Characterisation and biological activities of small molecules isolated from *Corymbia citriodora* (Hook) K.D.Hill & L.A.S. Johnson; and *Ceratopetalum hylandii* Rozefelds & R.W.Barnes.

Andres Felipe Ruiz Alvarez

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Supervisor:

Dr. Phurpa Wangchuk

Co-supervisor:

Dr. Kate Miller

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College of Public Health, Medical and Veterinary Science

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DECLARATION

I, *Andres Felipe Ruiz Alvarez*, declare that this thesis, submitted in fulfillment of the requirements for the award of the degree of *Master of Philosophy* from College of Public Health, Medical and Veterinary Science, James Cook University, is my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other institution.

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Andres Felipe Ruiz Alvarez

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Thesis Chapter	Published	Planned to publish	Target journal	Author list plan
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2	No	Yes	TrAC Trends in Analytical Chemistry	Ruiz A.F., Joost L.D. Nelis, Miller CM, Wangchuk P.
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Presentation at CPHMVS Seminar 3MT	AI in drug discovery
Presentation at TESS annual conference 3MT	Exploring drug discovery potential in FNQ through Australian Tropical Plants
3 minutes competition pitch at JCU Impact 10x AI innovation and entrepreneurship simulator	Molecules

List of abbreviation

Abbreviation	Meaning
AI	Artificial Intelligence
COSY	2D Correlation Spectroscopy
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DNN	Deep Neural Networks
ECD	Electronic Circular Dichroism
FT-IR	Fourier-transform Infrared Spectroscopy
GAE	Gallic Acid Equivalents
HMBC	2D Heteronuclear Multiple-Bond Correlation
HPLC	High Performance Liquid Chromatography
HRMS	High-Resolution Mass Spectrometry
HSQC	2D Heteronuclear Single Quantum Coherence
IL	Interleukin
LC-MS	Liquid Chromatography-Mass Spectrometry
LPS	Lipopolysaccharide
ML	Machine Learning
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NOSY	2D Nuclear Overhauser effect spectroscopy
NSAID	Non-Steroidal Anti-Inflammatory Drug
PBMC	Peripheral Blood Mononuclear Cell
PRA	Pattern Recognition Analysis
RA	Radical Scavenging Activity
RP-HPLC	Reverse Phase High-Performance Liquid Chromatography
RTCA	Real-Time Cell Analysis
S.E.M.	Standard Error of the Mean
SI	Selectivity Index
TFA	Trifluoroacetic Acid
TNF	Tumour Necrosis Factor

Abstract

The analysis of botanical documents describing the traditional use of plants by indigenous communities has been used as a basis for the discovery of new drugs for decades. To complement this traditional knowledge, compounds identification using mass spectroscopy and nuclear magnetic resonance (NMR) and *in-vitro* assays that demonstrated the bioactive potential for specific diseases, were required. This study includes two Australian tropical plants: the resin from *Corymbia citriodora*, which is used by aboriginals to treat cuts, burns, and wounds, and *Ceratopetalum hylandii*, an endemic plant in Far North Queensland (FNQ). This study aims to isolate, identify, and assess the bioactivity of select compounds derived from *C. citriodora and C. hylandii.*.

Techniques such as column chromatography, size exclusion, and multiple rounds of preparative high-performance liquid chromatography (HPLC) were employed to isolate compounds. The isolated compounds were characterised using mass spectrometry and NMR. Mass spectrometry was used to verify known compounds, while 1D NMR (^{1}H and ^{13}C NMR) and 2D NMR; correlation spectroscopy, heteronuclear multiple-bond correlation, heteronuclear single quantum coherence, and nuclear overhauser effect spectroscopy (COSY, HMBC, HSQC, NOESY respectively) experiments were used in this identification.

The indigenous Australian plant, *Corymbia citriodora*, which covers regions from northern Queensland down to New South Wales, has garnered significant interest for its medicinal attributes due to its aboriginal uses, and the essential oils and secondary metabolites identified. Five compounds were isolated and identified, namely; *p*-Coumaric acid **(1)**, 7-*O*-Methylaromadendrin **(2)**, Sakuranetin **(3)**, Mbabarametin **(4)**, HEMA **(5)**. Of these Mbabarametin was identified as a novel natural product. Using the xCELLigence Real-Time Cell Analysis (RTCA) system, the normalized cell index of liver cells treated with compounds from *C. citriodora* indicated no sign of toxicity at a concentration of 10 µg/ml. Additionally, compounds 4 and 5 derived from *C. citriodora* demonstrated notable *in-vitro* suppression of proinflammatory cytokines (IL-1β) in peripheral blood mononuclear cells (PBMCs), which indicates potential treatment for chronic inflammation diseases.

Ceratopetalum hylandii is a plant endemic to northeastern Australia, belonging to the Cunoniceae family. To our best knowledge, this is the first phytochemical study characterising this species. The compounds Shikimic Acid **(1)**, 3-dehydroshikimic acid **(2)**, gallic acid **(3)**, *trans*-melilotoside **(4)**, and *cis*-melilotoside **(5)** were isolated and identified from *C. hylandy*. Shikimic acid and phenylpropanoid acid pathways are likely the pathways for the synthesis of those compounds.

This research unlocks new knowledge about these two species, as a new compound was isolated for *C. citriodora*, and it is the first phytochemical study of *C. hylandii*. Each chapter of this thesis aims to provide new and relevant insights into the discovery of compounds with potential pharmaceutical

properties. Further investigations into the mechanism of actions of the compounds on specific targets should be focus of future studies.

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General Introduction

1.1 Background and significance

Natural products are an important source of new drug discovery. Since the isolation of the first compound with medicinal use, morphine in 1804 by Sertürner, the discovery of new compounds from natural products has been one of the most important fields of the pharmaceutical industry (Beutler, 2009; Wangchuk & Loukas, 2018). From 1981 to 2019, 1394 new small-molecules drugs received approval, with approximately 34% of these being derived from natural sources (Newman & Cragg, 2020). Tropical plants, in particular, play a vital role due to their unique diversity in species and in small molecules; although they only represent about 1% of drugs approved from plants, it is an area that has been explored in last decades (Newman & Cragg, 2020).

The Australian Wet Tropics Bioregion is one of the most diverse rainforests on the planet and its plant diversity is a potential resource for drug discovery. With an area of 894,420 hectares, it is the oldest surviving rainforest in the world which cover 0.12% of Australian territory and is the home of 20 Aboriginal Australian tribes (Roberts et al., 2021). This rainforest is the habitat of endemic plants with approximately 1,511 plant species have been reported to be used in Queensland Aboriginal bush medicine (Turpin et al., 2022). Traditional knowledge of medicinal plants by Aboriginal people has been documented as a basis for treating many disorders, such as wounds, cuts, skin infections, fractures, pain and inflammation (Turpin et al., 2022).

Inflammation is a physiological response to infections, toxic compounds, or damaged cells and it is associated with many types of diseases. When inflammation is unresolved, it can become a chronic disease such as inflammatory bowel disease (IBD), arthritis, Alzheimer's disease, Parkinson's disease, amongst others (Azab et al., 2016), where the immune cells produce a high amount of nitric oxide (NO) and pro-inflammatory cytokines during inflammation. Nitric oxide is a molecule that is involved in diverse biological processes that include immune defence, inflammation and neurotransmission (Sharma et al., 2007). Proinflammatory cytokines induce the expression of inducible NO synthase, which results in a significant increase of NO in inflammatory disorders. In inflammatory bowel disease, for example, NO levels increase in macrophages in the chronic phase (Sharma et al., 2007). Cyclooxygenase (COX) pathway plays an important role in inflammation. Arachidonic acid (AA) is metabolised by COX to produce prostaglandins (PGs) (Pezzuto et al., 2006). PGs play a role in the inflammatory response, as their biosynthesis significantly increased in inflamed tissue (Ricciotti & FitzGerald, 2011). Drugs like naproxen, a Non-Steroidal Anti-Inflammatory Drug (NSAID), inhibit COX activity (Oprea et al., 2015).

Any plant extracts or molecules that can block or inhibit the production of NO and proinflammatory cytokines has potential to become a novel drug for treating inflammation. For

example, *Corymbia terminalis* extract at 3 µg/mL inhibited COX-1 activity by 43% and COX-2 activity by 46% (Negahban, 2020). Likewise, ethyl acetate fractions from *Clerodendrum inerme* reduced the protein expression of inducible nitric oxide synthase in LPS-stimulated macrophages (Srisook et al., 2015). Recently, a study of the crude extract from seven plants from the Australian Wet Tropics inhibited the secretion of proinflammatory cytokines such as IL-23 and TNF in a PBMCs assay (Yeshi et al., 2022). A single compound called Galloyl-lawsoniaside A, which was isolated from *Uromyrtus metrosideros* one of these Wet Tropics plants, significantly suppressed IFN-γ secretion in peripheral blood mononuclear cells (PBMC) assay (Ritmejerytė et al., 2022). Similarly, costatamins A–C, which was isolated from *Angophora costata*, inhibited the NO production at 20-36 µg/mL and TNF-α at 20-36 µg/mL (Raju et al., 2019).

1.2 Rationale and aims.

Inspired by the potential bioactivities of Australian tropical plants for medical use, *Corymbia citriodora* and *Ceratopetalum hylandii* were selected for this study due to their anti-inflammatory activities. Essential oils from *Corymbia citriodora* have been previously studied and are reported to contain anti-inflammatory activities, significantly reducing carrageenan and dextran-induced rat hindlimb edema 1 to 4 h after administration (Lee et al., 2017; Silva et al., 2003). However, the resin of this species, used by Mbabaram Aboriginal community of Far North Queensland (FNQ) to treat infections, has not been studied in depth for its anti-inflammatory properties. Similarly, leaves from *C. hylandii*, which is endemic to the Wet Tropics of FNQ, contains alkaloids and its crude extracts have shown inhibitory effects on the secretion of proinflammatory cytokines, tumour necrosis factor (TNF), and interleukin-23 (IL)-23 in a lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMCs) assay (Yeshi et al., 2022).

Since pure compounds have not been isolated from these plants before and have the potential for identification of lead candidates with an anti-inflammatory property, I will endeavor to achieve the following objectives.

- To isolate and identify small molecules from *Corymbia citriodora* resin.
- To isolate and identify small molecules from *Ceratopetalum hylandii* leaves.
- To identify bioactivity from *C. citriodora* and *C. hylandii*'s isolated compounds.

The workflow for this project (Fig. 1) includes several key steps: collecting plant samples, drying the samples, extracting and concentrating the crude extracts, fractionating the extracts, isolating the compounds, identifying the compounds, and conducting bioactivity tests. To perform these procedures, various analytical techniques were employed, including High Performance Liquid

Chromatography (HPLC), Liquid Chromatography-Mass Spectrometry (LC-MS), and Nuclear Magnetic Resonance (NMR) spectroscopy.

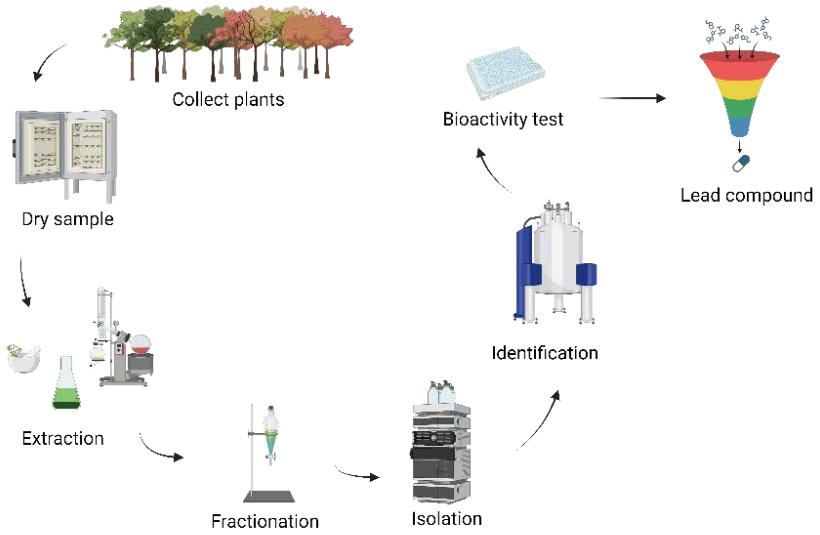

Figure 1. 1 General workflow for the project.

1.3 Scope of the thesis

The thesis is divided into five chapters focusing on the exploration of plants, for drug discovery purposes. Each chapter's scope is outlined as follows.

Chapter 2: From ethnobotanic to artificial intelligence: new perspectives for compounds identification in Australian medicinal plants. This section presents a review that discusses the perspectives, current advances, and potential benefits of fusing traditional knowledge with modern scientific methods in drug discovery. The goal is to showcase how this fusion can expedite the discovery of compounds potentially paving the way for pharmaceutical developments. This paper reviews how artificial intelligence (AI) is useful as a new tool for text mining, liquid chromatography mass spectroscopy (LC-MS), and nuclear magnetic resonance (NMR) analysis, in the context of drug discovery.

Chapter 3: Potential biological activity of chemical constituents isolated from resin of *Corymbia citriodora* (Hook) K.D.Hill & L.A.S. Johnson. This chapter shows isolated compounds with anti-inflammatory properties from *Corymbia citriodora* resin. It covers the methods used for extracting compounds the setup for testing inflammatory effects and the subsequent analysis of findings. This section illustrates how combining knowledge about plants with analytical techniques can identify promising compounds for further pharmaceutical research.

Chapter 4: Chemical constituents isolated from leaves extract from *Ceratopetalum hylandii* Rozefelds & R.W.Barnes. Focused on *Ceratopetalum hylandii* leaves, an endemic plant to Far North Queensland, this chapter outlines the research conducted to isolate and identify secondary metabolites with potential bioactivity. It explains the steps involved starting from collecting and preparing plant samples to using methods for isolating and identifying compounds. The chapter highlights the potential of this tropical species as sources of bioactive compounds that can aid in drug discovery efforts.

Chapter 5: Overall summary and future directions. The final chapter synthesizes the findings discussed in chapters 2-4 by providing an evaluation of results, within the context of existing literature and study objectives. It examines the research implications addressing both accomplishments and challenges faced.

In addition, it puts forward suggestions, for research paths proposing ways to expand on the findings presented in this thesis to advance the field of drug discovery by combining botanical knowledge with modern scientific methods. In general, the thesis supports a method to drug discovery highlighting the significance of botanical wisdom in supplementing and improving current compound identification approaches. By delving into inquiries and engaging in discussions the thesis adds to the ongoing conversation, about the importance of blending traditional and contemporary techniques in the pursuit of discovering new compounds for medicinal purposes.

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From Ethnobotany to Artificial intelligence: New Perspectives for compounds identification in Australian Medicinal Plants.

Abstract: Artificial intelligence (AI) is playing an increasingly prominent role in efficiently discovering new lead compounds derived from natural products with bioactive properties. The analysis of botanical treatises, documenting the traditional use of plants by indigenous communities, can now be expedited through cutting-edge techniques like text mining, where algorithm implementation accelerates the search process. Furthermore, the elucidation of potential compound structures using mass spectroscopy for metabolomics and nuclear magnetic resonance (NMR) spectra is continuously advancing, thanks to AI algorithms that can identify patterns and propose likely structures. This fusion of our indigenous knowledge concerning plant-based treatments with the power of AI may create the step change needed to uncover new medicines before they are lost through the biodiversity crisis.

Keywords: Natural products, indigenous knowledge, artificial intelligence, mass spectrometry, metabolomics, nuclear magnetic resonance.

2.1 Introduction

Plant biodiversity in Australia has been recognized for its endemism and significant medicinal properties. Among the oldest civilisations, Aboriginal Australians arrived 65,000 years ago in the Northern Territory, and since then, they have used plants to develop treatments for a diverse range of diseases (Clarkson et al., 2017). Approximately 1,511 plant species have been reported for medicinal use by indigenous Australian communities (Turpin et al., 2022). Consequently, knowledge of ethnobotany has been laying the foundation for the exploration and use of Australian medicinal plants to find new therapies in modern medicine (Packer et al., 2019).

Although closing the gap between indigenous knowledge and modern science has been a formidable challenge due to the epistemological differences, technological development has brought them closer. While ethnobotanical studies offer invaluable insights into the historical uses of medicinal plants, the identification and validation of bioactive compounds provides the mechanism of action of those natural products (Pirintsos et al., 2022). Protecting this traditional knowledge through intellectual property (IP) rights is essential, especially in the era of artificial intelligence.

Plants can produce from 100 000 to >1 000 000 of secondary metabolites, many of which have demonstrated therapeutic properties for treating various diseases (Amelia Palermo, 2023). For instance, quercetin, isolated from *Styphnolobium japonicum,* is currently in the phase 2 clinical trials for the treatment of liver fibrosis (Nieuwdorp, 2022). In the identification process, metabolomics plays a crucial role for lead compounds generation in early drug discovery. Metabolomics accelerates drug discovery by broadening chemical space for screening, predicting drug targets and actions, and optimizing conditions for production in synthetic biology (A. Palermo, 2023).. Traditionally, compounds identification derived from medicinal plants has used techniques such as nuclear magnetic resonance

(NMR) and mass spectrometry (MS) (Manickam et al., 2023). Today, machine learning (ML), a subset of artificial intelligence that uses algorithms to analyse data, has been integrated to meticulously analyse each spectrum acquired from NMR and MS, enhancing the accuracy and efficiency of compound characterization (Li et al., 2021). In addition, algorithms developed for AI, a science that develop computer system that mimic human intelligence, have managed to compare NMR and MS spectra with databases of known compounds, which accelerate the identification of molecules (Li et al., 2021). This integration of advanced analytical techniques with AI not only improves the efficiency of compound identification but also facilitates the discovery of new molecules from medicinal plants.

In recent years, the emergence of artificial intelligence (AI) has revolutionized the field of drug discovery and natural products research. Classical natural products drug discovery is both expensive and time-consuming due to the laborious process: the typical drug development cycle is at least 10 years with expenditure of around 2.5 billion US dollars (Deng et al., 2022; Mullard, 2014). AI has achieved complex data analysis, enabling the processing of large amounts of data rapidly, which may be useful for the elucidation of novel compounds (Nelis et al., 2023). New alternatives in developing drugs incorporating cutting-edge technology as the implementation of *artificial intelligence* (AI) will provide new pathways in this area (Deng et al., 2022).

This review paper discusses the synergies between ethnobotany and compound identification with NMR and MS, using artificial intelligence. The importance of intellectual property of ethnobotanical knowledge will be described, highlighting its cultural significance and therapeutic relevance and the inclusion of AI. Subsequently, the potential of AI in NMR and MS, two prominent methodologies for the identification of compounds, will be analysed.

2.2 Ethnobotany and Artificial Intelligence in Drug discovery

2.2.1 Ethnobotany, medicinal system, and intellectual property

Throughout history, the Aboriginal people of Australia have maintained a deep connection with the land and its ecosystems. Plants have held a crucial and multifaceted significance in the daily lives of indigenous Australian communities, serving purposes ranging from constructing canoes and crafting hunting spears to providing shelter coverings for huts, remedies for toothaches, headache, skin infections, pain, sores, swelling, among others(Fensham, 2021; Turpin et al., 2022). Similarly, the community residing in Madjedbebe, located in the Northern Territory, incorporates various plant species, such as *Buchanania sp.* (Green plum), and *Persoonia falcata* (Milky plum), into their dietary practices. (Florin et al., 2020). Both species can be eaten raw, the former contains high levels of protein, as well as been source of potassium, magnesium, calcium and phosphorous and the latter contains saponins and tannins which are used to treat gastrointestinal disorders (Fyfe et al., 2020; Zhang et al., 2023).

The use of medicinal plants to treat inflammation-related conditions has been an ancient practice of Aboriginal Australians. Ludwig Leichhardt's expedition in 1843–1844 reported that *Angophora subvelutina* (Broad-leaved Apple) was used by the Queensland Aboriginal people for treating a child's inflamed testicle (Fensham, 2021). In the same way, *Dodonaea polyandra* (Hop bush) was used for relieving mouth pain and inflammation by the Northern Kaanju (Kuuku I'yu) tribe of Cape York (Simpson et al., 2015). Remote communities in Queensland still use *Cocos nucifera* (oils and bark), *Dioscorea transversa* (tuber), *Euphorbia tirucalli* (latex), *Ipomoea pes-caprae* (leaves and stem), and *Plumeria rubra* (leaves) to treat swelling (Turpin et al., 2022).

Indigenous Australians possess a distinctive understanding and perspective regarding the origins of diseases. Their belief system recognizes four distinct causes that can contribute to the development and manifestation of health issues: natural, environmental, supernatural, and diseases introduced by Western society (Maher, 1999). Natural refers to different states of humans in every single day like emotions, diet, and physical activities. On the other hand, environmental variations such as wind, moon phases and climate can affect and alter our body, resulting in some diseases (Maher, 1999). For instance, the Thaayorre and Wik Ngantjera tribes residing on the western side of the Cape York Peninsula associate inflammation of the eyes and diarrhea with the natural cause of consuming meat with excessive fat (Taylor, 1977). Additionally, they assert that certain human behaviours, such as social and religious transgressions, can give rise to various illnesses and diseases (Taylor, 1977). Conversely, the arrival of British colonization in Australia increased the prevalence of diseases like cancer and diabetes due to lifestyles changes in the indigenes population as well as many infectious diseases (Maher, 1999).

The Aboriginal medical system encompasses a multitude of distinctive treatment practices that could be integrated into the Western medical system. One of the main principals in Chinese traditional medicine is preventing diseases by administering herbal formulas. For instance, 17 herbal formulas with slightly different ingredients have been recommended to prevent COVID-19 infection (Liu et al., 2022). While Western medicine primarily focuses on disease recognition and treatment through pharmaceutical interventions, all traditional medicine worldwide often follows a holistic approach whereby the patient is considered within the context of individual, familial, and communal aspects (Maher, 1999). Today, the concept of "One Health" has been incorporated into modern medicine, which refers to interdisciplinary incorporation to improve the health of people, animals and the environment (Riley et al., 2021). With this new approach, One Health will benefit health, conservation and development (Zinsstag et al., 2012).

Efforts are being made to ensure the protection and well-being of indigenous communities through various initiatives and policies. Although efforts have been made to maintain well-being in indigenous communities, there is still an inequity between this population and non-indigenous people.

For example, a study in Victoria-Australia demonstrates the highest prevalence in psychological distress and food insecurity that there are significant differences between indigenous and non-indigenous communities (14 to 19 social determinants) (Markwick et al., 2014). Furthermore, Aboriginals Victorians are significantly more likely to be diagnosed with cancer, asthma, depression, and anxiety (Markwick et al., 2014).

Different initiatives aim to preserve their cultural heritage, promote their rights, and address the unique challenges they face in a respectful and inclusive manner. Since the 1920s, groups like the International Labour Organization (ILO) has taken responsibility for the Indigenous and Tribal Peoples Convention, 1989 (No. 169), which stands as the sole international treaty available for ratification, focusing solely on safeguarding the rights of these peoples (Larsen & Gilbert, 2020). In addition, The United Nations Declaration enfaces the protection of cultural heritage, traditional knowledge, and traditional cultural expressions of Indigenous peoples in its different articles (Nations, 2008).

In the present day, modern society witness not only the preservation and protection of traditional indigenous medicine but also its integration into modern medical practices. Numerous studies and publications have explored this integration, shedding light on its benefits and challenges. For instance, the World Health Organization (WHO) WHO publication "Traditional Medicine Strategy 2014-2023" outlines the organization's strategy and initiatives for the integration of traditional medicine, highlighting the need for evidence-based practices, quality assurance, and regulation to ensure patient safety (World Health, 2013). Similarly, Y. L. Park and R. Canaway argue that the integration of traditional and complementary medicine can pave the way to enhance five crucial attributes of the health system required to achieve universal health coverage: quality, efficiency, equity, accountability, and sustainability and resilience. (Park & Canaway, 2019).

The preservation and integration of traditional indigenous medicine into modern practices represent a significant step towards comprehensive healthcare and cultural preservation. The Aboriginal people of Australia have long recognized the profound connection between their land, plants, and well-being, utilizing medicinal plants for various purposes, including the treatment of inflammation-related conditions. Efforts are underway to protect indigenous communities, promote their rights, and incorporate their valuable knowledge into the Western medical system. By embracing the holistic approach and wisdom of traditional medicine alongside evidence-based practices, we can foster a more inclusive and effective healthcare system that respects diverse cultural perspectives and promotes the well-being of all.

2.2.2 Incorporation of Artificial intelligence on the analysis of Ethnobotany knowledge

In the past, Aboriginal knowledge was primarily passed down through oral traditions from one generation to another or documented in ancient texts, which have now been digitized to enable computer analysis and extraction of pertinent information. The Biodiversity Heritage Library (BHL) digitized 115,124 titles on medicine, creating opportunities for conducting analyses over various time periods, making it an excellent initiative. (Sharma et al., 2017). By digitizing these texts, the BHL has paved the way for researchers to explore and study the rich Aboriginal knowledge and practices related to medicine in a more comprehensive and systematic manner.

However, the task of revising old books for data analysis can be time-consuming due to the diverse ways in which descriptions and concepts for specific diseases have been recorded throughout history (Thompson et al., 2016). A notable challenge arises from the existence of various synonyms for terms used in the past. For instance, the term "inflammation" has been documented under a wide array of synonyms such as dropsy, oedema, edema, swell, swollen growths, among others (Fensham, 2021). This variation in terminology necessitates careful navigation through different expressions and understanding their historical context to ensure accurate interpretation.

Fortunately, the utilization of artificial intelligence (AI) has revolutionized the computational analysis of data, leading to more efficient discovery of new information. By leveraging computational techniques that automatically analyse and identify patterns, relationships, and trends within a collection of documents, text mining (TM) allows for the extraction of a substantial amount of valuable information (Thompson et al., 2016). This approach has proven particularly powerful in various domains, including the exploration of traditional medicine. For instance, a study focused on 1,150 traditional Chinese medical books from the Ming (1369–1644) and Qing (1645–1911) dynasties utilized TM techniques to identify formulas employed in the treatment of rheumatoid arthritis (Xia et al., 2020). Furthermore, this study discovered 15 herbs with potential anti-inflammatory properties, highlighting the capacity of AI-driven analysis to reveal previously unknown insights.

The utilization of AI in TM not only accelerates the analysis process but also enables a more comprehensive exploration of Aboriginal knowledge in medicine. These algorithms are capable of processing vast volumes of data, identifying key concepts, relationships between terms, and patterns of usage across different time periods. Consequently, this approach saves considerable time and resources while facilitating deeper insights into traditional medicinal practices (Thompson et al., 2016).

The digitization of antique books on the uses of plants by Aboriginals, combined with the application of AI-driven text mining techniques, has significantly advanced the exploration and understanding of traditional knowledge concerning medicine (Figure 2.1). Institutions like the Biodiversity Heritage Library have played a crucial role in preserving and digitizing these texts, expanding access and enabling comprehensive analyses. By harnessing AI algorithms, researchers can efficiently navigate through digitized texts, extract valuable information, and uncover connections and patterns that were previously inaccessible. For instance, Ithaca, a epigraphic restauration model using neural network architecture, was trained to restore Greek inscriptions (Assael et al., 2022). This convergence of traditional wisdom and cutting-edge technology holds immense potential for advancing our understanding of medicine and exploring new avenues for therapeutic interventions.

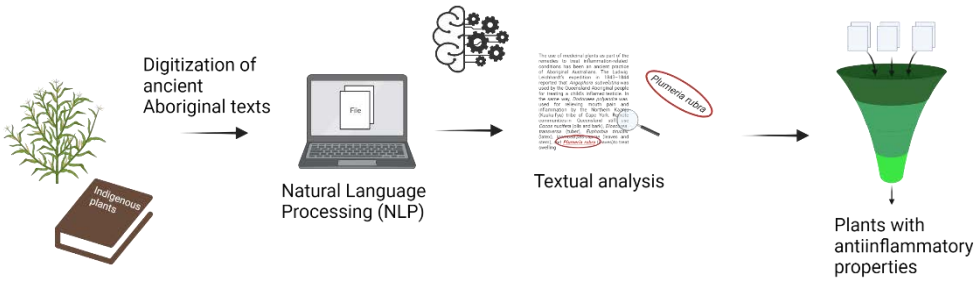

Figure 2. 1 Text mining process from ancient Aboriginal texts to extract valuable information about the plant uses. Created in BioRender.

2.3 MS and Metabolomics for the identification of compounds from medicinal plants using artificial intelligence.

The identification of compounds is undoubtedly the first step to connect traditional plant knowledge and modern chemistry. Over the last decades, countless compounds from natural products have been identified and isolated. This has led to the creation of large databases that include the chemical description of each molecule (Yates Iii, 2011). Furthermore, the application of metabolomics has become an essential part in the description of compounds present in cells and tissues (García-Pérez et al., 2021; Jean-Luc et al., 2020).

Metabolomics based on high resolution mass spectrometry (HRMS) is a fundamental area in drug discovery from natural products. Metabolomics studies the metabolome (amount of low molecular weight molecules in biochemical reaction) of cells, biofluids, tissues, or organisms, utilizing high-throughput analytical techniques (Manickam et al., 2023). Metabolomics for drug discovery is divided in targeted and untargeted approaches. The former require previous knowledge of molecules and the latter detects as many molecules as possible (Alarcon-Barrera et al., 2022). The identification of this

amount of compounds using HRMS requires the use of databases that have been develop along the years, such as IROA technologies, METLIN, and GNPS(Alarcon-Barrera et al., 2022). With the implementation of metabolomics, new drugs candidates for drug development has been founded (Noleto-Dias et al., 2023). HRMS has had a great prosperous in recent decades due to its sensitivity, selectivity, and resolution (Ledesma-Escobar et al., 2023). Liquid chromatography-mass spectrometry (LC-MS) is one of the most indispensable tools in metabolomics. LC-MS combines the precise separation of compounds and also measures the mass-charge ratio (m/z) of charged particles (ions) in each molecule (Tian et al., 2022). In addition, MS-DIAL an open-source software demonstrates how the data-independent acquisition (DIA) in liquid chromatography tandem mass spectroscopy (LC-MS/MS) improves identification and quantification of small molecules by mass spectral deconvolution (Tsugawa et al., 2015). As a result, the phytochemical characterization of many species, which have medical importance, has been completed (García-Pérez et al., 2021).

The incorporation of deep neural network, a type of machine learning model, has enabled the effective identification of molecules by analysing the complex spectral data (Asakura et al., 2018). For instance, MSNovelist uses a combination of fingerprint prediction and a neural network to create structures solely from tandem mass spectrometry (MS2) spectra (Stravs et al., 2022). This software exhibited accurate prediction of molecular structures, surpassing the effectiveness of existing database-dependent methodologies (Stravs et al., 2022).

Similarly, SIRIUS 4, another software that uses AI for the identification of molecular structures from MS data. SIRIUS 4 uses deep neural networks (DNN) for automated element detection from isotopic patterns (Dührkop et al., 2019). By combining formula prediction, tree-based algorithms, and machine learning, SIRIUS 4 achieves fast and reliable identification of molecular structures, making it highly efficient for metabolomics research. Integrates compound structure identification (CSI):FingerID to predict molecular fingerprints and search databases. This analysis allows SIRIUS 4 to process complex data sets quickly, demonstrating the importance of AI for chemical analysis (Dührkop et al., 2019). Finally, the use of tools such as Asari provides an innovative tool in the processing of LC-MS metabolomic data (Li et al., 2023). It implements "mass tracks" and "composite mass tracks", which improves the accuracy of mass alignment and simplifies feature detection processes (Li et al., 2023).

Metabolomics research of medicinal plants using artificial intelligence has emerged in recent years to accelerate this process. Advanced techniques such as MSNovelist, SIRIUS 4, MS-DIAL and Asari use AI for accurate identification of molecular structures. As a result, the discovery of lead comopounds for the development of new drugs is becoming faster.

2.4 Nuclear Magnetic Resonance and artificial intelligence

Nuclear Magnetic Resonance (NMR) plays a crucial role in compounds identification and with the advent of artificial intelligence (AI), there have been exciting developments in enhancing the acceleration of this process (Cortés et al., 2023). Industrial research and development (R&D) for drug discovery have implemented innovative methods that implement machine learning (ML) enables the establishment of correlations between experimental and calculated data (Berdigaliyev & Aljofan, 2020; Deng et al., 2022).

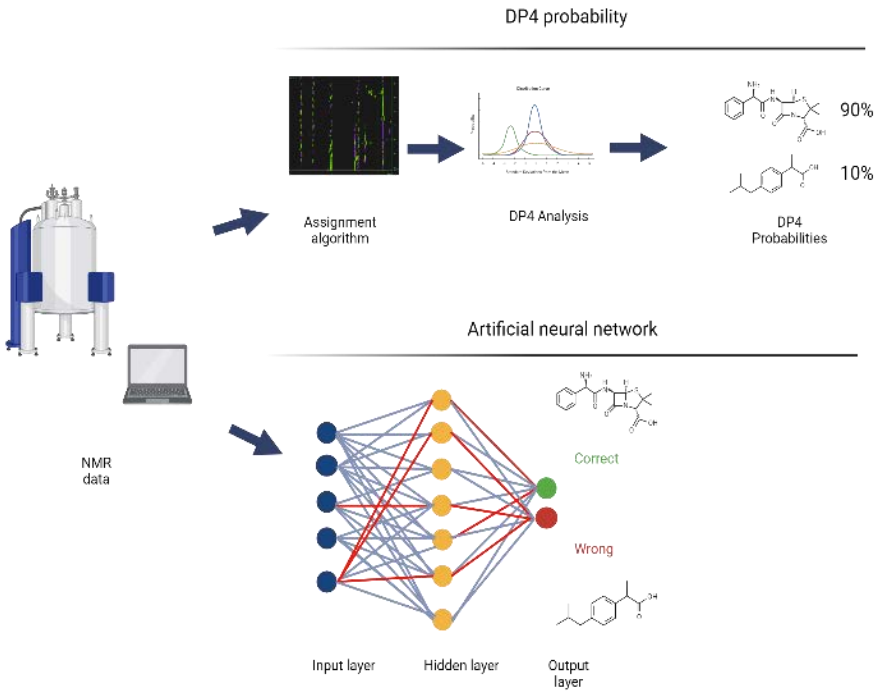

Figure 2. 2 Data analysis using DP4 and neural network for structure elucidation of different NMR spectrum. Created in BioRender.

DP4 probability is the most popular method to determine the structures. Its primary emphasis is on identifying the most probable structure from a limited collection of appropriately chosen candidates (Marcarino et al., 2020). By assuming independent random errors, the overall probability for the molecule is found by multiplying individual probabilities, followed by determining the accuracy of a candidate structure as the correct isomer using Bayes's theorem (Howarth et al., 2020; Smith & Goodman, 2010).

The implementation of neural networks is a tool that is being used not only in the analysis of MS/MS spectra but also in NMR spectra. The techniques developed by Sarotti's et al. (2013) aimed to solve the problem of structure elucidation. The technique used to identify potential molecular structures by analysing data relied on pattern recognition analysis (PRA), which was supported by neural networks

(Figure 2.2) (Sarotti, 2013). This methodology was tested on a diverse set of 200 molecules which demonstrated high efficiency in the correct classification of structures (Sarotti, 2013).

Another way to use neural networks from NMR is the deconvolution of 1D NMR spectra. This method implements algorithms that allow peaks in complex 1D NMR spectra to be accurately discerned and quantified (Schmid et al., 2023). A notable application of the neural network for NMR analysis is the prediction of chemical shifts. It has been used to reconstruct 2D spectra from down sampled data (Wei et al., 2022).

The integration of artificial intelligence (AI) into nuclear magnetic resonance (NMR) spectroscopy has catalysed notable advances in compound identification, significantly accelerating research and development processes in industries such as drug discovery from medicinal plants. Machine learning (ML) techniques are at the forefront of these innovations, establishing correlations between experimental data and theoretical predictions to streamline the identification process.

2.5 Conclusions

The integration of indigenous Australian ethnobotanical knowledge with the development of artificial intelligence (AI) provides a breakthrough in the identification of new compounds with pharmacological potential. For instance, text mining has been employed to analyse ancient ethnobotanical texts, uncover valuable insights preserved over centuries. Similarly, software like Ithaca, used for the epigraphic restoration of Greek inscriptions, demonstrates how AI can preserve and utilize traditional knowledge from diverse cultures throughout history. In this context, AI can accelerate the study of medicinal plants and their potential for treating diseases. This integration is allowing the identification of lead compounds of medicinal plants.

On the other hand, metabolomics has become a fundamental tool in the phytochemical study of medicinal plants. While metabolomics is being performed with programs that implement neural networks for the analysis of MS/MS and NMR spectra, significant challenges remain. Deconvolution is a critical bottleneck in metabolomics due to overlapping peaks and complex spectral data can limit the identification and quantification of metabolites. Additionally, the effectiveness of AI-based analysis depends on the quality and comprehensiveness of existing databases, making the curation and expansion of these databases fundamental to advancing plant metabolomics. Human intelligence, capable of discovering and understanding nature like no other species, has managed to advance and design an artificial intelligence capable of breaking the barriers of the unknown in the world of medicinal plants.

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Biological Activity of Chemical Constituents Isolated from Resin of *Corymbia citriodora* (Hook) K.D.Hill & L.A.S. Johnson.

Abstract: The indigenous Australian plant, *Corymbia citriodora*, which covers regions from northern Queensland down to New South Wales, has garnered significant interest for its medicinal attributes due to the small molecules from leaves demonstrating potential anti-inflammatory properties. Despite this, there exists a notable gap in research regarding the compounds extracted from its resin. This study aims to isolate, identify, and assess the bioactivity of select compounds derived from *C. citriodora.* Techniques such as column chromatography, size exclusion, and multiple rounds of preparative high-performance liquid chromatography (HPLC) were employed to isolate compounds. The isolated compounds were determined using mass spectroscopy and Nuclear Magnetic Resonance. 1D NMR (^{1}H and ^{13}C NMR) and 2D NMR; correlation spectroscopy, heteronuclear multiple-bond correlation, heteronuclear single quantum coherence, and nuclear overhauser effect spectroscopy (COSY, HMBC, HSQC, NOESY respectively) experiments were used in this identification. Five compounds from were isolated and identified. A new flavonoid named Mbabarametin (Compound 4 **(4))** is a novel natural product. Based on the normalized cell index acquired through xCELLigence Real-Time Cell Analysis (RTCA) of liver cells treated with the compounds from *C. citriodora*, they exhibited no toxicity at a concentration of 10 µg/ml. Additionally, compounds **4** and **5** derived from *C. citriodora* demonstrated notable *in-vitro* suppression of the proinflammatory cytokine IL-1β in peripheral blood mononuclear cells (PBMCs) which means potential anti-inflammatory properties.

Keywords: Indigenous Australians, HPLC, NMR, cytotoxicity, antioxidant.

3.1 Introduction

Corymbia citriodora (Hook) K.D.Hill & L.A.S.Johnson, commonly known as Lemon scented gum, is a Myrtacea plant originally from Australia (Goodine & Oelgemöller, 2020). It is distributed between Cooktown in north Queensland and as far south as Coffs Harbour in New South Wales (Goodine & Oelgemöller, 2020). This species produces resin which is an exudate used as analgesic, anti-inflammatory and antipyretic remedies by aboriginal Australians (Lee et al., 2017; Locher & Currie, 2010; Perry & Wangchuk, 2023). This synergism of knowledge between ethnobotany and the compounds isolated from this species has led to interest in discovery lead compounds for new treatments (Packer et al., 2019).

Although different compounds have been reported from *C. citriodora*, the resin remains a part of the plant with potential for exploration, as only a few compounds have been isolated from it. Currently, compounds such as kaempferol, 7-*O*-methyl aromadendrin, 6-[1-(*p*-hydroxyphenyl)ethyl] rhamnocitrin, and rhamnocitrin have been isolated form the resin (Lee et al., 2017). These are flavonoids which have shown inhibitory activity against 15-lipoxygenase, potentially contributing to the reduction of inflammation (Lee et al., 2017). In addition, 6-[1-

(*p*-hydroxy-phenyl)ethyl]-7-*O*-methyl aromadendrin demonstrates the inhibition of the proliferation of B16F10 cells via apoptosis (Duh et al., 2012).

Traditional medicine has functioned as a basis for the discovery of new drugs. By uniting traditional knowledge with sophisticated analytical techniques, such as HPLC, LCMS and NMR, the potential of natural products is being harnessed to advance drug discovery (Atanasov et al., 2021). In this sense, the traditional use of *C. citriodora* by indigenous communities has provided valuable knowledge for anti-inflammatory assay. This research aims to isolate, identify, and evaluate the cytotoxicity, antioxidant, and anti-inflammatory activity of *C. citriodora* resin compounds.

3.2 Materials and methods

3.2.1 Plant material and extraction

Corymbia citriodora resin was collected in Watsonville located in Shire of Mareeba (17.35749°S, 145.31268°E), Queensland, Australia. The plant resin (103.5 g) was washed under running water and air dried. After drying, resin was ground to powder using a NutriBullet. The resin was extracted with MeOH (MeOH: tissue, 2:1 v/w), filtered, and pooled. The extract was concentrated at 40 °C using a rotary evaporator (G5 Heidolf CVC 3000 Vacuubrand, John Morris Scientific), and fractionated using CH_2Cl_2/MeOH biphasic separation process. The focus of this study was the CH_2Cl_2 fraction (21.6 g).

3.2.2 Column Chromatography and RP-HPLC isolation

The CH_2Cl_2 fraction was dissolved in MeOH and then fractionated using flash column chromatography packed with Merck Kieselgel 60 PF254. The solvent system for the fractionation was ($CHCI_3$: MeOH, 85:15, 75:25, 70:30). Reverse Phase High-performance liquid chromatography (RP-HPLC) was used to isolate and purify the fraction I compounds. The mobile phases for preparative RP-HPLC were A, water with 0.05% (v/v) TFA; and B, 100% acetonitrile (HPLC grade, Fisher chemical™) with 0.045% (v/v) TFA. The sample were suspended in a solution of mobile phases A and B [1:1], and then filtered with a 0.22 µm PVDF Millipore Filter before injection. Agilent 1260 HPLC system coupled with a DAD detector (UV 254 nm) and preparative column (Synergi 10µ Fusion-RP 80 Å) at a flow rate 6ml/min. The gradient program was: 0-100 min, gradient elution 50%B. For purity checking, an analytical RP-HPLC column (Agilent 4µm EC-C18 120 Å) at a flow rate 1 ml/min was used . The gradient program was: 0-20 min, gradient elution 40% B (Figure 3.1).

3.2.3 NMR spectroscopy and analysis

The compounds were dissolved in CD_3OD. All NMR spectra were acquired on a Bruker 600 MHz AVANCE III NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a cryogenically cooled probe. 1D NMR (1H and 13C NMR) and 2D NMR (COSY, HMBC, HSQC, NOESY) experiments were used in this identification.

p-Coumaric acid **(1)**. Isolated as a yellow solid (5.4 mg). For ^1H NMR (CD_3OD, 600 MHz) δ 7.60 (d, J = 15.9 Hz, 1H), 7.45 (d, J = 8.6 Hz, 2H), 6.81 (d, J = 8.7 Hz, 2H), 6.28 (d, J = 15.9 Hz, 1H) and ^{13}C (CD_3OD, 600 MHz) δ 171.0, 161.2, 146.6, 131.1, 127.2, 116.8, 115.6; LRMS (ESI^-) *m*/*z* 163 (100%) $[M-H]^-$.

7-*O*-Methylaromadendrin **(2)**. Isolated as a yellow solid (7.9 mg). For ^1H NMR (CD_3OD, 600 MHz) δ 7.36 (d, J = 8.5 Hz, 2H), 6.83 (d, J = 8.6 Hz, 2H), 6.08 (d, J = 2.3 Hz, 1H), 6.04 (d, J = 2.2 Hz, 1H), 5.01 (d, J = 11.7 Hz, 1H), 4.67 (s, 1H), 4.58 (d, J = 11.7 Hz, 1H), 3.81 (s, 3H) and ^{13}C (CD_3OD, 600 MHz) δ 199.1, 169.8, 165.0, 164.4, 159.2, 130.4, 129.1, 116.1, 102.6, 96.0, 95.0, 85.1, 73.7, 56.4 (Ali et al., 2023).

Sakuranetin **(3)**. Isolated as a yellow solid (3.5 mg). For ^1H NMR (CD_3OD, 600 MHz) δ 7.32 (d, J = 8.6 Hz, 2H), 6.82 (d, J = 8.6 Hz, 2H), 6.07 – 6.03 (m, 2H), 5.38 (dd, J = 12.9, 3.0 Hz, 1H), 3.81 (s, 4H), 3.15 (dd, J = 17.1, 12.9 Hz, 1H), 2.74 (dd, J = 17.1, 3.0 Hz, 1H) and ^{13}C (CD_3OD, 600 MHz) δ 198.3, 169.5, 165.2, 164.7, 159.1, 131.0, 129.1, 116.3, 104.1, 95.7, 94.9, 80.6, 56.3, 44.1 (Zhang et al., 2006).

2,5-dihydroxy-6-[1-(4-hydroxyphenyl)ethyl]-7-methoxy-2,3-dihydro-4*H*-1-benzopyran-4-one. Mbabaramетin **(4)**. Isolated as a yellow solid (3.5 mg). mp 134.1 – 135.0°C; Optical Rotation 109.6 degrees at a concentration of 0.5 mg/mL in MeOH; UV data (ECD); FT-IR. For ^1H NMR (CD_3OD, 600 MHz) and ^{13}C (CD_3OD, 150 MHz) data, see (Table 3.1); LRMS (ESI^+) *m*/*z* 407 (100%) $[M+H]^+$; HRMS (ESI^+) Calculated for $C_{24}H_{21}O_6^-$ $[M-H]^-$ 405.1338, found 405.1342.

3,5-dihydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl)ethyl]-7-methoxy-chroman-4-one. HEMA **(5)**. Isolated as a yellow solid (7 mg). mp 134.1 – 135.0°C; Optical Rotation 6.77 degrees at 1.3 mg/mL in MeOH; UV data (ECD); FT-IR. For ^1H NMR (CD_3OD, 600 MHz) and ^{13}C (CD_3OD, 150 MHz) data, see (Table 3.1); LRMS (ESI^+) *m*/*z* 423 (100%) $[M+H]^+$; HRMS (ESI^+) Calculated for $C_{24}H_{21}O_7^-$ $[M-H]^-$ 421.1287, found 421.1289 (Duh et al., 2012; Freitas et al., 2007).

3.2.4 Cell culture and xCELLigence assay

Liver cells from humans were cultured in serum-free media at 37°C, following the manufacturer's instructions. All experiments utilized a 96-well gold-plated e-well plate from (ACEA Biosciences in Santa Clara, California, USA). The plate was prepared by seeding 3000 cells per well in 150 µL of media 24 hours before applying compounds to facilitate cell adherence. Each compound was dissolved in [10% DMSO (Dimethylsulfoxide)] and filtered with a 0.22 µm PVDF Millipore Filter

before the cytotoxicity assay. The *C. citriodora* compounds concentrations tested were 100, 50, and 10 µg/ml in triplicate.

The xCELLigence Real-Time Cell Analysis (RTCA) provided real-time bio-impedance measurements for the cell index, a measurements of cell viability, of liver cells dosed with the *C. citriodora* compounds. Data are presented as the mean normalized cell index at the point of treatment additions, along with the standard error of the mean (S.E.M.), where a cell index of 1 represents the measurement just before small molecule addition.

3.2.5 DPPH-HPLC for evaluation of the free radical-scavenging

The capability of compounds **4** and **5** to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured to evaluate their antioxidant potential, as this activity is indicative of their biological activity (Kumbhare et al., 2023; Yamaguchi et al., 1998). The blank was 0.1 mM DPPH in methanol. This blank was reacted with the compounds (3.5, 1.75, 0.87, 0.43, 0.218, 0.109 mg/ml) during 20 minutes in dark at room temperature. After the reaction each sample was measure per triplicate in analytical RP-HPLC, the mobile phase A was water with 0.05% (v/v) TFA; and B, 100% methanol (HPLC grade, Fisher chemical™) with 0.045% (v/v) TFA. Agilent 1260 HPLC system coupled with a DAD detector (UV 517 nm) and analytical column (Agilent 4µm EC-C18 120 Å). Isocratic elution was accomplished with methanol/water (80:20, v/v) at a flow rate of 1 mL/min for 5 minutes (Chandrasekar et al., 2006). It was used gallic acid as the positive control due to it is a well-known antioxidant molecule. The radical-scavenging (RA) activity was calculated using the Eq. (1).

$$RA\ (\%) = \left[\frac{PAb - PAs}{PAb}\right] \times 100 \qquad (1)$$

Where: PAb = Peak area of blank, PAs = Peak area of sample + DPPH

3.2.6 Human cytokine suppression assay.

For the cytotoxicity assay, inflammatory cytokines (IL-1β, TNF-α) were induced. Peripheral blood mononuclear cells (PBMCs) were separated from one donor. It was cryopreserved in Fetal Bovine Serum (FBS) containing 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich). PBMCs were stimulated with 10 ng/mL lipopolysaccharide (LPS) (SigmaAldrich). Stimulated PBMCs were incubated in triplicate with the compounds isolated from resin *C. citriodora* [10 µg/ml]. Culture plates were incubated for 20 h at 37 ◦C in a 5% CO2 incubator. Following overnight incubation, plates were centrifuged (277× g force, 4 ◦C for 5 min), and the culture supernatants were collected for cytokine analysis (Yeshi et al., 2022). Bacterial LPS is a strong stimulator of innate immune system in diverse eukaryotic cells including humans. The reduction in inflammatory cytokines triggered by the compounds was quantified using the LEGENDplexTM Multi-Analyte Flow Assay kit (BioLegendÒ) as detailed in the manufacturer's instruction in the assay kit (Ritmejerytė et al., 2022).

3.2.7 Data analysis

Statistical analyses were performed using RStudio (4.2.0), IBM SPSS statistics 27, and GraphPad Prism (version 6.0e). MestRenova 11 was the software to process the spectra from NMR.

3.3 Results and Discussion

After fractionated the sample using flash column chromatography, fraction I (0.51 g) was isolated and used to separate compounds in RP-HPLC. Analytical RP-HPLC demonstrates the purity of the five compounds isolated (Figure 3.1). Compounds 1-5 were identify using 1D and 2D NMR experiments. A novel compound was identified as 5-hydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl)ethyl]-7-methoxy-chroman-4-one, **()**. Compounds **1**, **2**, **3**, and **5** have been reported previously in the literature (Figure 3.2).

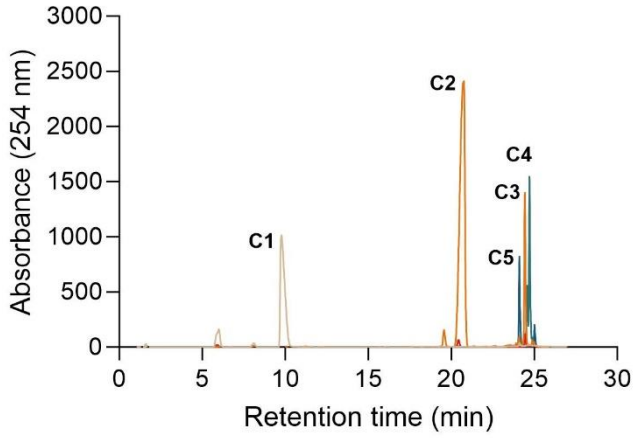

Figure 3. 1 Analytical RP-HPLC for purity of isolated compounds 1-5 from C. citriodora resin at 254 nm.

p-Coumaric acid (**1**) is a type of phenolic acid due to its structure contain a carboxyl group C1-COOH δ_C 171.04 and a hydroxyl group C4'-OH δ_C 161.16 bonded to aromatic ring (Cañadas et al., 2021). In terms of human health, diverse studies have been accomplished with this compound, finding properties such as inhibition of the development of ulcers (Barros et al., 2008), antimicrobial activity against gram-positive bacteria (Cho et al., 1998), and anti-inflammatory effect by the reduction in the TNF-α expression (Pragasam et al., 2013). 7-*O*-Methylaromadendrin and Sakuranetin (**2**, **3** respectively) are similar in their structure, and they are classified as flavonoids because the carbon skeletons are conform by two benzene rings (A, B), linked together with a heterocyclic pyrene ring (ring C) (Rakha et al., 2022). The difference between both is the presence of the hydroxyl group in the C3-OH δ_C 73.67 in 7-*O*-Methylaromadendrin and the absence of this in the same carbon C3 δ_C 44.06 in Sakuranetin. Both compounds showed significantly moderate anti-inflammatory activity *in-vitro* by suppressing of NF-kB (Ali et al., 2023).

Compound **4** was isolated as a yellow solid. Its molecular formula is $C_{24}H_{22}O_6$. Its NMR data (Table 3.1 and figure S19-S24, Supporting information) suggested a flavanone skeleton. Its ring B having *ortho*-coupled (*J*= 8.3 Hz) protons resonating at δ_H 7.32 (H-2') and δ_H 6.82 (H-3') with the corresponding carbons appearing at δ_c 129.2 (C-2') and δ_c 116.3 (C-3'). This ring is substituted at C-4' (δ_c 159.1) with hydroxy group. The C-1' (δ_c 131.1) of ring B is substituted with a Chromanone, which conforms the structure of ring A and C. The ring C protons resonate at δ_H 2.72 (H-3a) and δ_H 3.15 (H-3b) with the corresponding carbon C-3 (δ_c 4.19). The C-5 (δ_c 161.6) in the Chromanone is substituted with hydroxy group and a methoxy in C-7 (δ_c 167.4). The C-6 δ_C 115.6 is substituted with *p*-Ethylphenol which have *ortho*-coupled (*J*= 8.23 Hz H-3" and *J*= 8.32 H-4") protons resonating at δ_H 7.09 (H-3") and δ_H 6.63 (H-4"). Based on the above spectroscopy evidence, Mbabarametin **(4)**, was characterized as 2,5-dihydroxy-6-[1-(4-hydroxyphenyl)ethyl]-7-methoxy-2,3-dihydro-4H-1-benzopyran-4-one.

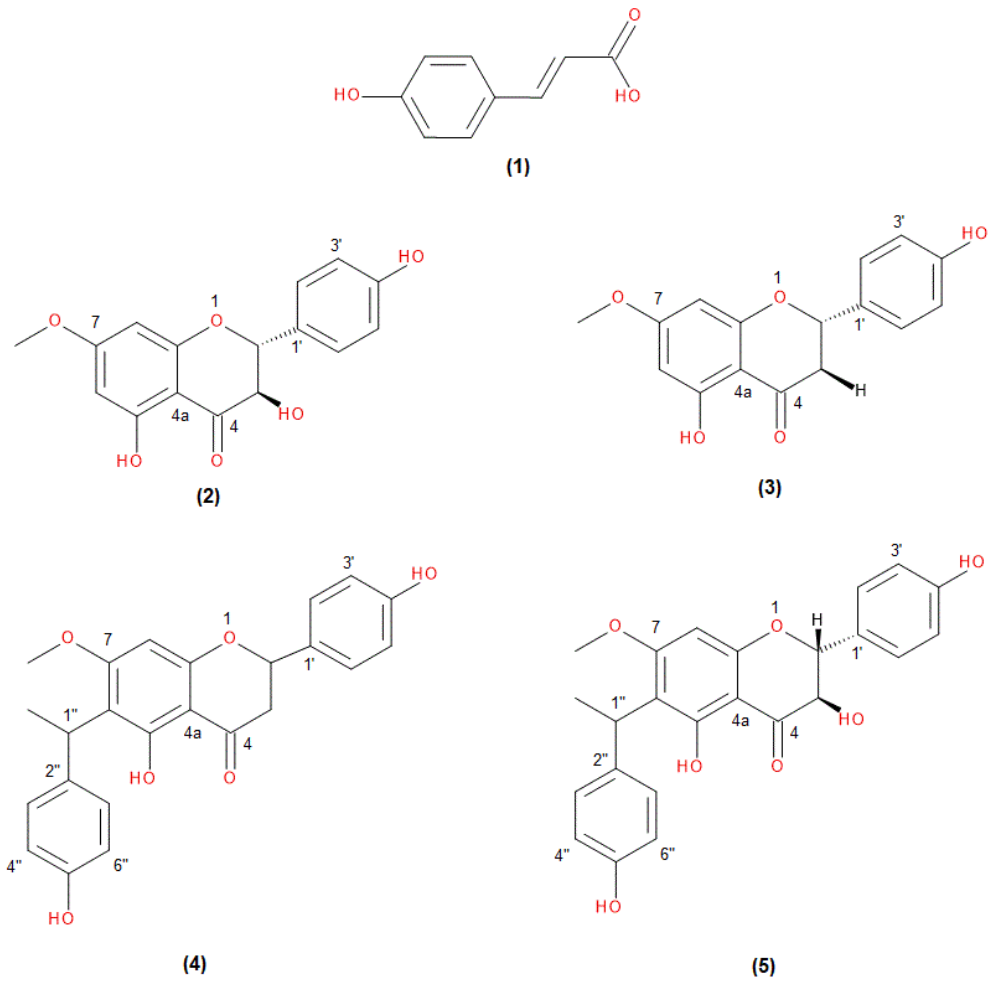

Figure 3. 2 Chemical structures of compounds 1-5 isolated from C. citriodora resin. p-Coumaric acid (1), 7-O-Methylaromadendrin (2), Sakuranetin (3), Mbabarametin (4), HEMA (5).

Compound **5** was isolated as a yellow solid. Its molecular formula is $C_{24}H_{22}O_7$. Its NMR data (Table 3.1 and figure S25-S30, Supporting information) suggested that is HEMA which was previously isolated from the kino of *E. citriodora* (Freitas et al., 2007; Lee et al., 2017). The C3 (δ_c 74.15) located in ring C is substituted with a hydroxy group, a feature does not present in **C4**.

Table 3. 1 NMR Spectroscopic Data (600 MHz, CD3OD) for Mbabarametin (4) and HEMA (5).

C4					C5				
Position	δH (*J* in Hz)	δC, type		HMBC	Position	δH (*J* in Hz)	δC, type		HMBC
2	5.36 dd (3.31, 13.21)	80.63	CH	7", 3"	2	5 dd (2.56, 11.77)	85.51	CH	3, 4
3a	2.72 dt (17.18, 2.83, 2.83)	44.19	CH2		3	4.58 dd (3.83, 11.77)	74.15	CH	2
3b	3.15 ddd (15.83, 13.20, 2.04)	44.19	CH2	2, 4, 1'					
4		198.52	C		4		199.77	C	
4a		103.86	C		4a		102.79	C	
5		161.56	C		5		161.73	C	
6		115.62	C		6		116.33 or 116.35	C	
7 OMe		167.36	C		7 OMe		168.05	C	
8	6.12 s	92.47	CH	4, 7OMe , 6, 9	8	6.1 s	93.05	CH	4a , 4, 9
9		163.5	C		9		163.58	C	
10	3.74 s	56.2	CH3	7OMe	10	3.73 m	56.7	CH3	7OMe
1'		131.05	C		1'		116.33 or 116.35	C	
2',6'	7.32 d (8.3)	129.18	CH	2, 7", 3", 4'	2',6'	7.36 d (8.52)	130.81	CH	2, 4'
3',5'	6.82 d (8.31)	116.31	CH	3', 1', 4'	3',5'	6.83 d (8.61)	116.55	CH	
4'		159.05	C		4'		159.66	C	
7'',3''	7.09 d (8.23)	129.05	CH	5", 7", 3"	7'',3''	7.09 m	129.61	CH	1'', 4'', 6''
6'',4''	6.63 d (8.32)	115.34	CH	5", 2"	6'',4''	6.63 dd (0.91, 8.61)	115.79	CH	4'
1''	4.61 q (7.39, 7.37, 7.37)	33.05	CH	8", 2", 5, 7MOe	1''	4.62 q (7.3, 7.31, 7.31)	33.5	CH	8'', 2'', 7OMe
2''		137.93	C		2''		138.2	C	
5''		155.8	C		5''		156.27	C	
8''	1.6 d (7.28)	18.05	CH3	6, 1", 2"	8''	1.6 dd (2.22, 7.22)	18.46	CH3	

3.3.1 Cytotoxicity activity

The compounds isolated from *C. citriodora* were tested for cytotoxicity with one human cell line, liver cells, using cell viability real-time assay, the xCELLigence system. Plots of average, normalized xCELLigence cell index data show the effect of C1-C5 in 10 µg/ml and all concentrations on liver cells (Figure 3.3).

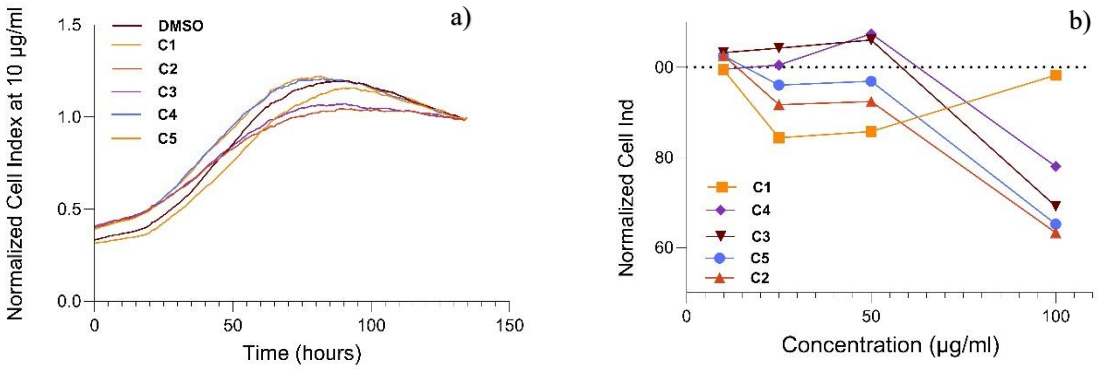

Figure 3. 2 Cytotoxicity assay of compounds 1-5 at different time (a) and different concentrations (b).

At a lower concentration, no compound has a tendency towards cellular cytotoxicity. C1 is the compound that has a tendency equal to DMSO. The trend between C2-C3 and C4-C5 compounds is very similar, possibly due to their molecular similarity. Nevertheless, it is observed that as the concentration of each compound increases, there is a considerable significant decrease ($p<0.05$; Dunn's Test) in C2 to C5, especially at 100µg/ml.

3.3.2 Antioxidant activity

Since compounds with antioxidant activities often exhibit strong ani-inflammatory activity, isolated compounds were first tested for their antioxidant activities using DPPH-HPLC assay. The DPPH-HPLC assay demonstrated that **C5** has better antioxidant activity that **C4** (Figure 3.4). The figure 6a illustrates that the intensity of DPPH at 517 nm using **C5** is close to zero absorbance unit (AU). In contrast, the DPPH intensity when reacted with **C4** is around 5 (AU).

In the same way, figure 3.4 b shows the percentage of radical scavenging activity at different concentrations. When the concentration is around 0.5 mg/ml, **C5** has more than 50% activity, while **C4** is more than 25%. 100% antioxidant activity is achieved at C5 around 1.7 mg/ml, while for C4 it must be greater than 3.5 mg/ml.

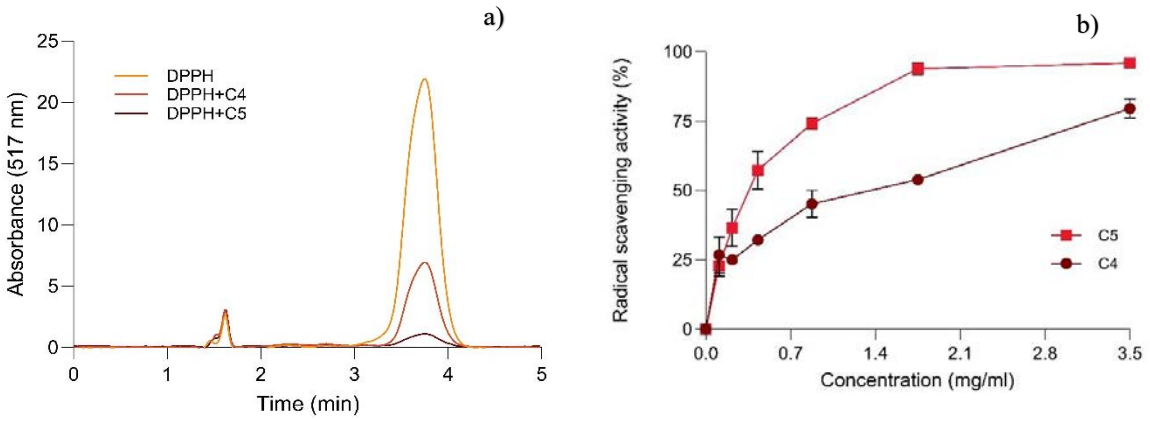

Figure 3. 3 DPPH-HPLC for C4 and C5 from C. citriodora. (a). Chromatogram of C4 and C5 at 3.5 mg/ml. (b) Radical scavenging activity of C4 and C5 in different concentrations.

3.3.3 Anti-inflammatory activity

Figure 3.5 shows the suppression of the cytokine IL-1β by compounds c1-c5 isolated from the *C. citriodora* resin using PBMC assay. The concentration of IL-1β (pg/ml) is lower when treatment with compounds C4 and C5 is applied. C1 presents the highest concentration of IL-1β in comparison with LPS. The cytokine concentration with compounds C2-C3 is approximately 150 pg/ml. This similarity could be due to the fact that these compounds, like C4-C5, differ only in the presence or absence of a hydroxyl group on carbon 3.

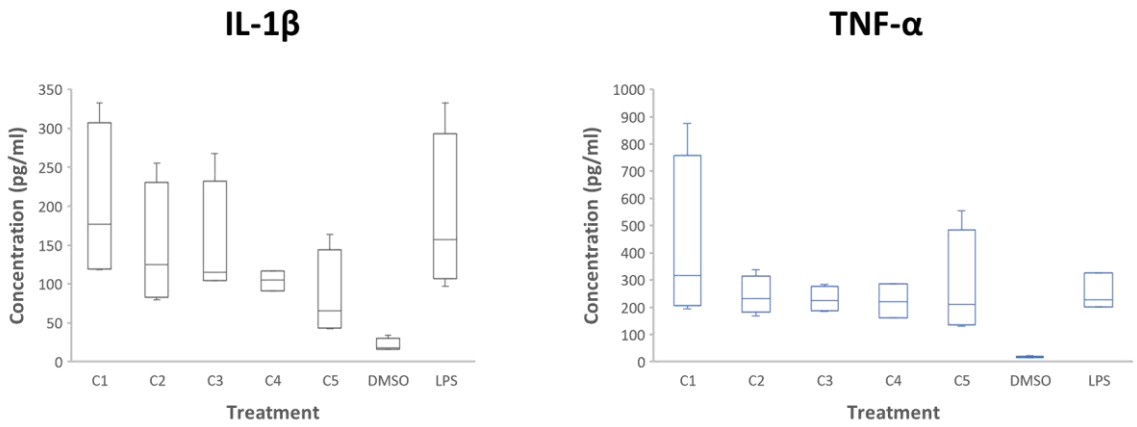

Figure 3. 4Inhibition of cytokines using PBMC assay at 10µg/ml of compounds 1-5.

3.4 Conclusion

Five compounds from *C. citriodora* resin have been studied. C1, C2, C3, and C5 are known compounds, however C4 has been isolated and determined as a new natural product for literature. All five compounds have demonstrated low toxicity at a concentration of 10 µg/ml. Although C4 and C5 have antioxidant activity, C5 exhibits the highest activity by scavenging 100% of DPPH radical at a concentration 3.5 mg/ml . Likewise, the suppression of the cytokine IL-1β is greater in the latter. Therefore, further research is required on the identification of other active compounds in *C. citriodora* resin to support the bioactivity of this species.

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Chemical constituents isolated from leaves extract from *Ceratopetalum hylandii* Rozefelds & R.W.Barnes.

Abstract: *Ceratopetalum hylandii* is a plant endemic to northeastern Australia. It belongs to the *Cunoniceae* family and there are mostly morphological records. To the best of our knowledge this is the first phytochemical study for this specie for the literature. This study aims to isolate, identify, and assess the bioactivity of select compounds derived from the leaves of *C. hylandii.* Techniques such as column chromatography, and multiple rounds of preparative high-performance liquid chromatography (HPLC) were employed to isolate compounds. The isolated compounds were determined using mass spectroscopy and Nuclear Magnetic Resonance. 1D NMR (1H and 13C NMR) and 2D NMR; correlation spectroscopy, heteronuclear multiple-bond correlation, heteronuclear single quantum coherence, and nuclear overhauser effect spectroscopy (COSY, HMBC, HSQC, NOESY respectively) experiments were used in this identification. The compounds Shikimic Acid **(1)**, 3-Dehydroshikimic acid **(2)**, Gallic acid **(3)**, *trans*-Melilotoside **(4)**, and *cis*-Melilotoside **(5)** from *C. hylandii* were isolated and identified. Shikimic acid and phenylpropanoid acid pathways are likely to be the pathways for the synthesis of phenolic compounds which would explain the high antioxidant capacity and possible anti-inflammatory properties.

Keywords: *Ceratopetalum hylandii,* Endemic, Australia, HPLC, NMR.

4.1 Introduction

Australia's tropical rainforests, particularly those in the northeastern region, are renowned for their unique biodiversity and high levels of endemism. These rainforests are home to a multitude of plant species, many of which have not been extensively studied for their chemical properties and potential pharmaceutical applications (Clarkson et al., 2017). The rich diversity of secondary metabolites produced by these plants, including alkaloids, terpenoids, flavonoids, and phenolic compounds, presents a vast resource for the discovery of new drugs and therapeutic agents (Palermo, 2023). The unique evolutionary history and isolated development of Australian flora have led to the emergence of novel bioactive compounds that are not found elsewhere, making these plants particularly valuable for bioprospecting and drug discovery efforts (Mani et al., 2022).

Ceratopetalum hylandii is a plant belonging to the Cunoniceae family and is native to the rainforest in northeastern Australia. Recent studies discovered that the genus *Ceratopetalum* exhibits a biogeographic pattern typical of a lake in Argentina (Gandolfo & Hermsen, 2017). This research suggests that the genus is extinct in South America today but was a component of the Paleogene to recent rain forest vegetation of Australasia (Gandolfo & Hermsen, 2017). This unique biogeographical history may have contributed to the evolution of distictive phytochemical profiles in this species. There are only morphological descriptions of *C. hylandii*, which highlight the trifoliate shape of the leaf and the midrib is raised on the adaxial surface above the leaf lamina (Rozefelds & Barnes, 2002).

The crude extract of *Ceratopetalum hylandii* leaves showed the presence of a high concentration of alkaloids, tannins, terpenoids, and cardiac glycosides (Yeshi et al., 2022). In addition, this crude extract significantly inhibited the production of proinflammatory cytokine TNF (Yeshi et al., 2022). These previously described results suggest that there is great potential for the discovery of new natural products from this plant. For this reason, this study aims to isolate, identify, and find small molecules with potential biological activity.

4.2 Materials and methods

4.2.1 Plant material and extraction

Ceratopetalum hylandii leaves was collected in Mount Lewis National Park, Queensland, Australia. The plant leaves (396.47 g) were washed under running water and then dried. After drying, leaves were ground to powder using a NutriBullet. The leaves were extracted in acetone per triplicate (solvent: tissue, 2:1, v/w), filter, and pooled. The extract was concentrated at 40 °C using a rotary evaporator (G5 Heidolf CVC 3000 Vacuubrand, John Morris Scientific).

For the fractionation, a separation funnel and different solvents were used with Hexane, Chloroform, and Ethyl acetate (EtOAc) added sequentially in a 1:1, v/v ratio. After each solvent addition, the layers were separated based on polarity, and the fractions were collected accordingly.

4.2.2 DPPH-HPLC for evaluation of the free radical-scavenging

The blank was 0.1 mM DPPH in methanol. This blank was reacted with the compounds (3.5, 1.75, 0.87, 0.43, 0.218, 0.109 mg/mL) during 20 minutes in dark at room temperature. After the reaction each sample was measured in triplicate in analytical Reverse Phase High-Performance Liquid Chromatography (RP-HPLC); buffer A was water with 0.05% (v/v) Trifluoroacetic Acid (TFA) and buffer B was methanol (HPLC grade, Fisher chemical™) with 0.045% (v/v) TFA. Agilent 1260 HPLC system coupled with a DAD detector (UV 517 nm) and analytical column (Agilent 4µm EC-C18 120 Å). The method used was isocratic elution with 20% buffer A/buffer B at a flow rate of 1 mL/min for 5 minutes. Gallic acid was used as the positive control. The radical-scavenging (RA) activity was calculated using the Eq. (1).

$$RA\ (\%) = \left[\frac{PAb - PAs}{PAb}\right] \times 100 \qquad (1)$$

Where: PAb = Peak area of blank, PAs = Peak area of sample + DPPH

4.2.3 RP-HPLC isolation and purification

Reverse Phase High-performance liquid chromatography (RP-HPLC) was used to isolated 528.02 mg of a fraction obtained from Ethyl acetate (EtOAc) fraction. The mobile phases for RP-HPLC

were water with 0.05% (v/v) TFA for buffer A; and buffer B was acetonitrile (HPLC grade, Fisher chemical™) with 0.045% (v/v) TFA. The sample were suspended in a solution of mobile phases A and B [1:1], and then filtered with a 0.22 μm polyvinylidene fluoride (PVDF) Millipore Filter before injection. Agilent 1260 HPLC system coupled with a DAD detector (UV 254 nm) and preparative HPLC column (Synergi 10μ Fusion-RP 80 Å) at a flow rate 6 ml/min. The gradient program was: 0-105 min, linear gradient 50% buffer B.

4.2.4 NMR spectroscopy and analysis

The compounds were dissolved in CD_3OD. All NMR spectra were acquired on a Bruker 600 MHz AVANCE III NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a cryogenically cooled probe. 1D NMR (^1H and ^{13}C NMR) and 2D NMR; correlation spectroscopy, heteronuclear multiple-bond correlation, heteronuclear single quantum coherence, and nuclear overhauser effect spectroscopy (COSY, HMBC, HSQC, NOESY, respectively) experiments were used in this identification.

Shikimic Acid **(1)**. White solid (100.4 mg). ^1H NMR (600 MHz, CD_3OD) δ 6.82 – 6.78 (m, 1H), 4.37 (h, *J* = 1.6 Hz, 1H), 3.99 (dt, *J* = 7.4, 5.3 Hz, 1H), 3.68 (dd, *J* = 7.3, 4.2 Hz, 1H), 2.70 (ddt, *J* = 18.2, 4.9, 1.9 Hz, 1H), 2.19 (ddt, *J* = 18.2, 5.7, 1.8 Hz, 1H) and ^{13}C NMR (600 MHz, CD_3OD) δ 170.0, 138.8, 131.7, 72.7, 68.4, 67.3, 31.6; LRMS (ESI^-) *m*/*z* 173 (100%) $[M-H]^-$; LRMS (ESI^-) *m*/*z* 347 (100%) $[2M-H]^-$.

3-Dehydroshikimic acid **(2)**. Yellow solid (2.1 mg).^1H NMR (600 MHz, CD_3OD) δ 6.71 (d, *J* = 3.0 Hz, 1H), 4.07 (d, *J* = 10.7 Hz, 1H), 3.87 (td, *J* = 10.1, 5.2 Hz, 1H), 3.08 (dd, *J* = 18.5, 5.2 Hz, 1H), 2.53 (ddd, *J* = 18.5, 9.7, 3.1 Hz, 2H) and ^{13}C NMR (600 MHz, CD_3OD) δ 201.2, 168.4, 148.4, 132.1, 80.2, 72.5, 34.4; LRMS (ESI^-) *m*/*z* 171 (100%) $[M-H]^-$, LRMS (ESI^-) *m*/*z* 375 (100%) $[2M-H_2O-H]^-$.

Gallic acid **(3)**. White solid (4.1 mg) ^1H NMR (600 MHz, CD_3OD) δ 7.06 (s, 1H) and ^{13}C NMR (600 MHz, CD_3OD) δ 170.4, 146.4, 139.6, 121.9, 110.3; LRMS (ESI^-) *m*/*z* 169 (100%) $[M-H]^-$.

trans-Melilotoside **(4)**. Yellow solid (3.5 mg). ^1H NMR (600 MHz, CD_3OD) δ 8.12 (d, *J* = 16.2 Hz, 1H), 7.63 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.38 (td, *J* = 7.9, 7.2, 1.6 Hz, 1H), 7.25 (d, *J* = 8.4 Hz, 1H), 7.06 (t, *J* = 7.5 Hz, 1H), 6.53 (d, *J* = 16.2 Hz, 1H), 5.00 (d, *J* = 7.8 Hz, 1H), 3.90 (dd, *J* = 12.2, 2.1 Hz, 1H), 3.71 (dd, *J* = 12.1, 5.4 Hz, 1H), 3.57 (t, *J* = 8.4 Hz, 1H), 3.48 (t, *J* = 8.8 Hz, 3H), 3.46 (dd, *J* = 5.4, 2.1 Hz, 1H), 3.43 (d, *J* = 9.0 Hz, 1H) and ^{13}C NMR (600 MHz, CD_3OD) δ 171.1, 157.5, 141.4, 132.8, 128.8, 125.4, 123.6, 119.5, 116.9, 102.3, 78.3, 78.1, 74.9, 71.3, 62.5. (Bayrakçeken Güven et al., 2023); LRMS (ESI^-) *m*/*z* 651 (100%) $[2M-H]^-$.

cis-Melilotoside **(5)**. Yellow solid (4.5 mg). ^1H NMR (600 MHz, CD_3OD) δ 7.59 (d, *J* = 7.6 Hz, 1H), 7.24 (t, *J* = 7.8 Hz, 1H), 7.17 (d, *J* = 8.2 Hz, 1H), 7.07 (d, *J* = 12.6 Hz, 1H), 6.95 (t, *J* = 7.5 Hz, 1H), 6.01 (d, *J* = 12.6 Hz, 1H), 4.93 (d, *J* = 7.3 Hz, 1H), 3.87 (d, *J* = 12.1 Hz, 1H), 3.70 (dd, *J* = 12.3,

4.4 Hz, 1H), 3.48 (dt, *J* = 21.8, 8.9 Hz, 2H), 3.40 (d, *J* = 6.4 Hz, 3H) and ^{13}C NMR (600 MHz, CD_3OD) δ 173.7, 156.4, 133.7, 131.2, 130.6, 124.8, 122.8, 116.5, 102.6, 78.2, 78.1, 74.9, 71.3, 62.5. (Bayrakçeken Güven et al., 2023). LRMS (ESI^-) *m*/*z* 325 (100%) $[M-H]^-$; LRMS (ESI^-) *m*/*z* 651 (100%) $[2M-H]^-$.

4.2.5 Data analysis

Statistical analyses were performed using RStudio (4.2.0), IBM SPSS statistics 27, and GraphPad Prism (version 6.0e). MestReNova 11 was the software to process the NMR spectra.

4.3 Results and Discussion

The crude extract of *C. hylandii* leaves was fractionated by different solvents from non-polar to polar. The selection of the fraction to isolate compounds was executed by comparing the antioxidant capacity (Figure 4.1). Antioxidants play a crucial role in reducing inflammation. For example, polyphenols, which have antioxidant properties, inhibit the activity of NF-κB, and reduce the production of proinflammatory cytokines such as Tumor Necrosis Factor-alpha and Interleukin-6 (TNF-α and IL-6 respectively). By mitigating oxidative stress, these antioxidants not only protect cellular structures but also prevent activation of inflammatory processes, which is essential to control chronic inflammatory conditions and improve overall health (Iddir et al., 2020). The DPPH assay demonstrates that Ethyl acetate fraction presented the highest radical scavenging, 70.06 %, in contrast with 0.75% hexane, and 2.21% chloroform fraction. The ascorbic acid and gallic acid were used as positives control, as they are well-known antioxidant molecules, with 94.9% and 94.2% respectively of radical scavenging.

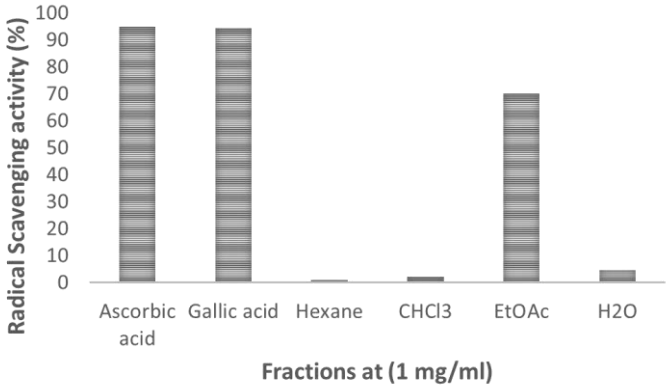

Figure 4. 1 Radical scavenging activity of Hexane, CHCl3, EtOAc, H20 fractions from leaves of C. hylandii.

Using preparative RP-HPLC, five compounds were separate from EtOAc fraction (528.02 mg) of *C. hylandii*. Preparative RP-HPLC demonstrates the different intensity of C1-C5 isolated (Figure 4.2), whit compound **(1)** is the most abundant and **(3)** the least. The structures of the isolated secondary metabolites were elucidated based on their NMR, and LC-MS spectroscopic (Fig. S31-S51,

Supplementary Material). The known compounds were identified as Shikimic acid **(1)**, 3-Dehydroshikimic acid **(2)**, Gallic acid **(3)**, *trans*-Melilotoside **(4)**, *cis*-Melilotoside **(5)** (Figure 4.3).

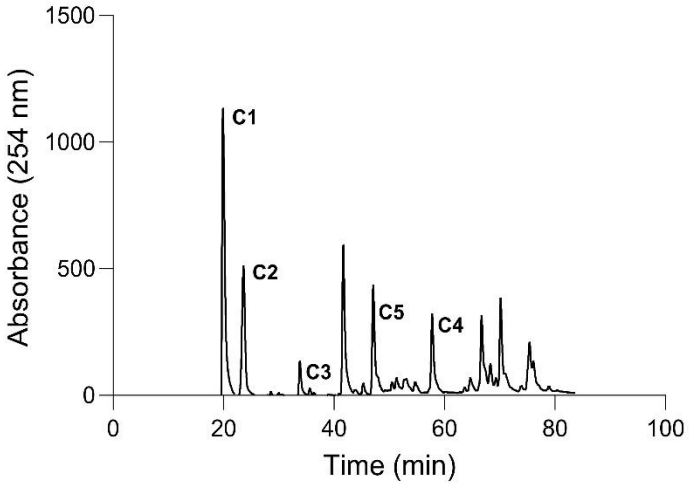

Figure 4. 2 Preparative RP-HPLC of isolated compounds from leaves C. hylandii at 254 nm.

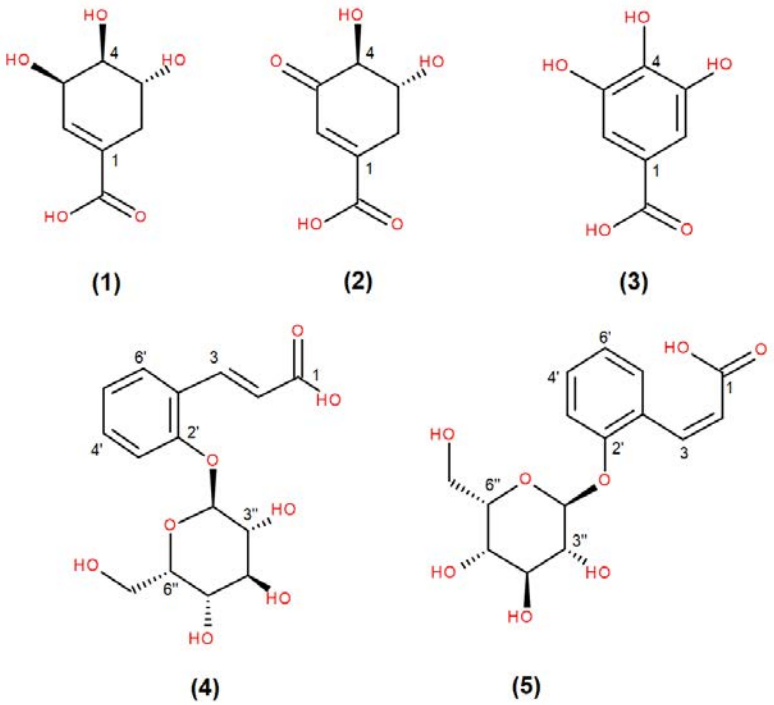

Figure 4. 3 Chemical structures of compounds 1-5 isolated from C. hylandii from leaves. Shikimic acid (1), 3-Dehydroshikimic acid (2), Gallic acid (3), trans-Melilotoside (4), cis-Melilotoside (5).

The synthesis of the isolated compounds in *C. hylandii* are secondary metabolites, which depends largely on the response of the plant to environmental conditions. These chemical strategies are synthetized, for example, when the plant is infested with pest, or when there is an infection with specific pathogens, or as a response for abiotic stress (Puupponen-Pimiä et al., 2001; Sambangi, 2022; Zandalinas et al., 2022). There are different pathways that plants use to synthesize secondary metabolites, such as Malonic-acid pathway, Mevalonic acid pathway, methylerythritol-phosphate (MEP) pathway, and shikimic acid pathway (Khare et al., 2020). Among those, the Shikimic acid **(1)** is an intermediate molecule for the synthesis of diverse type of phenolic compounds (Seigler, 2006a). In this way, the great abundance of shikimic acid **(1)** found in *C. hylandii* would be explained.

3-Dehydroshikimic acid **(2)** is the precursor of shikimic acid **(1)** and gallic acid **(3)** (Habashi et al., 2019). The enzyme shikimate dehydrogenase (SDH) catalyses the reduction of 3-Dehydroshikimic acid **(2)** to shikimic acid **(1)**, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH)

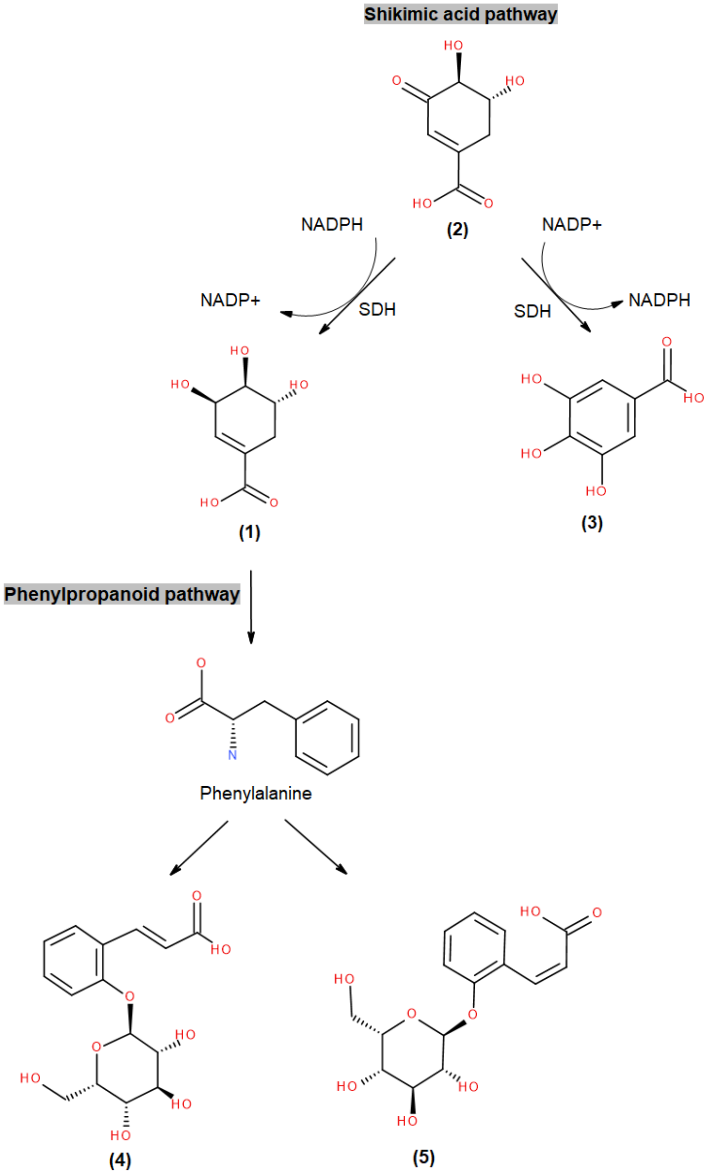

Figure 4. 4 . Shikimic acid and phenylpropanoid pathway for the synthesis of compounds in C. hylandii.

as a co-factor (Ye et al., 2003). On the other hand, in the presence of $NADP^+$ the enzyme SDH forms gallic acid **(3)** (Figure 4.4) (Dewick & Haslam, 1969; Habashi et al., 2019).

Phenylpropanoid pathway likely involves in the synthesis of *trans*-melilotoside **(4)** and *cis*-melilotoside **(5)** in *C. hylandii* leaves extract. One of the products of Shikimic acid pathway is phenylalanine which leads the phenylpropanoid pathway (Figure 4.4) (Dong & Lin, 2021). This pathway is directly correlated with the synthesis of phenolic secondary metabolites, which include compounds such as cinnamic acid and its derivates (Seigler, 2006b). The glycosylation of cinnamic acid

should derivatives in *trans*-melilotoside **(4)** and *cis*-melilotoside **(5)** (Tian et al., 2016). These compounds are phenolic compounds with a sugar substituted in C2' (δ_c 157.54) in *trans*-melilotoside. Both *trans*-melilotoside **(4)** and *cis*-melilotoside **(5)** shares similar chemical structure, but the spatial atoms arrangements are different which makes them isomers.

Although the compounds isolated in *C. hylandii* are known from the literature, they are characterized by their pharmacological potential. For instance, Shikimic acid **(1)** reduced glucose and glycated haemoglobin levels and has potential antioxidant properties (Al-Malki, 2019). Gallic acid **(3)** reduced the expressions of IL-21 and IL-23 in colitis model, likely due to is antioxidant property (Pandurangan et al., 2015). *trans*-melilotoside **(4)** and *cis*-melilotoside **(5)** showed moderate activity against *Trypanosoma cruzi*, the parasite responsible for Chagas disease, with IC50 values of 58 µg/mL and 78.2 µg/mL, respectively (Atay et al., 2016).

This thesis opens the door for future research in the discovery of lead compounds from *C. hylandii* leaves. Because it is the first work in phytochemical analysis, it is essential to perform a metabolic analysis to identify more compounds. Because shikimic acid and some of its derivatives were found, it is possible that this species has the presence of phenolic compounds. Which would explain the high antioxidant capacity and possible anti-inflammatory properties. It is essential to continue this type of work for this plant.

4.4 Conclusion

This study used the EtOAC fraction to isolate and characterized small molecules based on their antioxidant capacity. Five compounds were isolated and identified from *C. hylandii* leaves. Shikimic acid **(1)** was the most abundant and likely the precursors of the other isolated compounds. the shikimic acid pathway and phenylpropanoid pathway were probably the plant metabolic routes for the synthesis of the five compounds isolated in *C. hylandii* leaves. This study is the first chemical characterisation report of *C. hylandii* leaves. As a preliminary metabolomic study, further research is essential to characterize the complete metabolomes of *C. hylandii* leave and identify potential bioactive natural products.

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Overall summary and future directions

This thesis provides information base for the discovery of compounds with pharmaceutical potential, from Australian tropical plants. A paper review is presented with emphasis on the use of cutting-edge technologies such as AI, LC-MS and NMR to accelerate the process in the discovery of lead compounds from medicinal plants. Additionally, the use of traditional medicine as fundamental knowledge on this topic is emphasized. For the isolation, identification, and bioactivity of small molecules, this study utilized two distinct plantsincluiding the medicinal plant *Corymbia citriodora* and the Australian tropical plant *Ceratopetalum hylandii*. The medicinal plant *Corymbia citriodora* was studied with a focus on the bioactivity of its compounds, while for the Australian tropical plant *Ceratopetalum hylandii*, this study represents the first phytochemical analysis, concentrating on identifying its secondary metabolites.. This research reveals new knowledge for these two species because a new compound was isolated for *C. citriodora* and it is the first study in phytochemical terms for *C. hylandii*. Each chapter of this thesis tries to provide new and relevant knowledge for the discovery of new compounds with pharmaceutical properties.

5.1 New approach for drug discovery using AI

Chapter 2 presents a paper review that emphasizes the use of cutting-edge technology as a basis for drug discovery. It is evident that throughout human history the use of plants has been part of our society, as a food source and as medicine (Turpin et al., 2022). Today one of the concepts to incorporate traditional knowledge into modern medicine is “One Health”, which integrates human health, animals, and the environment (Riley et al., 2021). On the other hand, this traditional knowledge written in ancient books has been analysed using AI especially text mining (Xia et al., 2020).

In the same way, the development of technologies such as LC-MS and NMR for the structure elucidation of small compounds has provided fundamental knowledge for new drugs. Both the time and the cost of drug production have been quite high by the traditional method. Despite this, AI has been dramatically accelerating data analysis, leading to more efficient results (Deng et al., 2022). For example, the implementation of metabolomics in drug discovery has resulted in a deeper understanding of plant physiology (Palermo, 2023). With this information, neural network has been used to find lead compounds with enzyme inhibition for certain diseases (Sarotti, 2013). Integrating artificial intelligence with indigenous ethnobotanical knowledge offers a transformative approach to identifying novel bioactive compounds in Australian medicinal plants, bridging traditional wisdom with modern drug discovery techniques and revitalizing natural products as a key resource for new therapeutic developments.

5.2 Compounds from *C. citriodora*

Although compounds have been isolated from *C. citriodora* resin before, many of them and their bioactive properties still need to be thoroughly investigated (Ayinde, 2016; Perry & Wangchuk, 2023). This is because the process of isolation and purification focus on a subset of compounds, leaving a significant portion of potentially bioactive molecules uncharacterised. Chapter 3 precisely describes the compounds found in this research, which are phenolic compounds, mostly flavonoids. Mbabarametin **(4)** is a flavonoid like HEMA **(5)** and is the first time reported in the literature (Freitas et al., 2007; Lee et al., 2017). The difference between these two compounds is the absence of a hydroxy group at C3 for Mbabarametin **(4)**, which possibly explains the dissimilarities in terms of bioactivity. It was proven that these two compounds are not cytotoxic at 10μg/mL and C5 has better antioxidant activity than C4. In cytokines inhibition assays, the concentration of IL-1β was lower in the presence of **(4)** and **(5)**.

Some studies on the bioactivity of HEMA **(5)** have conclude that it had potential as an antimelanoma agent and exhibited inhibitory activity against 15-lipoxygenase, which is involved in inflammatory diseases (Duh et al., 2012; Lee et al., 2017). However, there are very few studies on the bioactivity that HEMA **(5)** may have. Because Mbabarametin **(4)** is quite like HEMA it is undoubtedly promising lead compounds for drug development. The results of this chapter open the door for future work on the potential of flavonoids as inhibitors of specific pro-inflammatory enzyme.

5.3 Compounds from *C. hylandii*

Chapter 4 describes the compounds isolated from *C. hylandii* leaves. The Ethyl acetate extract exhibited the highest antioxidant activity, for this reason compounds from this fraction were isolated. LC-MS and NMR analysis determined that compounds **1-5** are known to the literature, having been previously isolated from other plant species (Bayrakçeken Güven et al., 2023). Furthermore, the antioxidant, anti-inflammatory and antimalarial analysis of these compounds has been previously studied (Dong & Lin, 2021). Although they are well-known small molecules, this study identified possible phytochemical pathways, specifically the shikimic acid pathway and the phenylpropanoid pathway, that link these compounds through synthesis (Seigler, 2006; Tohge et al., 2013). This is the first phytochemical study conducted on *C. hylandii*, which serves as a reference for the search for lead phenolic compounds with pharmaceutical properties in this species.

5.4 Final words

The combination of traditional knowledge with the most advanced technology has resulted in an acceleration of the discovery of new treatments for humanity. It is essential that this type of academic work uses technologies and methods very similar to those of the industry because the possible results could trigger the start of a new medication. It is important to highlight that including metabolomics with

AI could be an important acceleration in this type of studies. The question: what are the mechanisms of action underlying the anti-inflammatory effects of compounds isolated from *C. citriodora* and C. *hylandii*? could open a new investigation based on these results. Ultimately, there is still much to learn about Australian tropical plants, both in terms of their physiology and possible uses. It is expected that the results in this thesis inspire future research to answer new questions for this topic.

5.5 References

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Appendix A. NMR experiments from isolated compounds of *C. citriodora*

Spectroscopy data of *p*-Coumaric acid (1)

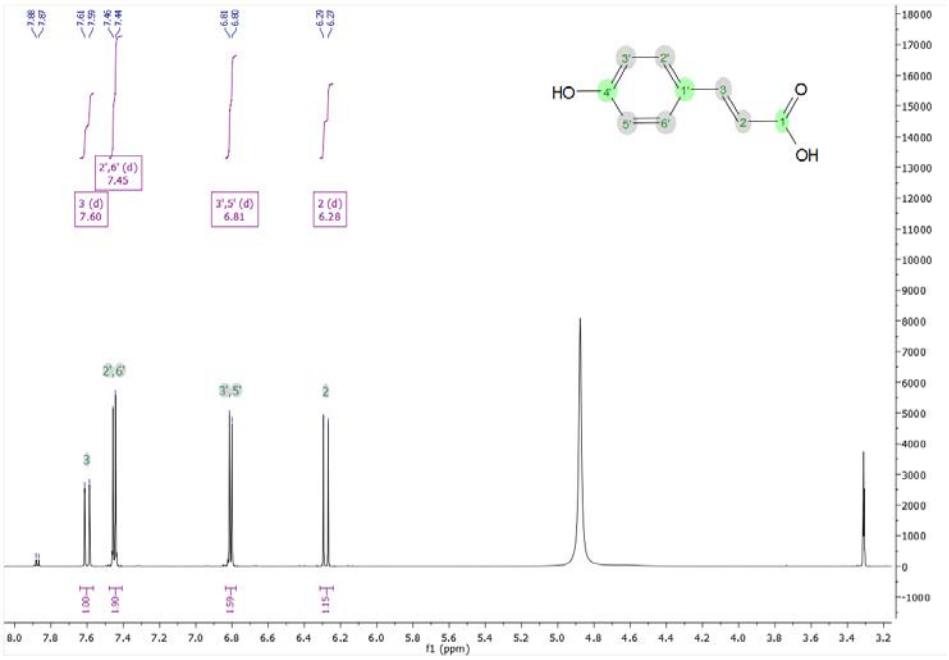

Figure SA 1. [1]H NMR (600 MHz, MeOD, 298°K) spectrum of P-Coumaric acid (1).

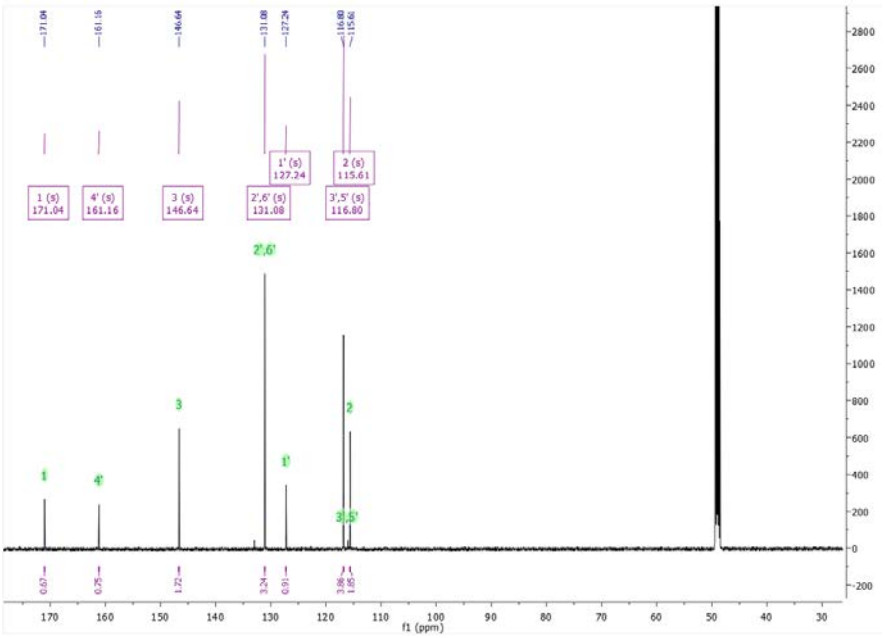

Figure SA 2. [13]C NMR (600 MHz, CD3OD, 298°K) P-Coumaric acid (1).

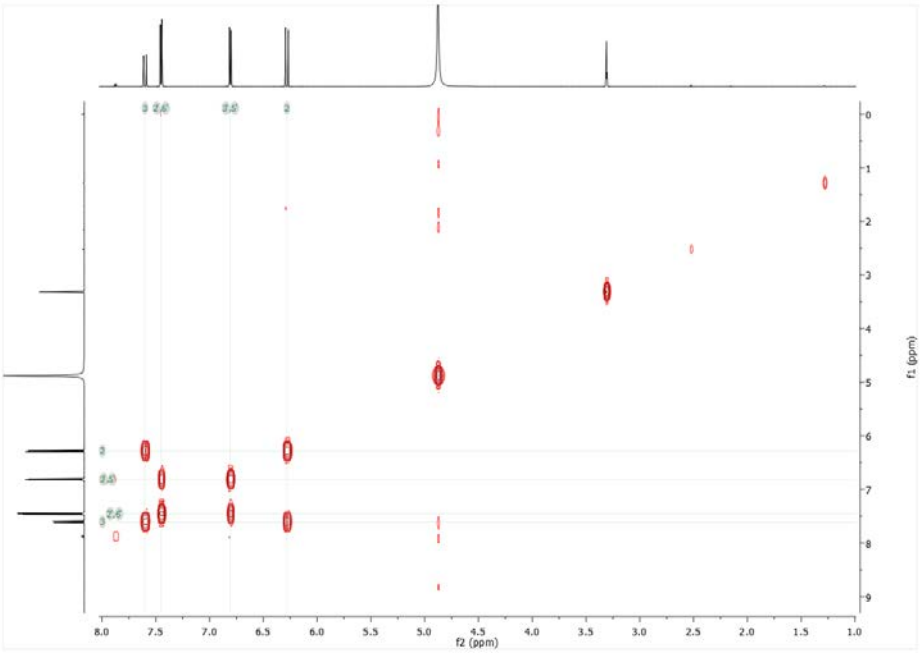

Figure SA 3. COSY (600 MHz, MeOD, 298°K) spectrum of P-Coumaric acid (1).

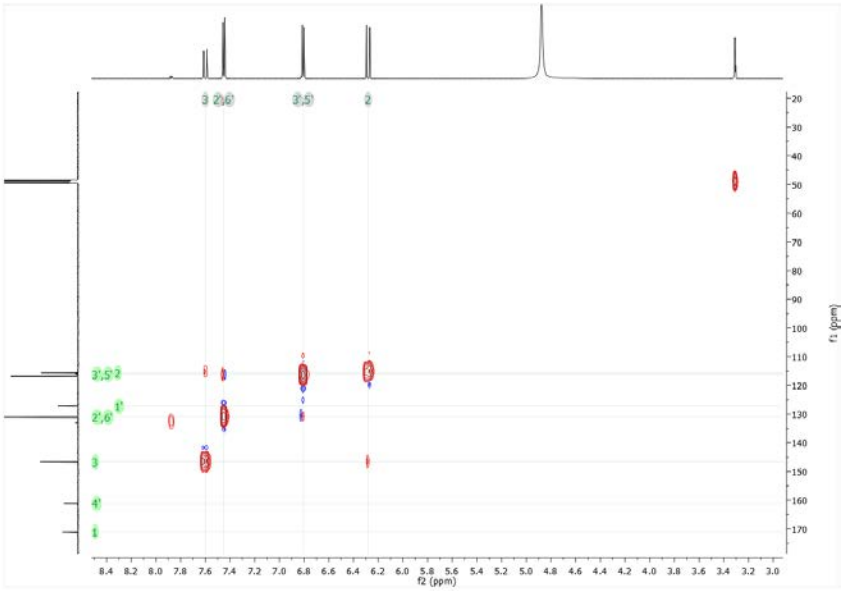

Figure SA 4. HSQC (600 MHz, MeOD, 298°K) spectrum of P-Coumaric acid (1).

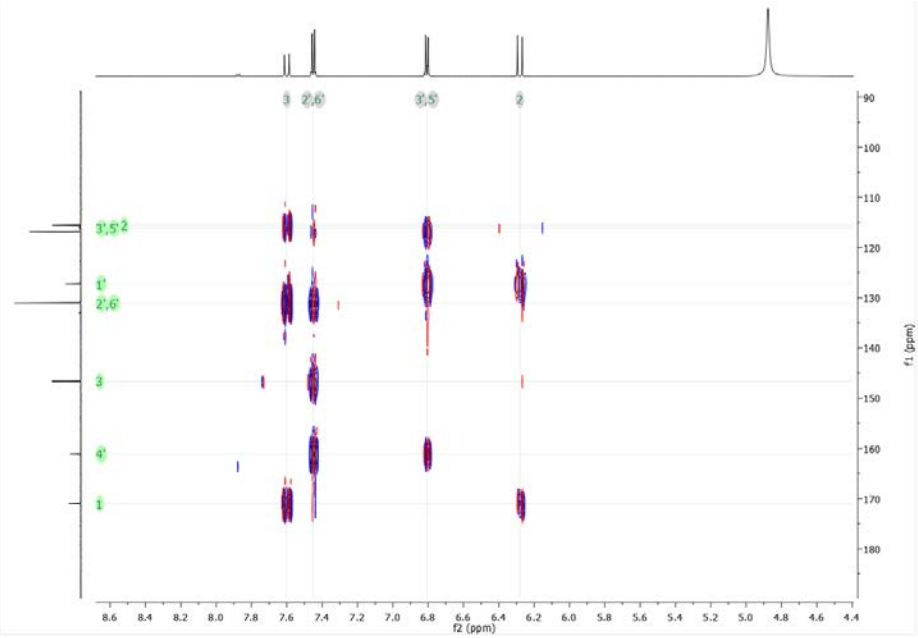

Figure SA 5. HMBC (600 MHz, MeOD, 298°K) spectrum of P-Coumaric acid (1).

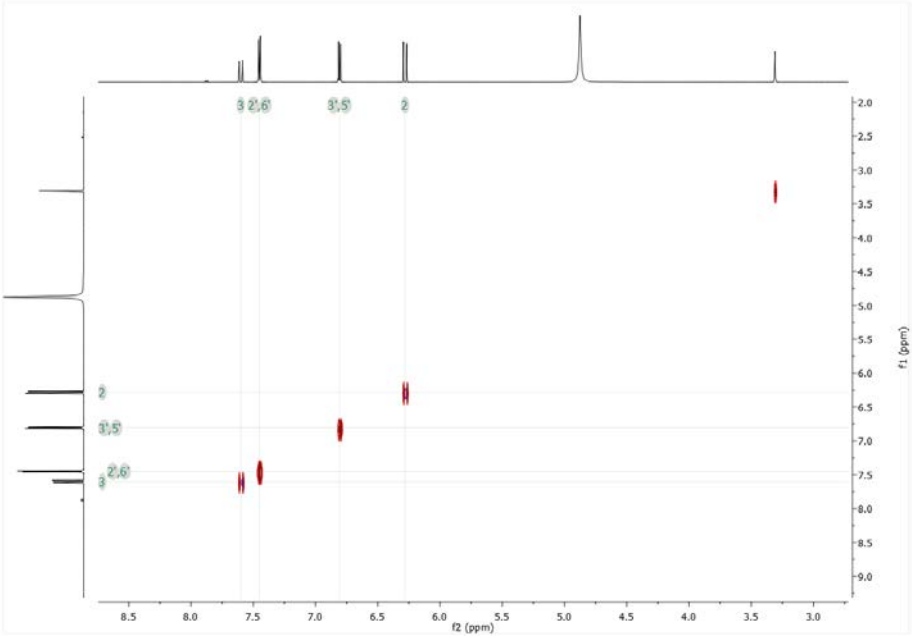

Figure SA 6. NOESY (600 MHz, MeOD, 298°K) spectrum of P-Coumaric acid (1).

Spectroscopy data of Folerogenin (2)

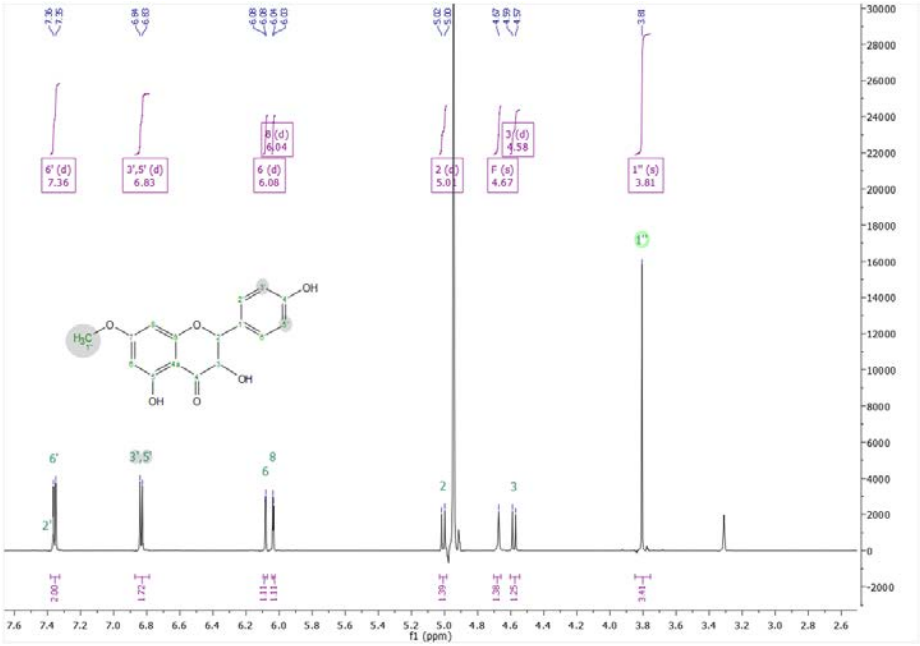

Figure SA 7. 1H NMR (600 MHz, MeOD, 298°K) spectrum of Folerogenin (2).

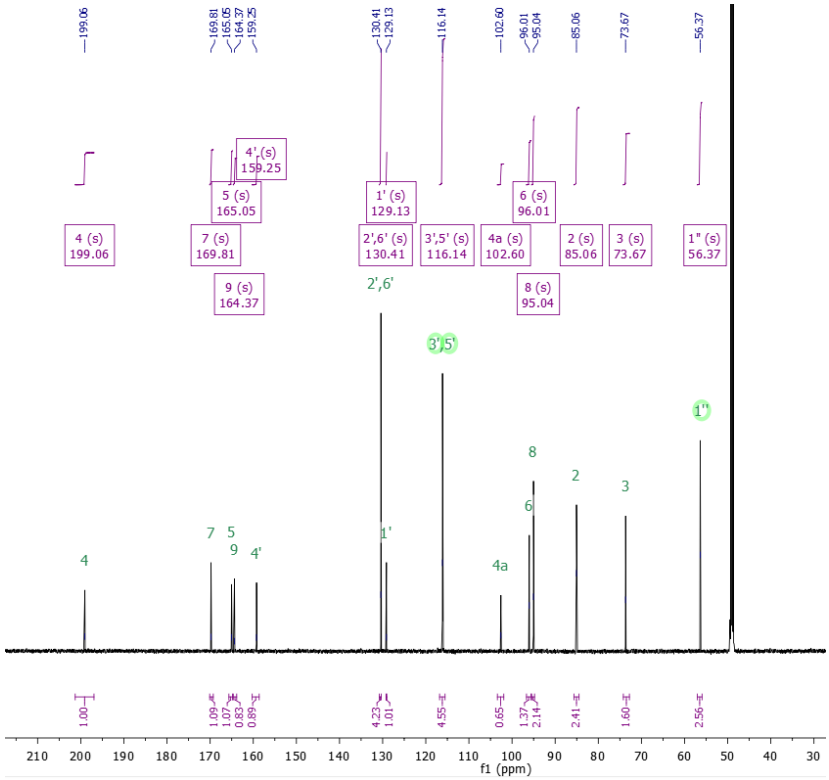

Figure SA 8. ^{13}C NMR (600 MHz, MeOD, 298°K) Folerogenin (2).

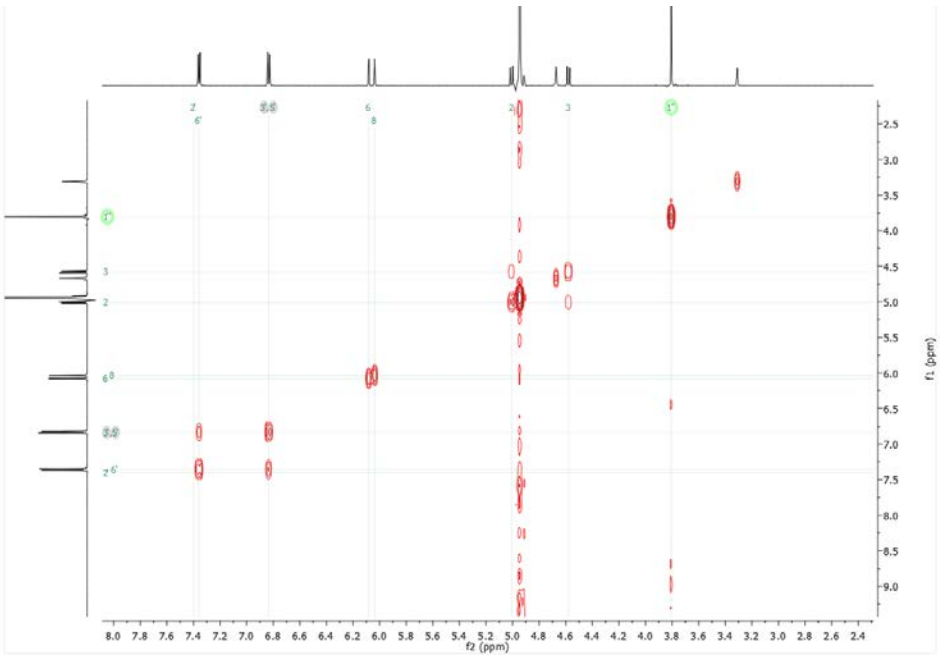

Figure SA 9. COSY (600 MHz, MeOD, 298°K) Folerogenin (2).

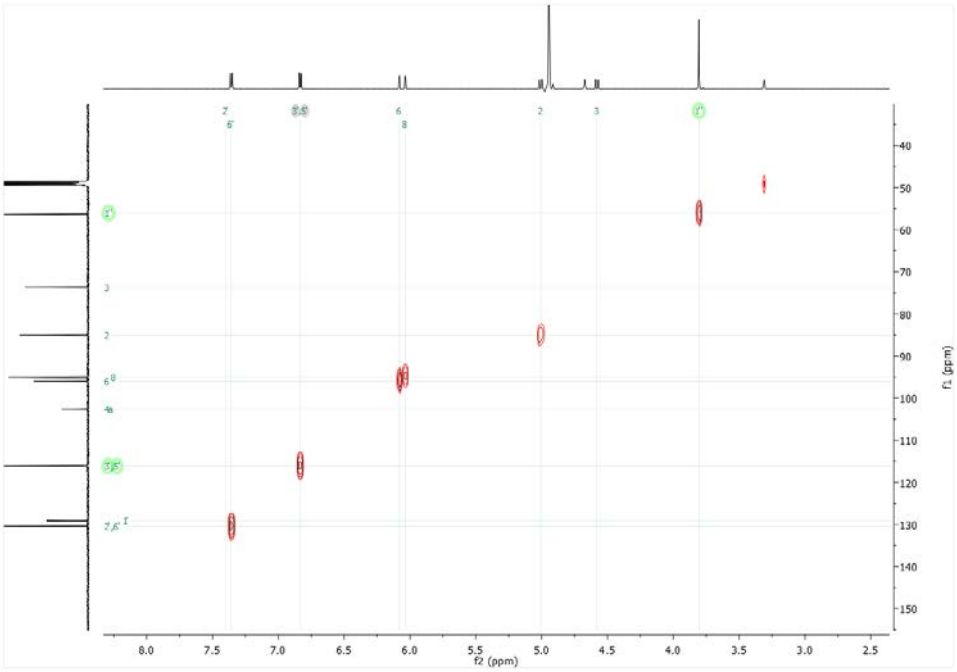

Figure SA 10. HSQC (600 MHz, MeOD, 298°K) Folerogenin (2).

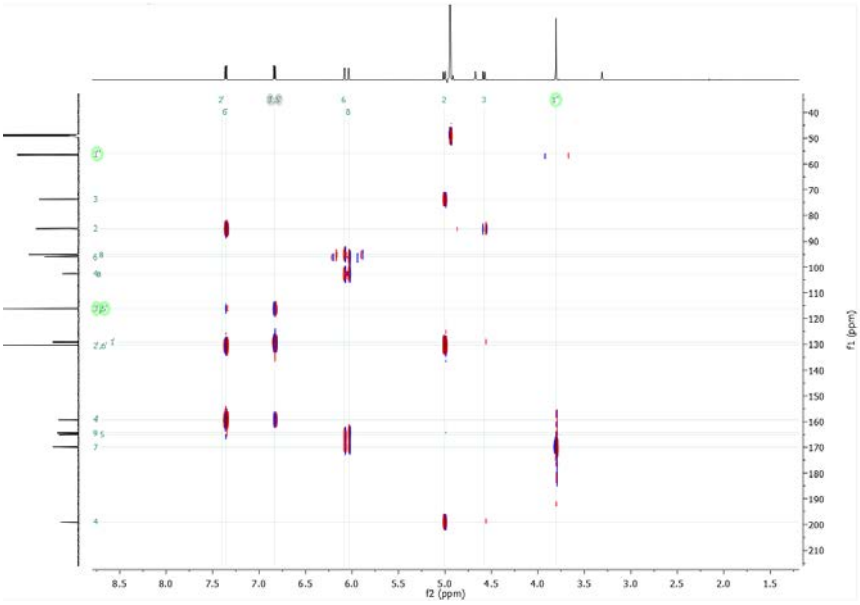

Figure SA 11. HMBC (600 MHz, MeOD, 298°K) Folerogenin (2).

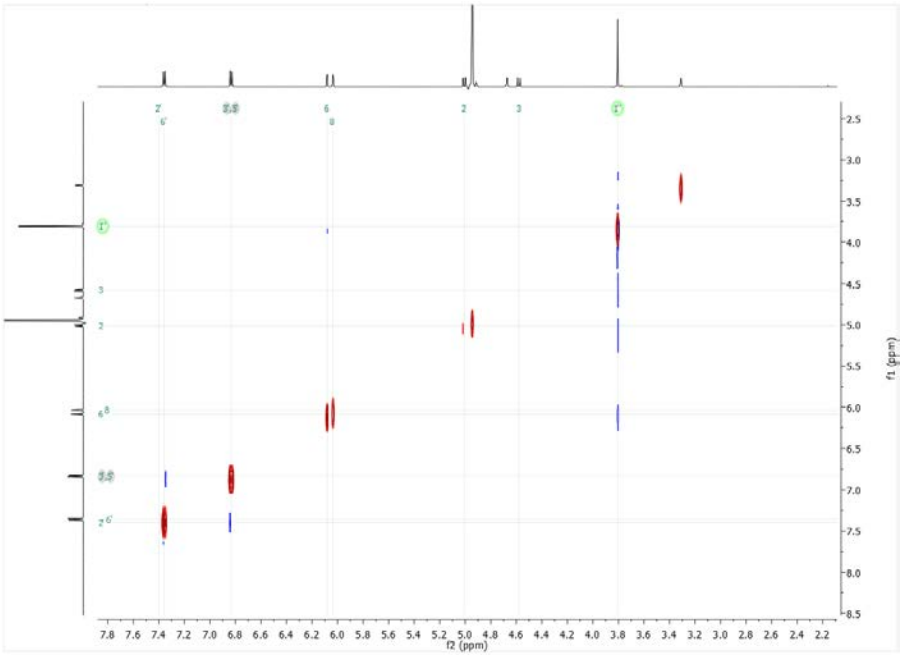

Figure SA 12. NOESY (600 MHz, MeOD, 298°K) Folerogenin (2).

Spectroscopy data of Sakuranetin (3)

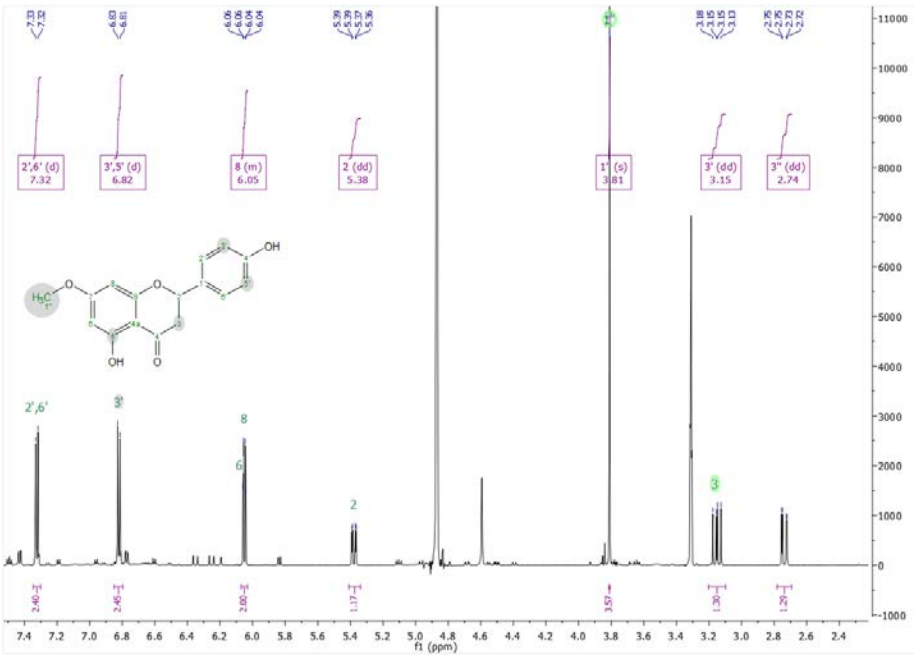

Figure SA 13. 1H NMR (600 MHz, MeOD, 298°K) Sakuranetin (3).

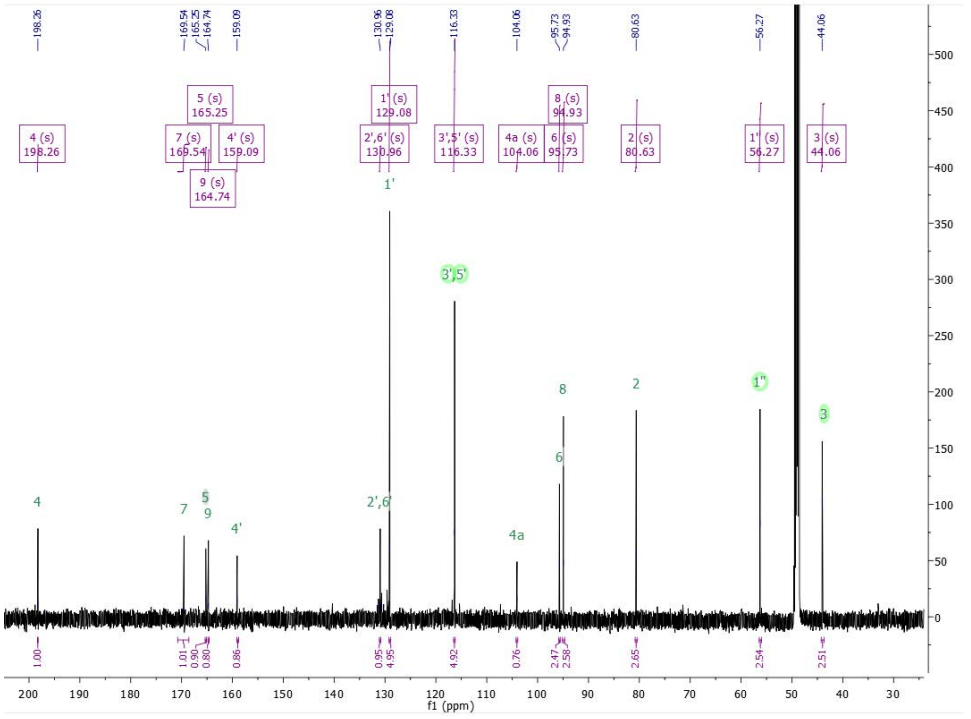

Figure SA 14. ^{13}C NMR (600 MHz, MeOD, 298°K) Sakuranetin (3).

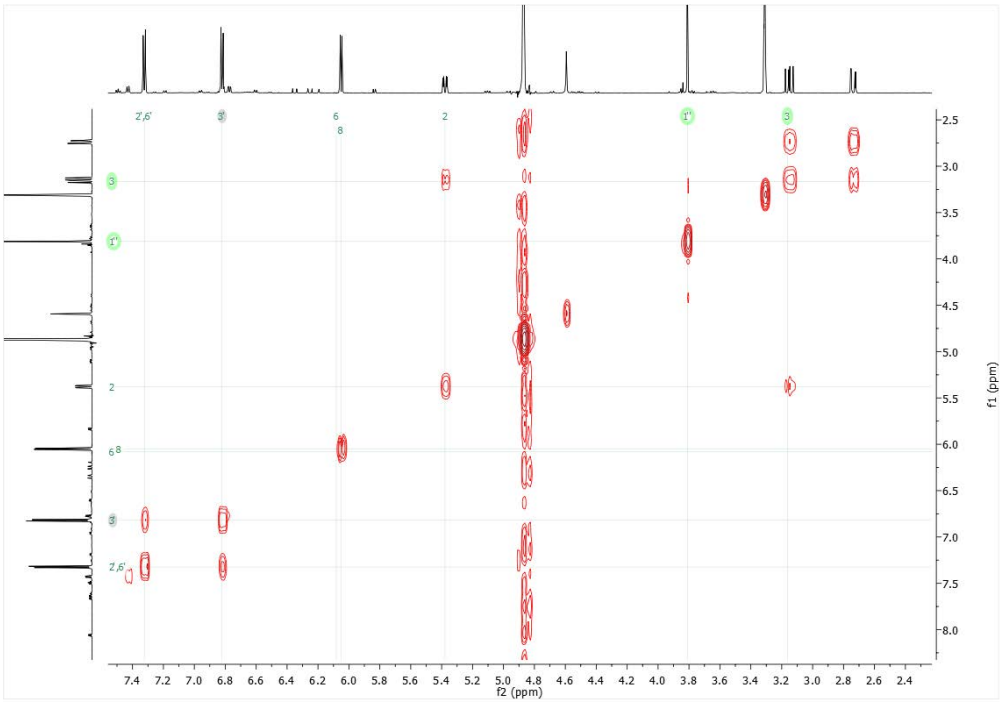

Figure SA 15. COSY (600 MHz, MeOD, 298°K) Sakuranetin (3).

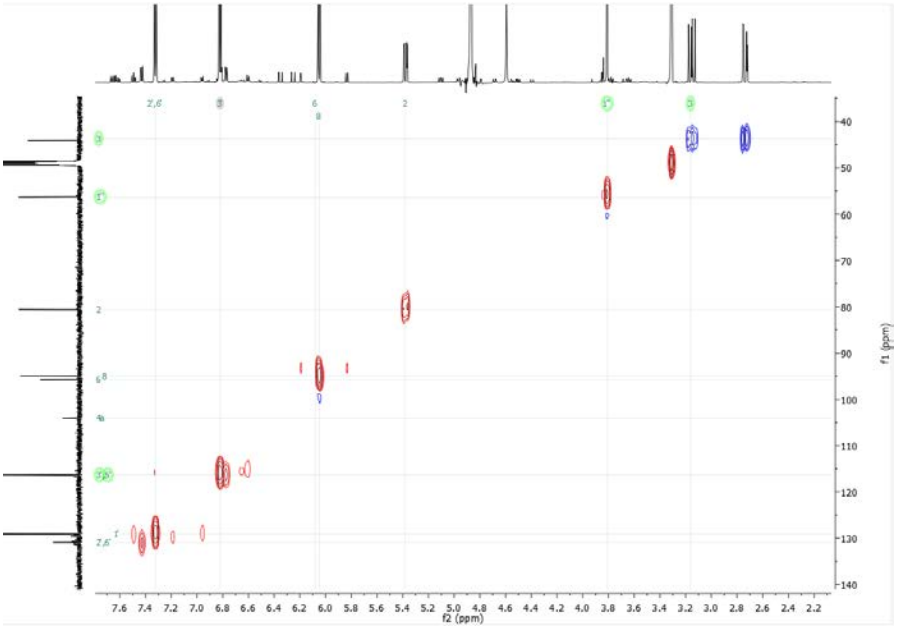

Figure SA 16. HSQC (600 MHz, MeOD, 298°K) Sakuranetin (3).

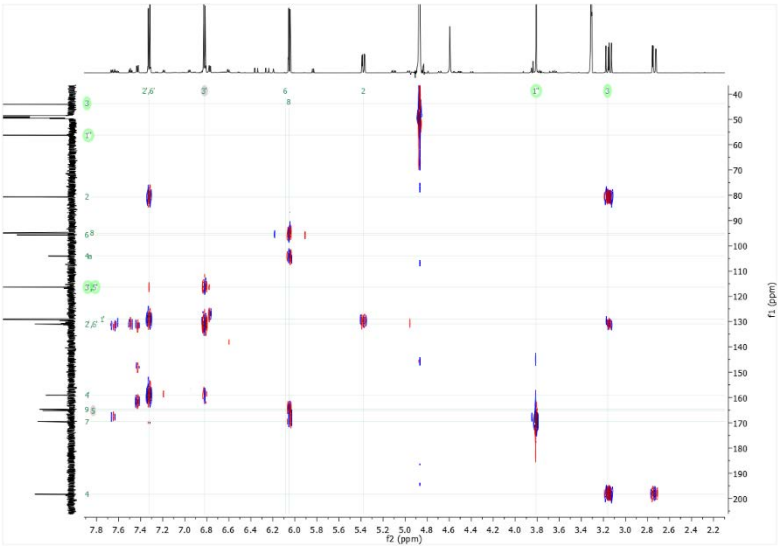

Figure SA 17. HMBC (600 MHz, MeOD, 298°K) Sakuranetin (3).

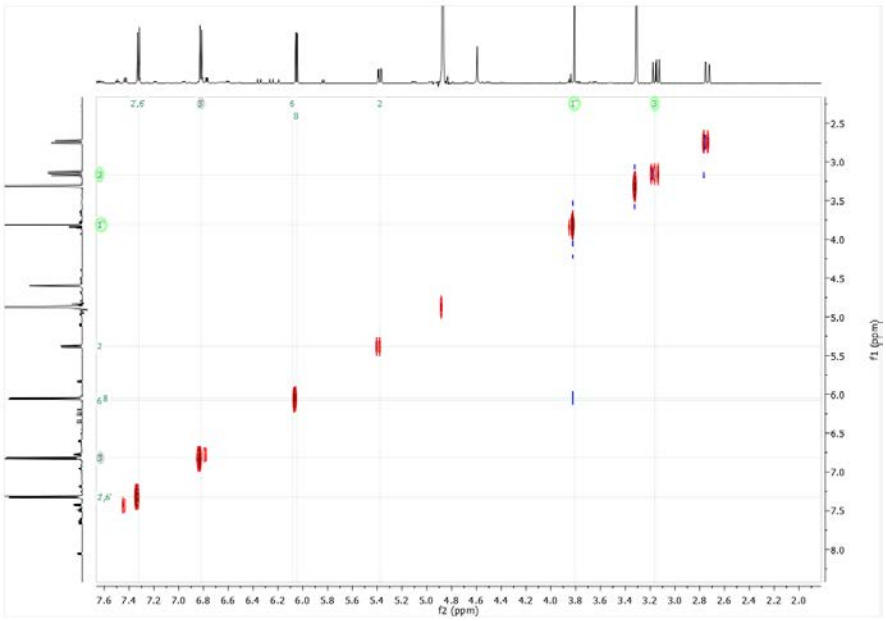

Figure SA 18. NOESY (600 MHz, MeOD, 298°K) Sakuranetin (3).

Spectroscopy data of 5-hydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (4)

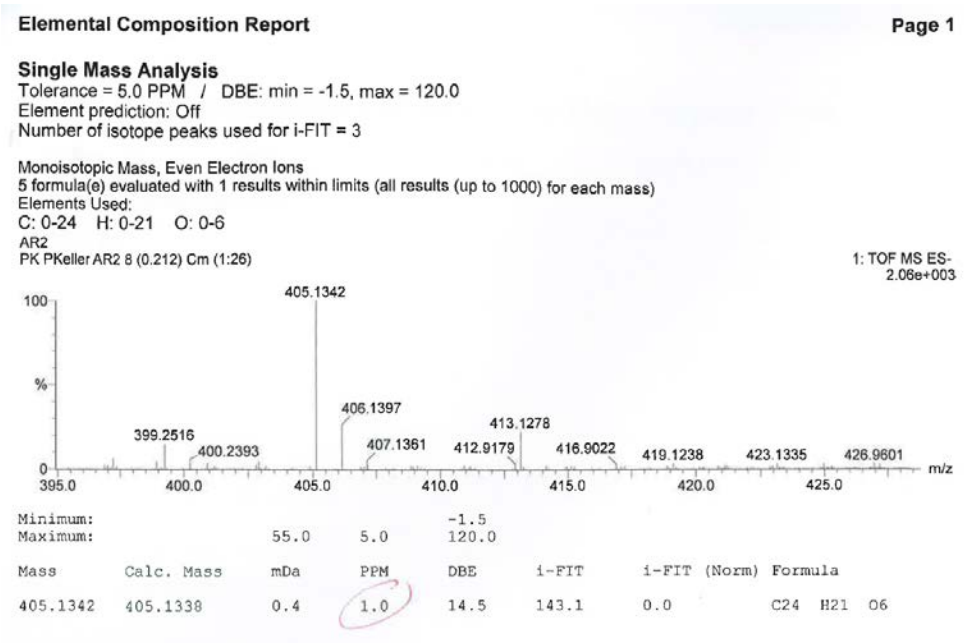

Figure SA 19.

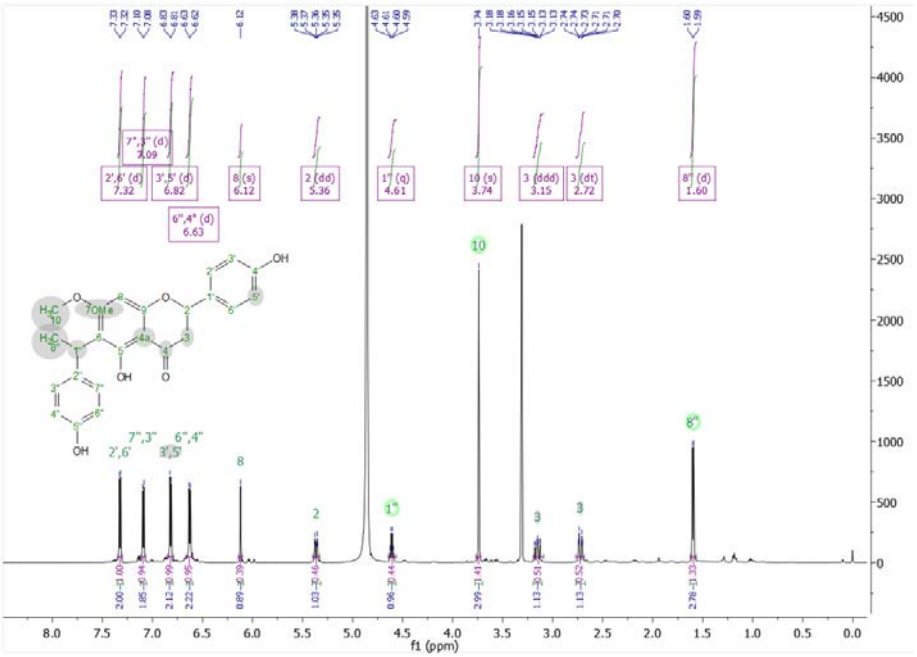

Figure SA 20. [1]H NMR (600 MHz, MeOD, 298°K) 5-hydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (4).

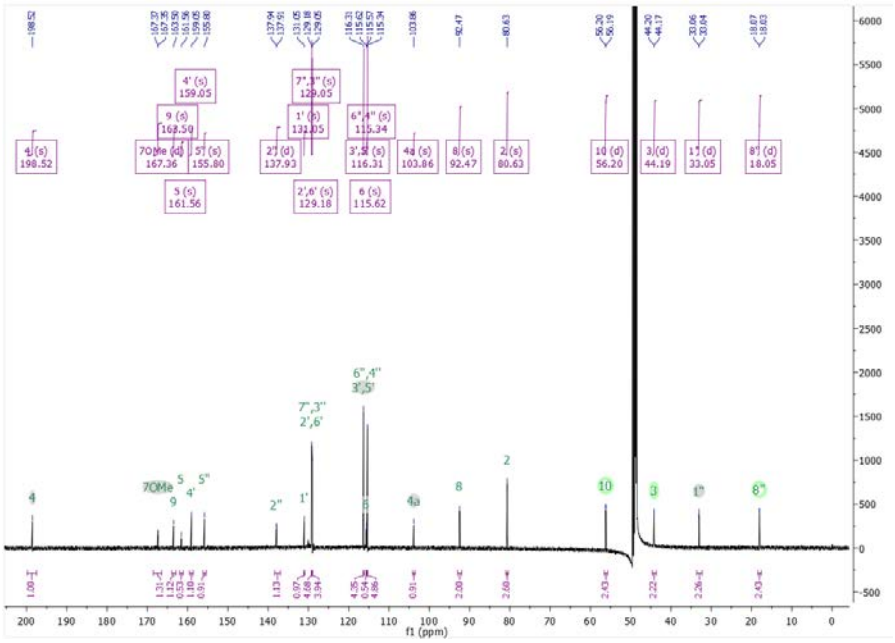

Figure SA 21. [13]C NMR (600 MHz, MeOD, 298°K) 5-hydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (4).

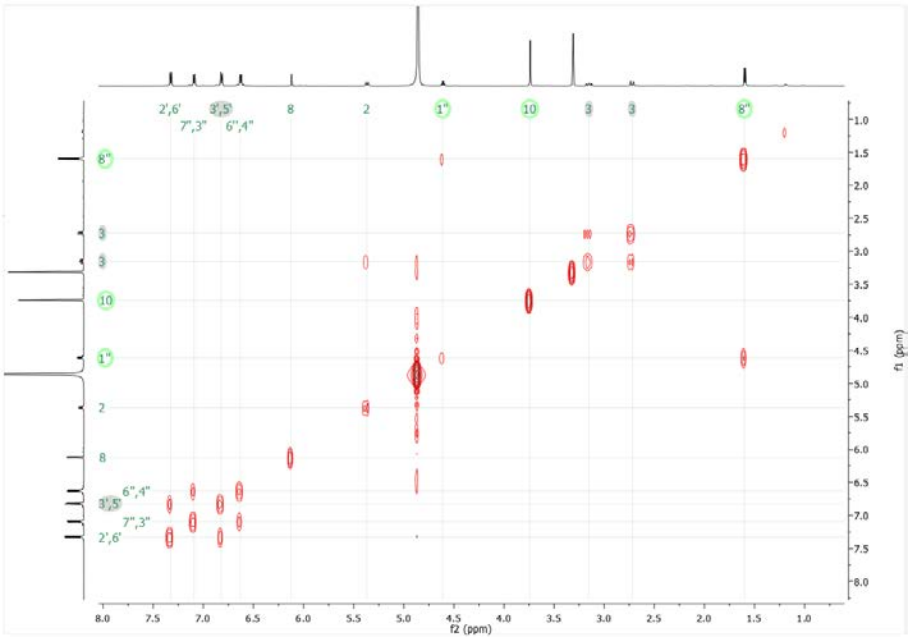

Figure SA 22. COSY (600 MHz, MeOD, 298°K) 5-hydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (4).

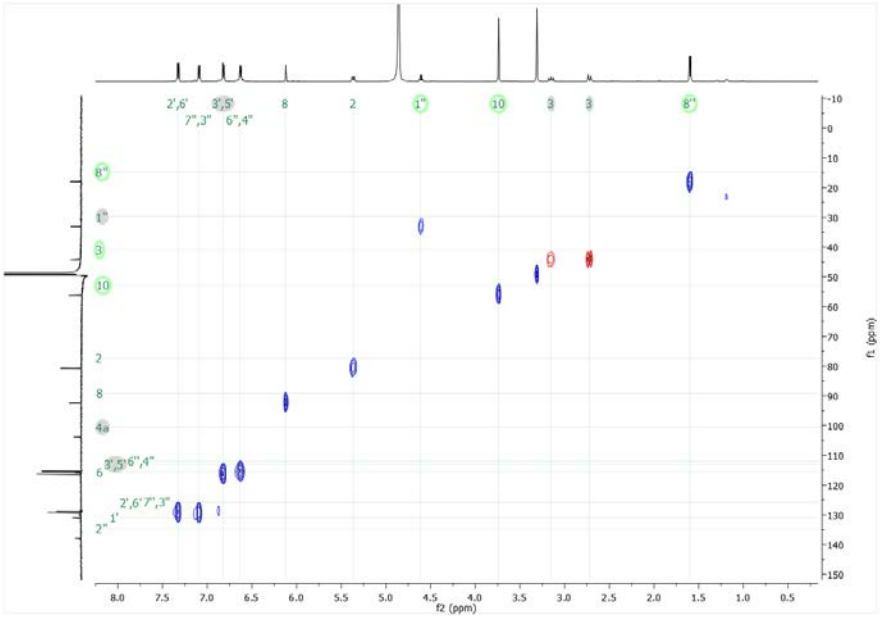

Figure SA 23. HSQC (600 MHz, MeOD, 298°K) 5-hydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (4).

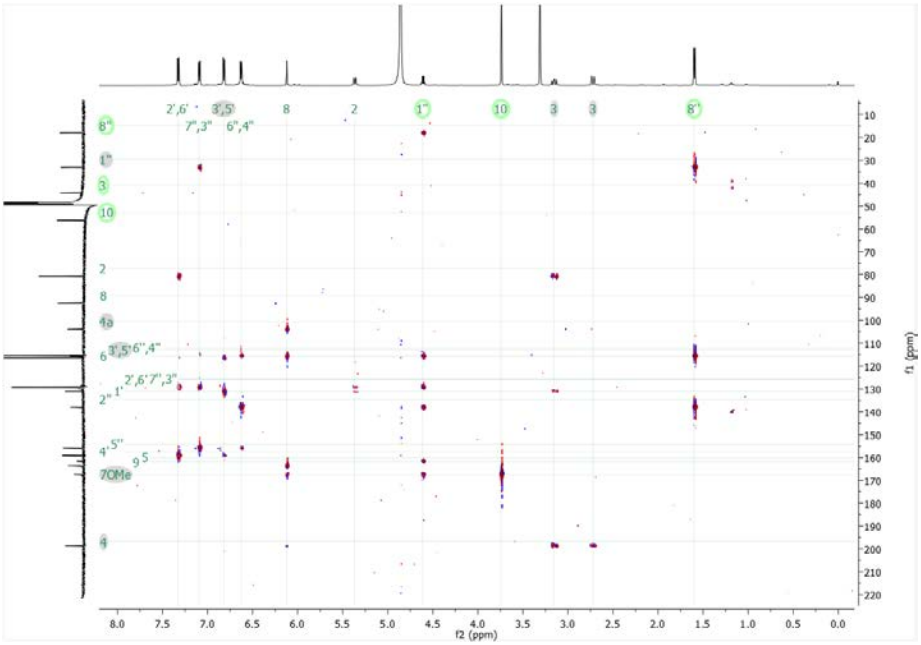

Figure SA 24. HMBC (600 MHz, MeOD, 298°K) 5-hydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (4).

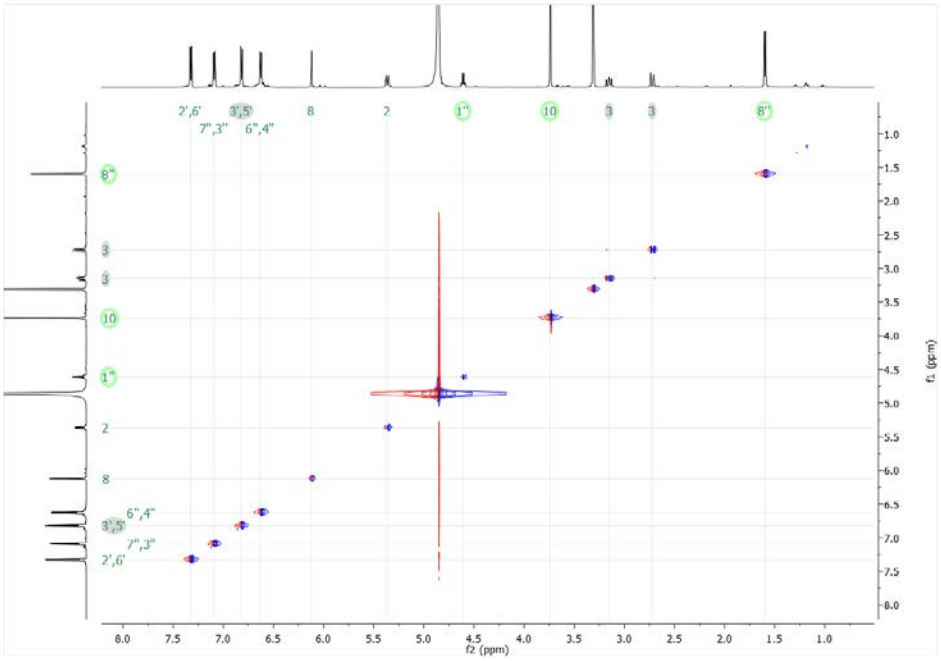

Figure SA 25. NOESY (600 MHz, MeOD, 298°K) 5-hydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (4).

Spectroscopy data of 3,5-dihydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (5)

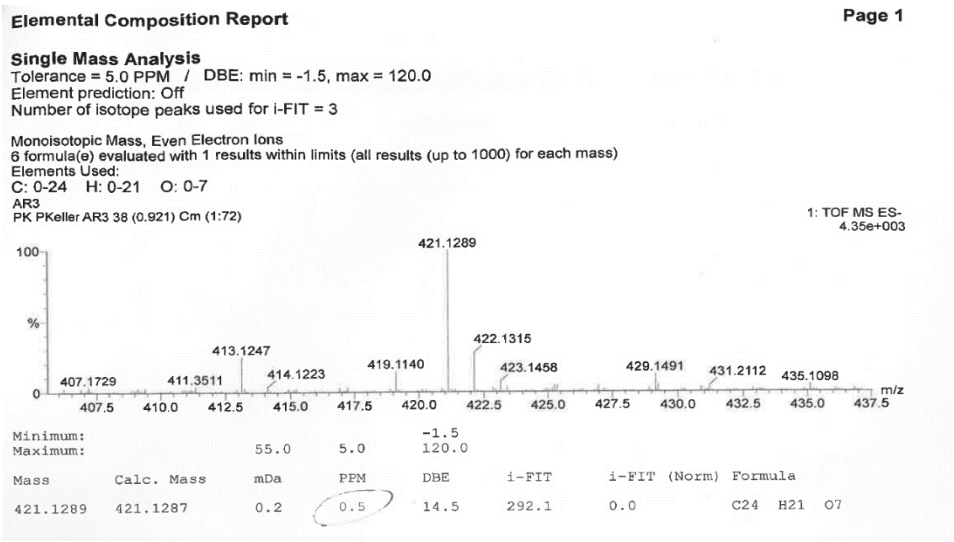

Figure SA 26.

Figure SA 27.. [1]H NMR (600 MHz, MeOD, 298°K) 3,5-dihydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (5).

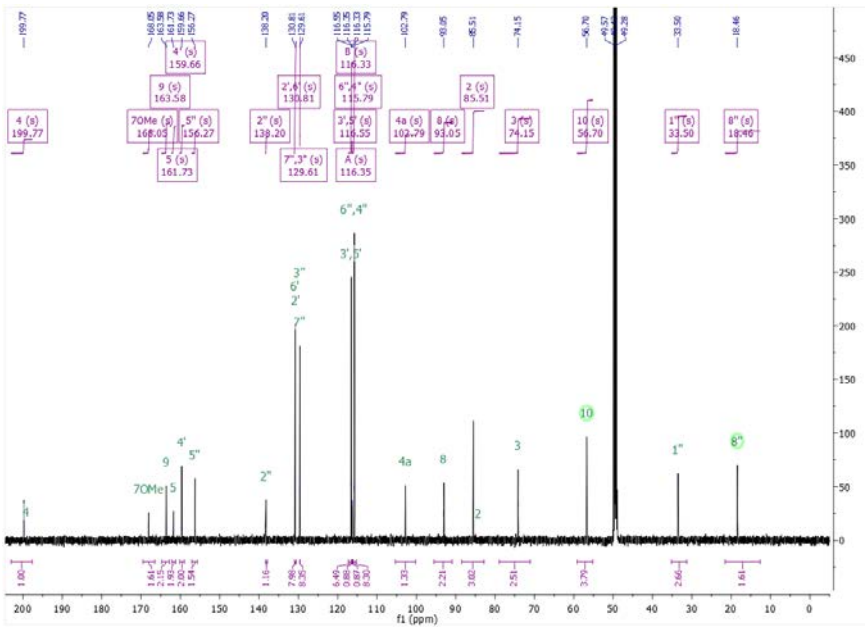

Figure SA 28. [13]C NMR (600 MHz, MeOD, 298°K) 3,5-dihydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (5).

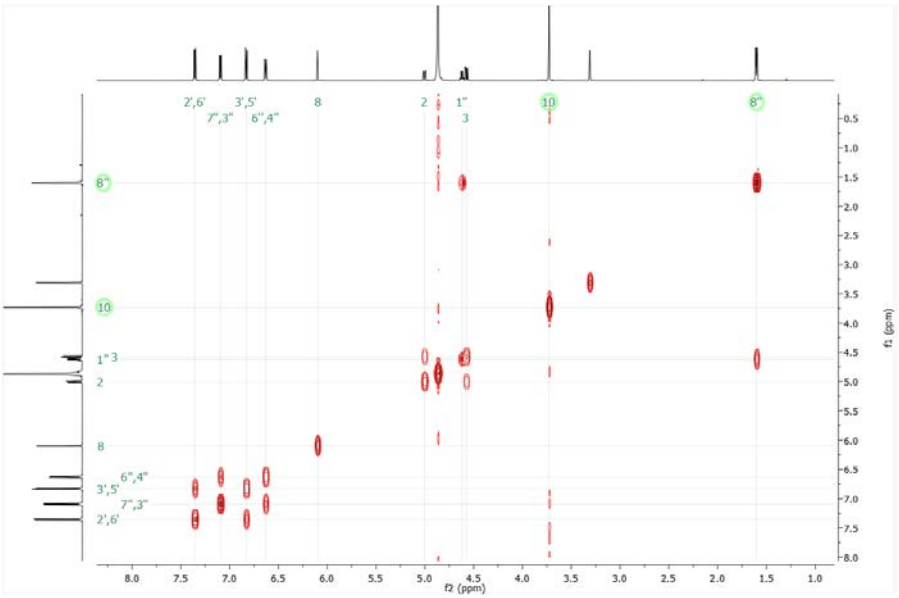

Figure SA 29. COSY (600 MHz, MeOD, 298°K) 3,5-dihydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (5).

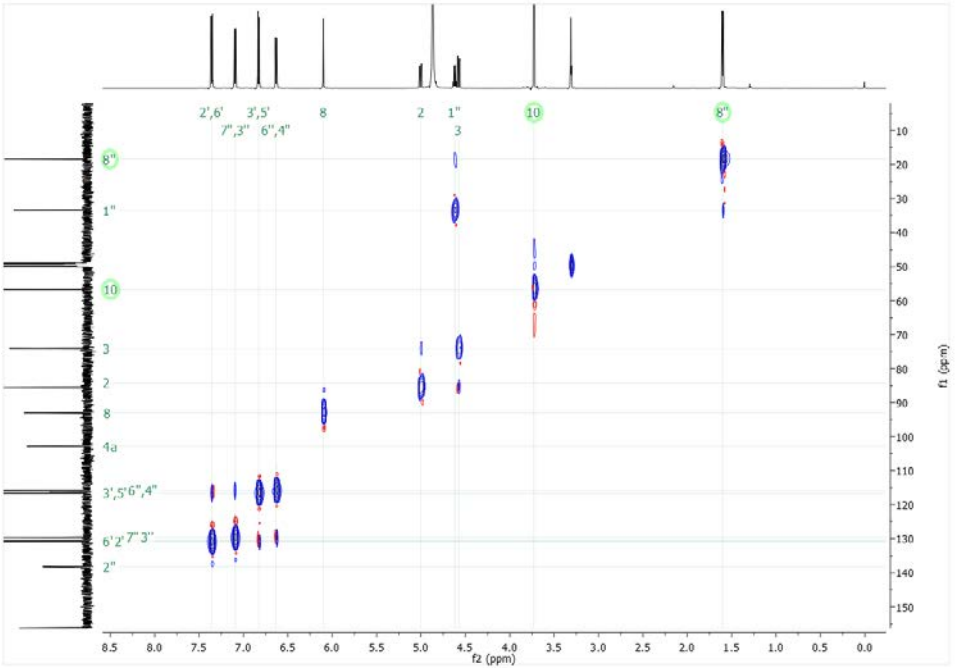

Figure SA 30. HSQC (600 MHz, MeOD, 298°K) 3,5-dihydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (5).

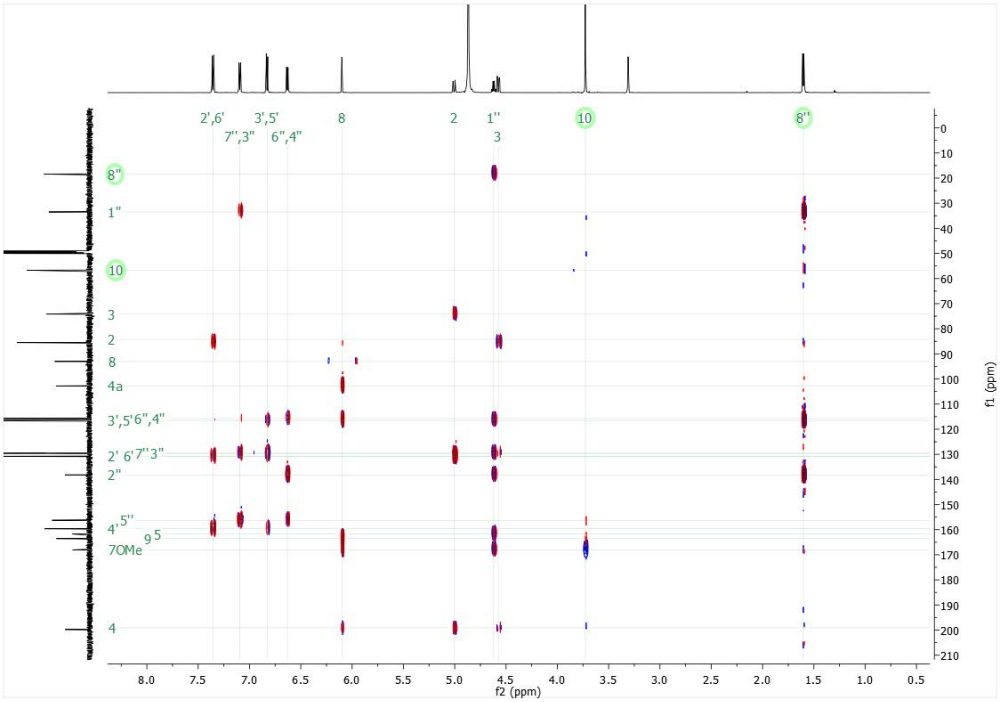

Figure SA 31. HMBC (600 MHz, MeOD, 298°K) 3,5-dihydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (5).

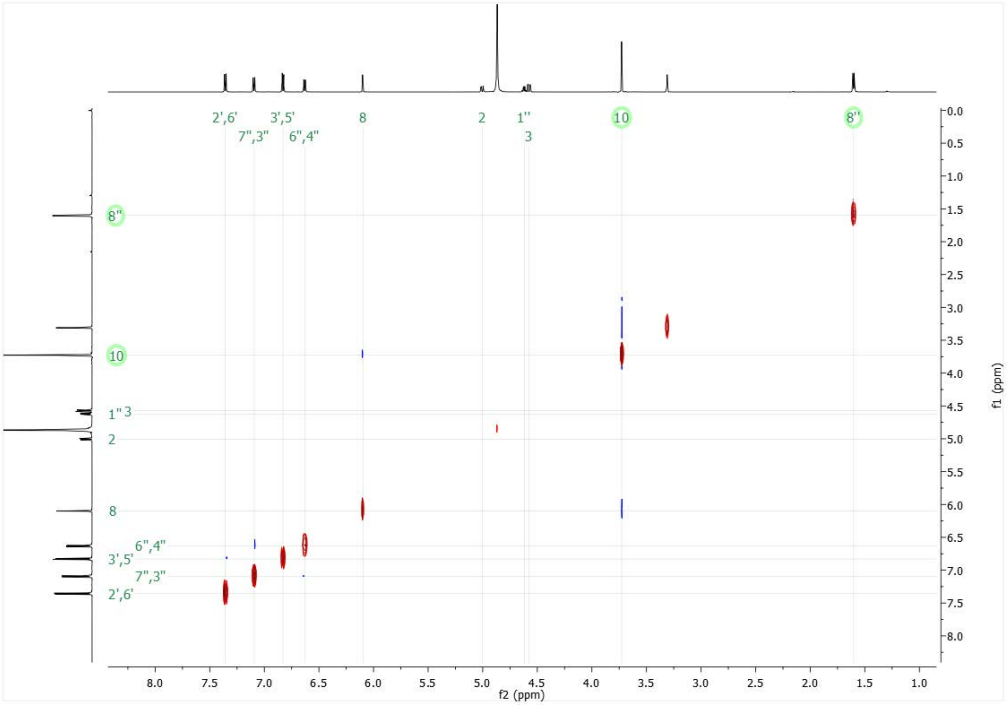

Figure SA 32. NOESY (600 MHz, MeOD, 298°K) 3,5-dihydroxy-2-(4-hydroxyphenyl)-6-[1-(4-hydroxyphenyl) ethyl]-7-methoxy-chroman-4-one (5).

Appendix B. Supporting information of NMR Experiments *C. hylandii*

Spectroscopy data of 4,5,6-trihydroxycyclohexene-1-carboxylic acid (1).

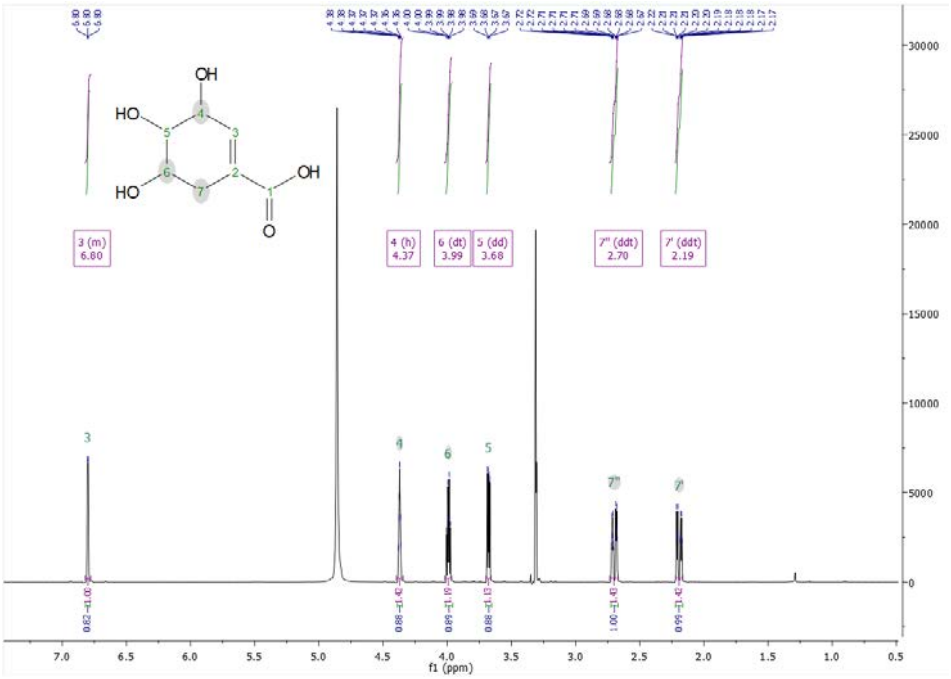

Figure SB 1. [1]H NMR (600 MHz, CD_3OD, 298°K) spectrum of (1).

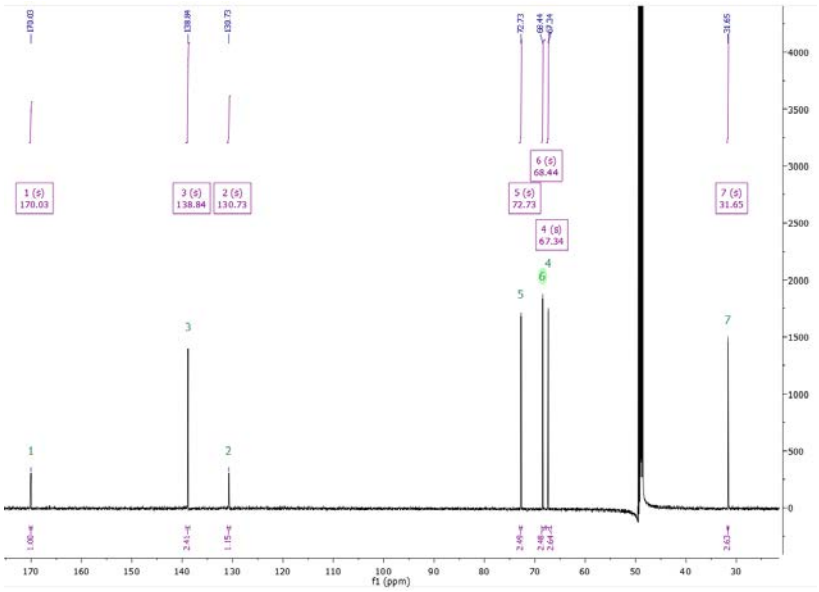

Figure SB 2. [13]C NMR (600 MHz, CD3OD, 298°K) spectrum of Compound 1.

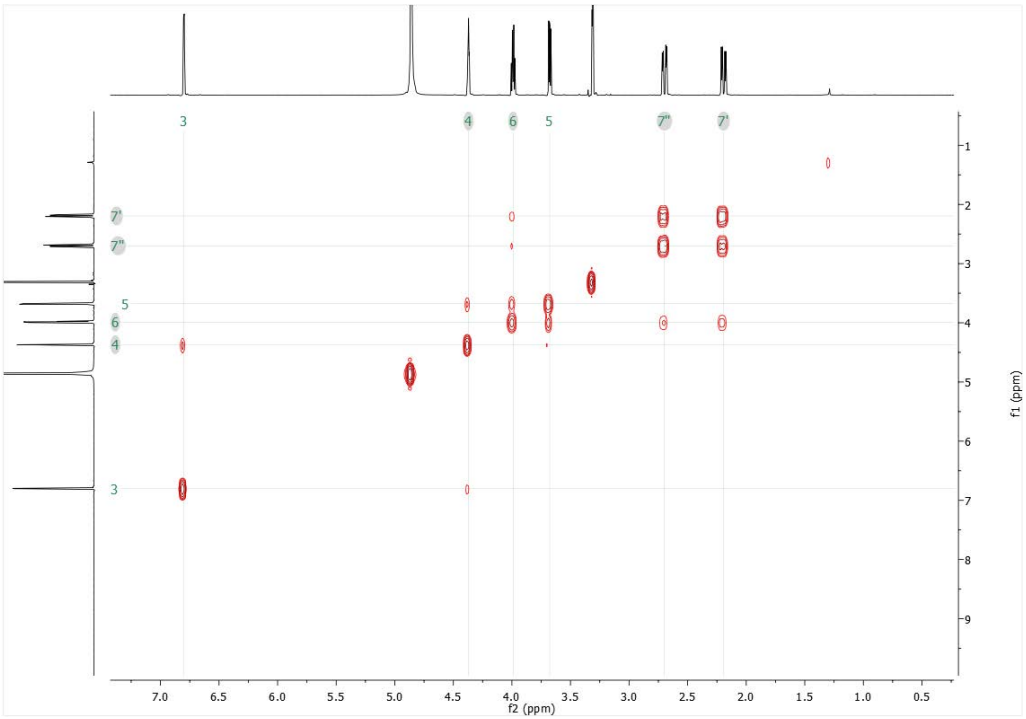

Figure SB 3. COSY (600 MHz, CD3OD, 298°K) spectrum of Compound 1.

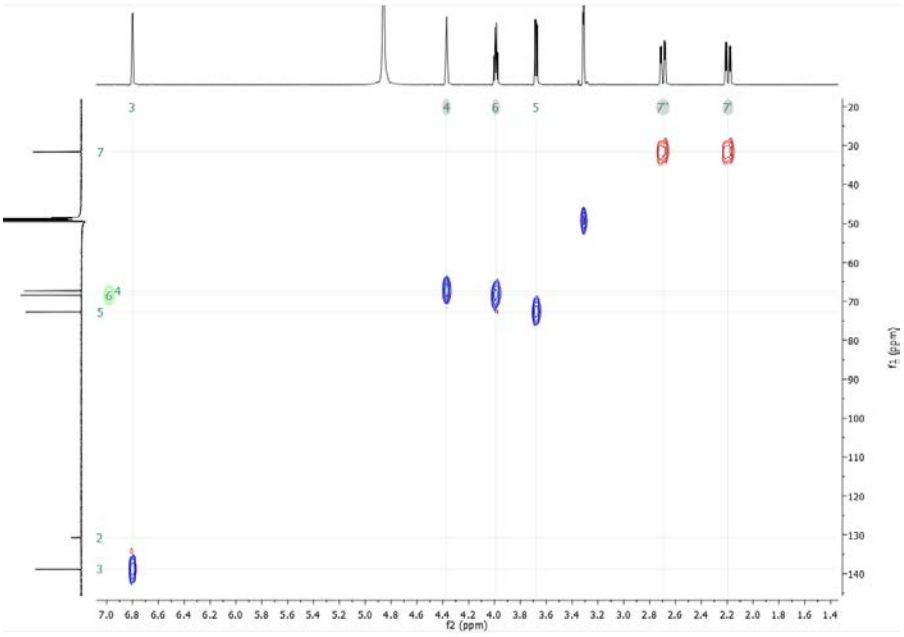

Figure SB 4. HSCQ (600 MHz, CD3OD, 298°K) spectrum of Compound 1.

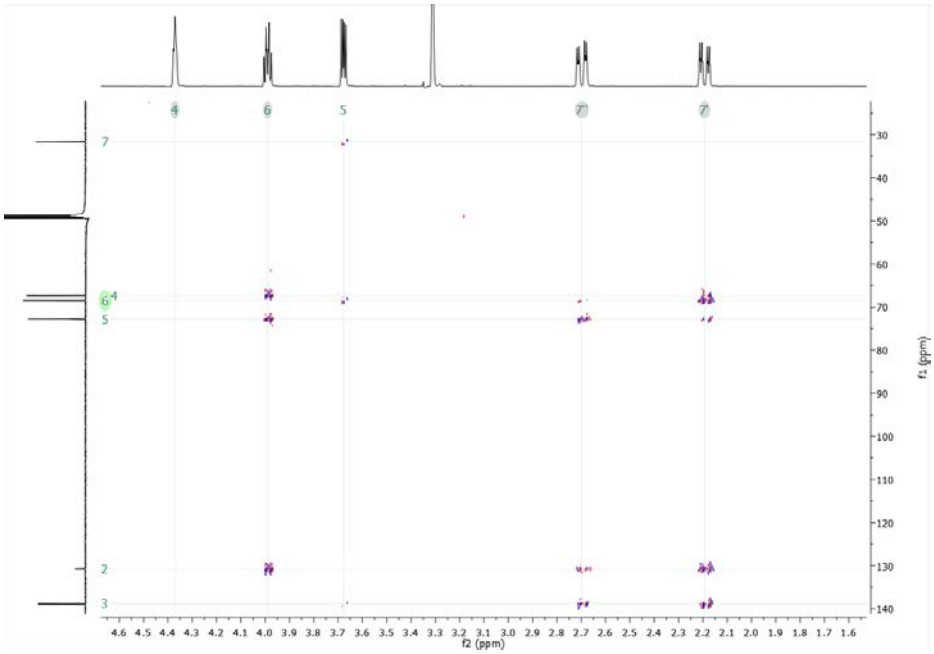

Figure SB 5. HMBC (600 MHz, CD3OD, 298°K) spectrum of Compound 1.

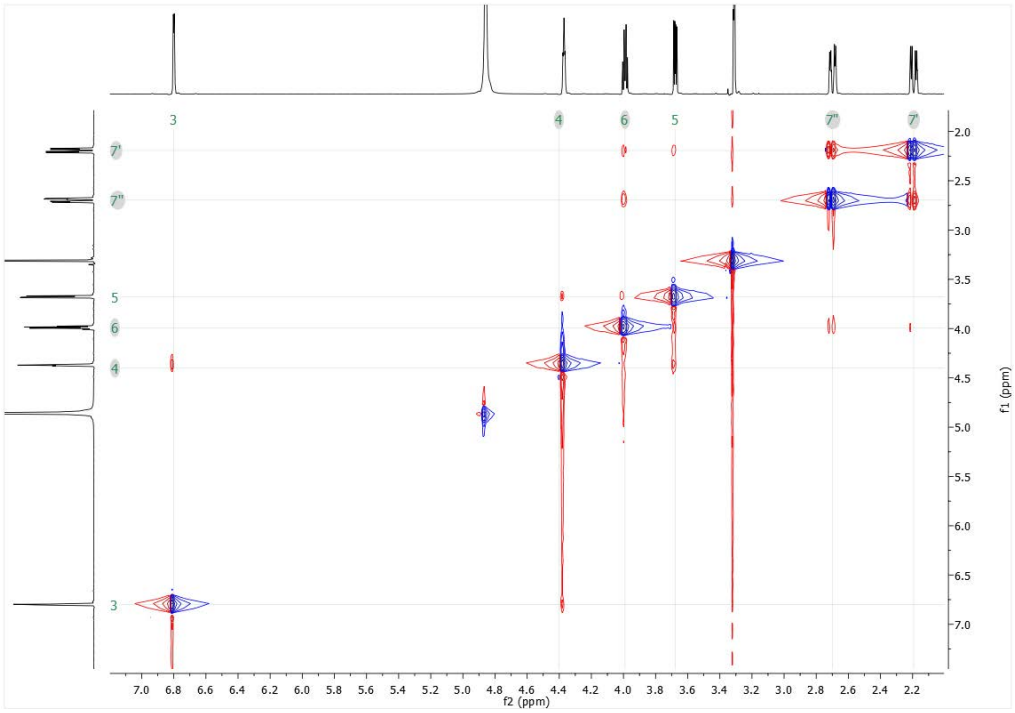

Figure SB 6. NOESY (600 MHz, CD3OD, 298°K) spectrum of Compound 1.

Spectroscopy data of (4*R*, 5*S*)-4,5 dihydroxy-3-oxo-cyclohexene-1-carboxylic acid (2).

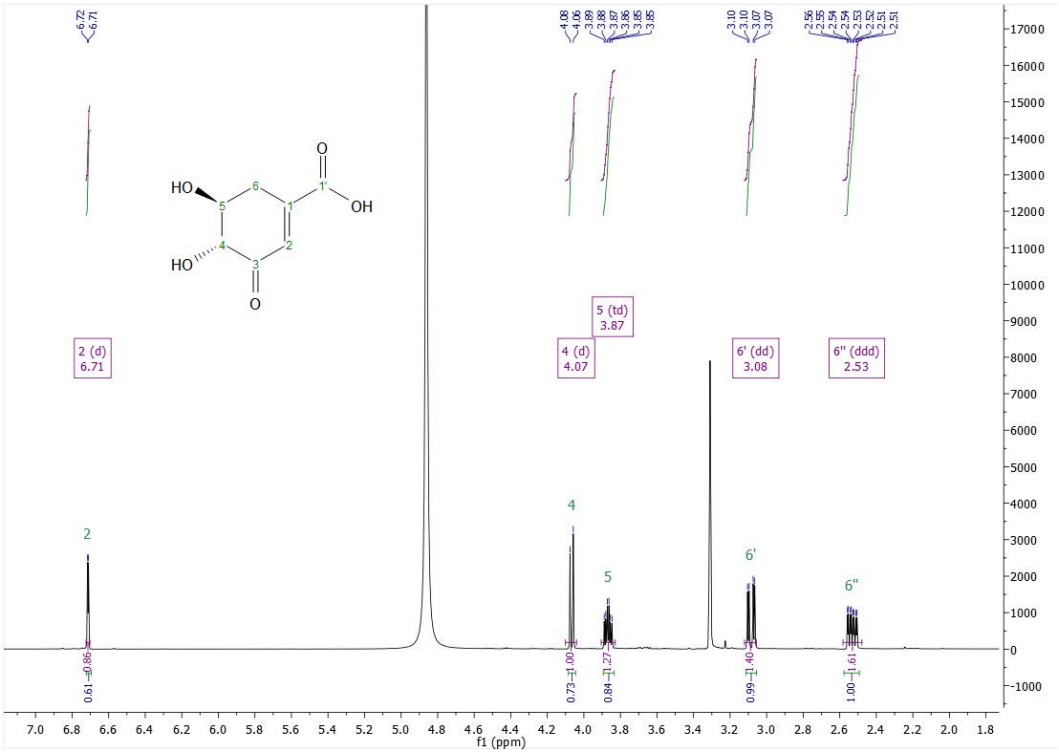

Figure SB 7. [1]H NMR (600 MHz, CD3OD, 298°K) spectrum of Compound 2.

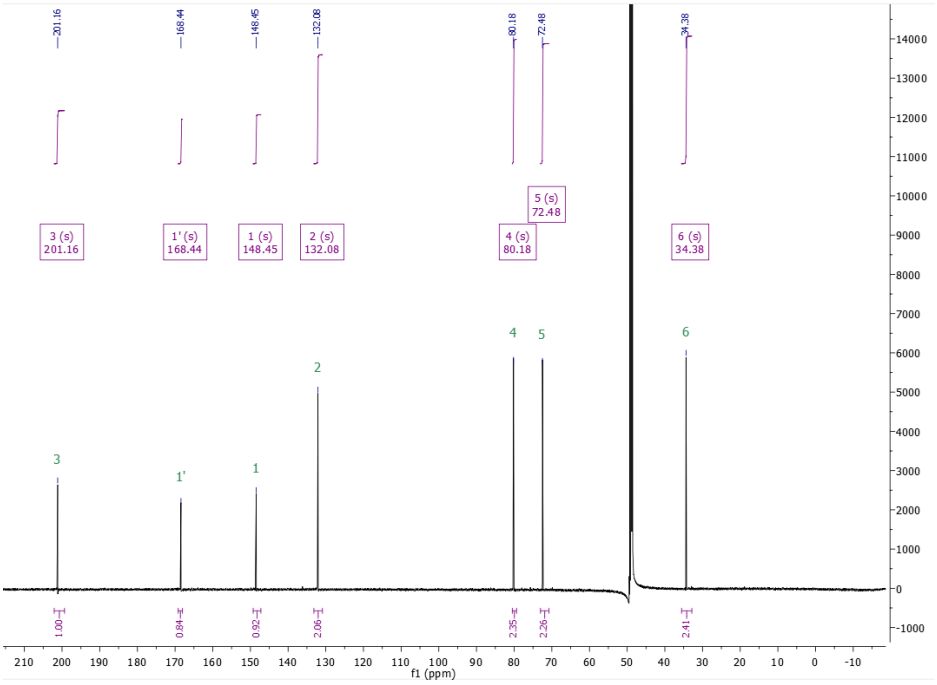

Figure SB 8. 13C NMR (600 MHz, CD3OD, 298°K) spectrum of Compound 2.

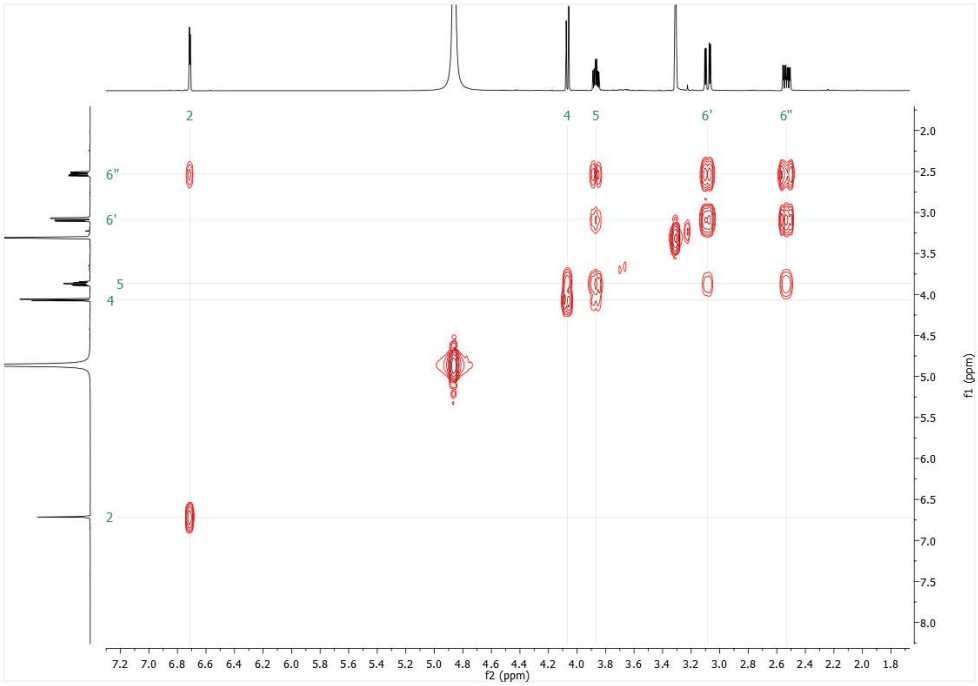

Figure SB 9. COSY (600 MHz, CD3OD, 298°K) spectrum of Compound 2.

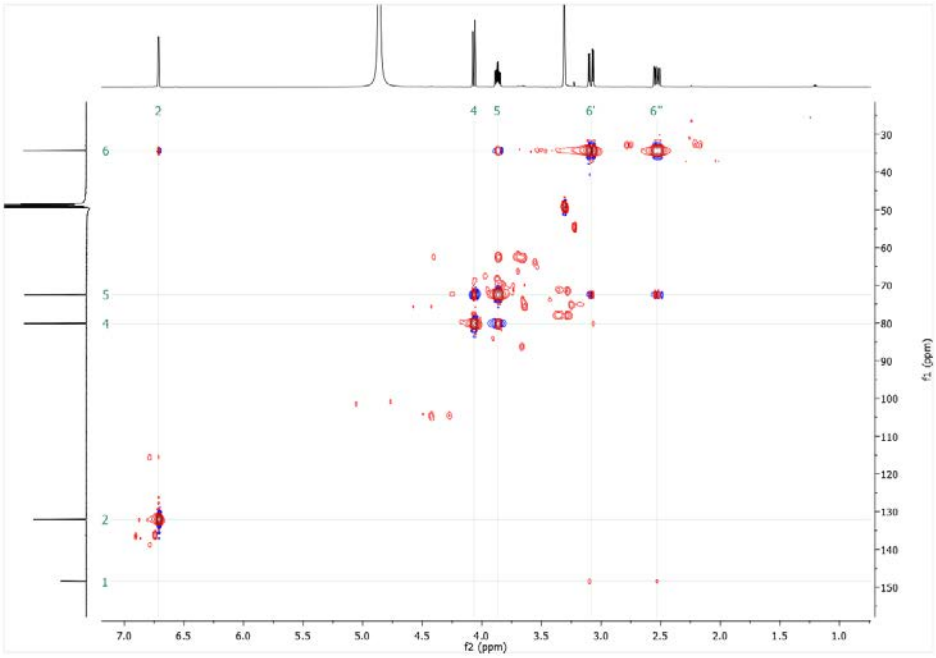

Figure SB 10. HSCQ (600 MHz, CD3OD, 298°K) spectrum of Compound 2.

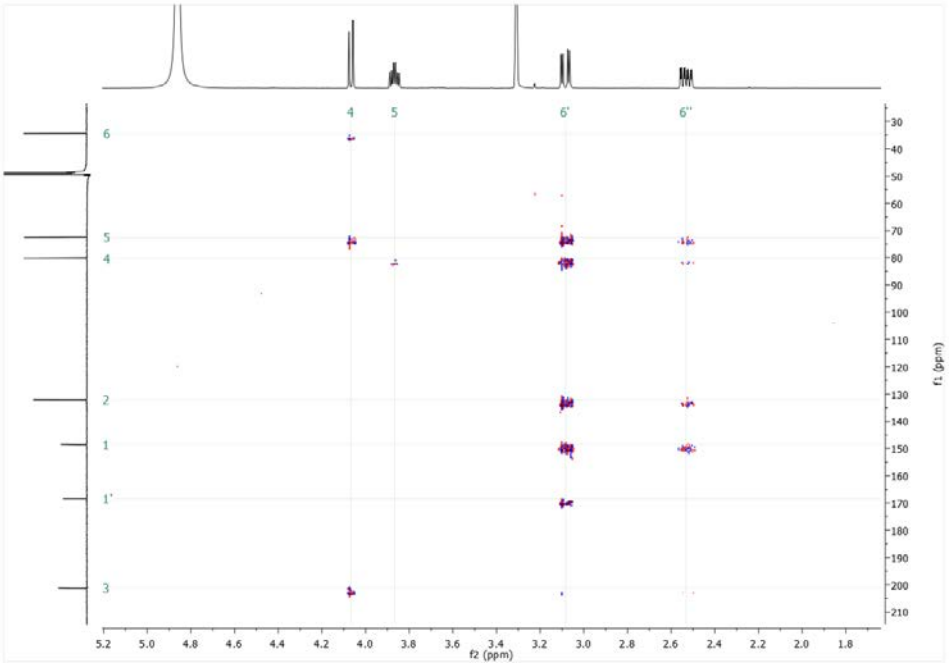

Figure SB 11. HMBC (600 MHz, CD3OD, 298°K) spectrum of Compound 2.

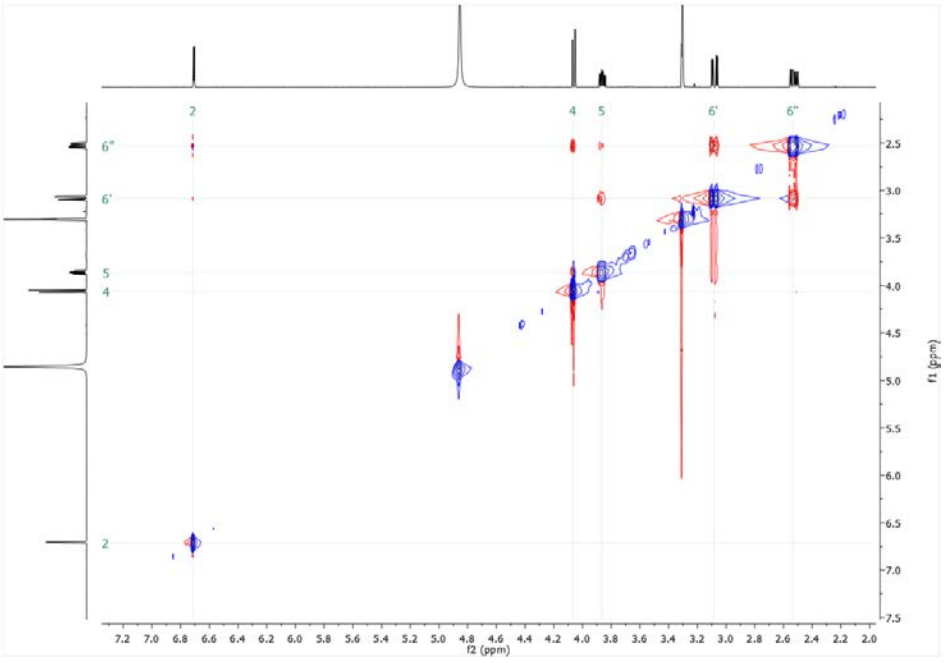

Figure SB 12. NOESY (600 MHz, CD3OD, 298°K spectrum of Compound 2.

Spectroscopy data of 3,4,5-trihydroxybenzoic acid (3).

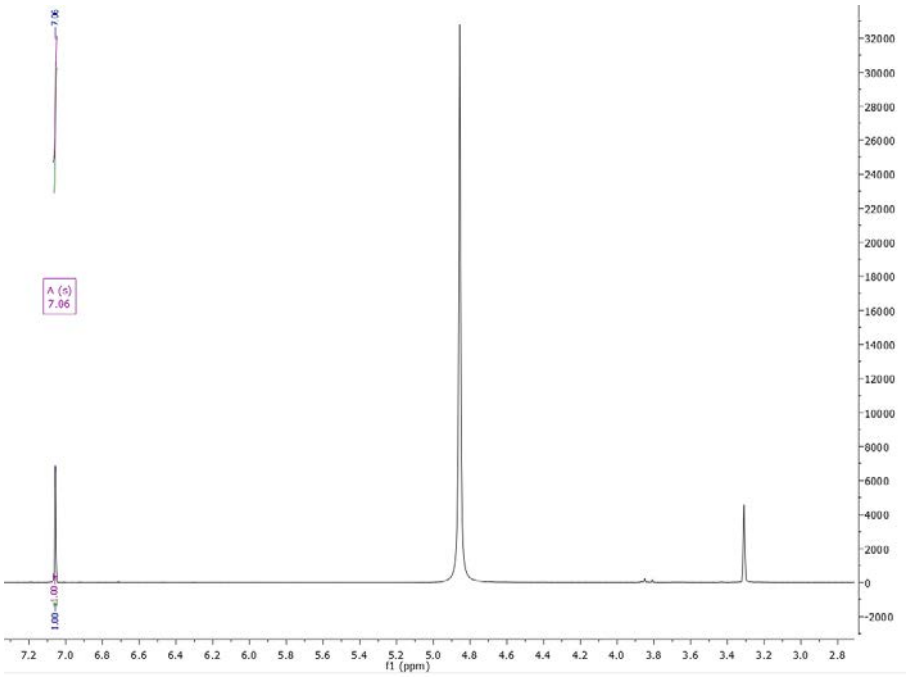

Figure SB 13. ^{1}H NMR (600 MHz, CD3OD, 298°K) spectrum of Compound 3.

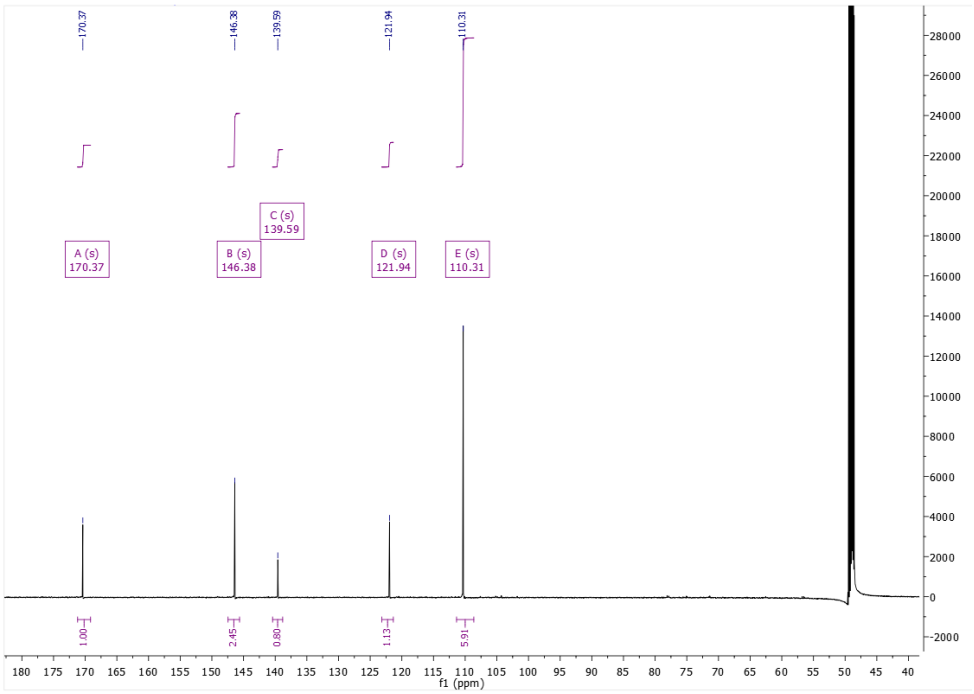

Figure SB 14. ^{13}C NMR (600 MHz, CD3OD, 298°K) spectrum of compound 3.

Spectroscopy data of (*E*)-3-[2-[(2*S*,3*R*,4*S*,5*S*,6*S*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxyphenyl]prop-2-enoic acid **(4)**.

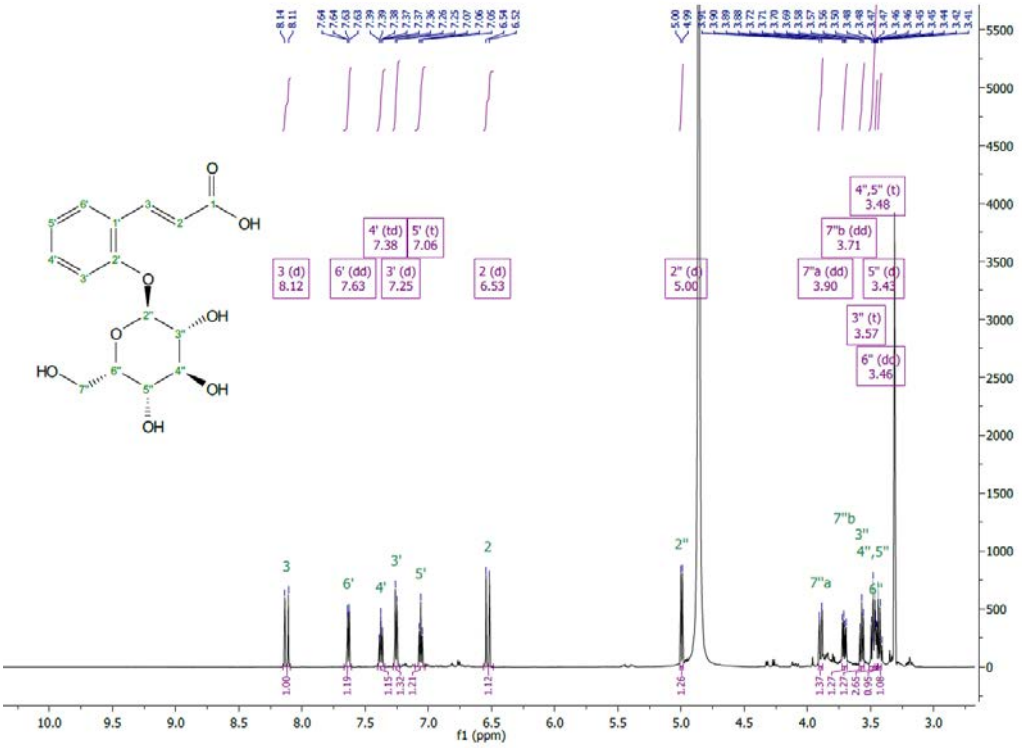

Figure SB 16. [1]H NMR (600 MHz, CD3OD, 298°K) spectrum of Compound 4.

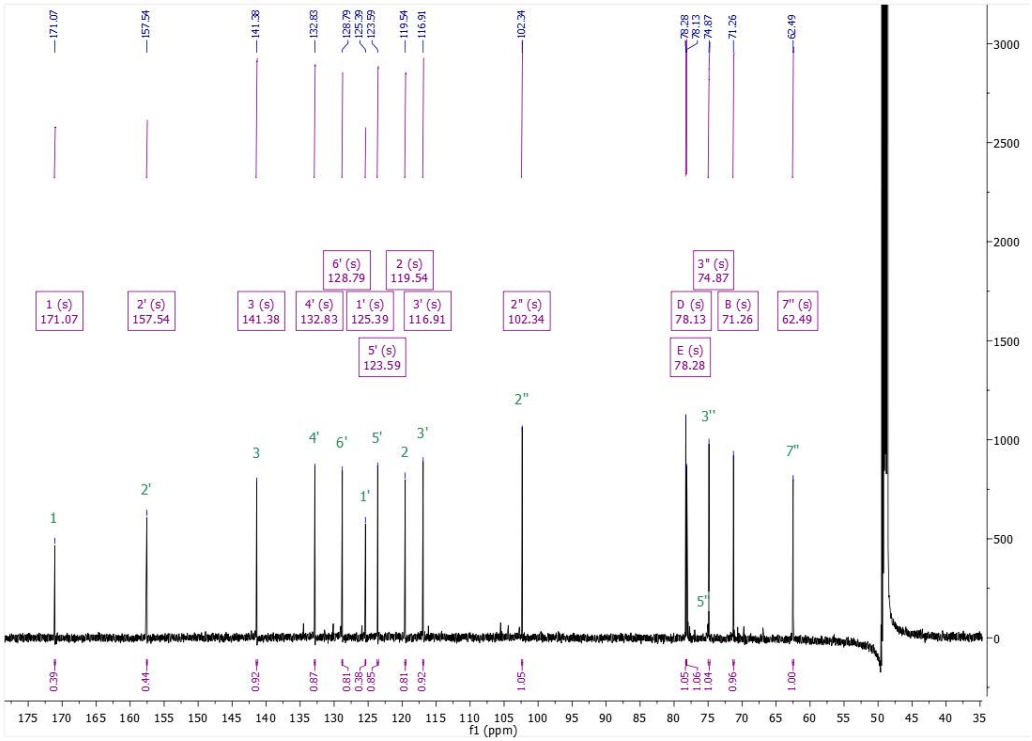

Figure SB 15. [13]C NMR (600 MHz, CD3OD, 298°K) spectrum of compound 4.

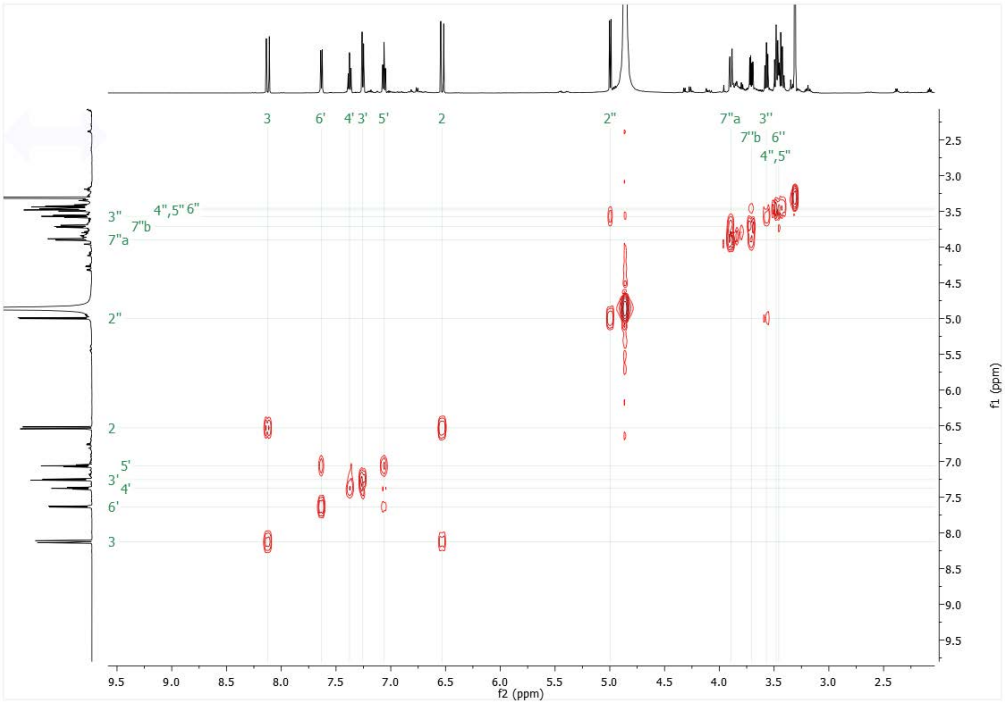

Figure SB 17. COSY (600 MHz, CD3OD, 298°K) spectrum of compound 4.

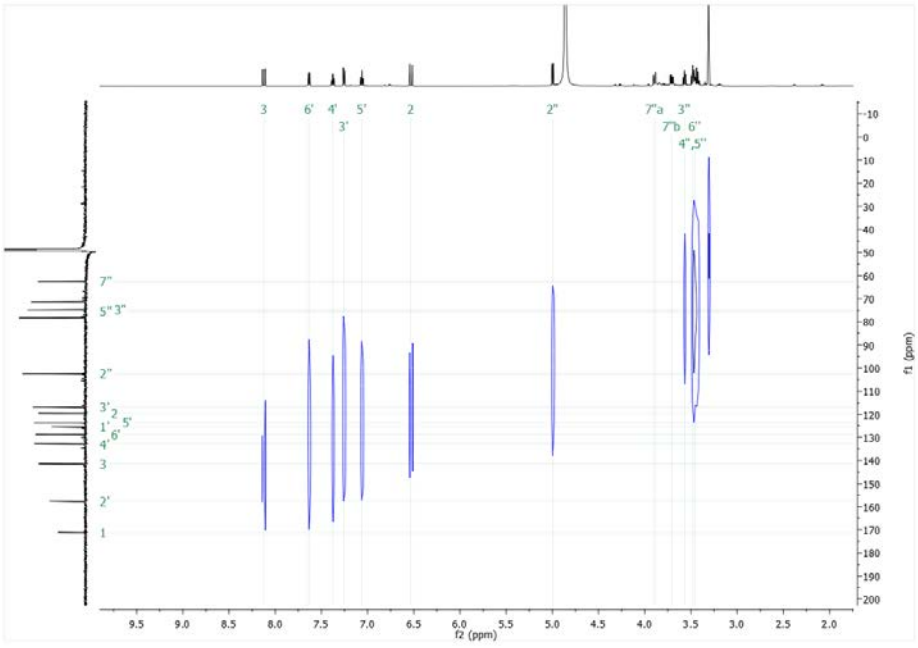

Figure SB 18. HSQC (600 MHz, CD3OD, 298°K) spectrum of compound 4.

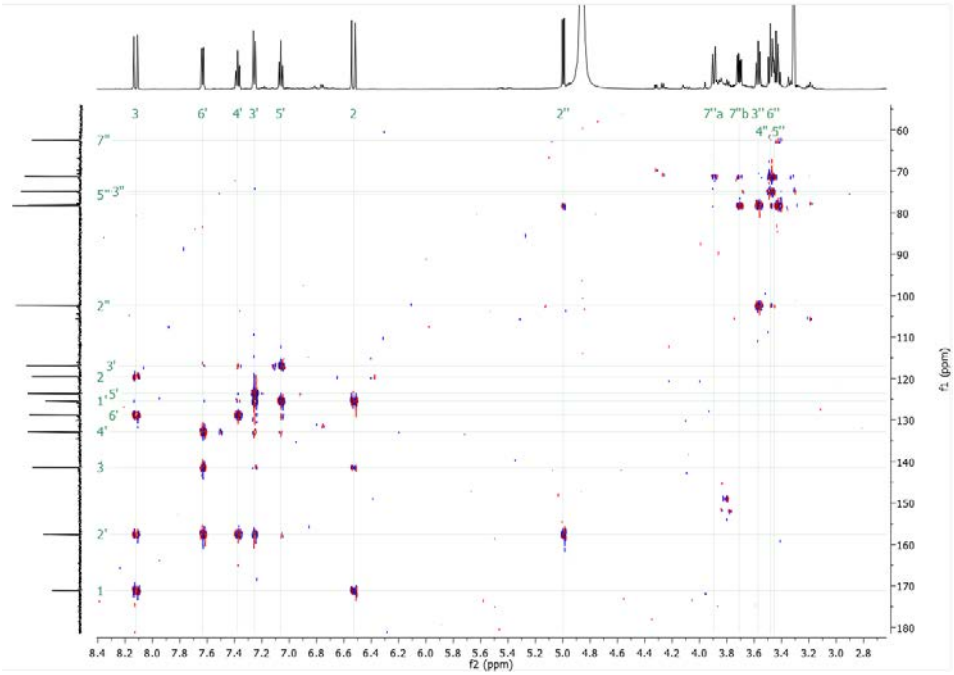

Figure SB 19. HMBC (600 MHz, CD3OD, 298°K) spectrum of compound 4.

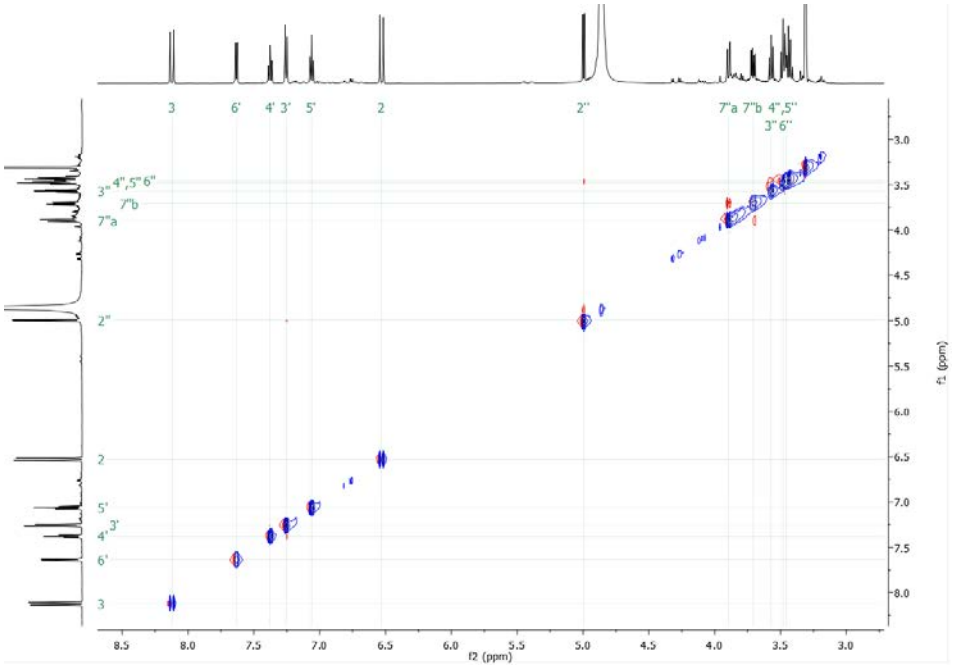

Figure SB 20. NOESY (600 MHz, CD3OD, 298°K) spectrum of compound 4.

cis-Melilotoside **(5)**.

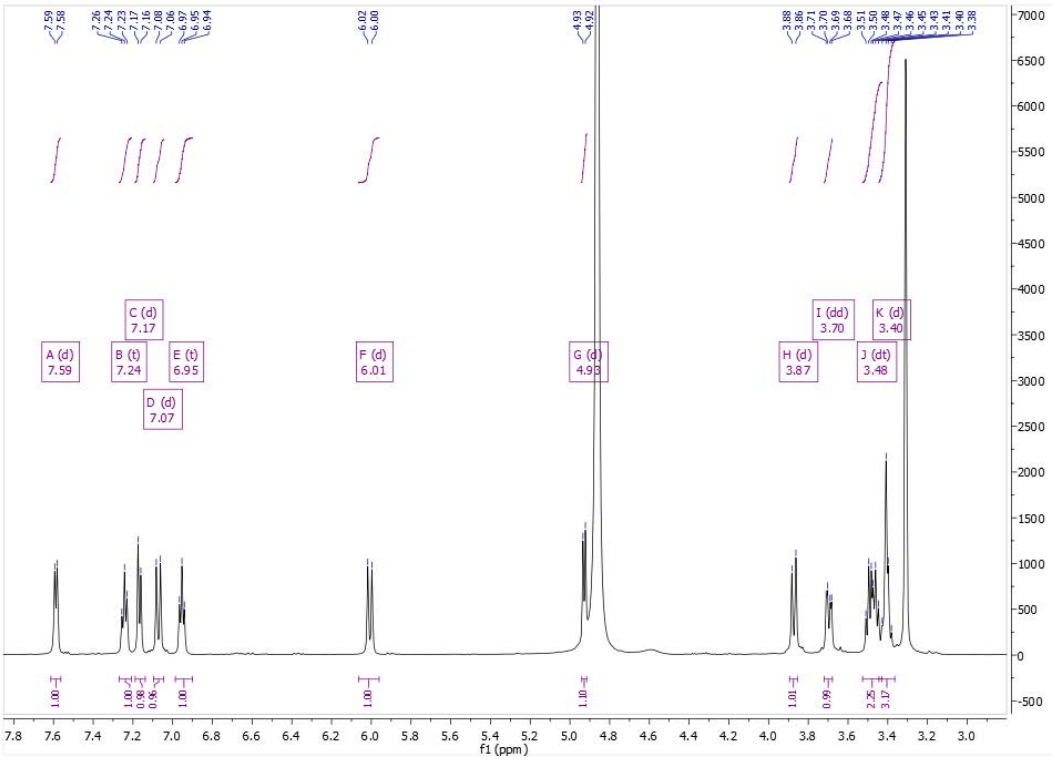

Figure SB 21. [1]H NMR (600 MHz, CD3OD, 298°K) spectrum of Compound 5.

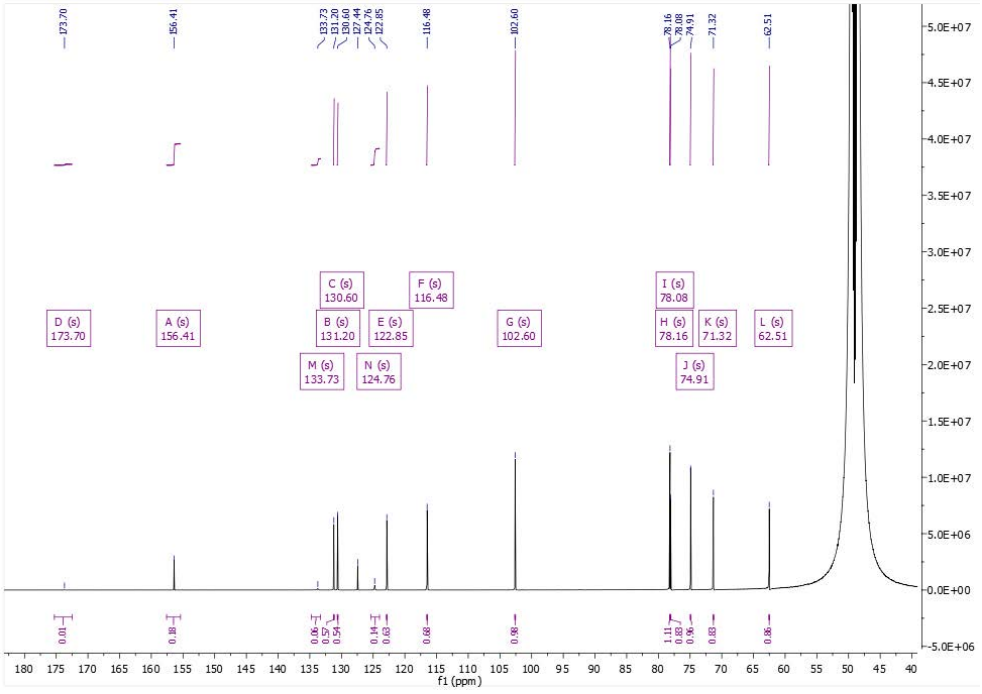

Figure SB 22. [13]C NMR (600 MHz, CD3OD, 298°K) spectrum of compound 5.

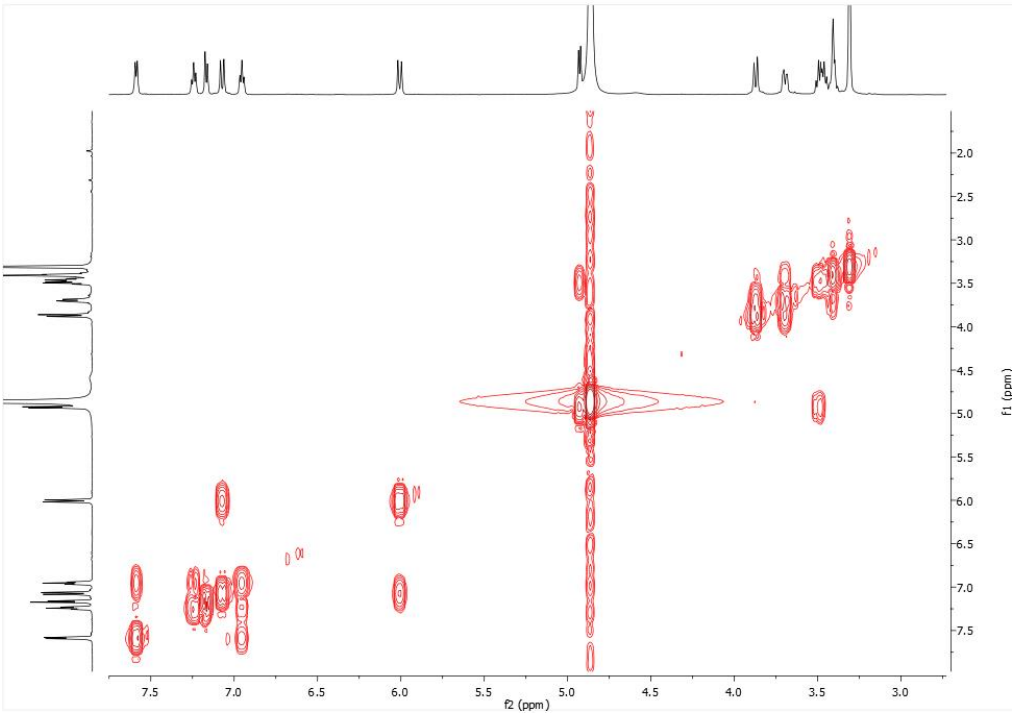

Figure SB 23. COSY (600 MHz, CD3OD, 298°K) spectrum of compound 5.

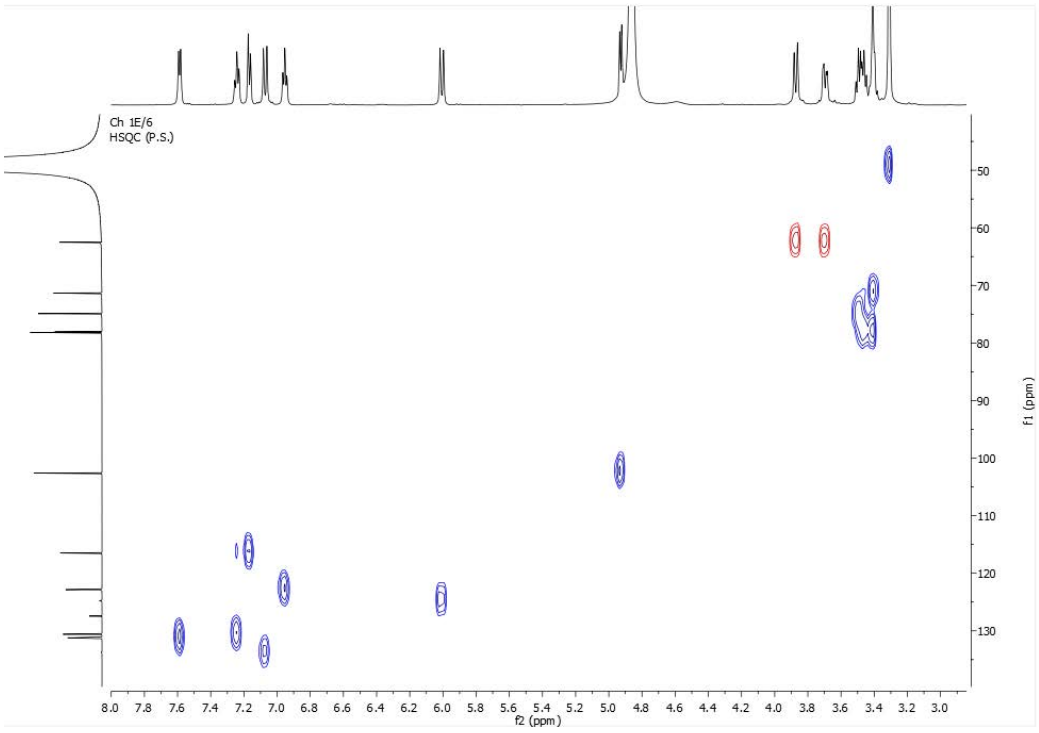

Figure SB 24. HSQC (600 MHz, CD3OD, 298°K) spectrum of compound 5.

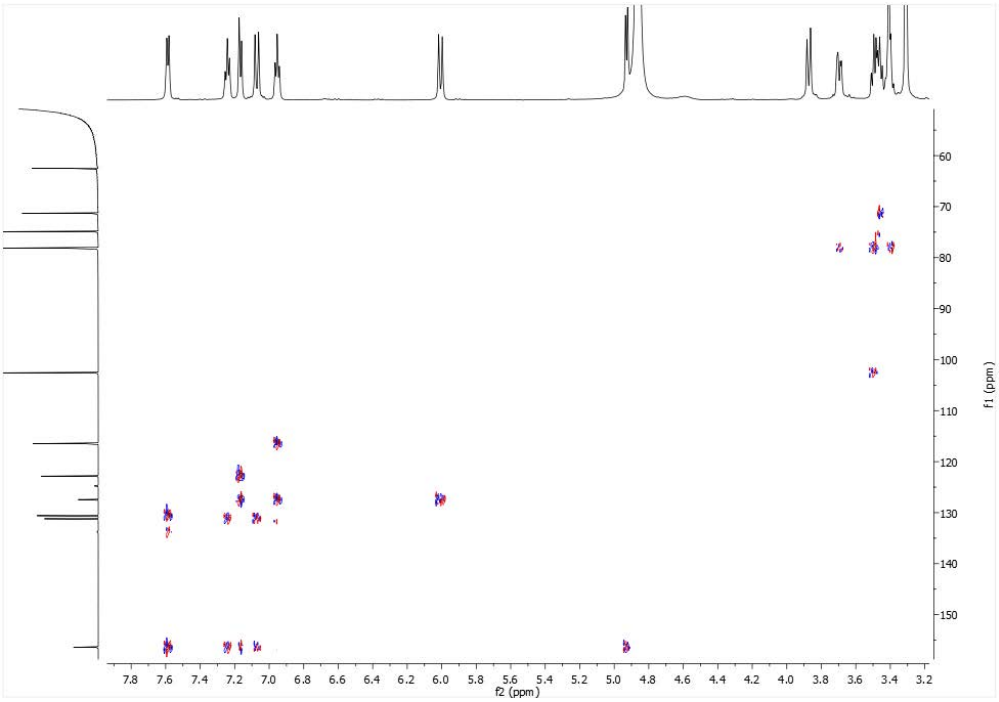

Figure SB 25. HMBC (600 MHz, CD3OD, 298°K) spectrum of compound 5.

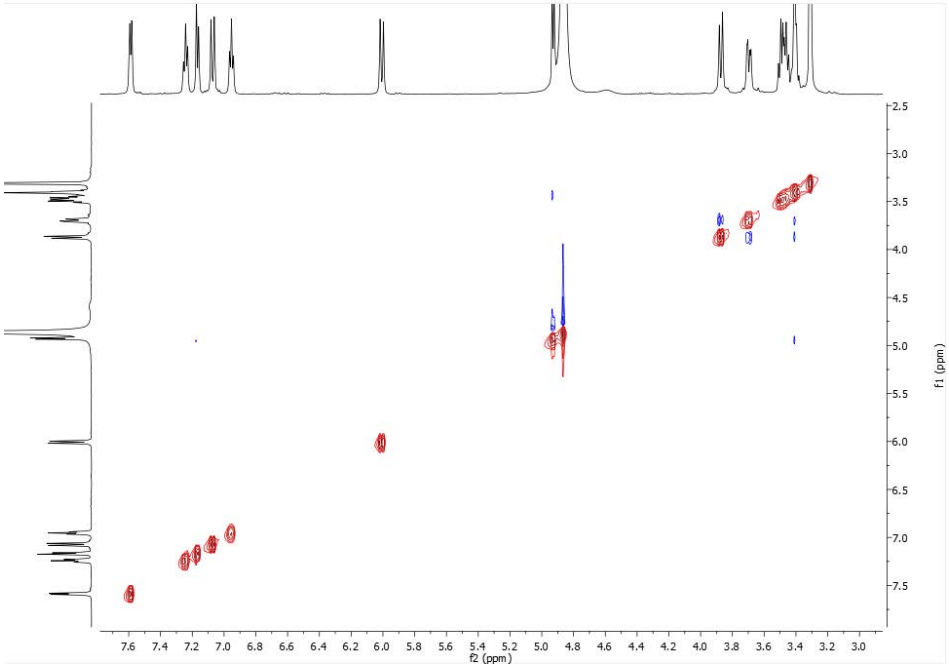

Figure SB 26. NOESY (600 MHz, CD3OD, 298°K) spectrum of compound 5.