cor das galhas. O estresse biótico altera a quantidade e a qualidade desses metabólitos. Como vimos nesta tese, é possível testar essas mudanças medindo a quantidade de transcritos que regulam o relógio circadiano, porque se este não estiver sincronizado com os sinais ambientais, as plantas hospedeiras podem ter seu *fitness* reduzido, produzindo menos compostos e tornando-se mais suscetíveis à ataques de outros herbívoros. É possível também que os metabólitos secundários protejam o relógio biológico por meio de suas funções antioxidantes e mantenham a sinconia entre os herbívoros galhadores e as plantas hospedeiras com seus diferentes formatos e cores.

1 Supplementary Material : Mass spectrometry LC-MS/MS

2 analysis

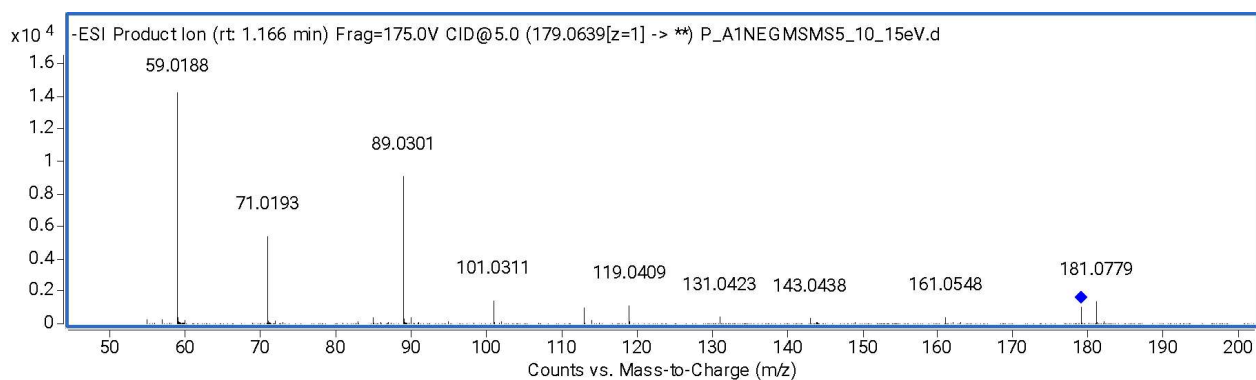


Figure 1: Espectro m/z 179.0568 $[M - H]^-$ (Hexose).

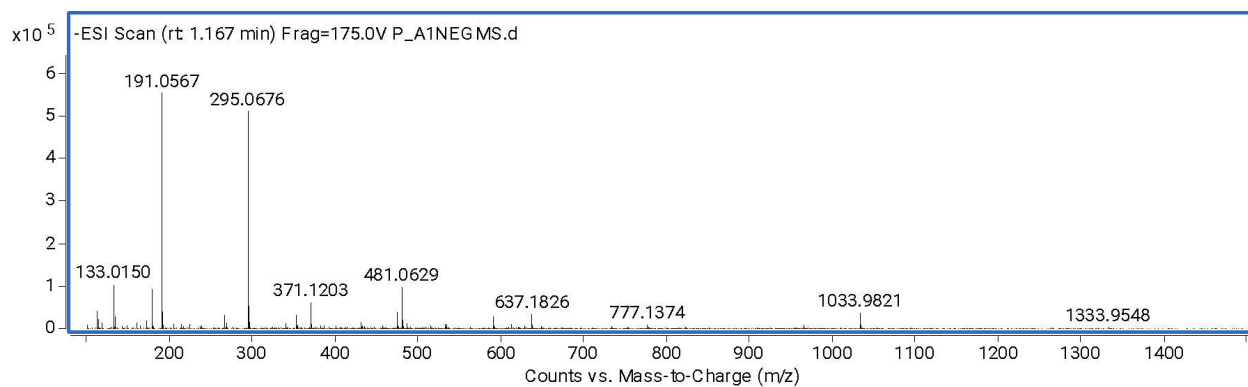


Figure 2: Espectro m/z 191.0567 $[M - H]^-$ (Quinic acid).

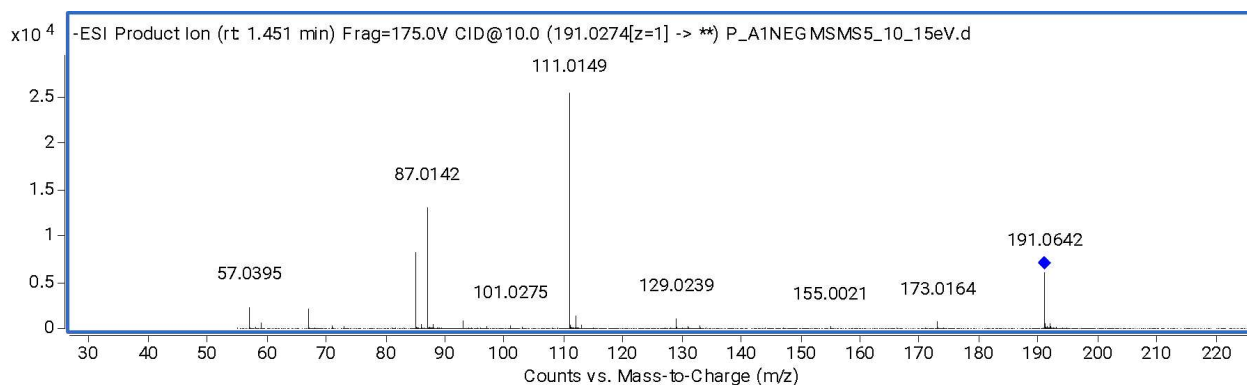


Figure 3: Espectro m/z 191.0567 $[M - H]^-$ (Quinic acid) in 10 eV.

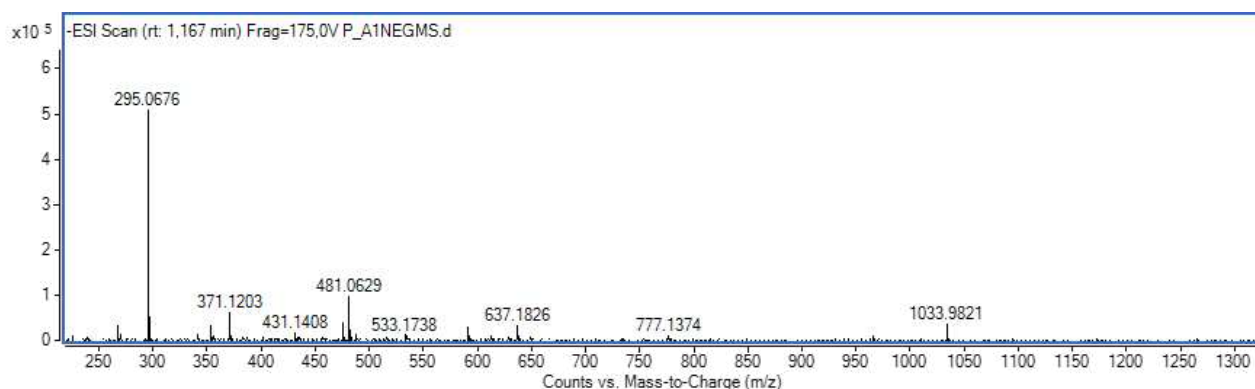


Figure 4: Espectro m/z 295.0676 $[M - H]^-$ (Maloyl hexose).

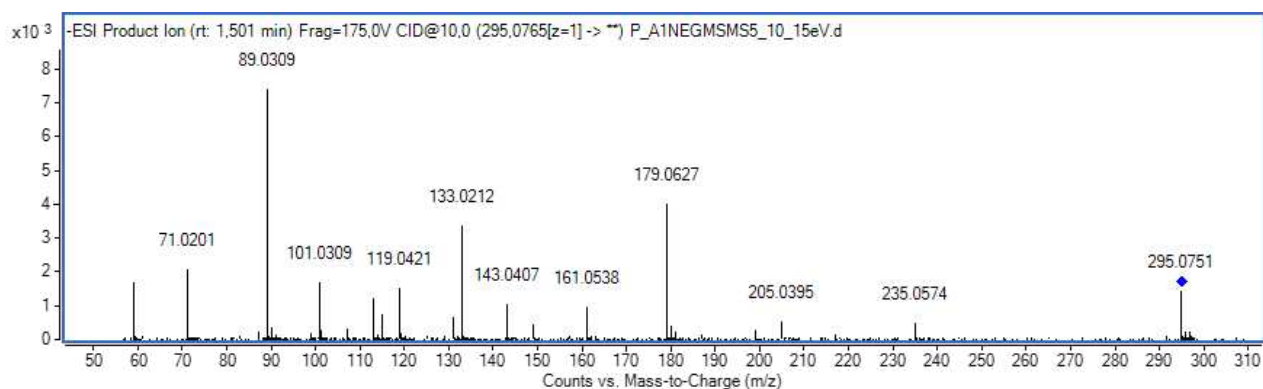


Figure 5: Espectro EM/EM m/z 295.0676 $[M - H]^-$ (Maloyl hexose) in 10 eV.

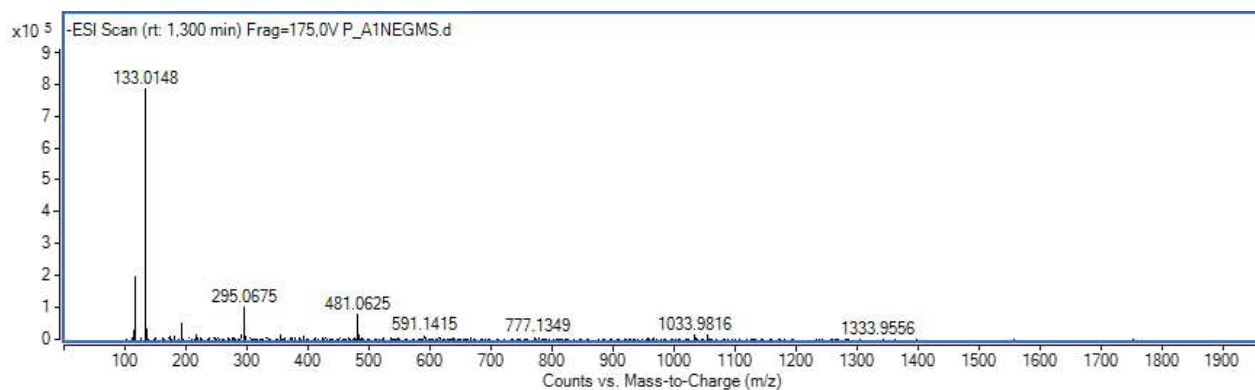


Figure 6: Espectro m/z 133.0148 $[M - H]^-$ (Malic Acid).

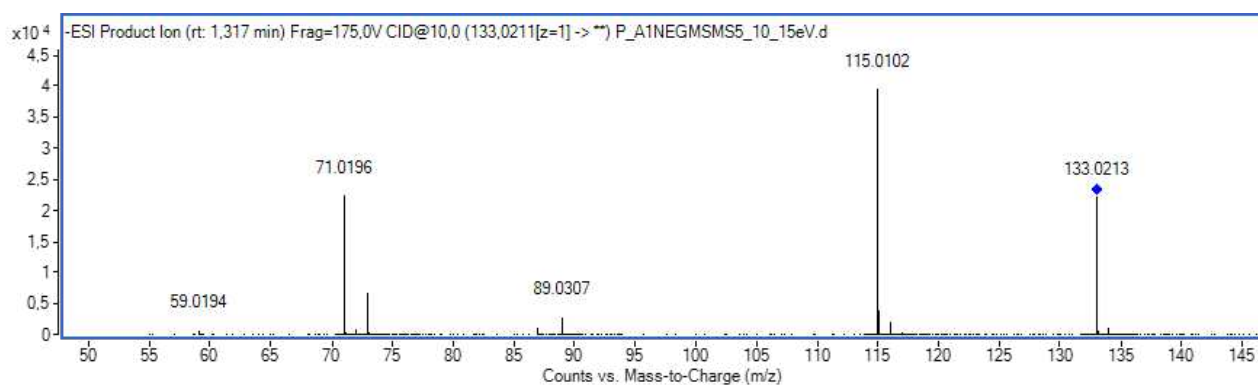


Figure 7: Espectro EM/EM m/z 133.0148 $[M - H]^-$ (Malic Acid) in 10 eV.

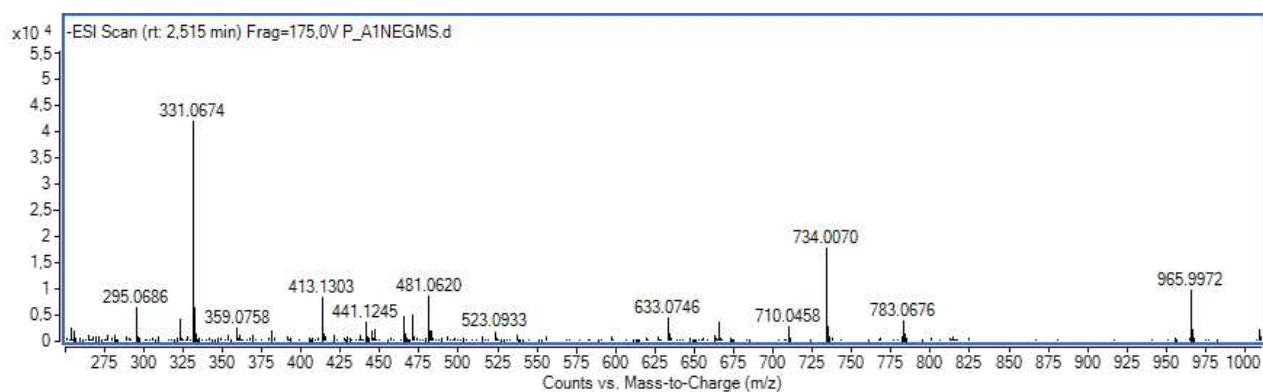


Figure 8: Espectro m/z 331.0674 $[M - H]^-$ (Galloylglucose).

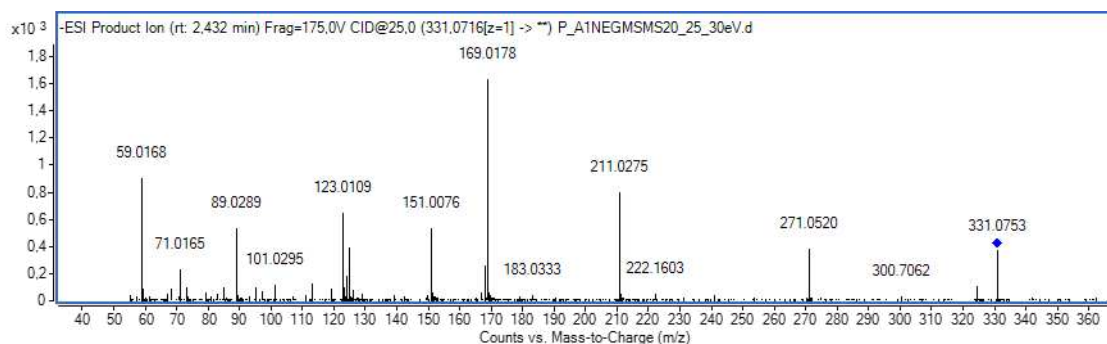


Figure 9: Espectro EM/EM m/z 331.0674 $[M - H]^-$ (Galloylglucose) in 25 eV.

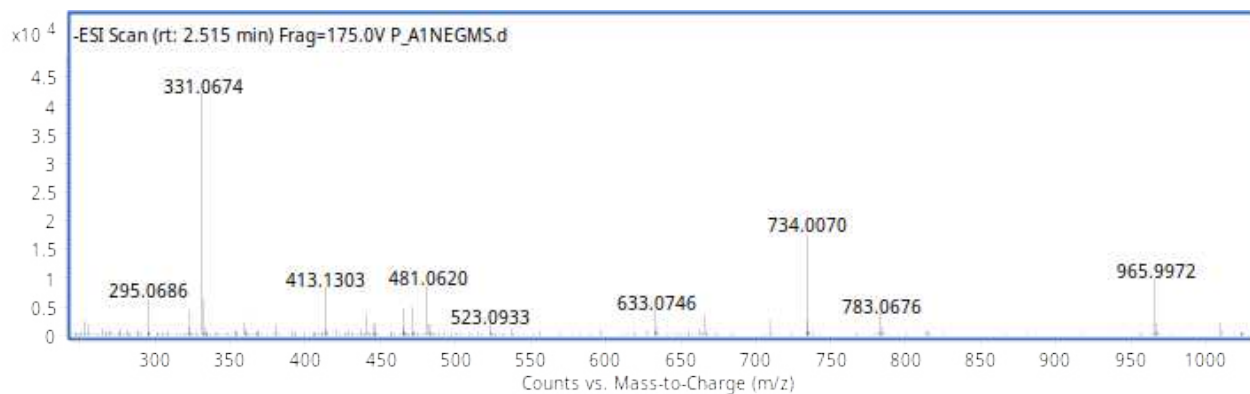


Figure 10: Espectro m/z 481.0620 $[M - H]^-$ (HHDP-hexose).

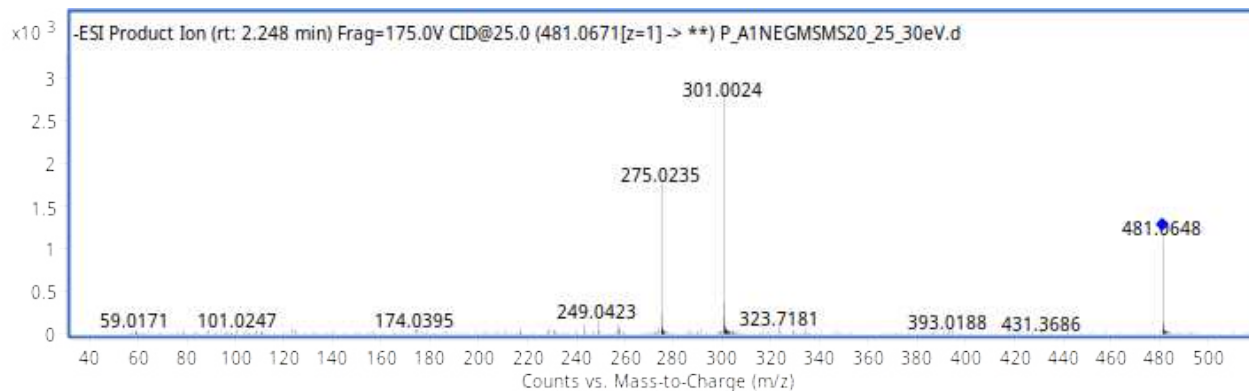


Figure 11: Espectro EM/EM m/z 481.0620 $[M - H]^-$ (HHDP-hexose) in 25 eV.

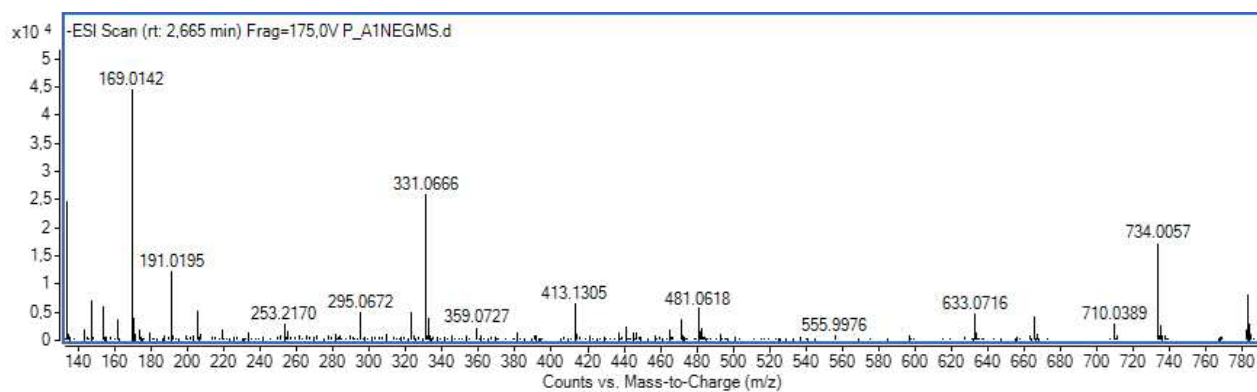


Figure 12: Espectro m/z 169.0142 $[M - H]^-$ (Galic acid)

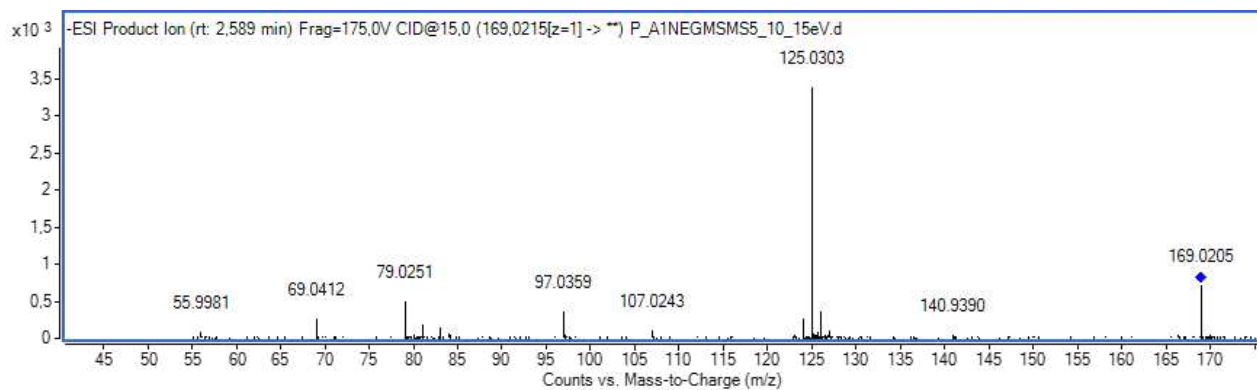


Figure 13: Espectro EM/EM m/z 169.0142 $[M - H]^-$ (Galic acid) in 15 eV.

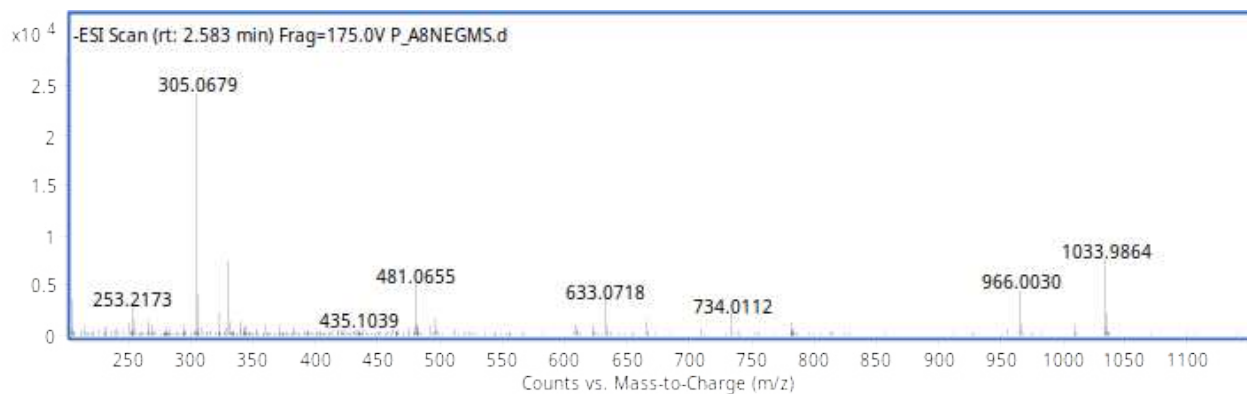


Figure 14: Espectro m/z 305.0679 $[M - H]^-$ ((Epi)gallocatechin).

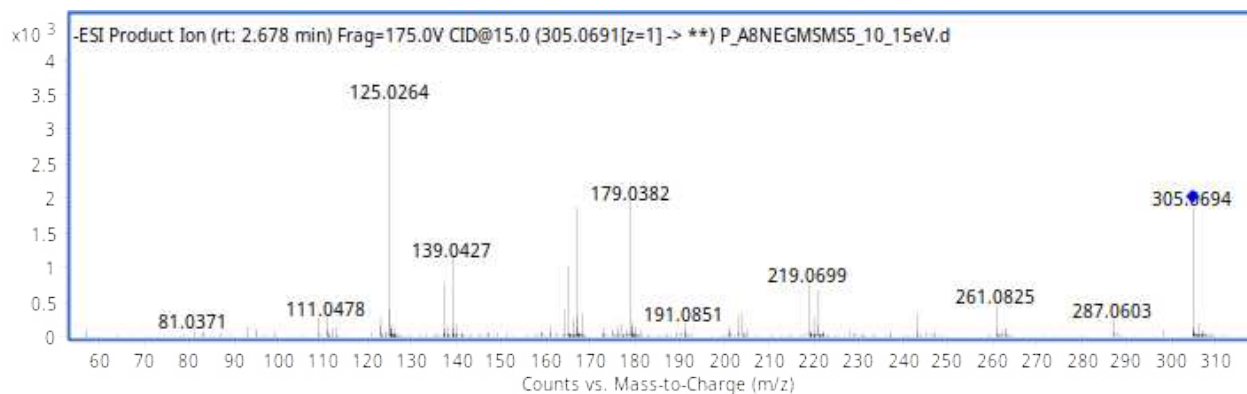


Figure 15: Espectro EM/EM m/z 305.0679 $[M - H]^-$ ((Epi)gallocatechin) in 15 eV.

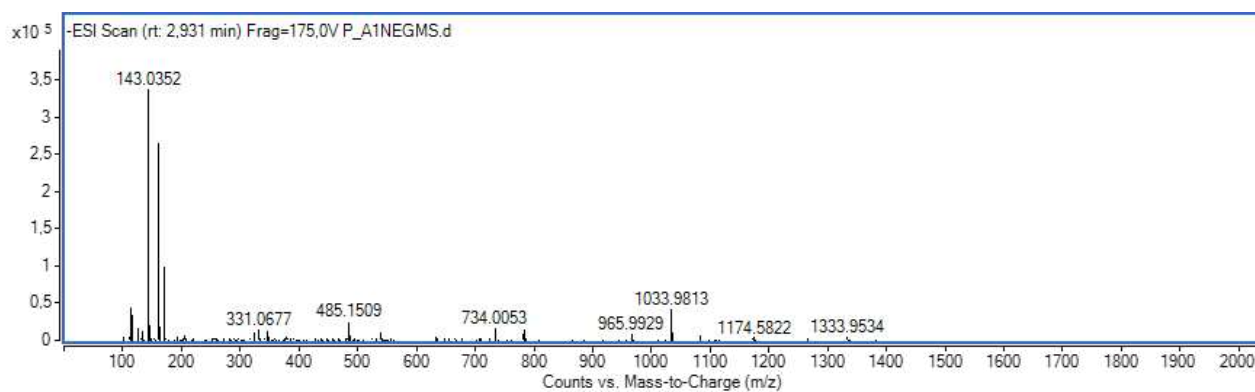


Figure 16: Espectro m/z 143.0352 $[M - H]^-$ (4-Ethoxy-4-oxobut-2-enoic acid).

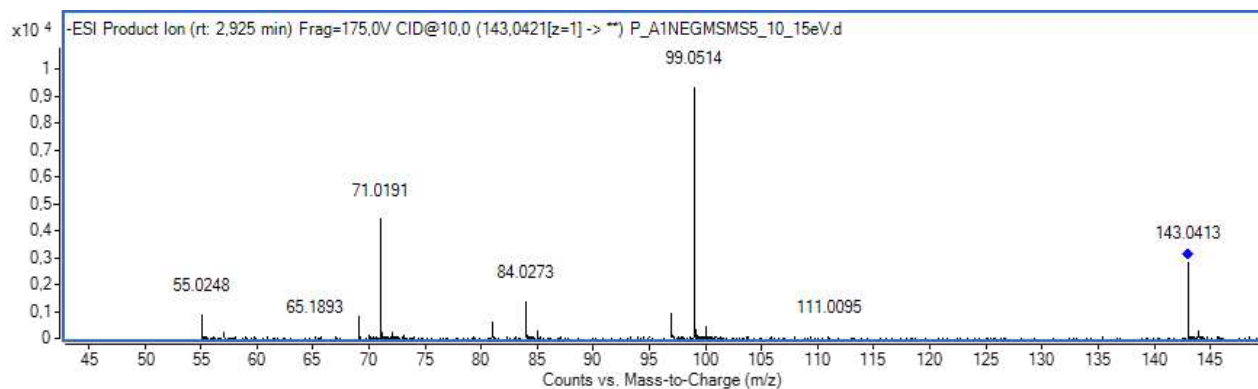


Figure 17: Espectro EM/EM m/z 143.0352 $[M - H]^-$ (4-Ethoxy-4-oxobut-2-enoic acid) in 10 eV.

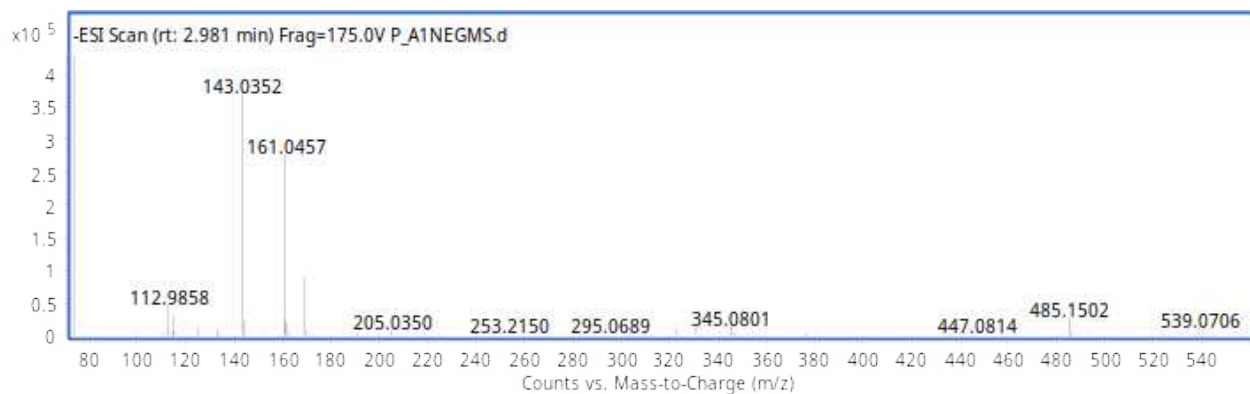


Figure 18: Espectro m/z 161.0457 $[M - H]^-$ (2,3-Dimethylmalate).

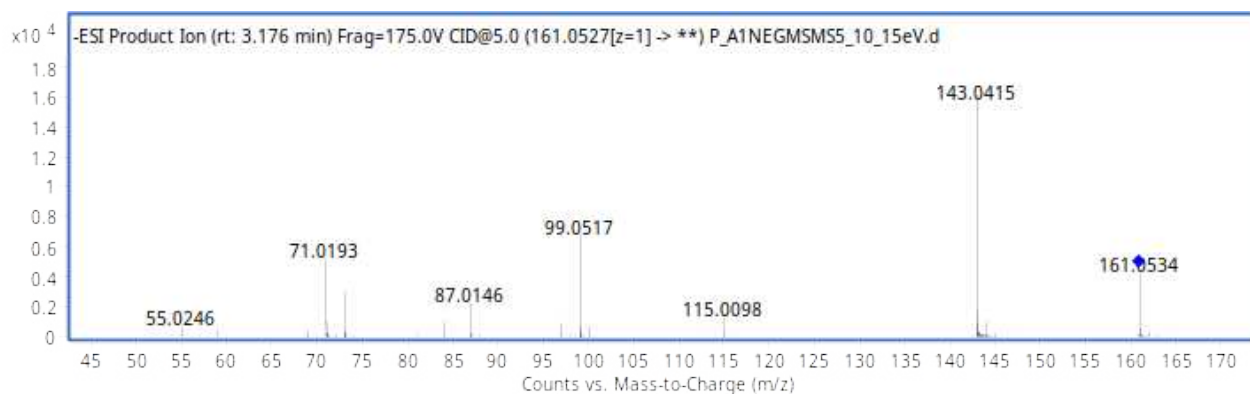


Figure 19: Espectro EM/EM m/z 161.0457 $[M - H]^-$ (2,3-Dimethylmalate) in 5 eV.

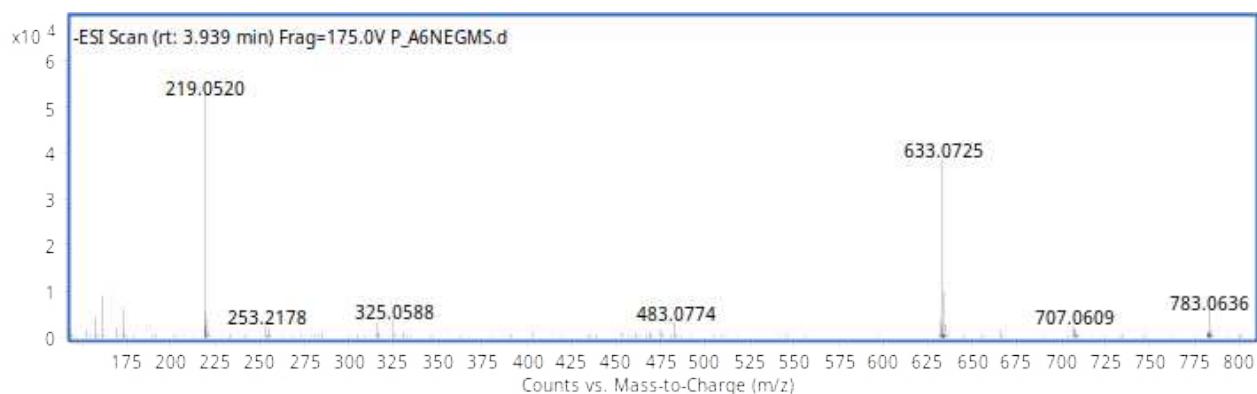


Figure 20: Espectro m/z 219.0512 $[M - H]^-$ (Unknown) and m/z 633.0725 $[M - H]^-$ (Galloyl-HHDP-glucose isomer I).

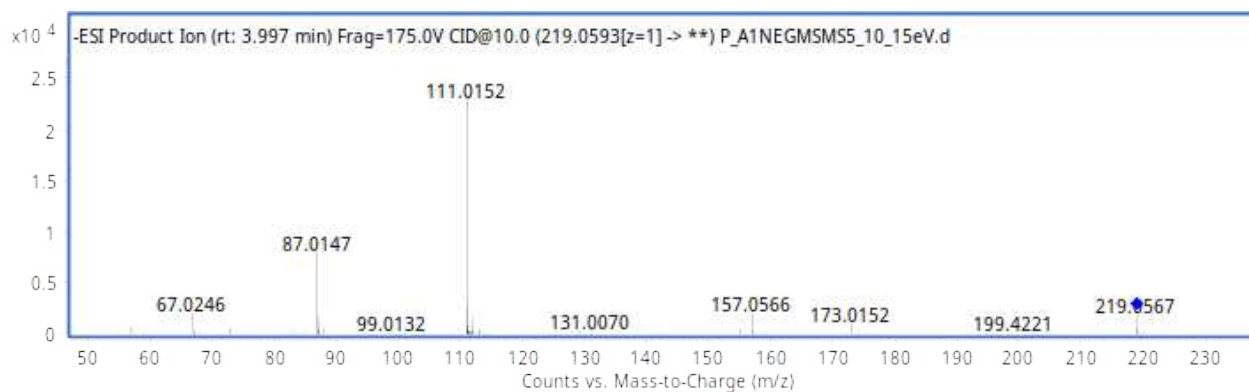


Figure 21: Espectro EM/EM m/z 219.0512 $[M - H]^-$ (Unknown) in 10 eV.

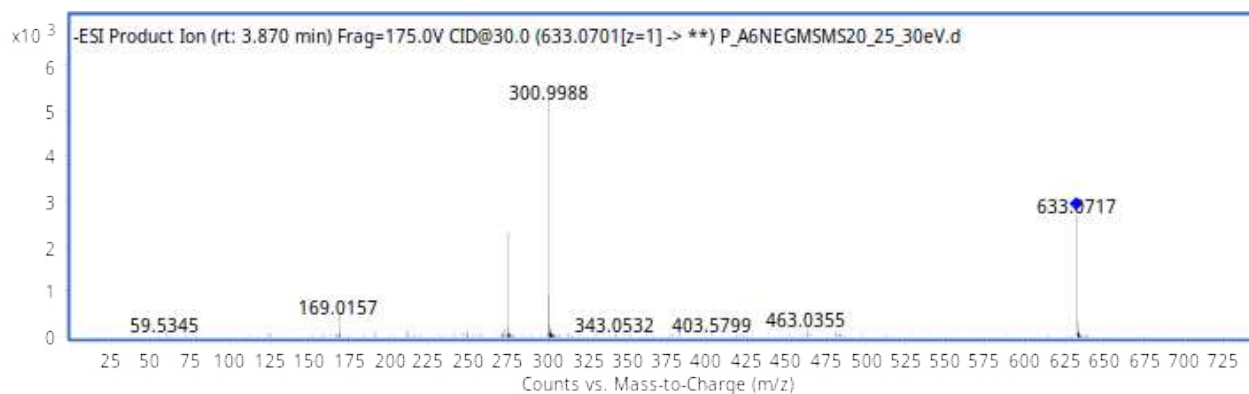


Figure 22: Espectro EM/EM m/z 633.0725 $[M - H]^-$ (Unknown) in 30 eV.

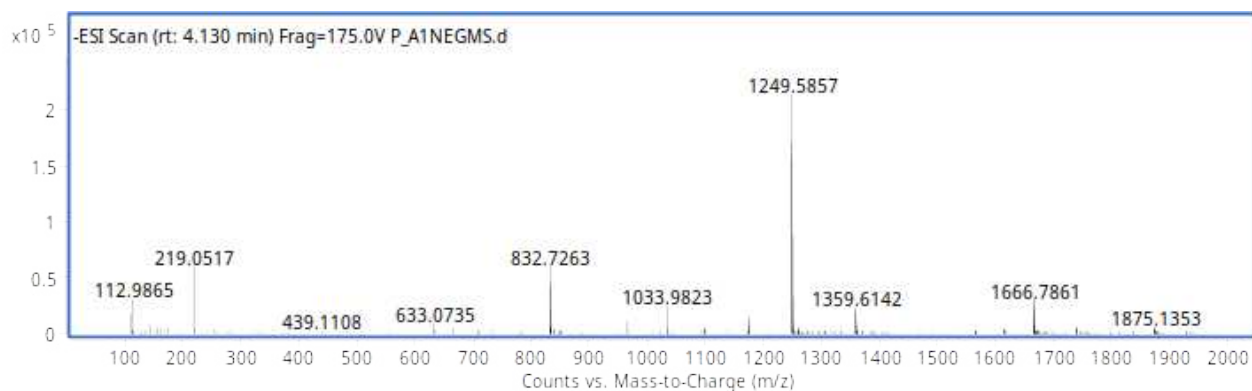


Figure 23: Espectro m/z 1249.5857 $[M - H]^-$ (unknown)

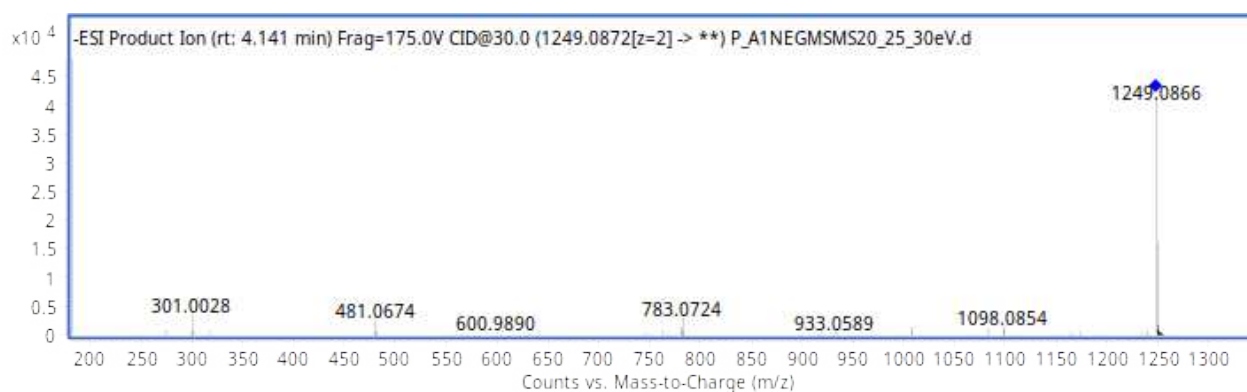


Figure 24: Espectro EM/EM m/z 1249.5857 $[M - H]^-$ (unknown) in 30 eV.

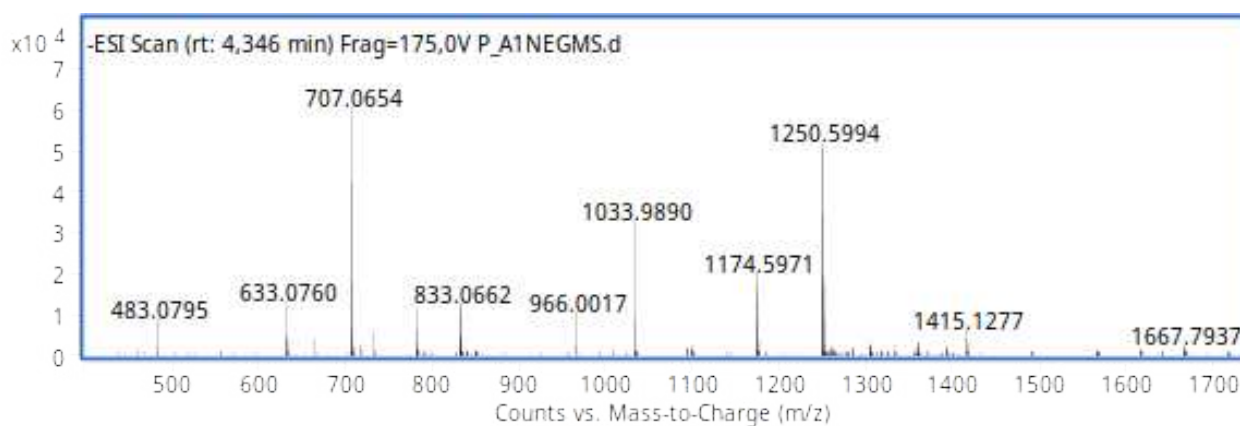


Figure 25: Espectro m/z 1415.12 $[M - H]^-$ and 707.0654 $[M - 2H]^{2-}$ (HHDP-galloyl-HHDP).

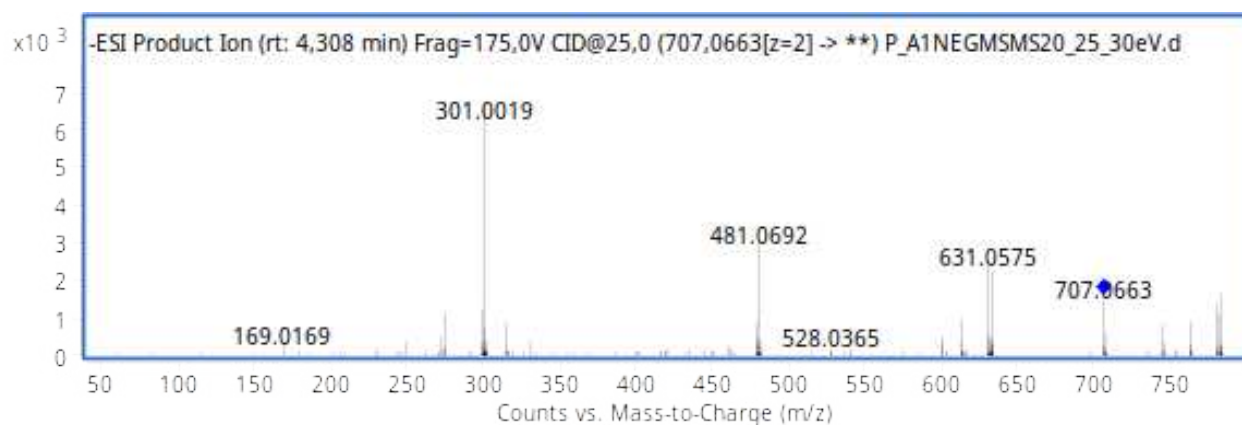


Figure 26: Espectro m/z 707.0654 $[M - H]^-$ (HHDP-galloyl-HHDP) in 25 eV.

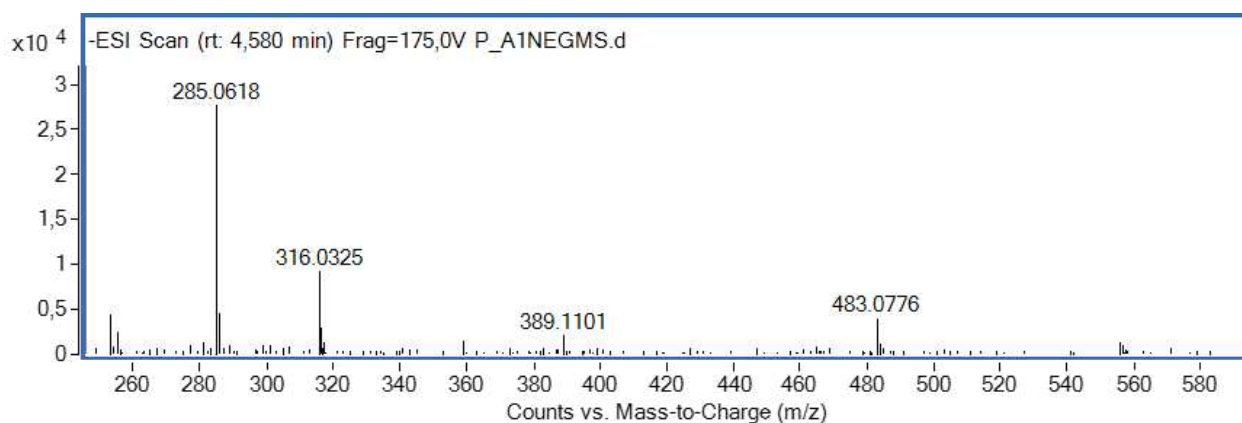


Figure 27: Espectro m/z 285.0618 $[M - H]^-$ (Dihydroxybenzoic acid pentose).

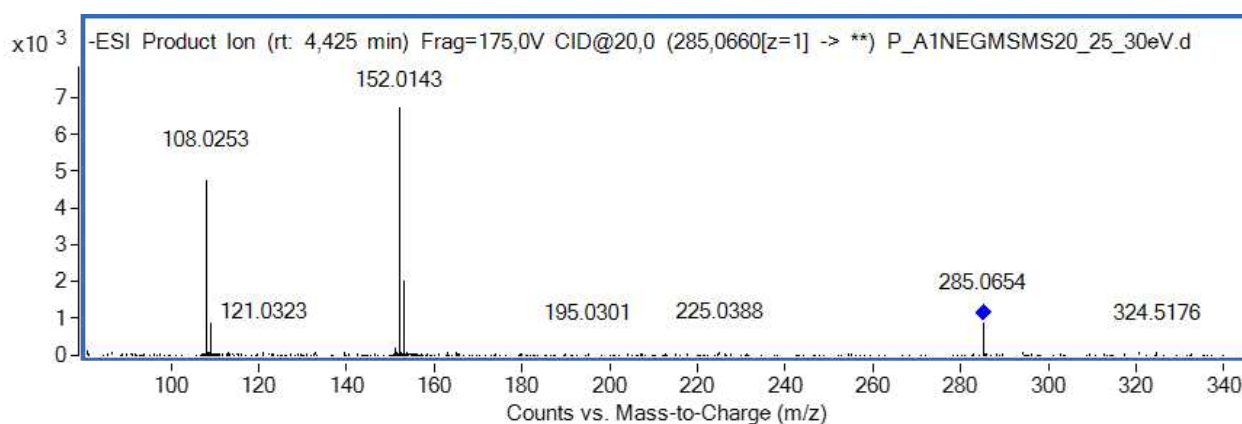


Figure 28: Espectro EM/EM m/z 285.0618 $[M - H]^-$ (Dihydroxybenzoic acid pentose) in 20 eV.

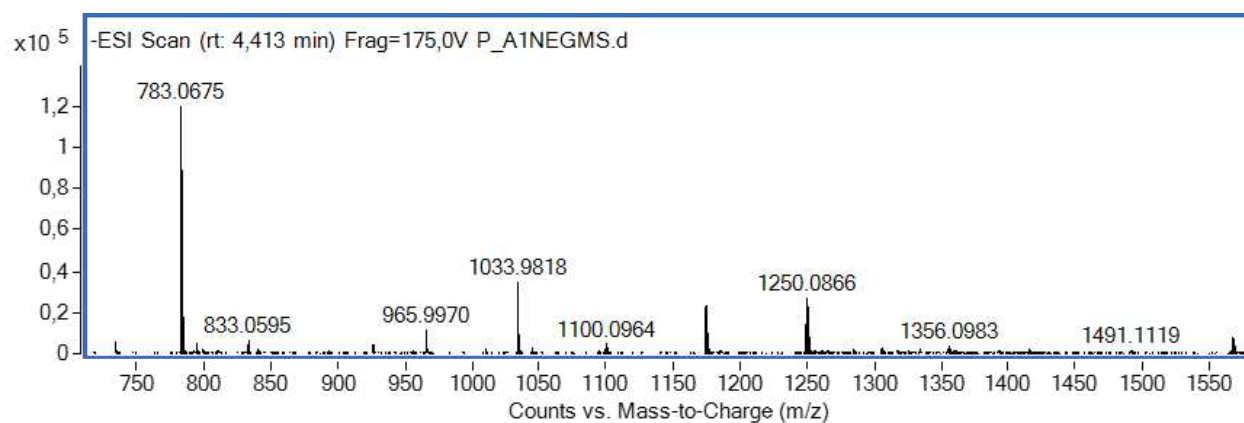


Figure 29: Espectro m/z 783.0675 $[M - H]^-$ (Pedunculagin)

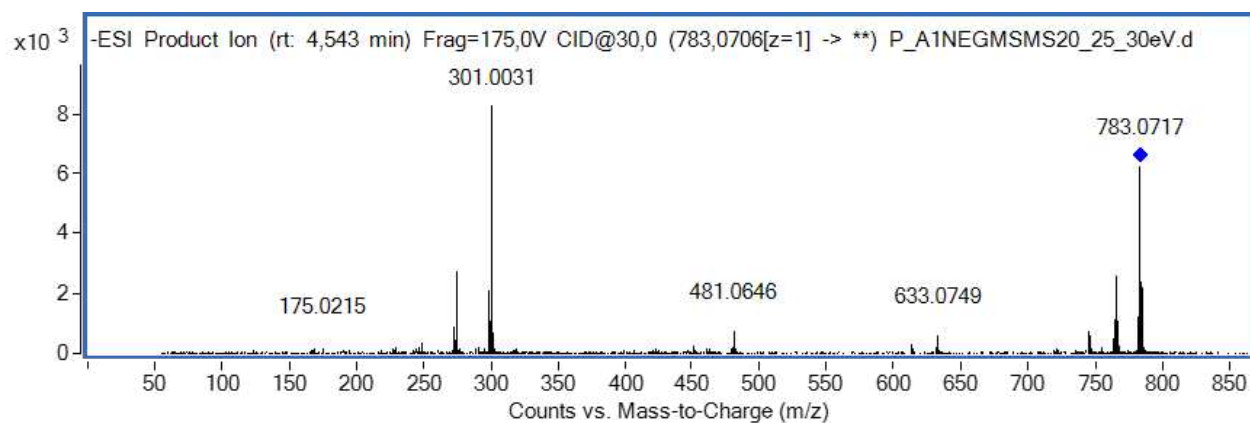


Figure 30: Espectro EM/EM m/z 783.0675 $[M - H]^-$ (Pedunculagin) in 30 eV.

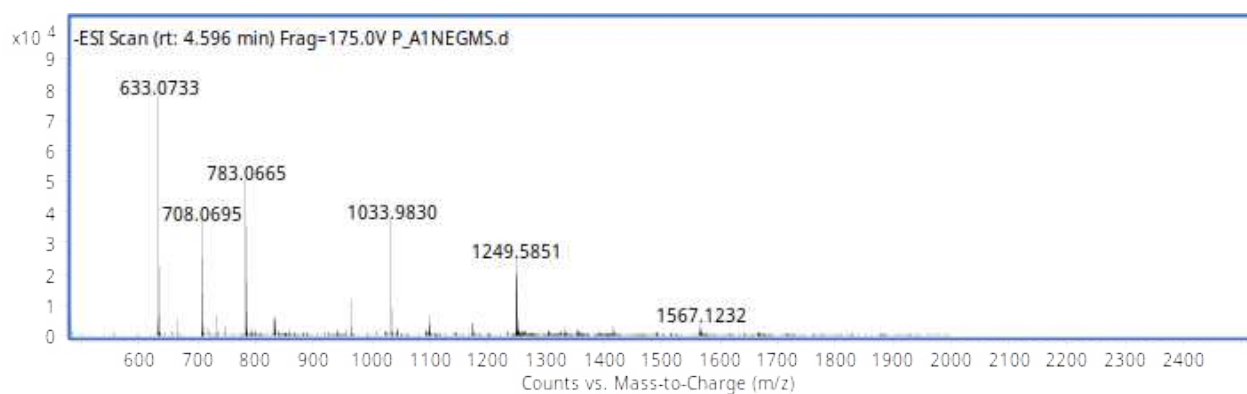


Figure 31: Espectro m/z 633.0733 $[M - H]^-$ (Galloyl-HHDP-glucose).

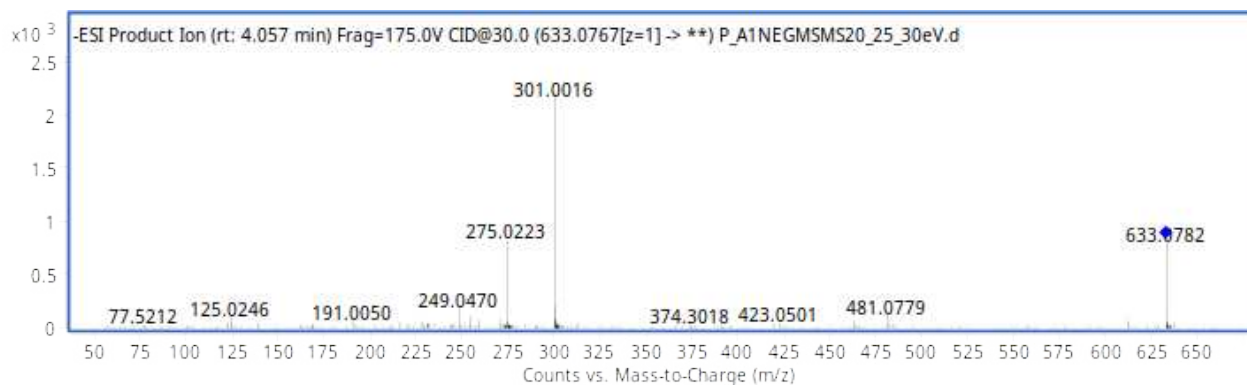


Figure 32: Espectro EM/EM m/z 633.0733 $[M - H]^-$ (Galloyl-HHDP-glucose) in 30 eV.

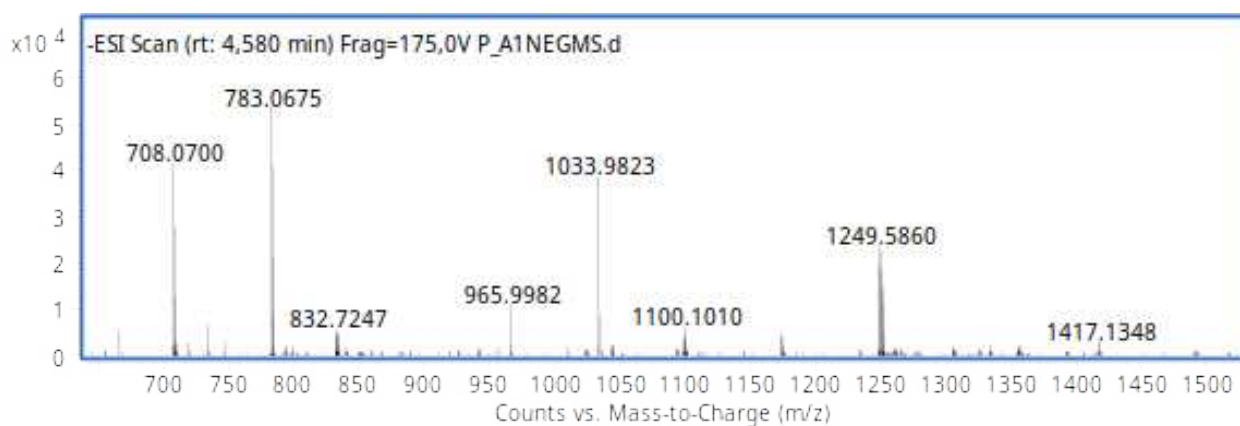


Figure 33: Espectro m/z 708.0700 $[M - H]^-$ (Pedunculagin-trigalloyl-glucose).

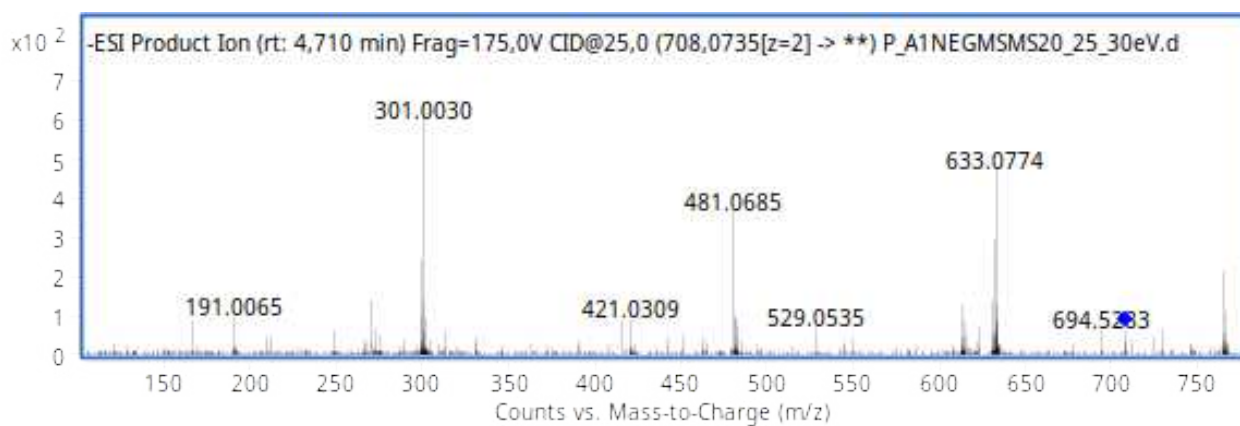


Figure 34: Espectro EM/EM m/z 708.0700 $[M - H]^-$ (Pedunculagin-trigalloyl-glucose) in 25 eV.

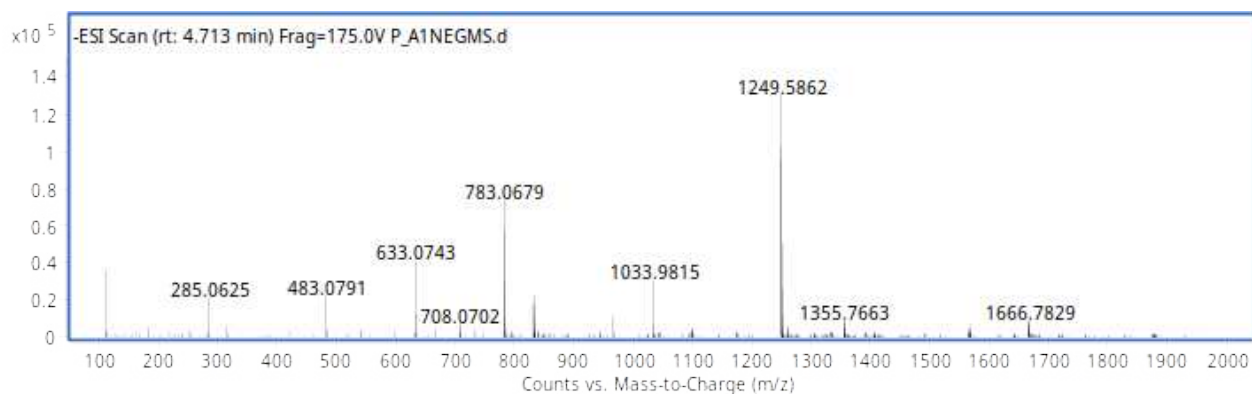


Figure 35: Espectro m/z 1249.5862 $[M - H]^-$ (Unknown).

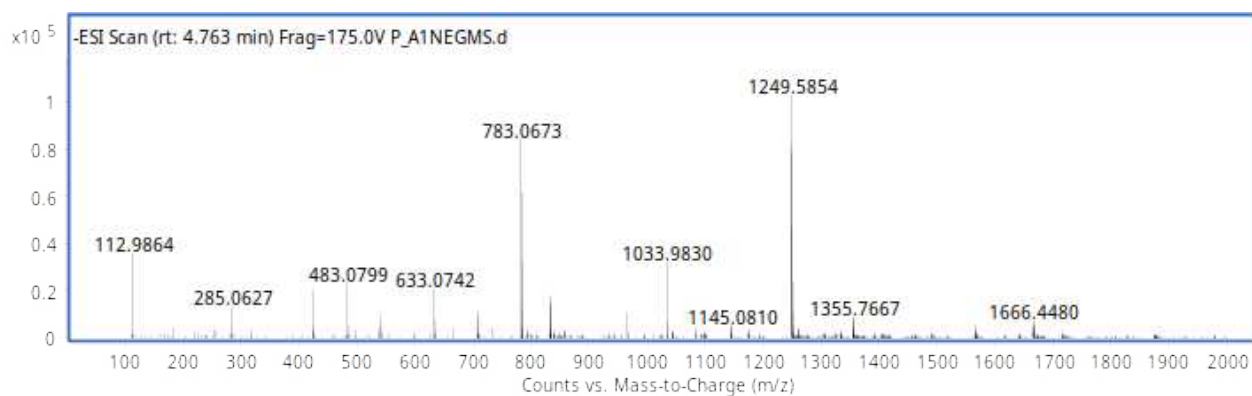


Figure 36: Espectro m/z 783.0673 $[M - H]^-$ (Pedunculagin isomer II).

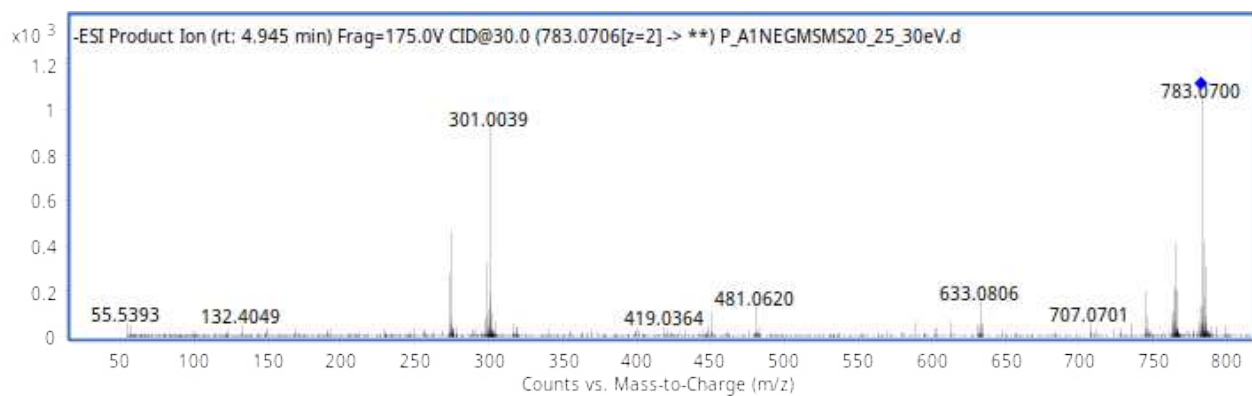


Figure 37: Espectro EM/EM m/z 783.0673 $[M - H]^-$ (Pedunculagin isomer II) in 30 eV.

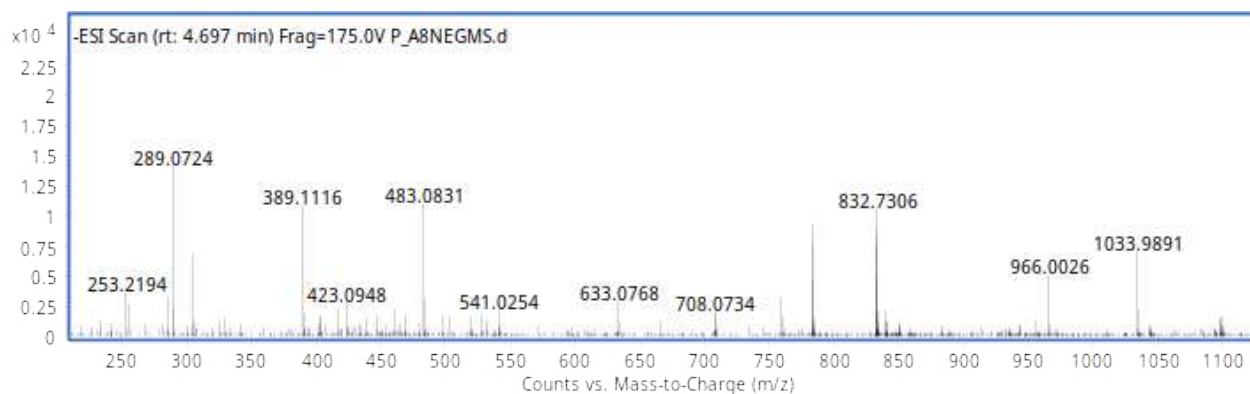


Figure 38: Espectro m/z 289.0724 $[M - H]^-$ ((Epi)catechin).

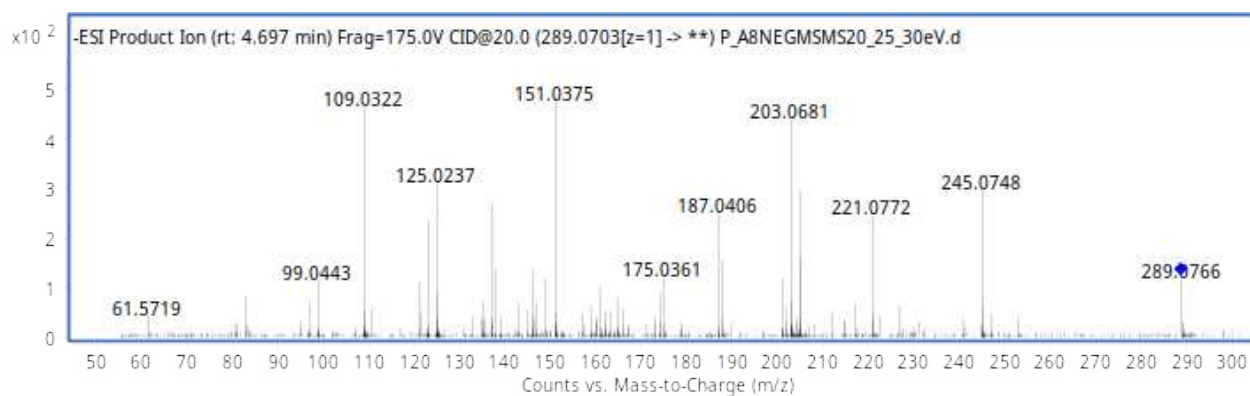


Figure 39: Espectro EM/EM m/z 289.07 $[M - H]^-$ ((Epi)catechin) in 20 eV.

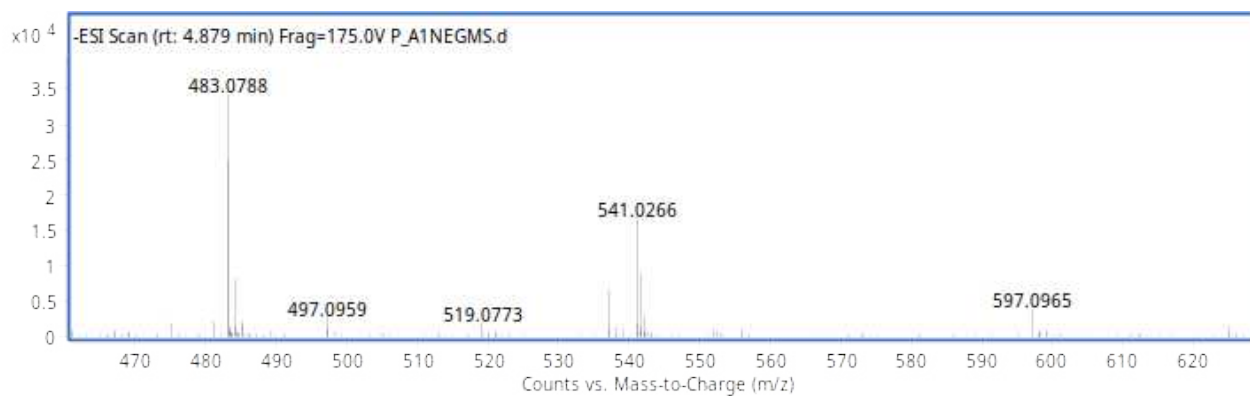


Figure 40: Espectro m/z 483.0788 $[M - H]^-$ (Digalloylglucose isomer I)

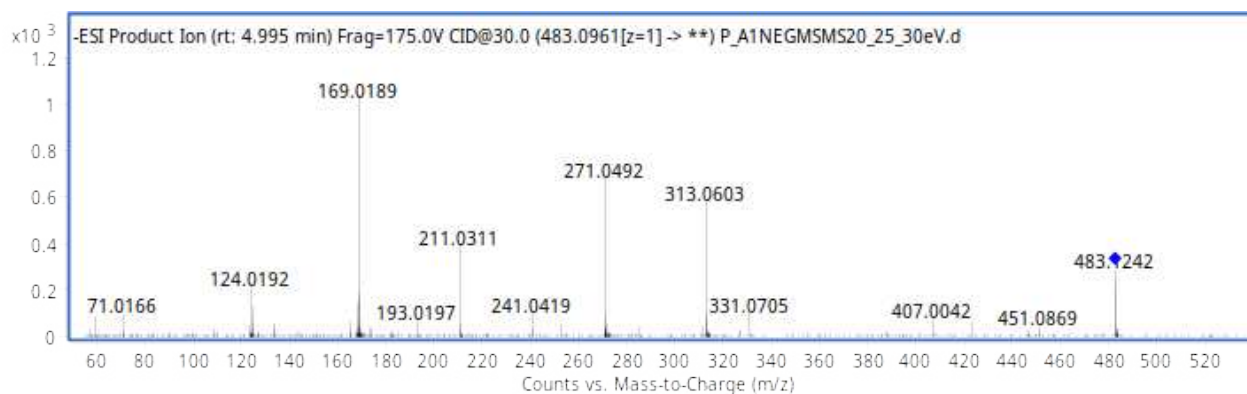


Figure 41: Espectro EM/EM m/z 483.0788 $[M - H]^-$ (Digalloylglucose isomer I) in 30 eV.

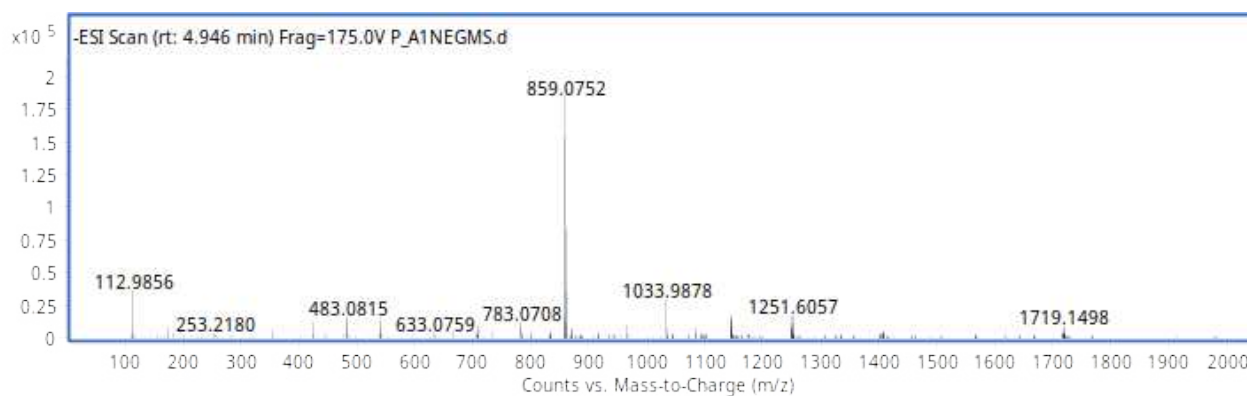


Figure 42: Espectro m/z 859.0752 $[M - H]^{2-}$ and m/z 1719.1498 $[M - H]^-$ (Camellioferin A).

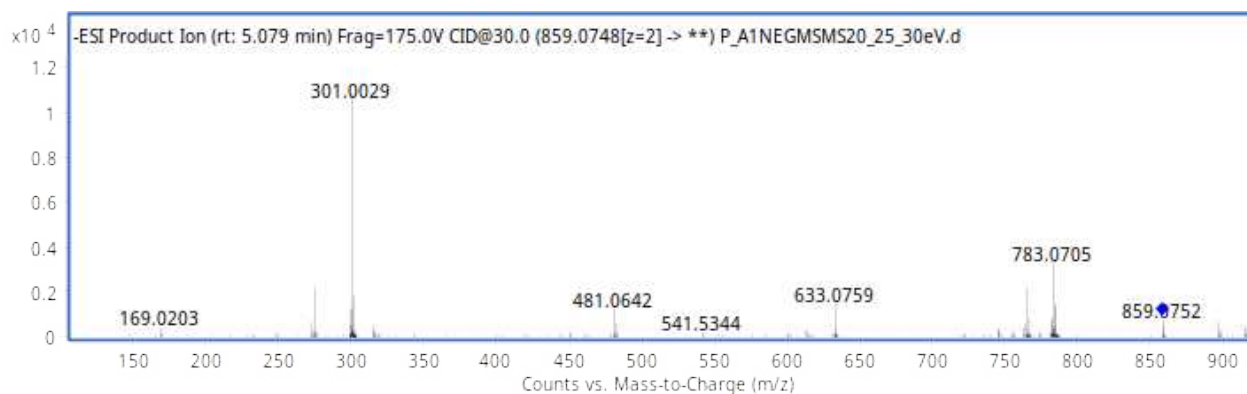


Figure 43: Espectro EM/EM m/z 859.0752 $[M - H]^{2-}$ (Camellioferin A) in 30 eV.

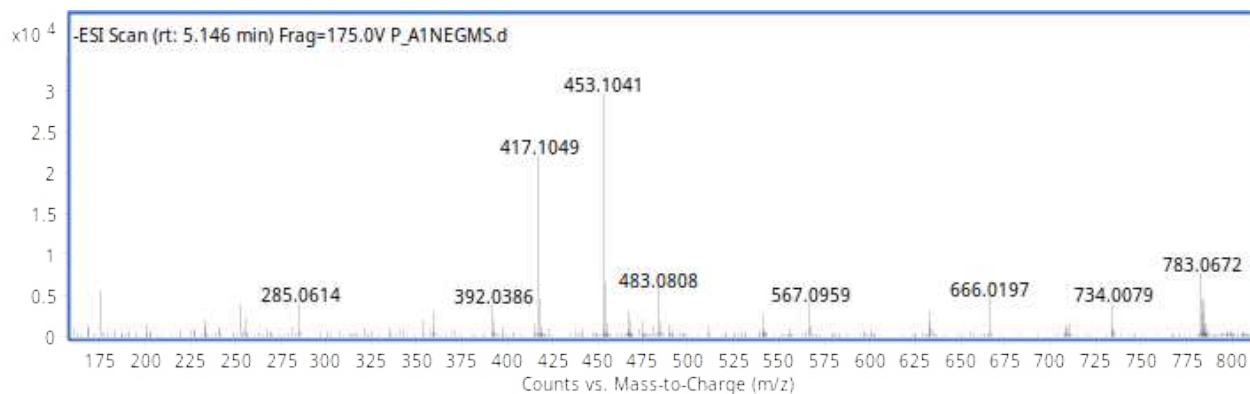


Figure 44: Espectro m/z 453.1041 $[M - H]^-$ (3-Methoxy-4-hydroxyphenol-1-O-(6'-O-galloyl)-glucoside) and m/z 417.1049 $[M - H]^-$ (Unknown).

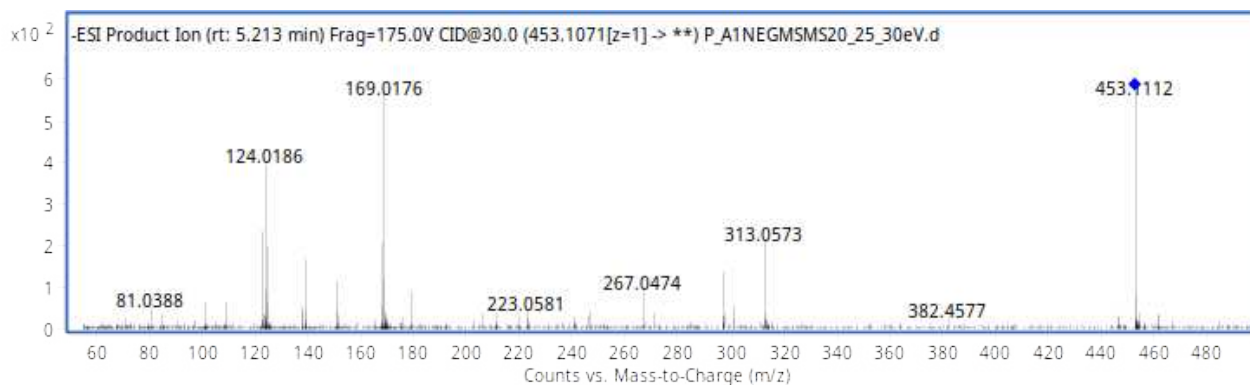


Figure 45: Espectro EM/EM m/z 453.1041 $[M - H]^-$ (3-Methoxy-4-hydroxyphenol-1-O-(6'-O-galloyl)-glucoside) in 30 eV.

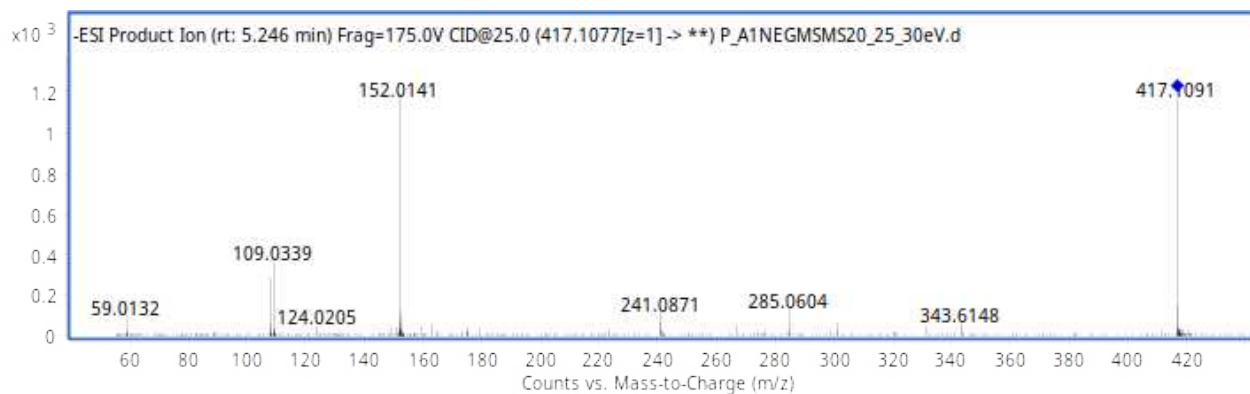


Figure 46: Espectro EM/EM m/z 417.1049 $[M - H]^-$ (Unknown) in 25 eV.

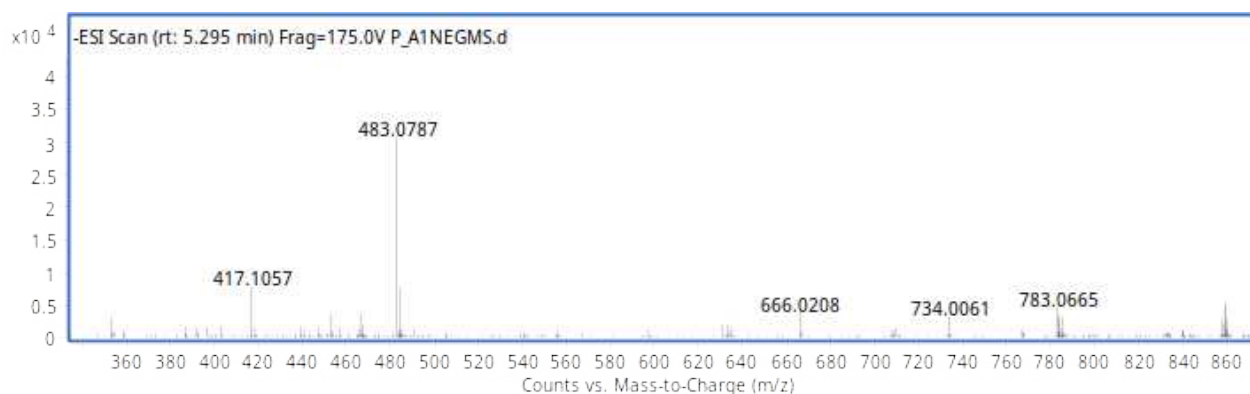


Figure 47: Espectro m/z 483.0787 $[M - H]^-$ (Digalloylglucose isomer II).

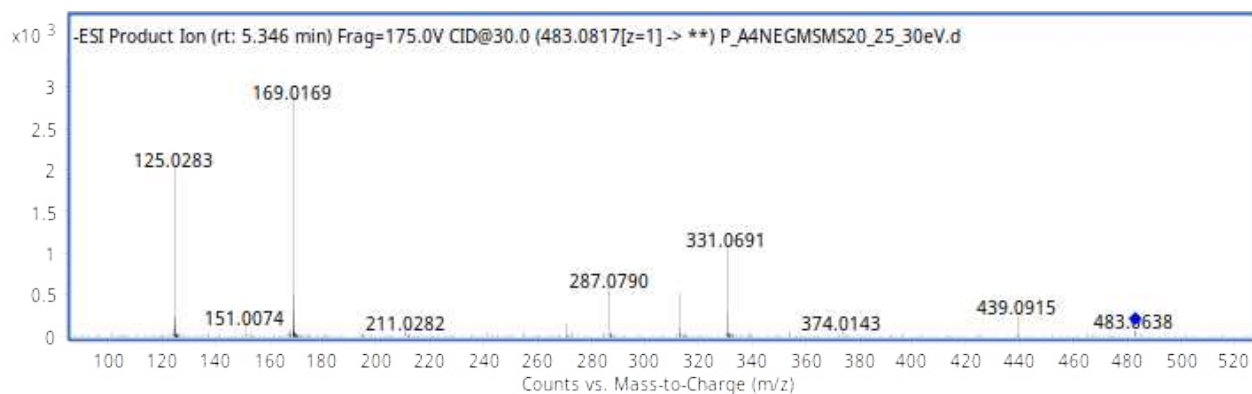


Figure 48: Espectro EM/EM m/z 483.0787 $[M - H]^-$ (Digalloylglucose isomer II) in 30 eV.

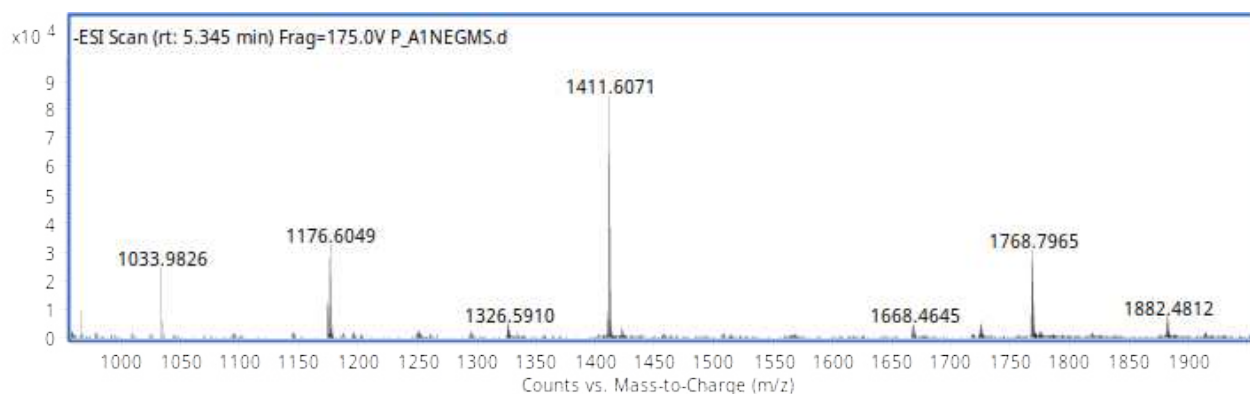


Figure 49: Espectro m/z 1176.6049 $[M - H]^-$ (Unknown) and m/z 1411.6071 $[M - H]^-$ (Unknown).

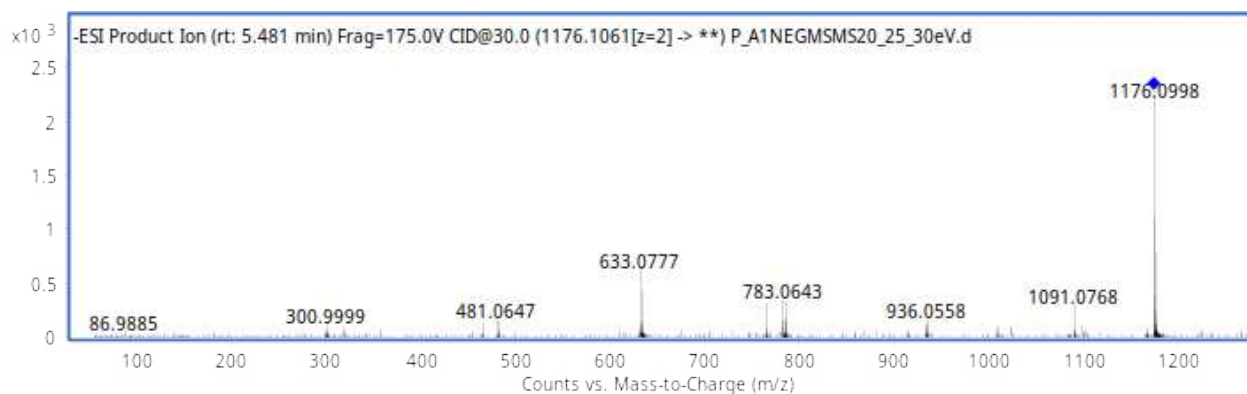


Figure 50: Espectro EM/EM m/z 1176.6049 $[M - H]^-$ (Unknown) in 30 eV.

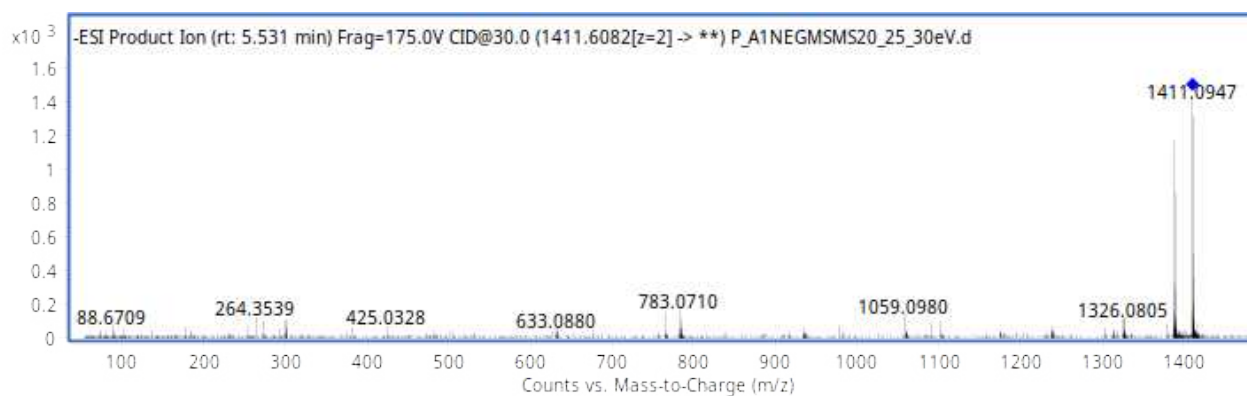


Figure 51: Espectro EM/EM m/z 1411.6071 $[M - H]^-$ (Unknown) in 30 eV.

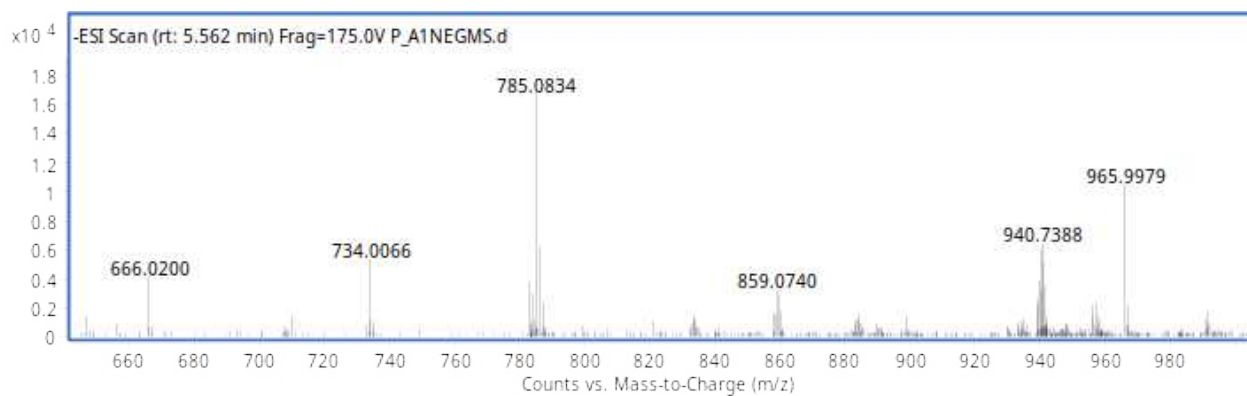


Figure 52: Espectro m/z 785.0834 $[M - H]^-$ (Digalloylglucose).

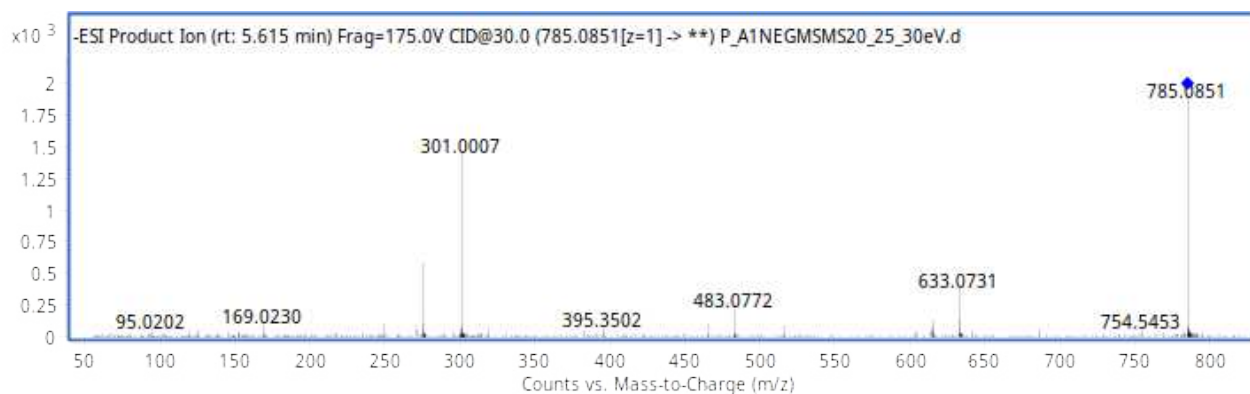


Figure 53: Espectro m/z 785.0834 $[M - H]^-$ (Digalloylglucose).

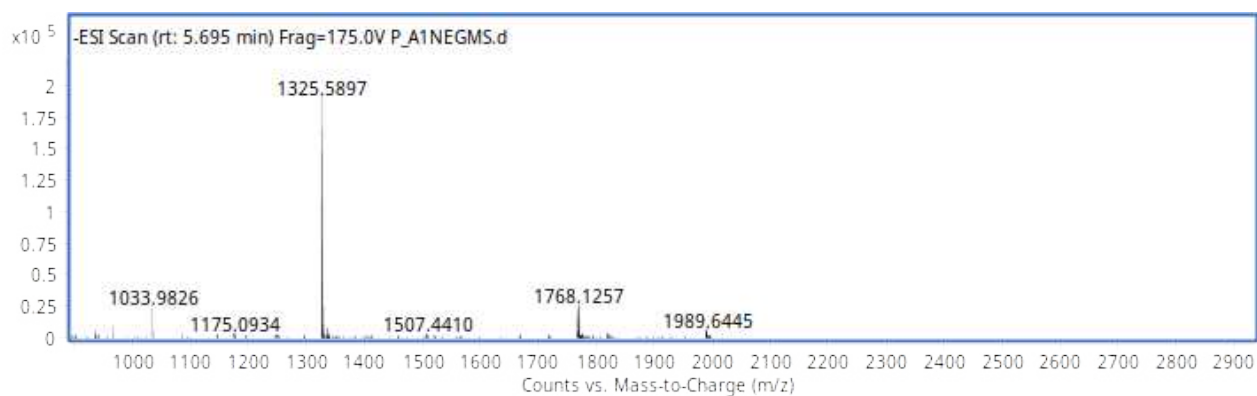


Figure 54: Espectro m/z 1325.5897 $[M - H]^-$ (Unknown).

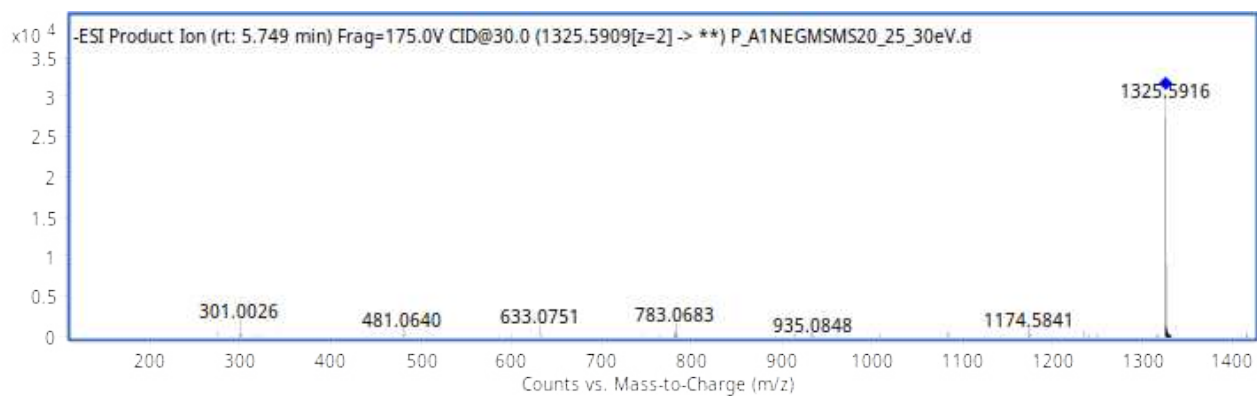


Figure 55: Espectro EM/EM m/z 1325.5916 $[M - H]^-$ (Unknown) in 30 eV.

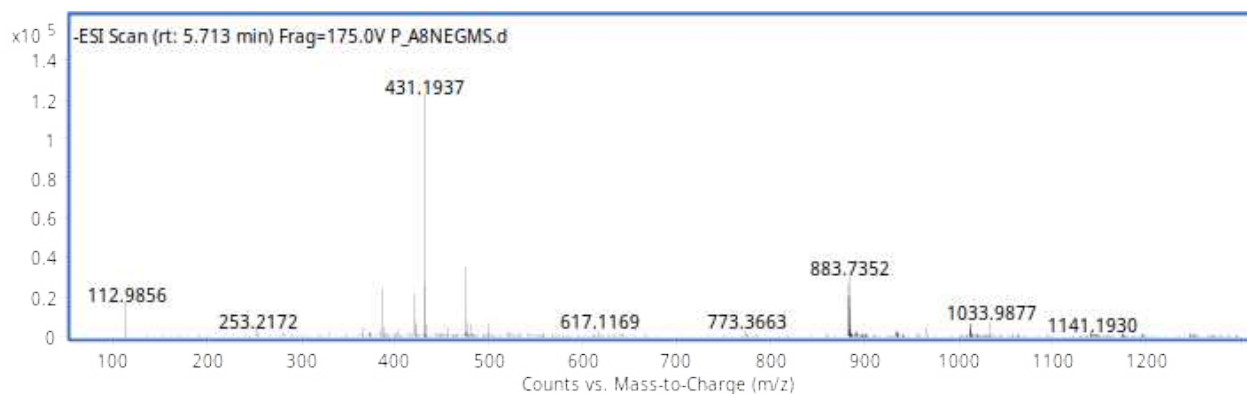


Figure 56: Espectro m/z 431.1937 $[M - H]^-$ (Roseoside (formate adduct)).

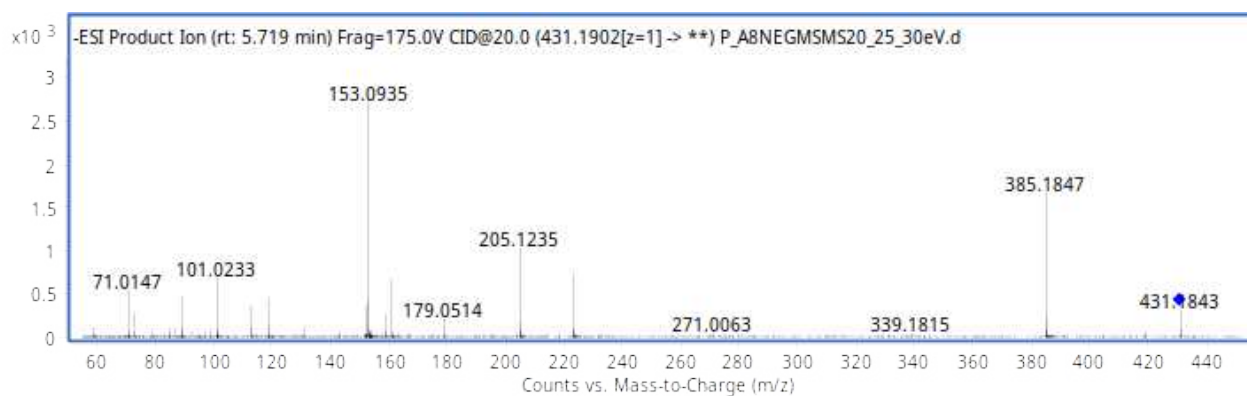


Figure 57: Espectro EM/EM m/z 431 $[M - H]^-$ (Roseoside (formate adduct)) in 20 eV.

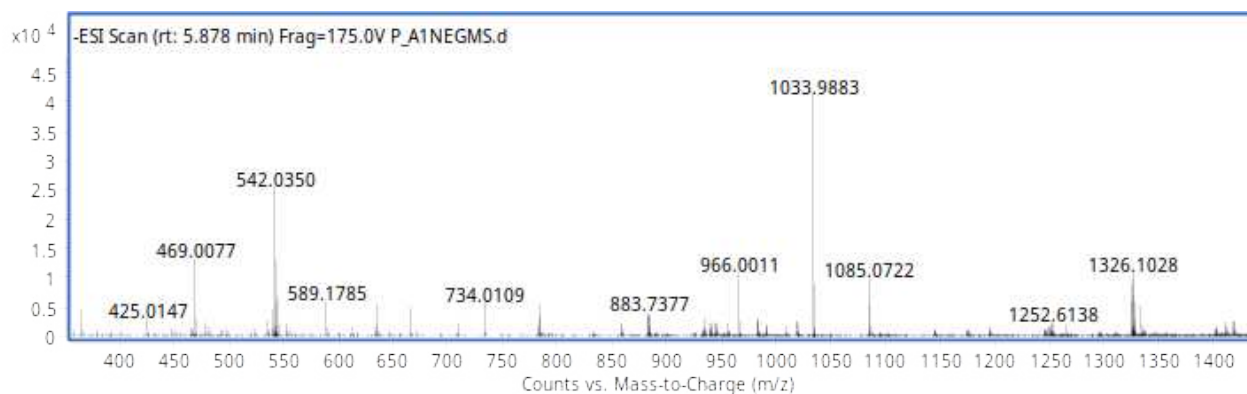


Figure 58: Espectro m/z 542.0350 $[M - H]^{2-}$ and m/z 1085.0722 $[M - H]^-$ (Terflavin A).

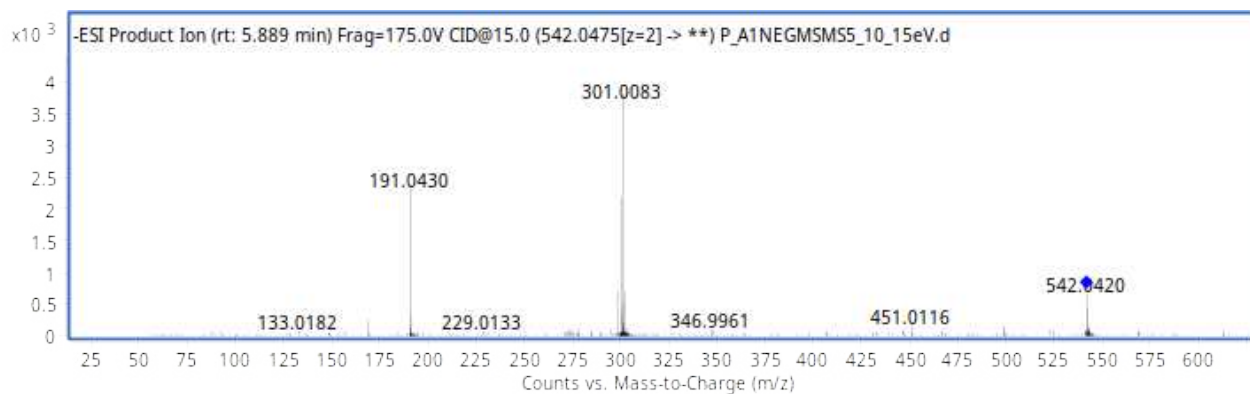


Figure 59: Espectro EM/EM m/z 542.0350 $[M - H]^{2-}$ (Terflavin A) in 15 eV.

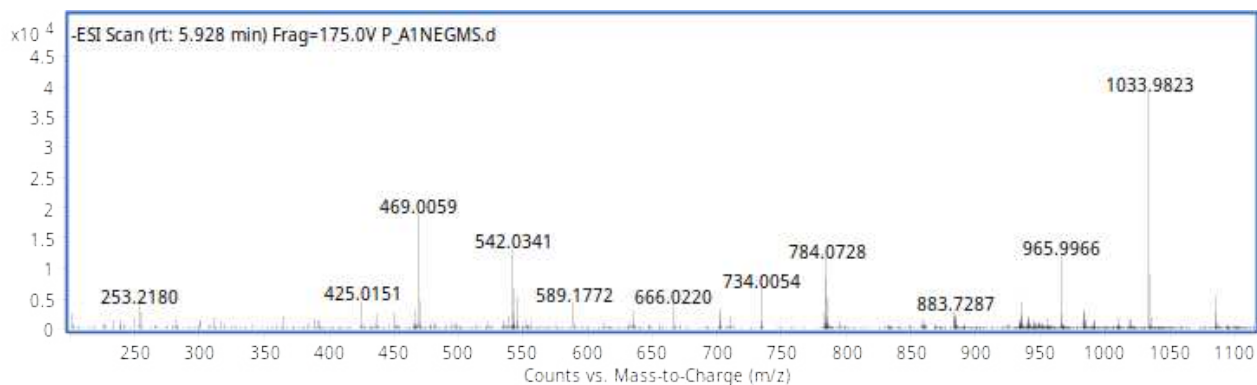


Figure 60: Espectro m/z 469.0059 $[M - H]^-$ (Valoneic acid dilactone).

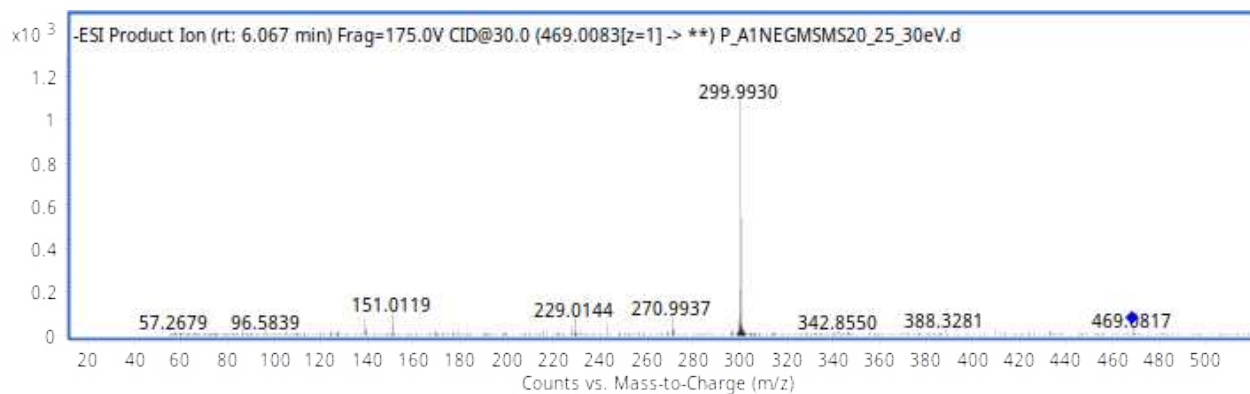


Figure 61: Espectro EM/EM m/z 469.0059 $[M - H]^-$ (Valoneic acid dilactone) in 30 eV.

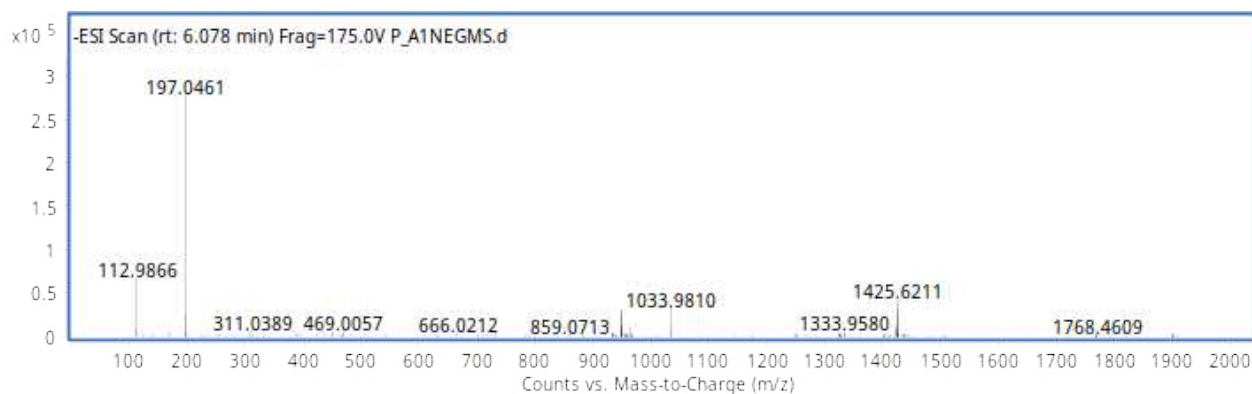


Figure 62: Espectro m/z 197.0461 $[M - H]^-$ (Gallic acid ethyl ester).

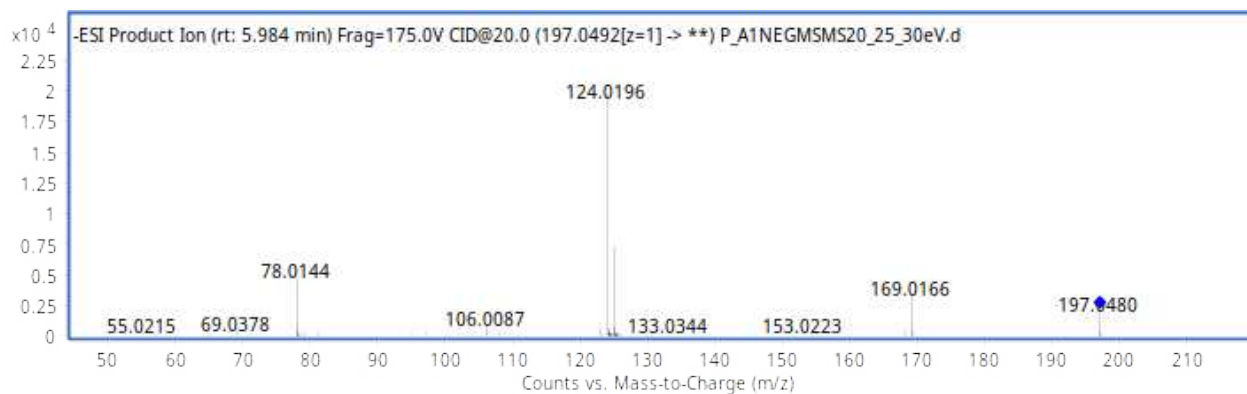


Figure 63: Espectro EM/EM m/z 197.0461 $[M - H]^-$ (Gallic acid ethyl ester) in 20 eV.

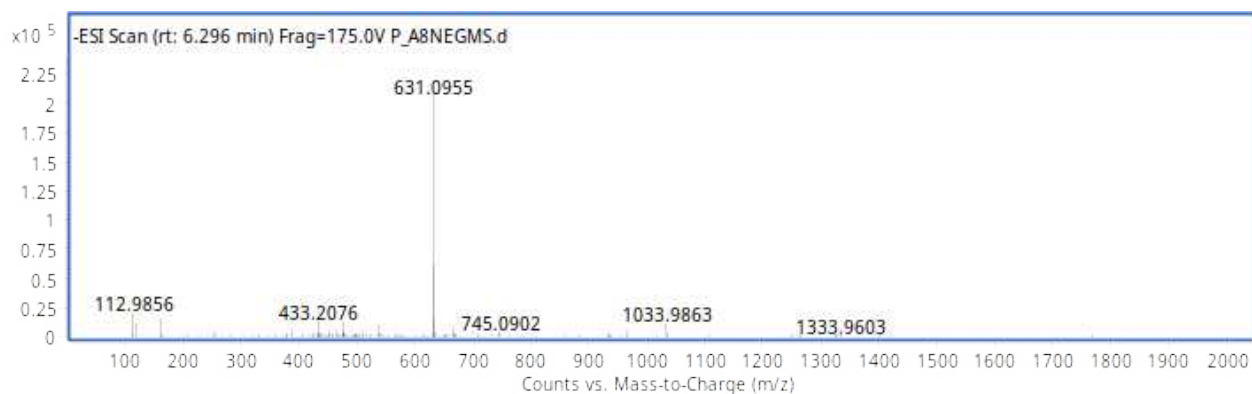


Figure 64: Espectro m/z 631.0955 $[M - H]^-$ (Myricetin-3-O-(2''-O-galloyl) glucoside).

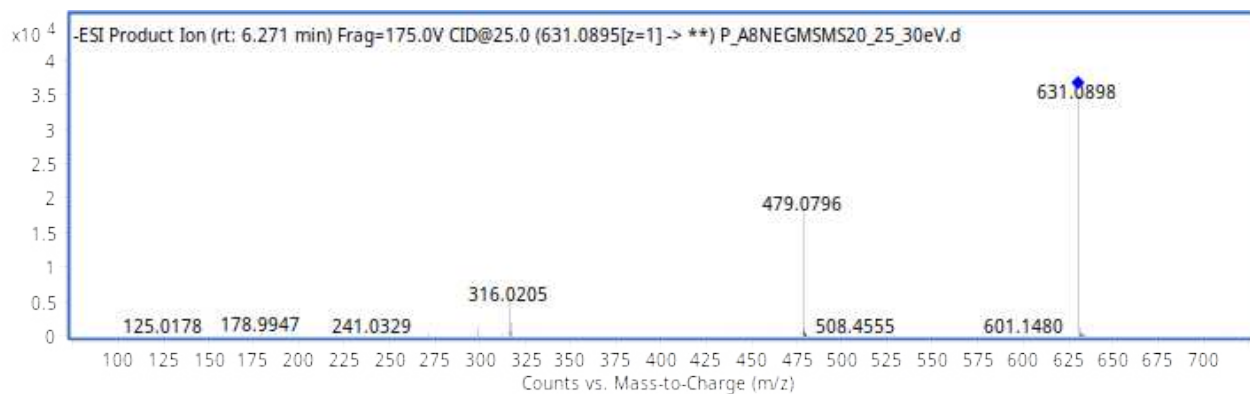


Figure 65: Espectro EM/EM m/z 631 $[M - H]^-$ (Myricetin-3-O-(2''-O-galloyl) glucoside) in 25 eV.

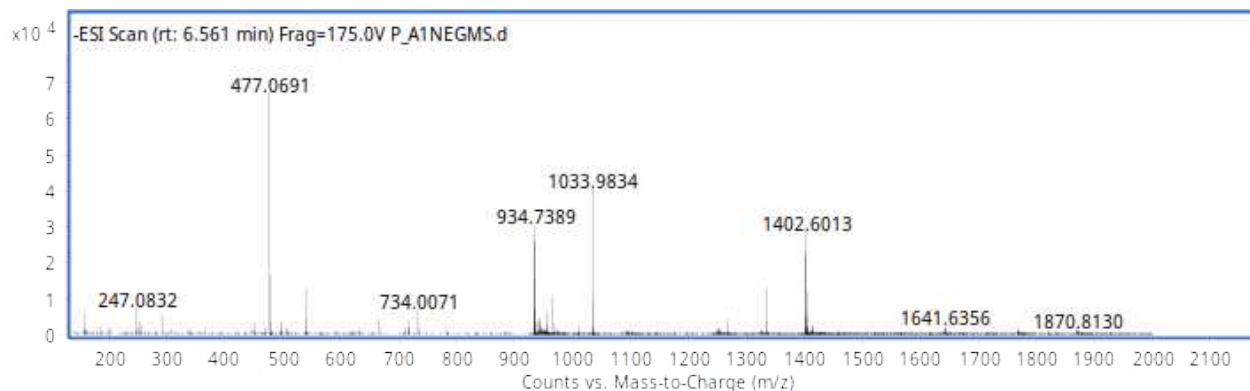


Figure 66: Espectro m/z 477.0691 $[M - H]^-$ (Unknown).

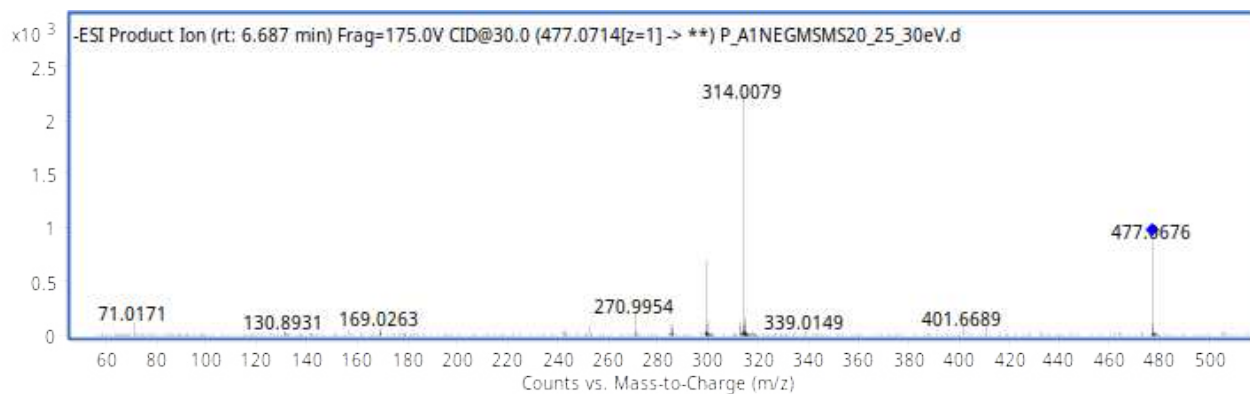


Figure 67: Espectro EM/EM m/z 477.0691 $[M - H]^-$ (Unknown) in 30 eV.

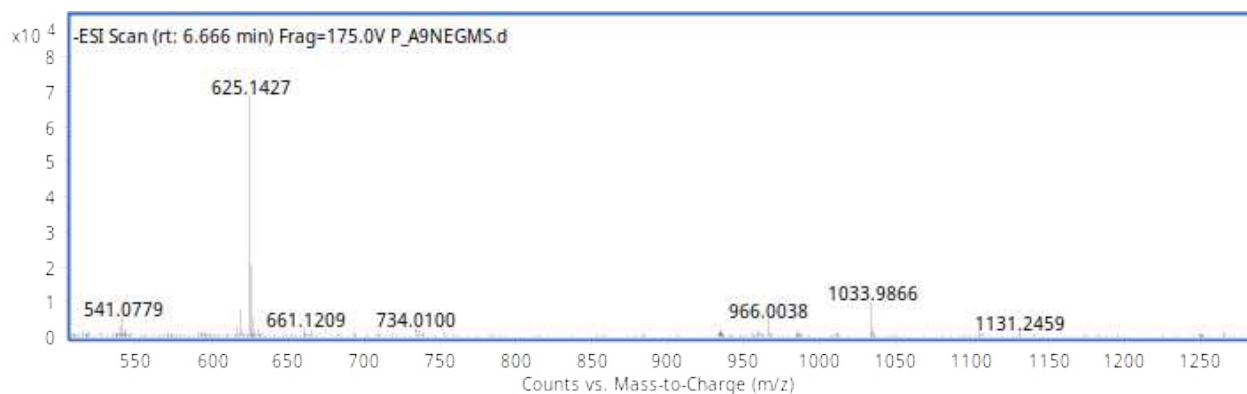


Figure 68: Espectro m/z 625.1427 [$M - H$]⁻ Myricetin rhamno-hexoside).

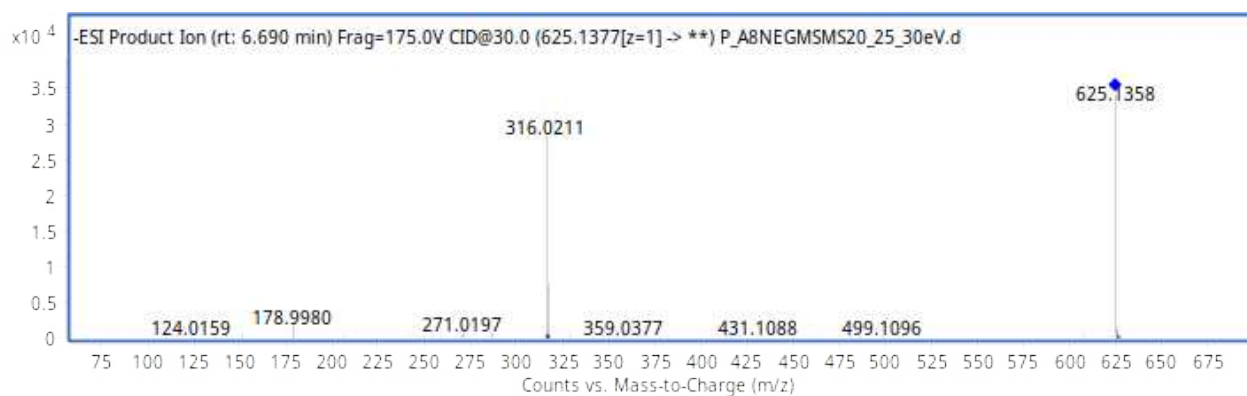


Figure 69: Espectro EM/EM m/z 625.1427 [$M - H$]⁻ (Myricetin rhamno-hexoside) in 30 eV.

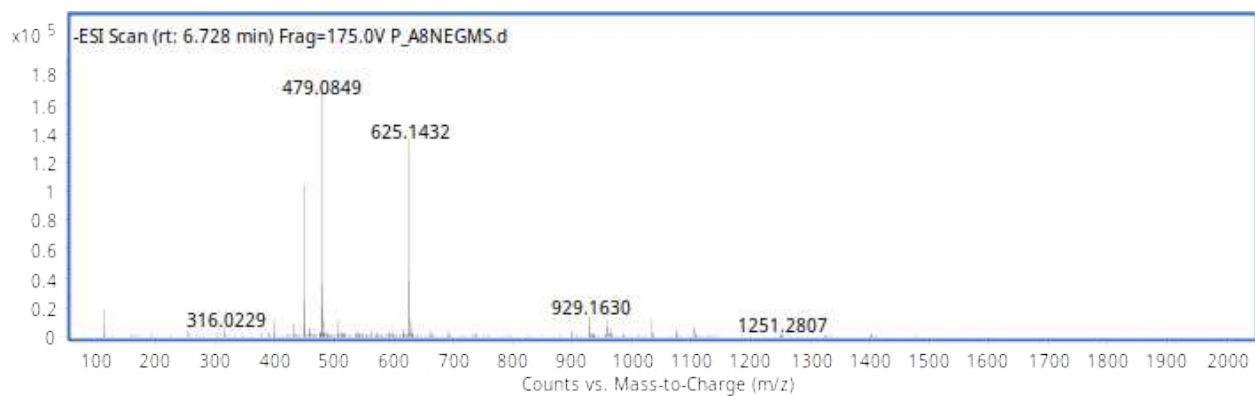


Figure 70: Espectro m/z 479.0849 [$M - H$]⁻ (Myricetin-hexoside).

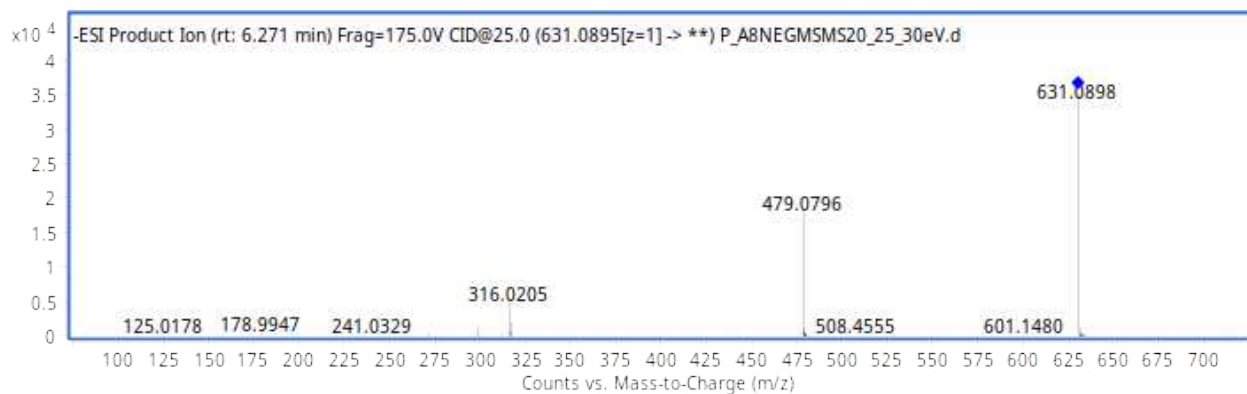


Figure 71: Espectro EM/EM m/z 479.0849 $[M - H]^-$ (Myricetin-hexoside) in 25 eV.

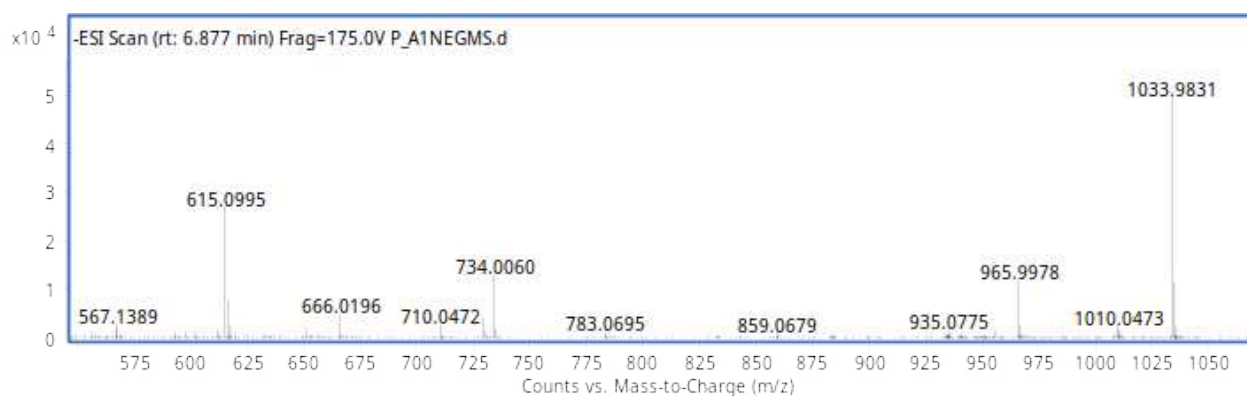


Figure 72: Espectro m/z 615.0995 $[M - H]^-$ (Quercetin-3-O-galloyl hexoside)

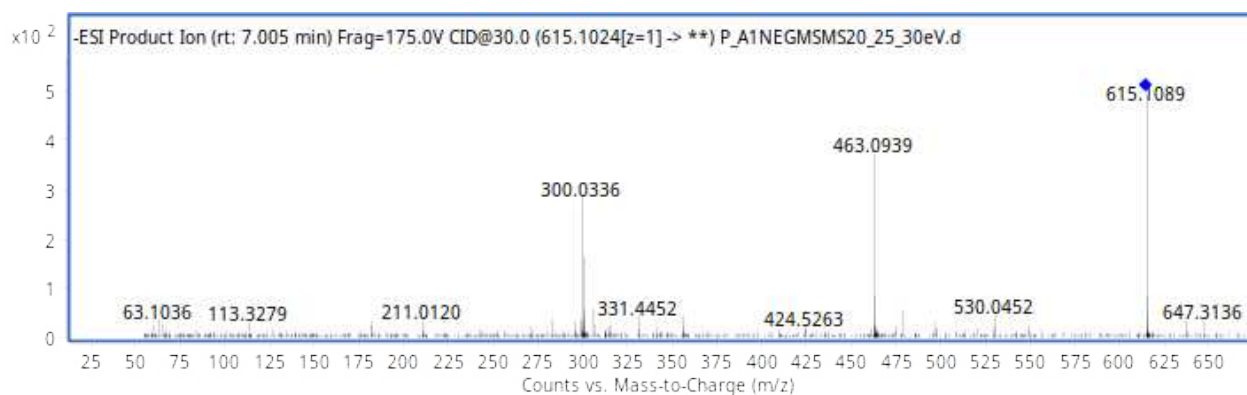


Figure 73: Espectro EM/EM m/z 615.0995 $[M - H]^-$ (Dehydro-galloyl-HHDP-hexoside) in 30 eV.

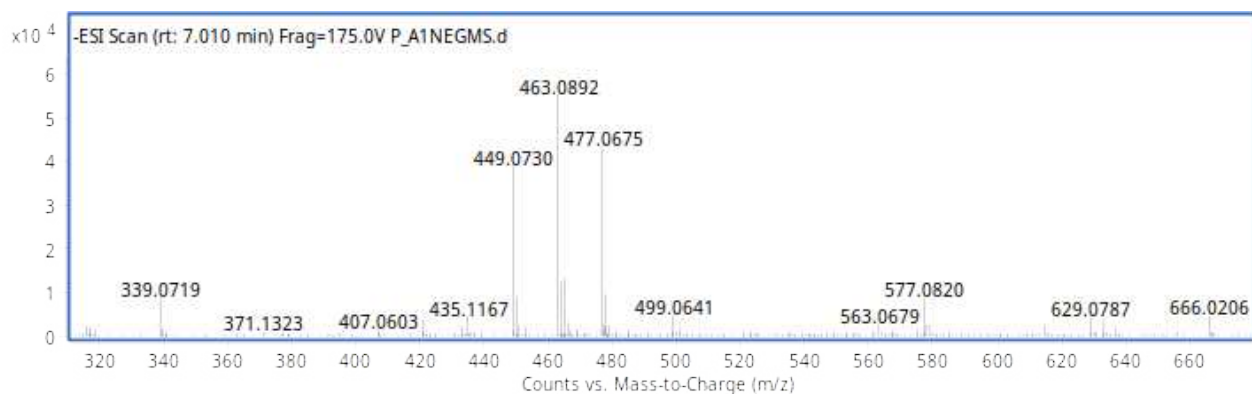


Figure 74: Espectro m/z 463.0892 $[M - H]^-$ and m/z 449.0730 $[M - H]^-$ (Unknown).

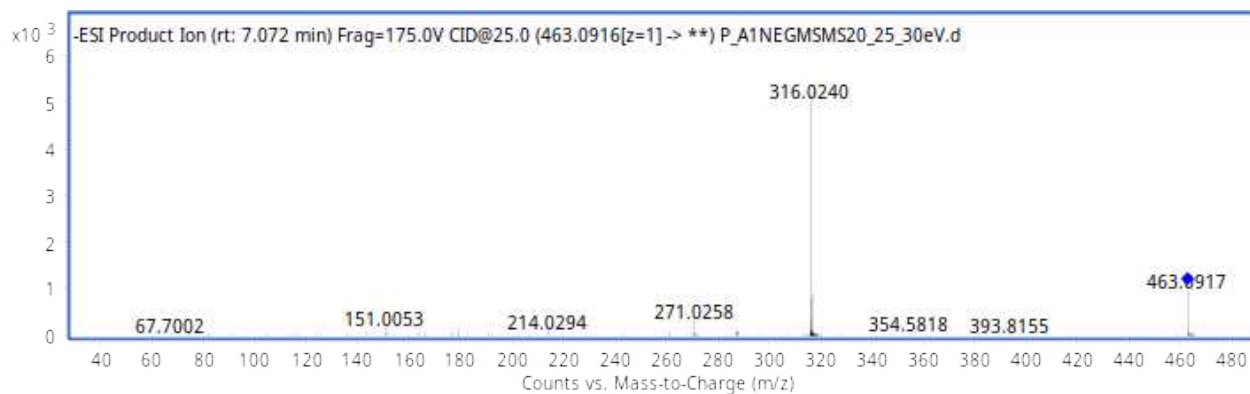


Figure 75: Espectro EM/EM m/z 463.0892 $[M - H]^-$ (Unknown) in 25 eV

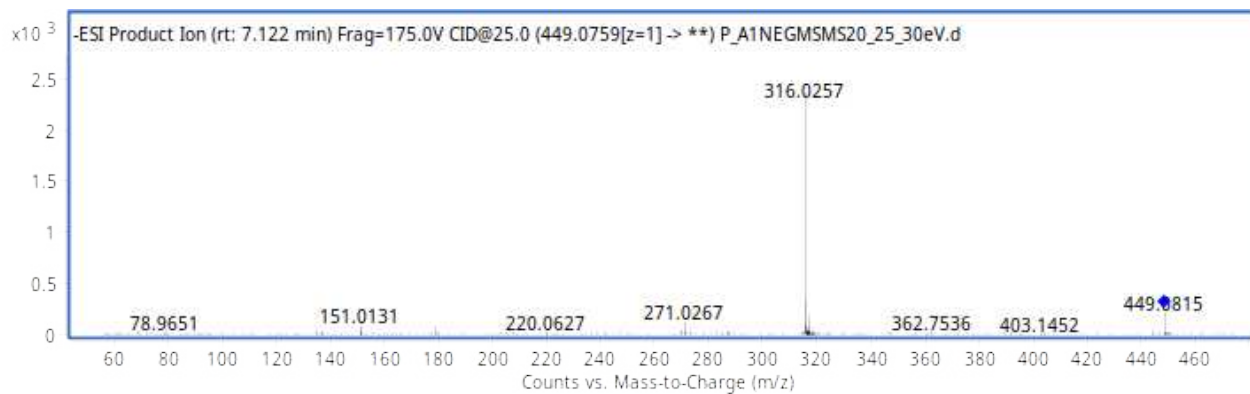


Figure 76: Espectro EM/EM m/z 449.0730 $[M - H]^-$ (Unknown) in 25 eV.

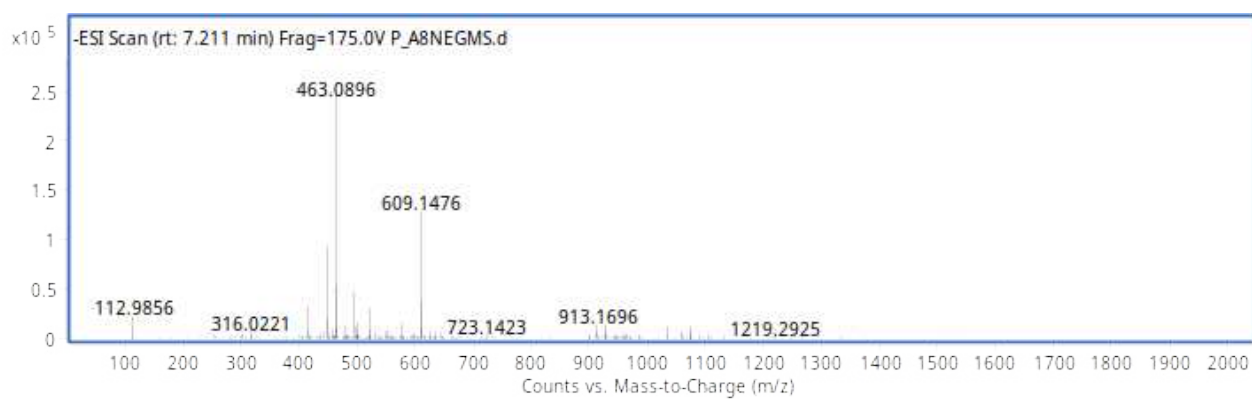


Figure 77: Espectro EM/EM m/z 609.1476 $[M - H]^-$ (Quercetin-3-O-hexose-deoxyhexose) in 25 eV.

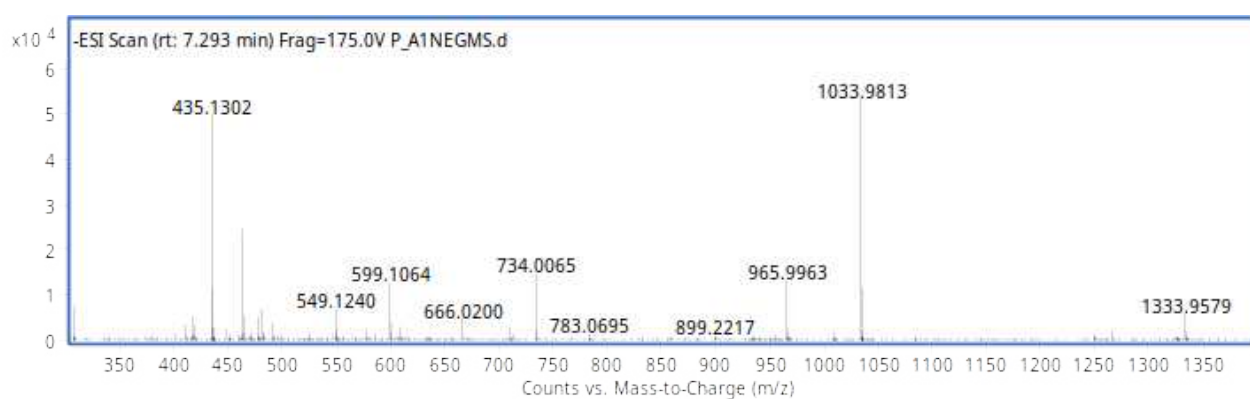


Figure 78: Espectro m/z 435.1302 $[M - H]^-$ (Unknown).

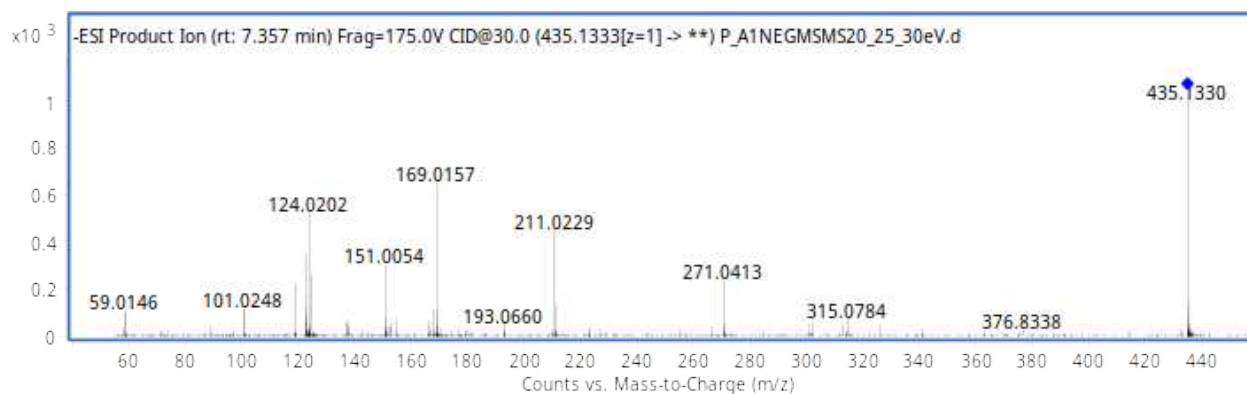


Figure 79: Espectro EM/EM m/z 435.1302 $[M - H]^-$ (Unknown) in 30 eV.

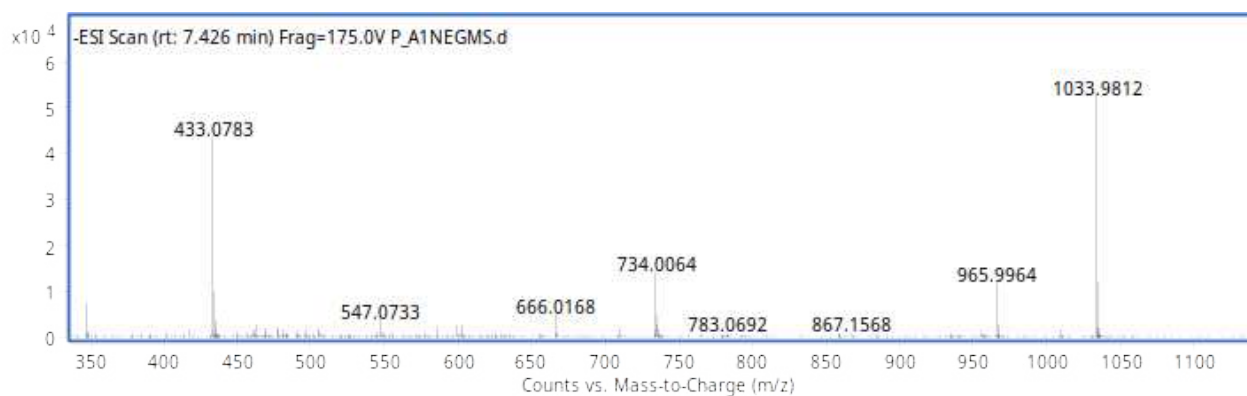


Figure 80: Espectro m/z 433.0783 $[M - H]^-$ (Quercetin-3-O-pentoside).

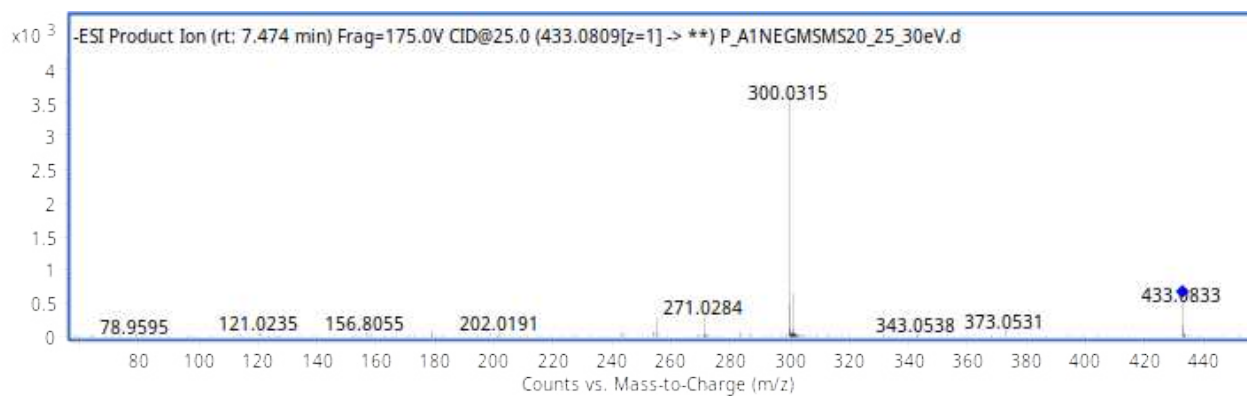


Figure 81: Espectro EM/EM m/z 433.0783 $[M - H]^-$ (Quercetin-3-O-pentoside) in 25 eV.

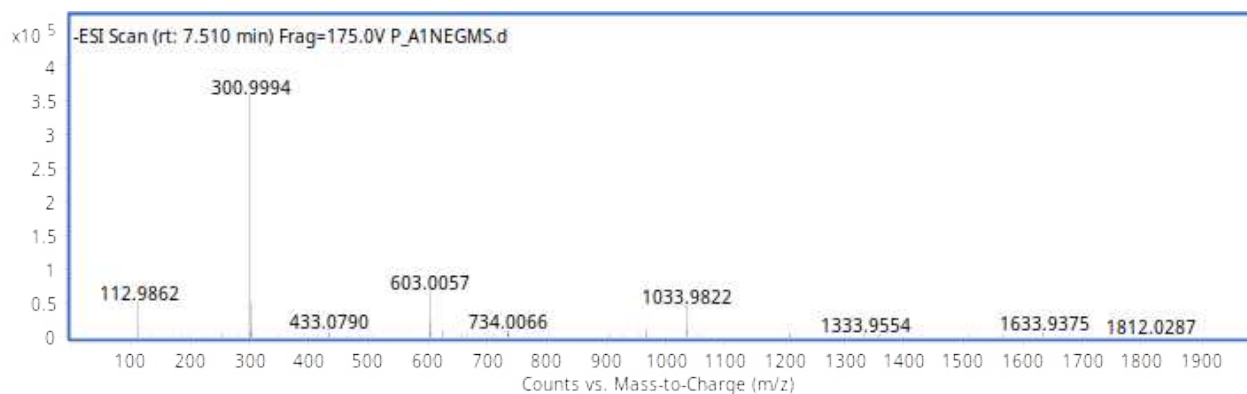


Figure 82: Espectro m/z 300.9994 $[M - H]^-$ (Ellagic acid).

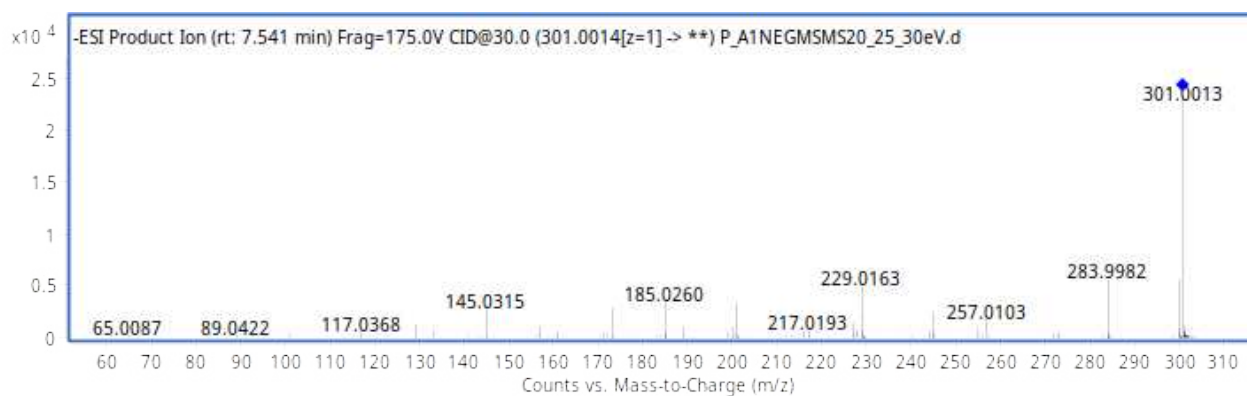


Figure 83: Espectro EM/EM m/z 300.9994 $[M - H]^-$ (Ellagic acid) in 30 eV.

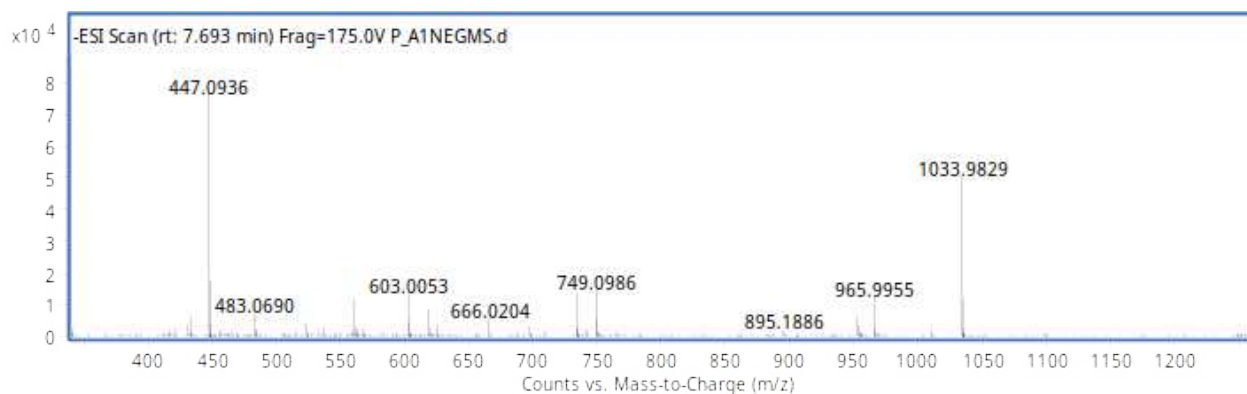


Figure 84: Espectro m/z 447.0936 $[M - H]^-$ (Quercetin-3-O- rhamnoside).

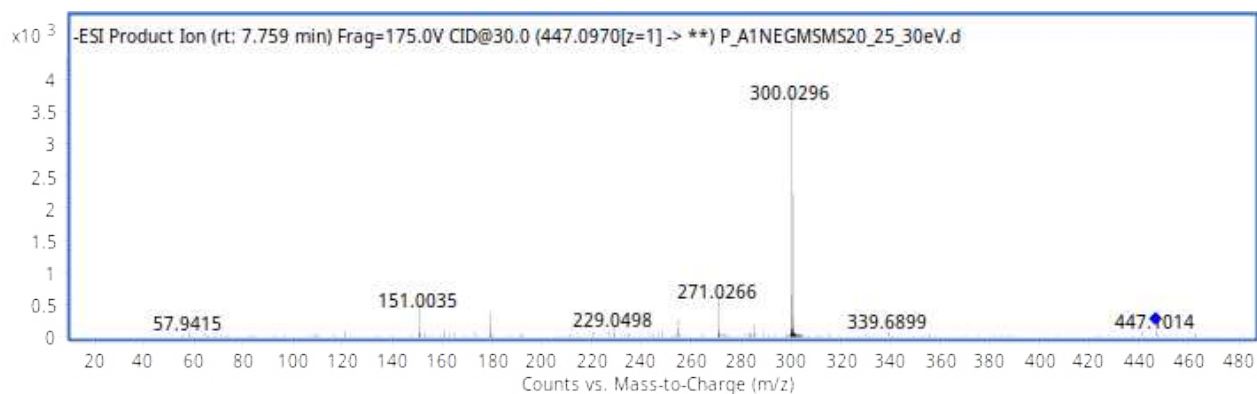


Figure 85: Espectro EM/EM m/z 447.0936 $[M - H]^-$ (Quercetin-3-O- rhamnoside) in 30 eV.

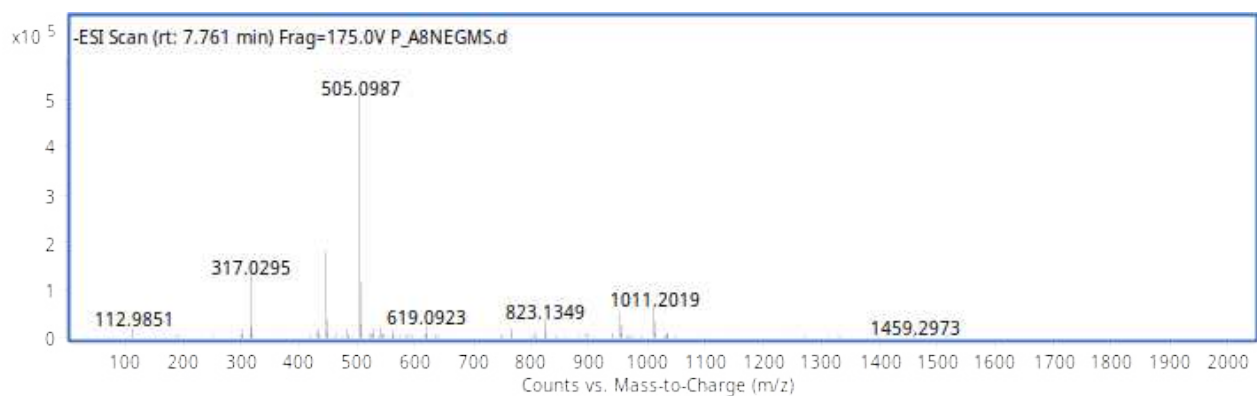


Figure 86: Espectro m/z 505.0987 $[M - H]^-$ (Myricetin-acetylramnoside).

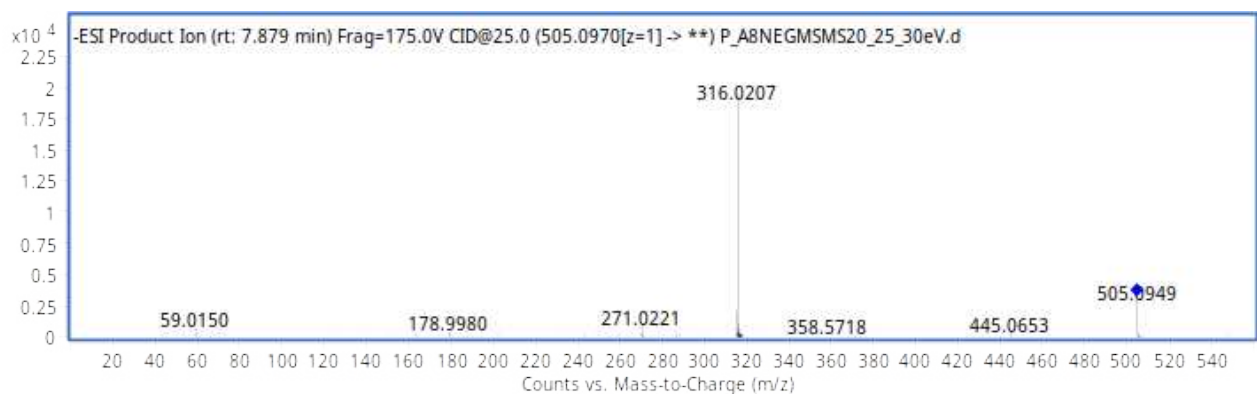


Figure 87: Espectro EM/EM m/z 505.0987 $[M - H]^-$ (Myricetin-acetylramnoside) in 30 eV.

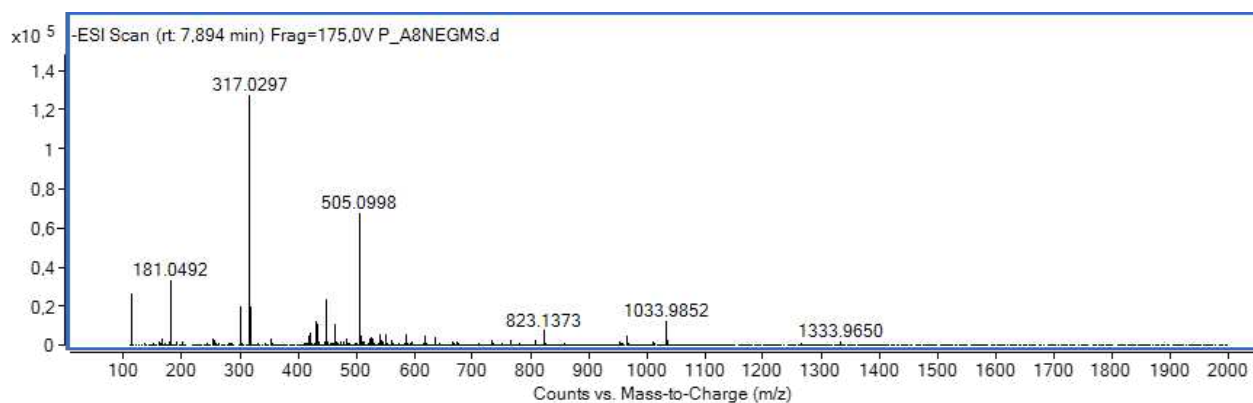


Figure 88: Espectro m/z 317.0297 $[M - H]^-$ (Myricetin).

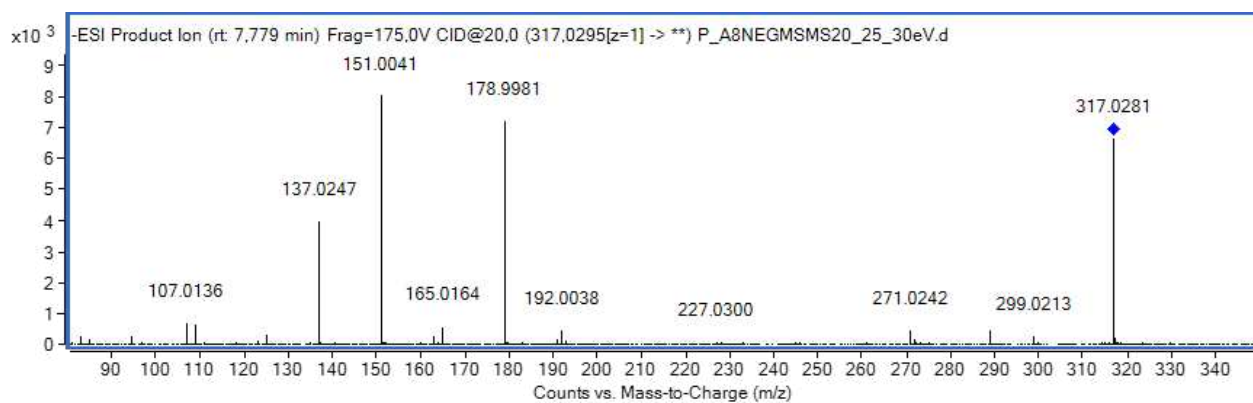


Figure 89: Espectro EM/EM m/z 317.0297 $[M - H]^-$ (Myricetin) in 20 eV.

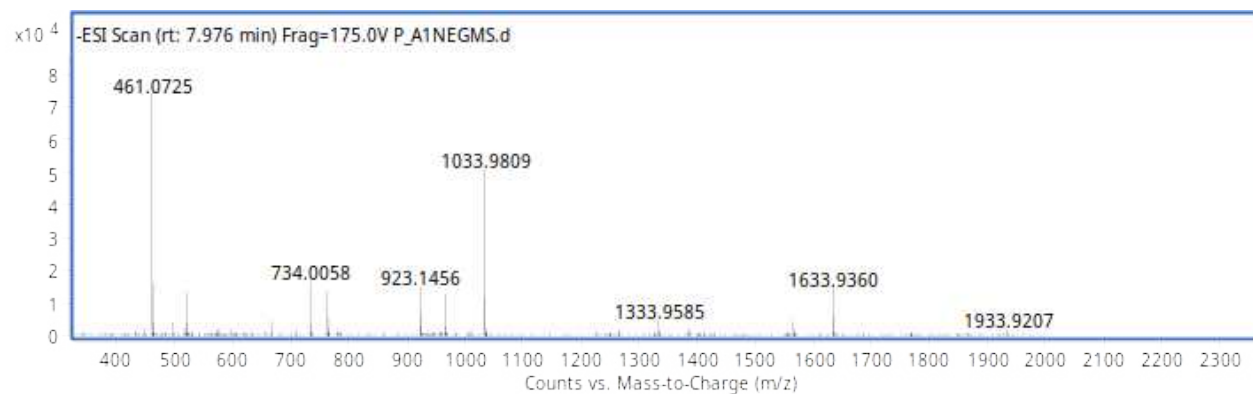


Figure 90: Espectro m/z 461.0725 $[M - H]^-$ (3'-O-Methyl-4-O-(rhamnopyranosyl) ellagic acid).

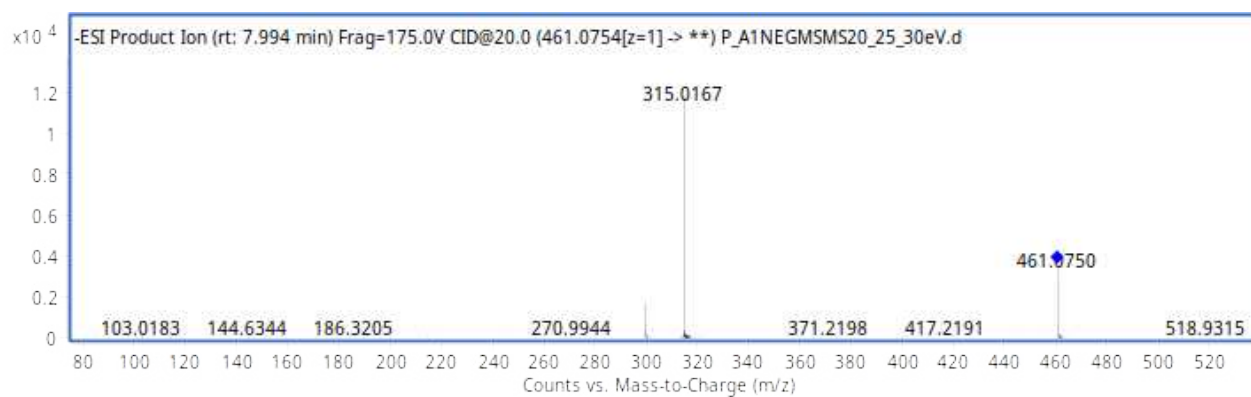


Figure 91: Espectro EM/EM m/z 461.0725 $[M - H]^-$ (3'-O-Methyl-4-O-(rhamnopyranosyl) ellagic acid) in 20 eV.

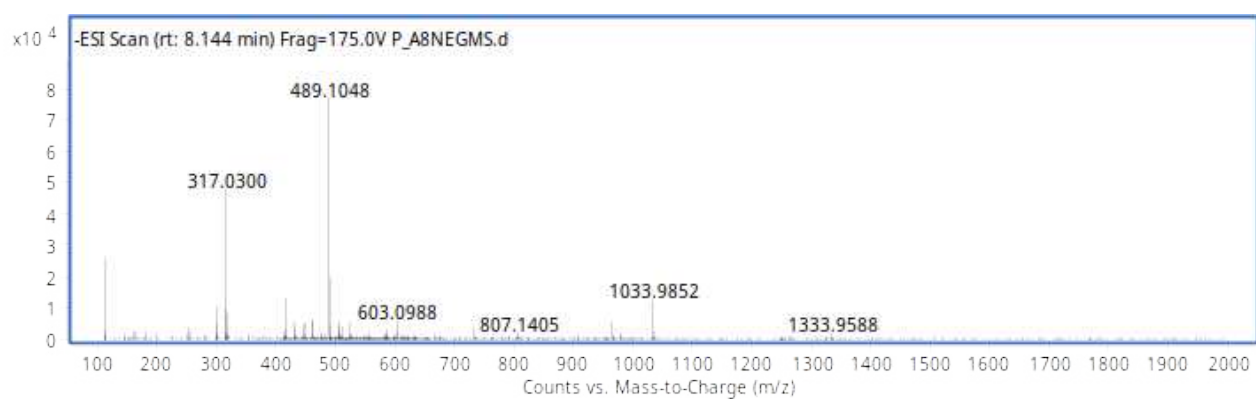


Figure 92: Espectro m/z 489.1048 $[M - H]^-$ (Acetylquercitrin).

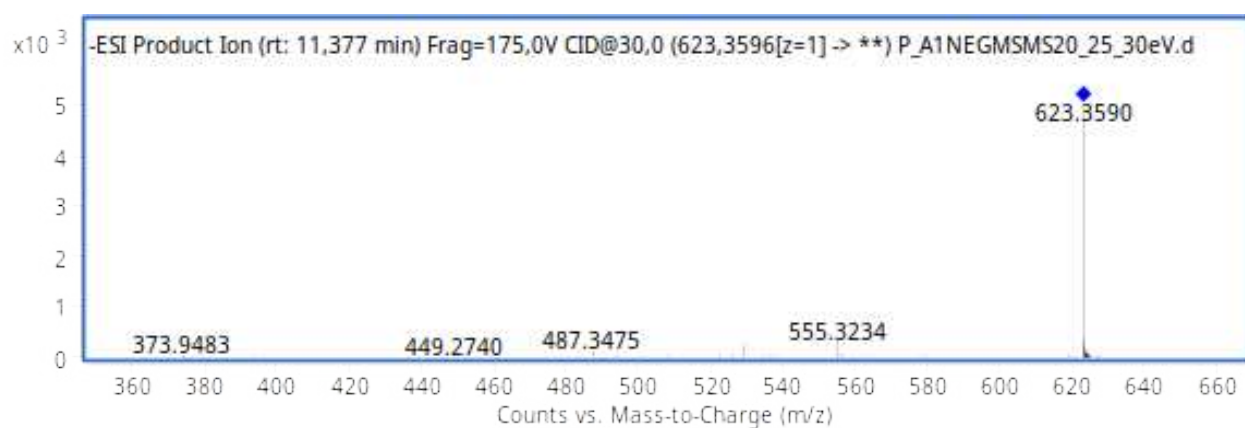
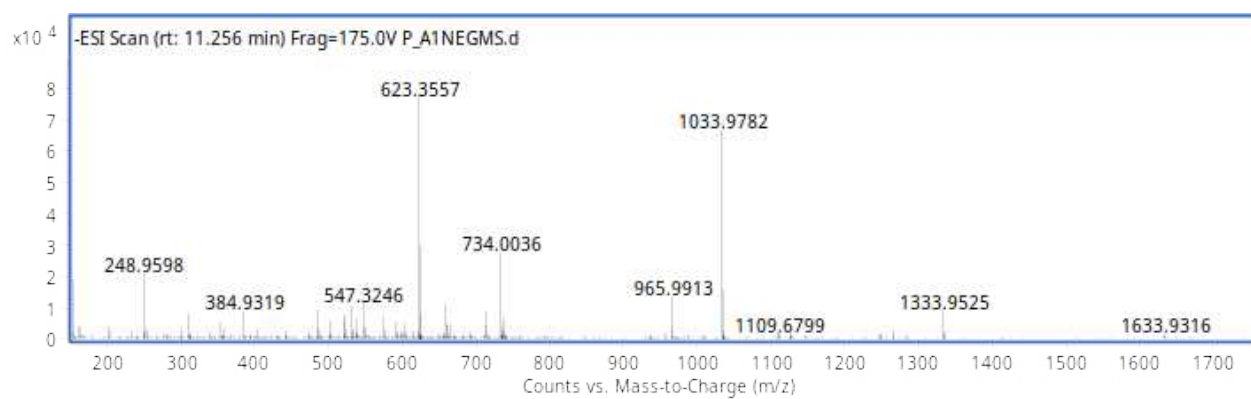


Figure 93: Espectro EM/EM m/z 623.3557 $[M - H]^-$ (Unknown) in 30 eV.

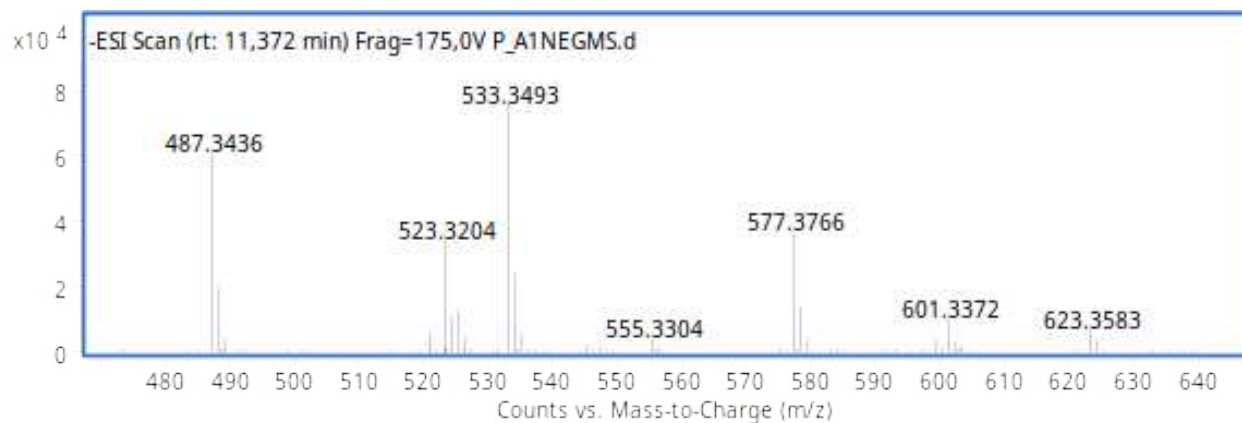


Figure 94: Espectro m/z 487.3436 $[M - H]^-$ (Triterpene pentacyclic I).

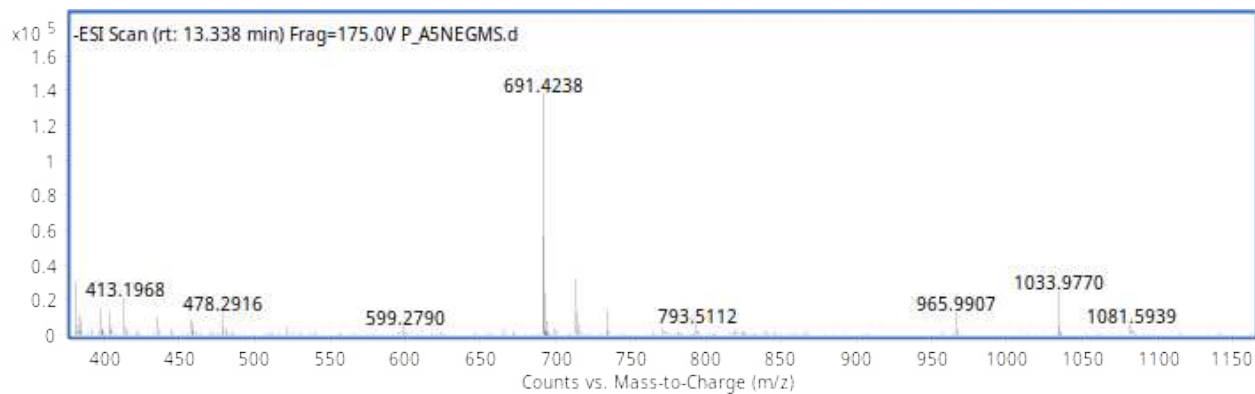


Figure 95: Espectro m/z 691.4238 $[M - H]^-$ (Unknown).

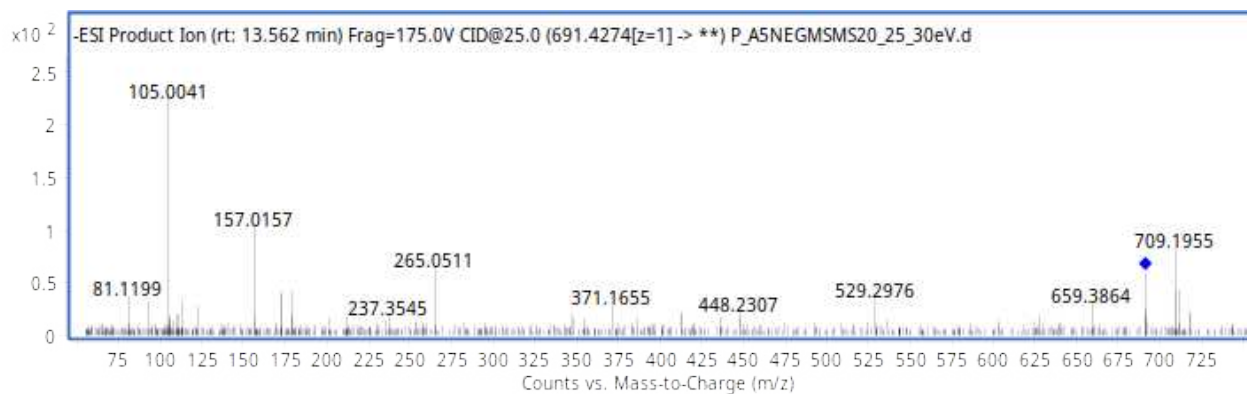


Figure 96: Espectro EM/EM m/z 691 $[M - H]^-$ (Unknown) in 25 eV.

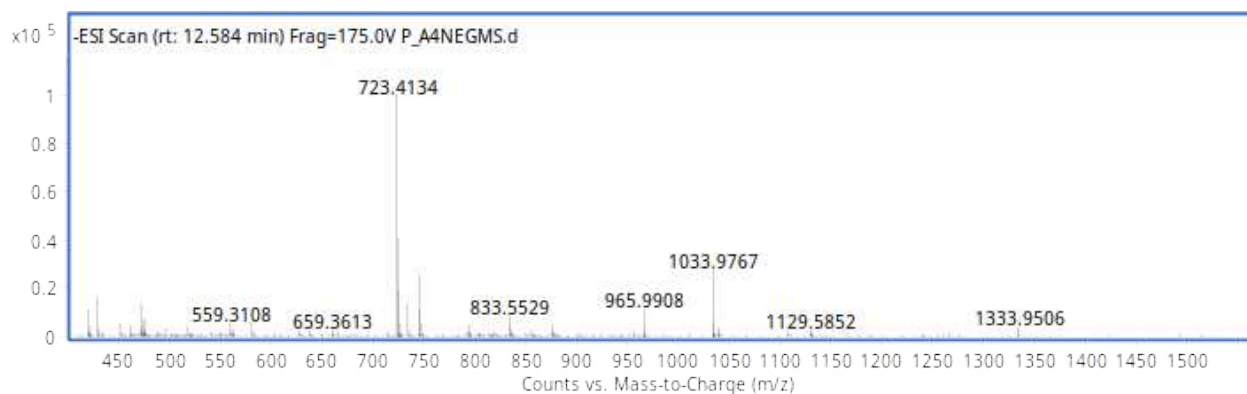


Figure 97: Espectro m/z 723.4134 $[M - H]^-$ (Unknown).

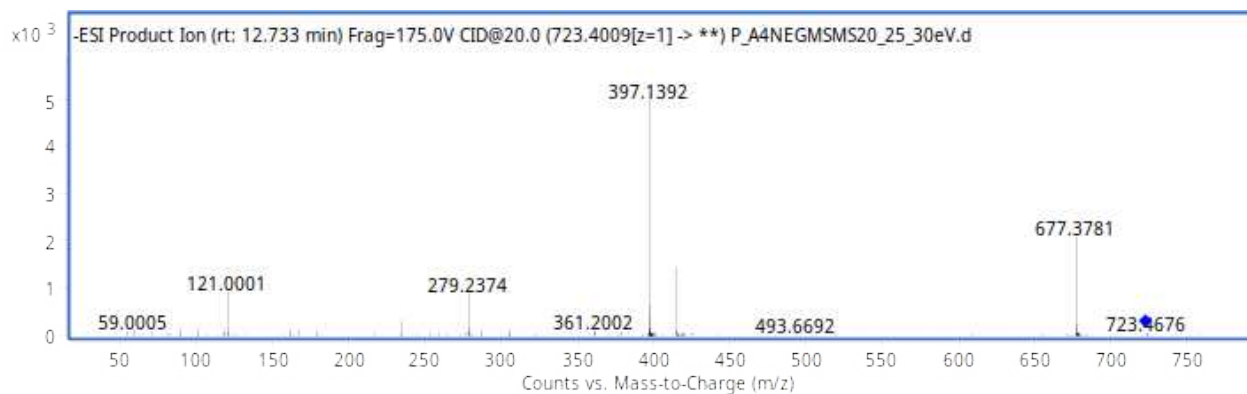


Figure 98: Espectro EM/EM m/z 723.4131 $[M - H]^-$ (Unknown) in 20 eV.

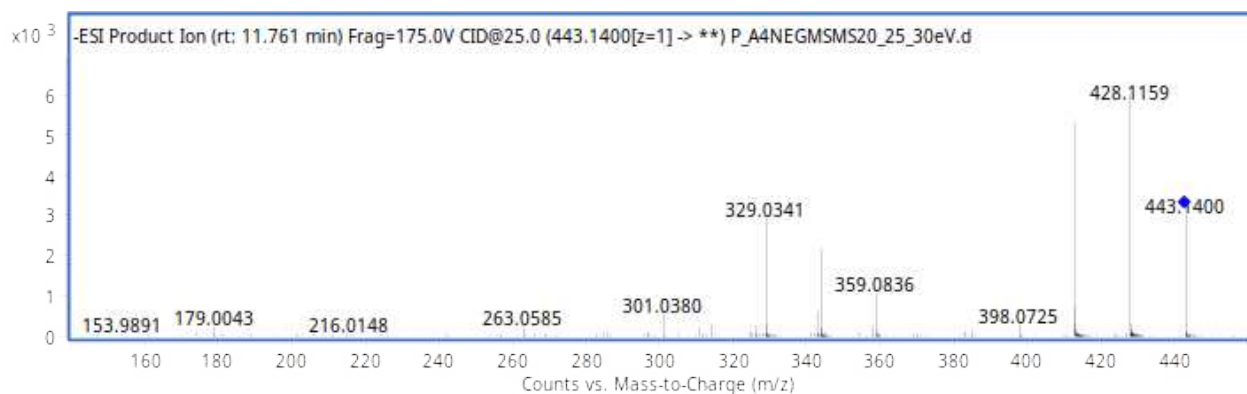


Figure 99: Espectro EM/EM m/z 443.0000 $[M - H]^-$ (Unknown) in 30 eV.

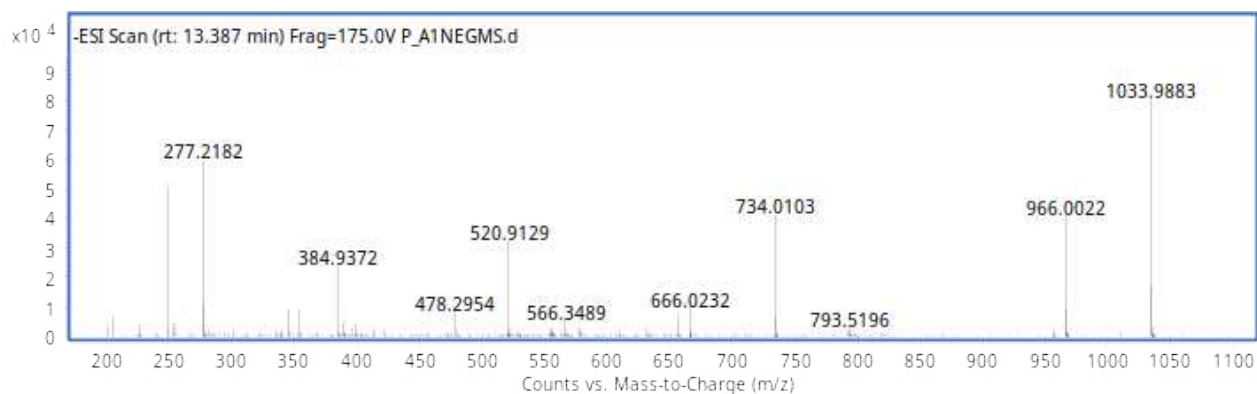


Figure 100: Espectro m/z 277.2162 $[M - H]^-$ (Linolenic acid).

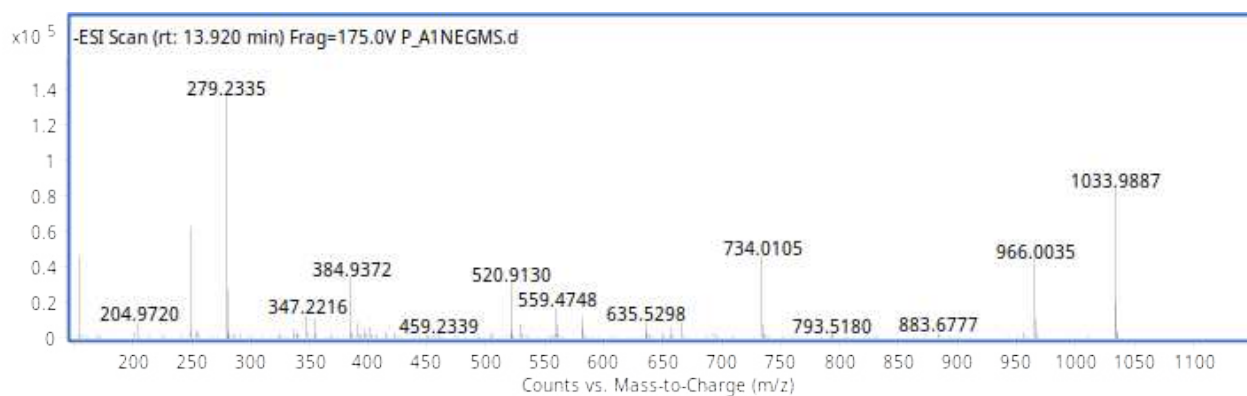


Figure 101: Espectro m/z 279.2335 $[M - H]^-$ (Linoleic acid).

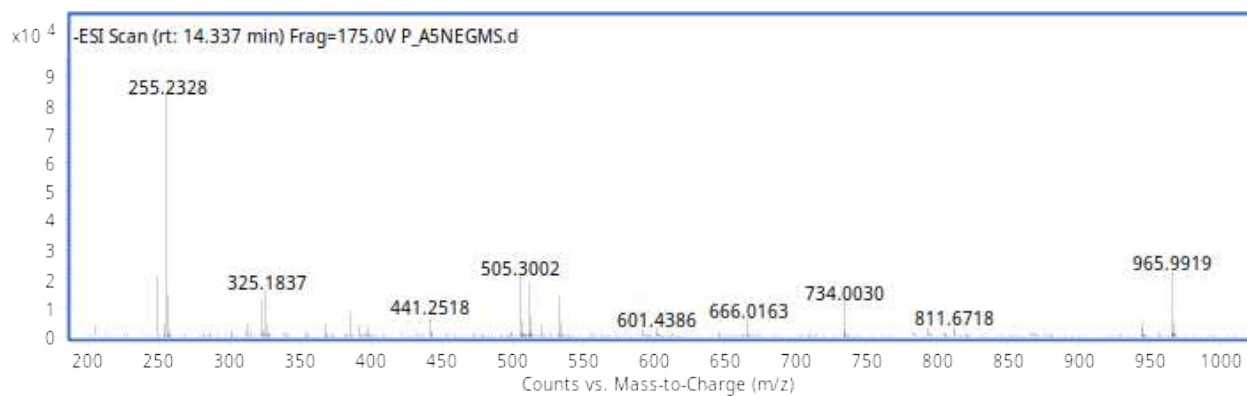


Figure 102: Espectro m/z 255.2328 $[M - H]^-$ (Palmitic acid).

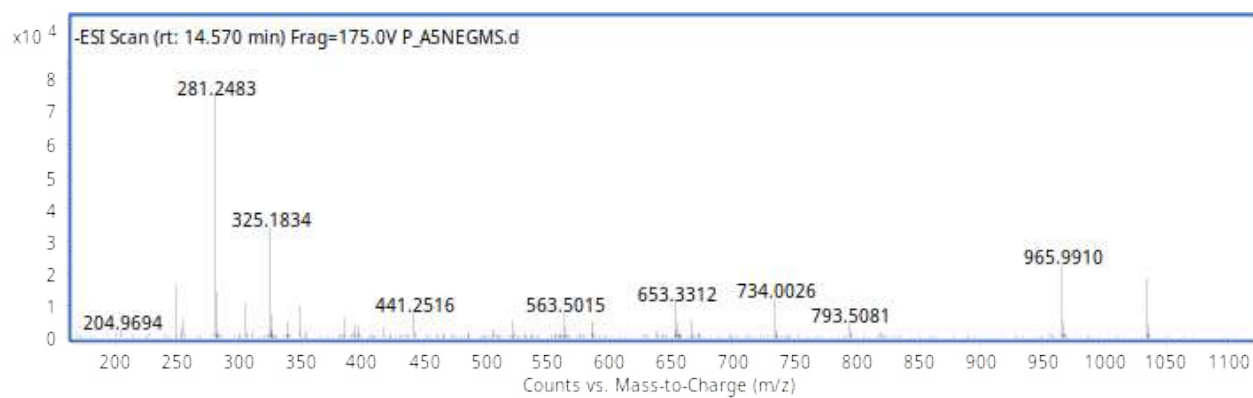


Figure 103: Espectro m/z 281.2483 $[M - H]^-$ (Oleic acid)