

Phytochemical, antimicrobial and antiplasmodial investigations on Guinean plant species

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Proefschrift voorgelegd tot het behalen van de graad van doctor in de farmaceutische wetenschappen  
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Phytochemical, antimicrobial and antiplasmodial investigations on Guinean  
plant species

Fytochemisch, antimicrobieel en antiplasmodiaal onderzoek van  
plantensoorten uit Guinea

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verdedigen door

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***To My Grand Mother "Kadiatou Baldé"***  
*May Allah keep her soul in peace. Amen...*



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## LIST OF ABBREVIATIONS

<b>ABS</b>	access and benefit sharing
<b>APAD</b>	3-acetyl pyridine adenine dinucleotide
<b>ACT</b>	artemisin combination therapy
<b>AMR</b>	antimicrobial resistance
<b>ATCC</b>	american type culture collection
<b>CBD</b>	convention on biological diversity
<b>DAD</b>	diode array detector
<b>DCM</b>	dichloromethane
<b>DEPT</b>	distortionless enhancement by polarization transfer
<b>DHODH</b>	dihydroorotate dehydrogenase
<b>DMSO</b>	dimethylsulfoxide
<b>COSY</b>	correlation spectroscopy
<b>ELSD</b>	evaporating light scattering detector
<b>ESI</b>	electrospray ionization
<b>FA</b>	formic acid
<b>FDA</b>	food and drug administration
<b>FCM</b>	flow cytometric
<b>GNPS</b>	global natural product social molecular networking
<b>HEPES</b>	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
<b>HM</b>	herbal medicines
<b>HMBC</b>	heteronuclear multiple bond correlation
<b>HPLC</b>	high-performance liquid chromatography

<b>HR-ESI-MS</b>	High-resolution electrospray ionization mass spectrometry
<b>HSQC</b>	heteronuclear single quantum coherence
<b>IPR</b>	intellectual property rights
<b>IRS</b>	indoor residual spraying
<b>ITNs</b>	insecticide-treated nets
<b>LAMP</b>	loop-mediated isothermal amplification
<b>LDH</b>	lactate dehydrogenase
<b>MIC</b>	minimal inhibitory concentration
<b>MoA</b>	mechanism of action
<b>MDR</b>	multi-drug resistant
<b>MEM</b>	minimum essential medium
<b>MIC</b>	minimum inhibitory concentration
<b>MN</b>	molecular network
<b>MRSA</b>	methicillin resistant <i>S. aureus</i>
<b>MS</b>	mass spectrometry
<b>NADPH</b>	reduced nicotinamide adenine dinucleotide phosphate
<b>NP</b>	Nagoya Protocol entity
<b>NCE</b>	new chemical
<b>NBT</b>	nitroblue tetrazolium
<b>NMR</b>	nuclear magnetic resonance
<b>NP</b>	natural product

<b>OPLS-DA</b>	orthogonal partial least-squares discrimination analysis
<b>PDA</b>	photo diode array
<b>PCP</b>	<i>Pneumocystis carinii pneumonia</i>
<b>PES</b>	phenazine ethosulfate
<b>PCR</b>	polymerase chain reaction
<b>QC</b>	quality control
<b>QTOF</b>	quadrupole time-of-flight
<b>RDTs</b>	rapid diagnostic tests
<b>SAR</b>	structure–activity relationships
<b>SI</b>	selectivity index
<b>SPE</b>	solid-phase extraction
<b>SSTI</b>	superficial skin and soft tissue infections
<b>TFA</b>	trifluoroacetic acid
<b>TLC</b>	thin-layer chromatography
<b>TOF</b>	time-of-flight
<b>UPLC</b>	ultra-performance liquid chromatography
<b>UV</b>	ultraviolet
<b>WHO</b>	world health organization



# **CHAPTER 1**

## **Introduction**



## **1.1. Ethnopharmacological approach in drug discovery**

Ethnopharmacology is a highly diversified approach in drug discovery which involves the observation, description and experimental investigation of indigenous drugs and their biologic activities<sup>1,2</sup>. The main aim of this method is to develop natural medicines in the form of standardized crude extracts as well as to discover the active components of these natural products. According to Bruhn and Holmstedt (1981), ethnopharmacology is defined as “the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by human”<sup>2</sup>. A classic example of ethnopharmacological research that has led to new medicines is the ‘discovery’ of curare. In fact, the exploration of the botanical origin of the arrow poison curare, its physiological (as well as toxic) effects and the compound responsible for these properties provides a fascinating example of an early ethnopharmacological approach<sup>3,4</sup>. Several standardized methods have been developed in order to obtain reproducible results in ethnopharmacological studies. These methods, involving the combination of disciplines such as botany, pharmacognosy, pharmaceutical biology, natural product chemistry, plant physiology, biochemistry, pharmacology, toxicology, clinical research, anthropology,... have significantly contributed to the discovery of novel drugs from natural sources<sup>1,2,5</sup>. Many of these compounds have been approved as therapeutic agents for the treatment of several diseases. Some of these therapeutics would be challenging, if not impossible, to identify without the ethnopharmacological information<sup>1</sup>. Prominent examples of approved drugs that were initially discovered by the use of ethnopharmacological data are: khellin from *Ammi visnaga* (L.) Lam., the sodium salt of which is used as mast cell stabilizer in allergy and asthma, galegine from *Galega officinalis* L., quinine from the bark of Peruvian *Cinchona* species and artemisinin isolated from *Artemisia annua* L.<sup>5</sup>.

The ethnopharmacological approach begins with the collection of materials, which are recognized through their use in traditional medicine. Indeed the raw material of ethnopharmacology is the vast diversity of plants, animals and microorganisms (both terrestrial and marine) that grow on our planet in natural or semi-natural ecosystems<sup>1</sup>. The collection of raw materials is followed by preclinical studies during which biological, phytochemical and pharmacological studies are carried out in order to determine the

active compounds. After purification, chemical structure elucidation studies, followed by various chemical syntheses are carried out to determine Structure–Activity Relationships (SAR). On the other hand, various compounds may also exhibit synergism in biological activity, which is also of interest for development of new therapeutically active medicines. Nowadays, the ethnopharmacological research approach is commonly employed in developing countries of Asia, South America and Africa where traditional medicine knowledge exists. Likewise, during the last decade, ethnopharmacological studies have multiplied dramatically in Europe, focusing especially on the Mediterranean region including Turkey, Spain and Italy. Scientific studies on traditionally used medicinal plants in these countries do not only provide essential information for community-based health management but also introduce poorly known local natural products<sup>2</sup>.

In several African countries including Guinea, popular ethnobotanical information has been gathered through surveys and provides a solid foundation for many ethnopharmacological studies. Using the ethnobotanical approach, many medicinal plants have been extensively studied for both their biological and phytochemical properties. It is estimated that over 15,000 phytochemical compounds exhibiting significant pharmacological activity have been isolated from African medicinal plants<sup>6,7,8,9,10</sup>. Although significant progress has been made in ethnopharmacological research, it is important to stress that few plants have undergone detailed biological, phytochemical and clinical studies.

## **1.2. Ethnopharmacology and Intellectual Property Rights**

The global challenges related to access and benefit sharing (ABS) of biological resources have become a key concern in several areas of research including herbal medicines, ethnopharmacology, drug discovery, and the development of other high value products for which intellectual property protection can be secured<sup>11</sup>. The value of plants for medicines is more widely recognised and the “intellectual property rights” (IPR) connected with their use have been debated worldwide<sup>12</sup>. The convention on Biological Diversity (CBD) was the first international treaty in 1992 to deal with ownership of biological resources. Its three objectives are: the conservation of biological diversity; the sustainable use of its components; the fair and equitable sharing of the benefits arising

from the utilization of genetic resources. The CBD treaty was intended as a response to centuries of extensive exploitation of biodiversity and lack of recognition of the ownership rights of source countries. Quinine from Peruvian *Cinchona* species, can be cited as prominent example in this sense. Another example, also from Peru, is the resin of dragon's blood (*Croton lechleri* Müll.Arg.), used traditionally especially in the Amazon area, and sustained by a good clinical and pharmacological evidence-base, but no benefits to local communities are known<sup>13</sup>. The CBD was later reinforced by the Nagoya Protocol (NP), adopted in October 2010, which entered into force on 12 October 2014. The Nagoya protocol aims to facilitate creation of greater legal certainty and transparency for both providers and users of genetic resources by establishing more predictable conditions for access to genetic resources and helping to ensure benefit sharing when genetic resources leave the borders of the providing country. With facilitated access and ascertaining benefit-sharing, the Nagoya Protocol creates incentives for conservation and sustainable use of biological resources, and therefore enhances the contribution of biodiversity in development and human well-being<sup>14</sup>. The adoption of the Nagoya Protocol on Access to Genetic Resources and Benefit Sharing was seen as a significant step forward in the recognition of indigenous peoples and local communities' rights over their genetic resources and traditional knowledge. Indeed biodiversity-rich countries, indigenous cultures with their knowledge of the use of biosources as medicines and companies that seek to discover new therapeutics through medicinal plants and traditional knowledge are on the way sharing common interests<sup>12</sup>. The benefit sharing can be both monetary and non-monetary in the form of royalties, joint ventures, technology transfer, capacity building, etc., thus contributing to poverty alleviation and sustainable development of the developing countries.

### **1.3. Medicinal plants in drug discovery**

Medicinal plants have historically proven their value as sources of molecules with therapeutic potential, and nowadays still represent an interesting pool for the discovery of novel drug-leads<sup>5</sup>. The identification of biologically active extracts and compounds from nature has led to the discovery of new therapeutics, prompting the improvement of the human health and pharmaceutical sectors<sup>15</sup>. Indeed, plant-derived metabolites have been used as drugs, either in their original or semi-synthetic form. These metabolites can

also serve as drug precursors, drug prototypes, and pharmacological probes. Despite the increasing interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, it is important to note that natural products (NPs), and particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (NCEs)<sup>16</sup>. For instance, more than one-third of all the US FDA-approved therapeutic agents over the past 20 years are metabolites derived from or inspired by NPs, and more than 50% of the developed small-molecule drugs from 1981 to 2014 were originated from unaltered natural products, semi-synthetic natural products and natural products-derived mimetics<sup>17</sup>. Approximatively 25% of all conventional medicines currently in use as modern medicine are of plant origin<sup>2</sup>. For instance, in the oncology sector, plants have contributed more than 60% of the anti-cancer drugs, directly or indirectly<sup>18</sup>. Several plant extracts or “phytomedicines” are currently in clinical trials for the treatment of various diseases<sup>15</sup>.

Nowadays, plant based-metabolites are being actively investigated for direct use as therapeutic agents and as prototype lead compounds for the development of new synthetic or semisynthetic drugs. Globally, the margin of herbal medicine utilization is quite fascinating due to the side effects of using their synthetic counterparts for an extended period. Over time, the necessity of using herbal medicines (HM) became integral, and the drug development progressed accordingly. According to the World Health Organization (WHO) medicinal plants provide primary healthcare for approximately 3.5 to 4 billion people worldwide, and about 85% of traditional medicine involves the use of plant extracts, which may be called “modern herbal medicine”<sup>19</sup>. In 2016, the value of the global HM market was USD 71.19 billion, compared to USD 63.05 billion in 2014. Therefore, the level of the global HM market is expected to grow significantly in the future<sup>20</sup>. The most powerful and promising constituents of plants are their secondary metabolites, on which humans depend. Indeed, these metabolites are considered as the end-products of the gene expression, generally not essential for the reproduction, the growth or the development of the plant and produced as the results of the adaptation to the environment or as a possible defence mechanism against predators; in both cases, secondary metabolites are produced to assist and to improve the survival of the plant<sup>21</sup>. In fact the biosynthesis of these secondary metabolites is derived from the

fundamental processes of photosynthesis, glycolysis and the Krebs cycle to afford biosynthetic intermediates which, ultimately, results in the formation of secondary metabolites<sup>17</sup>.

According to available estimates, the total number of described plants species (comprising angiosperms and gymnosperms) is approximately 310,000 species, among which 60,000 species or about 20% have already been screened; and have provided 135 known drugs. Therefore, these numbers suggest that the screening of the remaining plant species could lead to detect around 700 new drug candidates<sup>21</sup>. The most under-explored source of such materials lies in the tropical and subtropical regions of the world. In these areas, a long tradition of ethnobotanical medicine often exists and offers a rich and relatively untapped source for the discovery of novel drugs from natural products. Furthermore, most of the already screened plants in these areas were investigated for their effects against a limited number of disease targets and there are still chances to find supplementary effects against new targets<sup>21</sup>. Despite the tremendous progress made in discovering drugs to treat various diseases over the past decades, the scourge of communicable and non-communicable diseases remains a major challenge for global public health. Thus, finding safe and effective drug candidates that can protect patients from these diseases is an essential mission for both pharmaceutical industry and academia<sup>17</sup>.

Current research in drug discovery from medicinal plants involves a multifaceted approach combining several methods and techniques. The availability of high-throughput screening for target-based drug discovery, libraries containing a large number of highly pure phytochemicals, laboratory animal models simulating human diseases, profiling kits for drug toxicity studies, and bioinformatics databases for long-term safety prediction have renewed research interest in herbal medicine globally towards the discovery of new and important leads against various pharmacological targets including microbial diseases and malaria<sup>22</sup>.

#### **1.4. Medical plants as antimicrobial agents.**

Microbial pathogens are responsible for more than 400 million years of life lost annually across the globe, a higher burden than either cancer or cardiovascular diseases. The continued growth of the world's population and increased interconnectivity heighten the risk that infectious diseases pose for human health worldwide<sup>23</sup>. In 2013, 9.2 million deaths have been reported because of infections i.e. about 17% of total deaths<sup>24</sup>. Although the use of antimicrobial agents has significantly increased human lifespan over the past century, it is important to note that the emergence of pathogen strains that are resistant to antimicrobials threatens to reverse these gains. The heavy use and misuse of antibiotics has led to the development of multidrug-resistant, extremely drug-resistant and even pan-resistant pathogens across the world<sup>23</sup>. Therefore, these pathogens, including those caused by fungi, viruses, and bacteria, are becoming more challenging to treat. Bacterial pathogens gain resistance to antibiotics due to three major reasons<sup>25</sup>. The impact of antimicrobial resistance (AMR) is a huge concern, which results in the greatest loss to individual and social economy. It is estimated that by 2050, the death rate due to AMR will balloon to 10 million lives per year at an expense of one hundred trillion dollars<sup>26,27</sup>. Among the resistant bacteria, staphylococcal infections, particularly those caused by methicillin resistant *Staphylococcus aureus* (MRSA), have increased the morbidity and mortality of patients due to the cumbersome treatment required. Therefore, the WHO has recently considered this strain as a high priority pathogen that urgently requires new antibiotic targeting<sup>28</sup>. Furthermore, it is well known that *S. aureus* is a major cause of community and health care associated infections, ranging from superficial skin and soft tissue infections (SSTI) to invasive infections, sepsis and death<sup>29</sup>.

Despite the considerable progress in terms of research and development of new treatment and prevention procedures over the last decades, infectious diseases still remain the leading cause of death in many developing countries<sup>30</sup>. Meanwhile, many infectious diseases have emerged or re-emerged in Africa in the 21st century. Some of them are associated with newly discovered microorganisms; others are known, historical diseases. It should also be noted that there are diseases linked to previously known microorganisms which have recently been implicated for the first time in massive epidemics with global impacts (such as Ebola virus, Zika virus, Chikungunya virus and

severe acute respiratory syndrome (SARS) coronavirus). Research on emerging infectious diseases needs to be identified as a priority<sup>23,31</sup>. The re-emergence of these pathogens coupled with the increasing incidence of certain drug-resistant pathogens have drawn the attention of the pharmaceutical and scientific communities to discover new effective and selective antimicrobial agents from natural origin including plant extracts. Current research on extracts and natural products primarily focuses on plants, since they can be sourced more easily and be selected on the basis of their ethno-medicinal use in different countries<sup>32</sup>. It is important to point out that several secondary metabolites isolated from medicinal plants have already proven their effectiveness against several microbial diseases (Table 1.1). For instance, the essential oils derived from aromatic medicinal plants (e.g., fennel, peppermint, thyme and lavender) and containing mixtures of volatile substances, such as monoterpenes, sesquiterpenes and/or phenylpropanoids, have been reported to be active on Gram-positive and Gram-negative bacteria and on fungi and viruses.

Likewise, in the past few years the effects of combinations of antibiotics and plant-derived metabolites have been widely studied. The results of these investigations have shown that phytochemicals modulate or modify resistance mechanisms in bacteria, suggesting that phytochemicals can be used in combination with antibiotics to increase the activity and minimize the toxic side effects of antibiotics<sup>33,34</sup>. Indeed, polyphenols, a widely studied group of aromatic compounds, have been suggested to have antimicrobial potential alone or in combination with currently available antibiotics<sup>35</sup>. Moreover, the synergistic activity of epigallocatechin gallate and quercetin when used in combination has been shown to be particularly effective against a topical MRSA infection<sup>36</sup>. It has long been known that sanguinarine, a benzophenanthridine alkaloid isolated from the rhizomes of *Sanguinaria canadensis*, has broad antimicrobial activity and anti-inflammatory properties. In fact, *in vitro* studies have demonstrated that sanguinarine is able to inhibit bacterial adherence to the surface of teeth, exerting an anti-plaque action<sup>33</sup>. Thus, previous studies have shown that a combination of sanguinarine, EDTA and vancomycin and a combination of sanguinarine, EDTA and streptomycin have additional and synergistic effects against Gram-positive and Gram-negative bacteria, including Multi-Drug Resistant (MDR) bacteria. Berberine, an isoquinoline-type alkaloid, contributes to oral health benefits. Several studies have demonstrated the efficacy of berberine against oral streptococcal

growth and selected endodontic pathogens using a multispecies biofilm tooth model. The most pronounced antibacterial effect was observed against *Streptococcus sanguinis* and the most significant synergistic effect was found in combination with penicillin, clindamycin and erythromycin<sup>33</sup>. The antibacterial activity of other alkaloids, such as reserpine isolated from *Rauwolfia* spp., piperine from *Piper nigrum*, cinchonidine and cinchonine from *Cinchona* spp. has been demonstrated. Reserpine showed inhibitory activity against *Klebsiella pneumoniae* biofilms. Furthermore, the effectiveness of reserpine and piperine against *Escherichia coli* has been demonstrated. In addition, piperine increased penetration of the antibiotics azithromycin and ciprofloxacin into *E. coli* biofilms, and promoted the effect of these antibiotics in dispersing biofilms. Cinchonidine and cinchonine were active against *Staphylococcus aureus* (strain ATCC 25923)<sup>33</sup>. Chanoclavine, a tricyclic ergot alkaloid isolated from *Ipomoea muricata*, has exhibited synergistic effects when co-administered with tetracycline against MDR *Escherichia coli*<sup>24</sup>. *Holarrhena antidysenterica* belongs to the Apocynaceae family and has been traditionally employed for the treatment of different diseases such as dysentery, diarrhea, fever and bacterial infections. *H. antidysenterica* bark contain alkaloids, particularly the steroid alkaloid conessine.

**Table 1.1.** Overview of several plants and natural products with antimicrobial activity<sup>24</sup>

Common (name)	Scientific name	Compound	Active against
Barberry	<i>Berberis vulgaris</i> subsp. <i>australis</i> (Boiss.) Heywood	Berberine	Bacteria, protozoa
Black pepper	<i>Piper nigrum</i> L.	Piperine	Fungi, <i>Lactobacillus</i> , <i>Micrococcus</i> ,
Burdock	<i>Arctium lappa</i> L.		Bacteria, fungi, viruses
Caraway	<i>Carum carvi</i> L.		Bacteria, fungi, viruses
Cascara sagrada	<i>Rhamnus purshiana</i> DC.	Tannins	Bacteria, fungi, viruses
Chamomile	<i>Matricaria chamomilla</i> L.	Anthemic acid	<i>M. tuberculosis</i> , <i>S. typhimurium</i> , <i>S. aureus</i>
Clove	<i>Syzygium aromaticum</i> L.	Eugenol	Broad antimicrobial activity
Cranberry	<i>Vaccinium</i> spp.	Phenolic compounds	Bacteria
Eucalyptus	<i>Eucalyptus globulus</i> Labill.	Tannin	Bacteria, viruses
Garlic	<i>Allium sativum</i> L.	Allicin, ajoene	Broad antimicrobial activity
Goldenseal	<i>Hydrastis canadensis</i> L.	Berberine, hydrastine	Bacteria, <i>Giardia duodenale</i>
Green tea	<i>Camellia sinensis</i> (L.) Kuntze	Catechin	Broad antimicrobial activity
Licorice	<i>Glycyrrhiza glabra</i> L.	Glabrol	<i>S. aureus</i> , <i>M. tuberculosis</i>
Oak	<i>Quercus rubra</i> L.		
Onion	<i>Allium cepa</i> L.	Allicin	Bacteria, <i>Candida</i>
Oregon grape	<i>Mahonia aquifolia</i> Gagnep.	Berberine	<i>Plasmodium</i> sp.
Senna St. John's wort	<i>Hypericum perforatum</i> L.	Hypericin, others	Broad antimicrobial activity

Thyme

*Thymus vulgaris* L.

Caffeic acid, thymol, Viruses, bacteria, fungi  
Tannins

Turmeric

*Curcuma longa* L.

Curcumin, turmeric Bacteria, protozoa

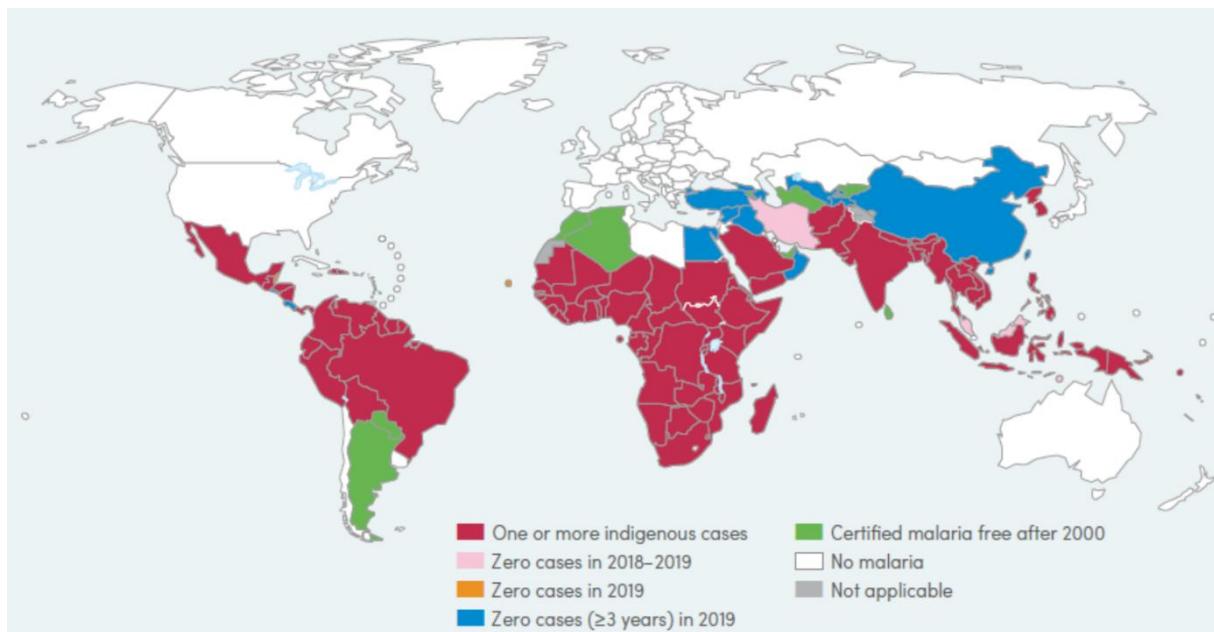
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## 1.5. Malaria

Malaria is the most important parasitic disease of human beings and remains a leading cause of illness and death worldwide. It is transmitted in 108 countries inhabited by roughly 3 billion people<sup>37</sup>. Despite a gradual decline in the global incidence rate, estimated to 18% from 2010 to 2016, malaria remains a global public health concern with approximately 228 million new cases (range 206–258 million), which resulted in an estimated 405.000 disease-induced deaths in 2018<sup>27</sup>. The causative agents for malaria infections are *Plasmodium* protozoans (i.e. *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi*)<sup>37,38</sup>. The most widespread and severe malaria is caused by *P. falciparum*, which transiently infects the liver before invading red blood cells of the mammalian host (Figure 1.1). Clinical manifestations occur at the erythrocytic stage and can include fever, chills, prostration and anaemia, as well as delirium, metabolic acidosis, cerebral malaria and multi-organ system failure, which may be followed by coma and death. It is well established that the incidence of the disease depends on environment suitability for local vectors in terms of altitude, climate, vegetation and implementation of control measures, and is therefore inextricably linked to poverty, natural disasters and war<sup>37</sup>. Malaria is considered as an endemic disease in most tropical countries (Africa, Asia, and Latin America), with about half of the world's population at risk of infection according to the WHO. Children aged under 5 years are the most vulnerable group affected by malaria; in 2018, they accounted for 67% (272 000) of all malaria deaths worldwide<sup>39</sup>. The WHO African Region carries a disproportionately high share of the global malaria burden. Indeed, indigenous malaria cases have been recently detected in several countries (Figure 1.1). In 2018, the region was home to 93% of malaria cases and 94% of malaria deaths<sup>40</sup>.

Of the 87 countries where malaria was endemic in 2019, 29 accounted for 95% of malaria cases globally. Amongst these countries, Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%) and Niger (3%) accounted for about 51% of all cases globally. On the other hand, about 95% of malaria deaths were in 32 countries. Nigeria (23%), Democratic Republic of the Congo (11%), Tanzania (5%), Burkina Faso (4%), Mozambique (4%) and Niger (4%) accounted for about 51% of all malaria deaths globally in 2019<sup>41</sup>.

In Guinea the entire estimated population is at risk of malaria with a global prevalence of 44%, and regional malaria prevalence ranging from 39% to 61%. According to the Ministry of Health, malaria is the first cause of all outpatient visits (34%) and the first cause of death (28%) among Guinean children. The malaria transmission is year-round with a high frequency from July through October in most areas. The majority of infections are caused by *Plasmodium falciparum*<sup>42</sup>.

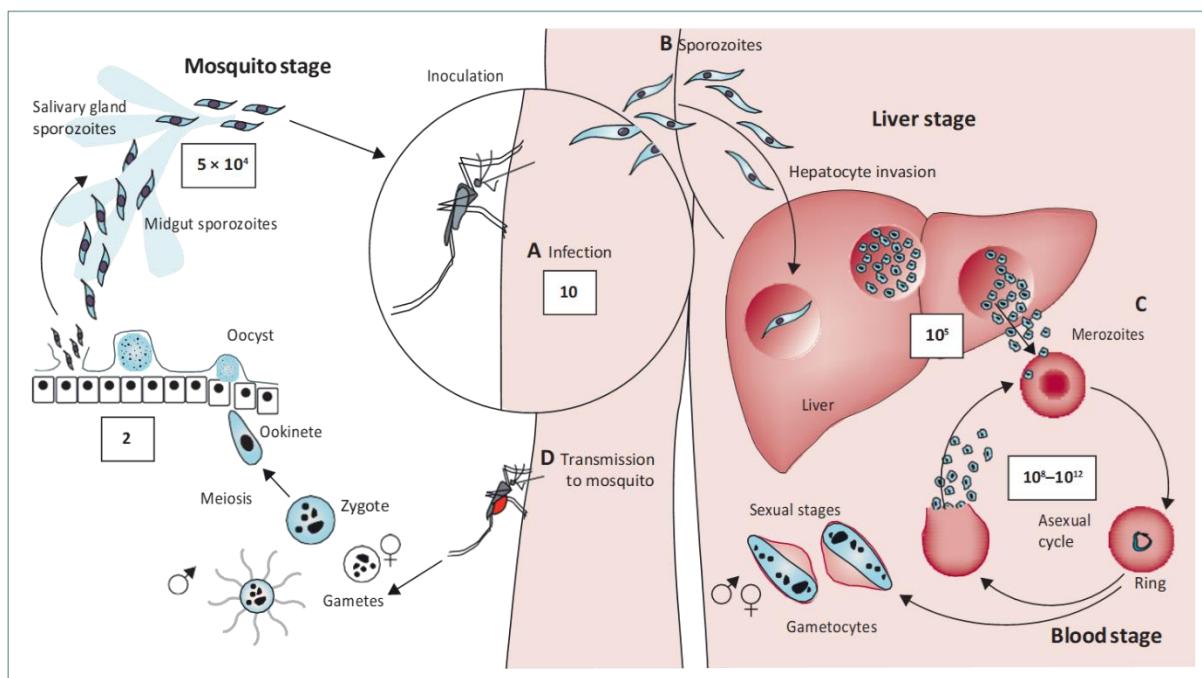


**Figure 1.1.** Countries with indigenous cases in 2000 and their status by 2019 Countries with zero indigenous cases over at least the past 3 consecutive years are considered to have eliminated malaria<sup>42</sup>.

### 1.5.1. Lifecycle of *Plasmodium*

The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host. Sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony). Merozoites infect red blood cells. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites. Some parasites differentiate into sexual erythrocytic stages (gametocytes).

Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal. The parasite's multiplication in the mosquito is known as the sporogonic cycle C, while in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated (ookinetes) which invade the midgut wall of the mosquito where they develop into oocysts (figure 1.1). The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle<sup>43,37</sup>.



**Figure 1.1.** Lifecycle of *Plasmodium falciparum* in the human body and the anopheline mosquito<sup>38</sup>.

### 1.5.2. Diagnosis

Early and accurate diagnosis is essential both for effective management of the disease and for strong malaria surveillance. Parasite-based diagnostic testing significantly reduces illness and death by enabling health providers to swiftly distinguish between malarial and non-malarial fevers and select the most appropriate treatment. It improves the overall

management of patients with febrile illnesses and may also help reduce the emergence and spread of drug resistance<sup>44</sup>.

The clinical diagnosis of malaria is based on the patients' signs and symptoms, and on physical findings during the examination by medical doctors. This method is least expensive and most widely practiced in areas where the required resources are not available. The earliest symptoms of malaria are very nonspecific and variable, and include fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting, anorexia, and pruritus. A clinical diagnosis of malaria is still challenging because of the non-specific nature of the signs and symptoms, which overlap considerably with other common, as well as potentially life-threatening diseases, e.g. common viral or bacterial infections, and other febrile illnesses.<sup>45</sup>

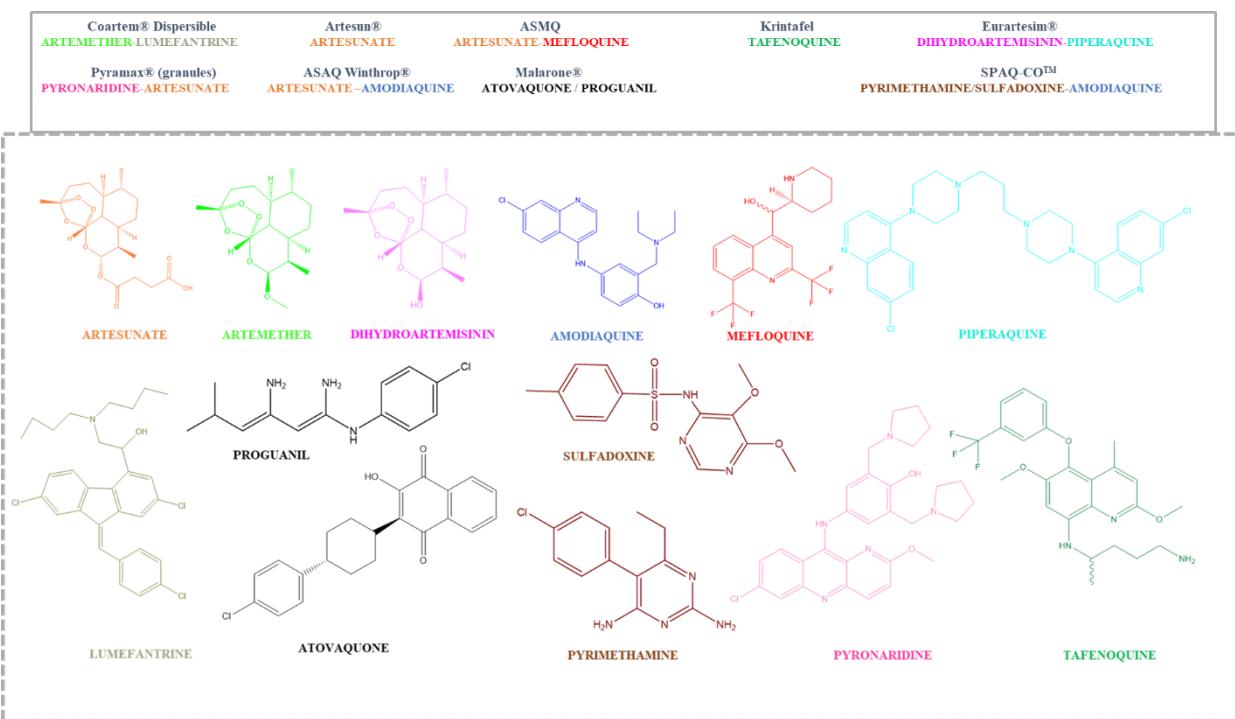
Prompt malaria diagnosis either by microscopy or rapid diagnostic tests (RDTs) is recommended by WHO for all patients with suspected malaria before they are given treatment. The microscopic detection and identification of *Plasmodium* species in Giemsa-stained thick blood films (for screening the presenting malaria parasite), and thin blood films (for species' confirmation) remains the gold standard for laboratory diagnosis. Recent developments in molecular biological technologies, e.g. polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS), and flow cytometric (FCM) assay techniques, have permitted extensive characterization of the malaria parasite and are generating new strategies for malaria diagnosis<sup>45,46</sup>.

### **1.5.3. Profylaxis and treatment**

The management of malaria requires the rigorous application of existing tools and approaches, as well as the development and use of new interventions<sup>47</sup>. Preventive treatment strategies currently target *falciparum* malaria and need to be developed for other types of human malaria. The strategies for preventive treatment vary, depending on the intensity of transmission and the level of parasite resistance to antimalarial medicines in a given region. Indeed, the preventive approaches currently in use focus mainly on chemoprophylaxis, vaccination, bite prevention and vector control measures<sup>38,37</sup>. The two core interventions for malaria vector control are insecticide-treated nets (ITNs) and

indoor residual spraying (IRS)<sup>48</sup>. The WHO-recommended preventive treatment against malaria presently includes intermittent preventive treatment of pregnant women, intermittent preventive treatment of infants, and seasonal chemoprevention for children aged under 5 years. Moreover, chemoprophylaxis should be given to individuals exposed to high malaria risk in combination with advice about measures to reduce vector bites, particularly non-immune travellers, who are more susceptible to malaria illness and death. It is also recommended for travellers within countries from malaria-free areas to areas with high malaria risk. The main goal of malaria treatment is to ensure the rapid and complete elimination of *Plasmodium* parasites from a patient's bloodstream to prevent an uncomplicated case of malaria from progressing to severe disease or death<sup>49</sup>. Several strategies are currently being deployed in parallel to fight this disease. These strategies include the use of vaccines, novel drugs, and vector control methods. Currently, the most advanced malaria vaccine, RTS,S/AS01, is in clinical development<sup>50,47</sup>. Results from a phase III clinical trial have shown that RTS,S/AS01 is able to prevent malaria cases (67.6% effectiveness)<sup>51</sup>, particularly among children in high impact areas. Strategies to control the mosquito (insecticide-treated nets and indoor residual spraying) have been very effective in the past but are currently at risk due to increased resistance to the insecticides used (mainly pyrethroids).

Nowadays, several classes of compounds are clinically used for the management of malaria (figure 3.1). The most common are: quinolines (quinine, chloroquine, amodiaquine, mefloquine), antifolates (sulfadoxine, pyrimethamine), artemisinin derivatives (artesunate, artemether, dihydroartemisinin) and antimicrobials (doxycycline). These compounds are used alone or in combination depending on the geographical area and the condition of the affected people<sup>52</sup>. The first-line drug based treatments including artemisinin combination therapies (ACTs) are at risk due to the emergence of resistance<sup>53,54</sup>. This highlights the need for discovery of new drugs with novel mechanisms of action that can be used to control, eliminate, and eradicate malaria<sup>55</sup>.



**Figure 1.2.** New anti-malarial drug combinations/formulations that have been approved for use. Brand name of the drug (in bold) and drug combinations (colour-coded to the structures) are listed<sup>52</sup>.

#### 1.5.4. Natural product as anti-malarial agents.

Natural products have played a pivotal role in malaria chemotherapy, progressing from quinine and artemisinin to ozonide-based compounds. Many of these natural products have served as template for the design and development of antimalarial drugs currently in the clinic or in the development phase<sup>54</sup> (Table 1.2). Like many other infectious diseases, malaria drug discovery has greatly benefited from nature and its products. In fact, medicinal plants, have been used in the traditional treatment of malaria for thousands of years due to their efficacy and availability<sup>56</sup>. Indeed, two of the most effective antimalarial drugs (quinine and artemisinin) were sourced from medicinal plants, and many synthetic antimalarial drugs are analogues of these two natural products. In addition to the use of isolated natural products as antimalarial agents, many plants are used in ethnomedicine for the treatment of malaria<sup>56</sup>.

#### **1.5.4.1. Quinine and its analogues**

The alkaloid quinine, isolated from the bark of the South American tree *Cinchona officinalis* and other species of the same genus, was the first effective antimalarial agent to be discovered. It served humanity well for about 300 years, although resistance to the drug was first noted in 1910, and it is no longer recommended by the WHO as a first line treatment for malaria. It was largely replaced in the mid-20th century by the synthetic analogue chloroquine. However, resistance to this drug emerged in 1957, and it is no longer of value in many areas of the world. Several other synthetic antimalarial agents have been based on the quinine pharmacophore, including mefloquine, primaquine, mepacrine, and amodiaquine (Table 1.2)<sup>54,57</sup>. These drugs are thought to act by interfering with the digestion of hemoglobin in the blood stages of the malaria life cycle. Complexation with heme and inhibition of hemozoin formation is the most accepted mechanism<sup>58,59</sup>. Although the mechanism of action (MoA) of piperaquine is not completely understood, studies have suggested that it acts by accumulating in the digestive vacuole and inhibiting heme detoxification through the binding of heme-containing species<sup>52</sup>.

#### **1.5.4.2. Artemisinin and its analogues**

The current antimalarial drug of choice artemisinin (Qinghaosu, IV) was originally obtained from the leaves of *Artemisia annua* L. (Asteraceae) in the 1970s. The plant Qinghao has been used as a traditional medicine in China for the treatment of fever for about 2000 years<sup>57,60</sup>. The active metabolite (artemisinin) isolated from the plant is a sesquiterpene lactone with an endoperoxide bridge, which is necessary for antimalarial activity during multiple stages of parasite development (Table 1.2)<sup>61</sup>. This compound is known to be particularly active *in vivo* against chloroquine-resistant *P. falciparum* and is reported to have relatively low toxicity. Several derivatives of artemisinin have been prepared in attempts to improve its activity and utility. The analogues dihydroartemisinin, artemether, artemotil and artesunate have all found clinical use, while the fully synthetic arterolane and various oxime and other dimers have promising activities<sup>57,61,62</sup>. Owing to its rapid action and high effectiveness against malaria, the combination of artemisinin derivatives and other antimalarial drugs, so-called artemisinin-based combination therapies (ACTs), has been recommended as the first-line

treatments against malaria since 2006<sup>61</sup>. The mechanism of action (MoA) through which artemisinin acts has been widely debated. The most accepted theory is that the molecule is activated by heme to generate free radicals, which in turn damage proteins required for parasite survival. Still, evidence for a number of other possible mechanisms have been found. Indeed, in 2013, a computational approach was taken to determine the MoA based around previous studies which identified haem and *PfATP6* (Ca<sup>2+</sup> transporter) as potential MoAs. In 2015, artemisinin was shown to be associated with the up-regulation of the unfolded protein response (UPR) pathways which may be linked to decreased parasite development. Another study showed that artemisinin is a potent inhibitor of *P. falciparum* phosphatidylinositol-3-kinase (*PfPI3K*)<sup>52,63</sup>.

#### **1.5.4.3. Quinones and quinolones.**

Lapachol firstly isolated from *Tabebuia avellanedae* was considered the compound that provided the clues for the latter development of naphthoquinones as antimalarials (Table 1.2). This prenyl naphthoquinone has been found in several families, such as Bignoniaceae, Verbenaceae, Proteaceae, Leguminosae, Sapotaceae, Scrophulariaceae and Malvaceae<sup>54</sup>. The interest in 2-hydroxy-3-alkyl-1,4-naphthoquinones as antimalarial agents commenced in the early 1940s as a result of intensive research programmes to find a replacement for quinine<sup>64</sup>. Amongst the various structural types evaluated, the hydroxyl naphthoquinone (hydrolapachol) was found to be active. Subsequently hundreds of analogues of this compound were synthesised and the 2-hydroxy-3-(9-hydroxy-9-pentyltetradecyl)1,4-naphthoquinone (lapinone) was found to be the most effective against *P. lophurae* in ducks<sup>54,64</sup>. It is important to emphasize that atovaquone currently marketed as antimalarial drug was discovered while exploiting the lateral chain of lapinone<sup>54</sup>. Atovaquone is a lipophilic hydroxynaphthoquinone which has broad-spectrum antiprotozoal activity. In combination with proguanil, atovaquone is currently used for the treatment and prevention of malaria<sup>65</sup>. Atovaquone is a competitive inhibitor of ubiquinol, specifically inhibiting the mitochondrial electron transport chain at the *bc<sub>1</sub>* complex. Inhibition of *bc<sub>1</sub>* activity results in a loss of mitochondrial function. During the intra-erythrocytic stage of infection, a key role of the parasite mitochondrion is to provide orotate for pyrimidine biosynthesis through the activity of dihydroorotate dehydrogenase (DHODH). Consistent with this, inhibition of the *bc<sub>1</sub>* complex by

atovaquone affects the concentrations of metabolites in the pyrimidine biosynthetic pathway<sup>66</sup>.

#### **1.5.4.4. Spiroindolones.**

Spirooxindole alkaloids initially isolated from plants of the Apocynaceae and Rubiaceae families were found to have a common scaffold, spiro[pyrrolidine-3,3'-oxindole]<sup>67</sup>. The *Uncaria* genus (Rubiaceae) is known as a prolific producer of the bioactive spirocyclic oxindole alkaloids<sup>68</sup>. In addition to the interesting molecular architecture and densely functionalized core, several natural products possessing this heterocyclic motif exhibit significant bioactivity<sup>69</sup>. The starting point of this antimalarial class of compounds (known as spiroindolones) was identified by Novartis through the screening of a small library of 12000 compounds from natural products and fully synthetic drugs with structural features found in natural products. An NCE named cipargamin (NITD609), inspired on a spirocyclic oxoindole scaffold, has recently entered in clinical trials (Table 1.2)<sup>54</sup>. Rosling et al (2018) have proved that cipargamin targets the *Plasmodium falciparum* P-type ATPase (*PfATP4*) by characterisation of the *PfATP4*-associated ATPase activity in membranes of blood-stage *P. falciparum* parasites<sup>70</sup>. The *PfATP4* is a plasma membrane P-type ATPase that has been proposed to function as a Na<sup>+</sup> eflux pump, extruding Na<sup>+</sup> ions from the parasite cytosol while importing H<sup>+</sup> ions. It is well known that *PfATP4* inhibitors cause swelling and lysis of infected erythrocytes, attributed to the accumulation of Na<sup>+</sup> inside the intracellular parasites.

#### **1.5.4.5. Further natural product scaffolds identified from medicinal plants with antimalarial activity**

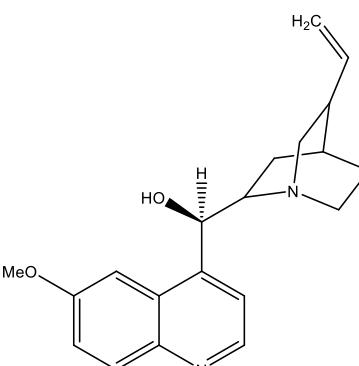
Traditional medicinal plants have provided the sources of several antimalarial drugs currently in use, and many researchers are screening plants for novel chemical entities to develop them as “lead compounds” for new anti-malarial drugs. However, conventional drug development is slow and expensive, taking up to 15 years and up to \$800m to develop a new drug. Furthermore, the finished products are often unavailable and unaffordable to the poorest patients in remote areas, unless they are part of a heavily subsidized schemes. To overcome this obstacle, parallel development of standardized,

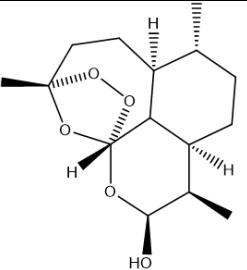
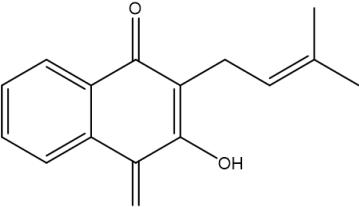
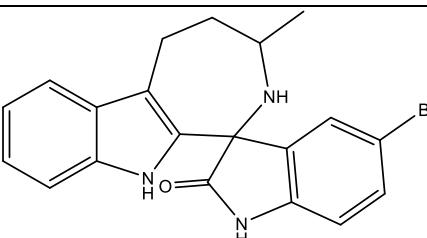
safe, effective and low-cost herbal phytomedicines has been undertaken in areas where traditional medicine is widely used. For instance, *Argemone mexicana*, from the Papaveraceae family, has been recognized as a phytomedicine for home-based malaria treatment. The decoction of *A. mexicana* has been shown to be effective to treat non-severe *Plasmodium falciparum* cases of malaria in children from Mali, with minor side effects. These effects were related to the presence of benzylisoquinoline alkaloids berberine, protopine, and allocryptopine<sup>54</sup>. Indeed, berberine inhibits *in vitro* nucleic acid and protein synthesis, as well as telomerase activity in *P. falciformis*. Structure-function analyses have revealed that the antiplasmoidal activity of berberine is mainly related to the presence of the quaternary nitrogen and the oxygen functions. However, it was shown recently that berberine has a low bioavailability, suggesting that other alkaloids, such as protopine and allocryptopine, both of the protopine-type, may account for such effects<sup>71</sup>. Species of Simaroubaceae are used for the treatment of malaria *in vivo*. Antimalarial activity has been demonstrated for a number of quassinoids. In fact bruceantin, simalikalactone D, glaucarubinone, soularubinone and sergeolide have been demonstrated to be active against *P. falciparum* *in vitro*. Sergeolide reduced virulence in *P. berghei*-infected mice when administered subcutaneously at 0.26 mg/kg/day but its high toxicity, with a LD<sub>50</sub> of 1.8 mg/kg, is indicative of its unsuitability for the curative treatment of malaria<sup>62</sup>. *Nauclea pobeguinii* is a plant traditionally used in Africa for the management of malaria, which has been the subject of clinical trials. Daily oral administration of the 80% EtOH extract of *Nauclea pobeguinii*, at 300 mg/kg, resulted in 86% reduction of parasitaemia in the 4-day *P. berghei* mouse model, and 75% reduction in the *P. yoelii* N67 model. Strictosamide, the putative active constituent, may be metabolically activated in the gastrointestinal tract after oral administration<sup>54,72</sup>.

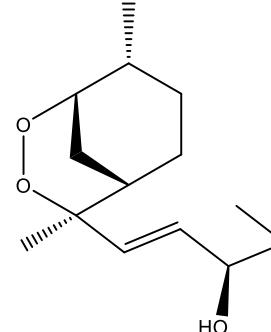
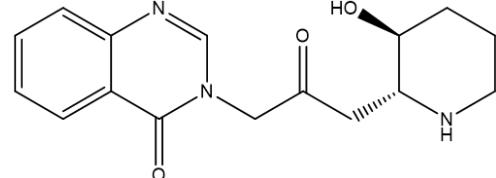
Yingzhaosu isolated from *Artabotrys uncinatus* showed efficacy in *P. berghei*-infected mice comparable to that of mefloquine or quinine but was 10-fold less potent than artemisinin. Yingzhaosu was studied as a potentially more stable artemisinin-like antimalarial scaffold. As a result of a structure–activity study published in 1994, artelene was identified as a compound with improved efficacy and reduced toxicity. Artelene reached clinical trials; however, when compared with mefloquine, it did not present advantages in a single dose monotherapy treatment<sup>54,73</sup>.

Another species used as an antimalarial drug in Chinese traditional medicine is *Dichroea febrifuga* (Saxifragaceae). The active principle, febrifugine, has been used clinically against *P. vivax* and *P. ovale* but its liver toxicity makes it unacceptable as a useful antimalarial drug<sup>74</sup>. Because the high antimalarial activity was accompanied by gastrointestinal toxicity associated with, e.g., diarrhea, vomiting, and liver toxicity, the structure of febrifugine was used as a lead compound in the synthesis of some active molecules with lower toxicity<sup>75</sup>. In this regard several febrifugine analogs have been synthesised and halofuginone was among the most active compounds against *P. falciparum* growth *in vitro*, and displayed curative effects in *P. berghei*-infected mice. Two modes of halofuginone actions have been described: (1) Inhibition of Smad3 phosphorylation downstream of the TGFβ signaling pathway results in inhibition of fibroblasts-to-myofibroblasts transition and fibrosis. (2) Inhibition of prolyl-tRNA synthetase (ProRS) activity in the blood stage of malaria and inhibition of Th17 cell differentiation thereby inhibiting inflammation and the autoimmune reaction by activation of the amino acid starvation and integrated stress responses<sup>76</sup>.

**Table 1.2.** Some clinically used antimalarial drug molecules derived from plant products<sup>54,77</sup>

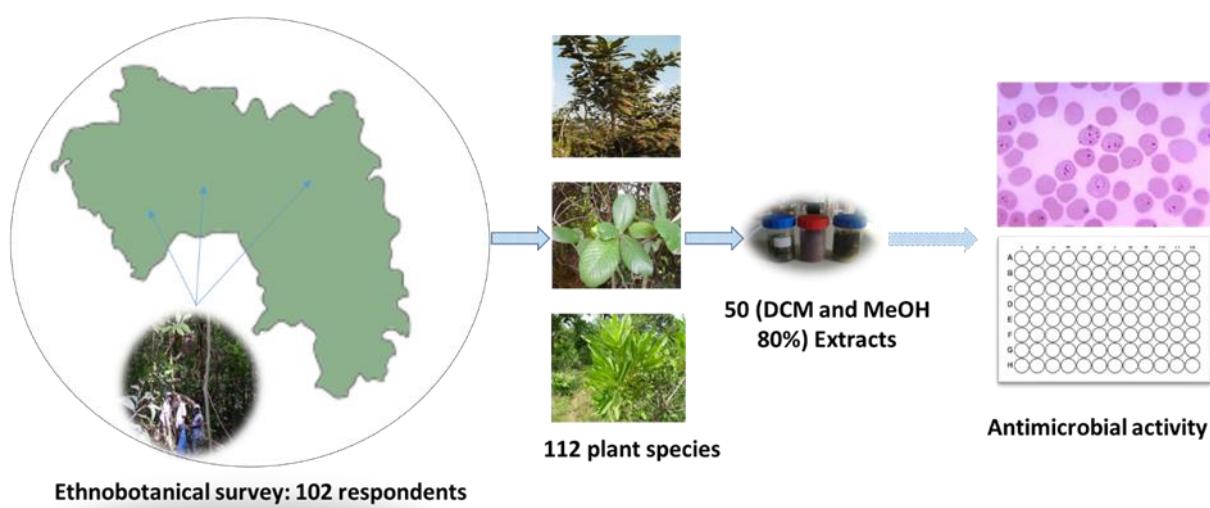
Natural Product	Molecules derived	Mechanism of action	Source
 <p><b>Quinine</b></p>	Chloroquine AQ13 Amodiaquine Isoquine Fluoroamodiaquine Ferroquine Piperaquine Mefloquine Pyronaridine (azaacridine quinoline relative)	Inhibition of hemozoin formation	<i>Cinchona Sp.</i>
	Pamaquine Bulaquine Primaquine Tafenoquine	Mitochondrial damage potentially due to generation of redox active intermediates	

 <b>Artemisinin</b>	Dihydroartemisinin Artemether Arteether Artesunate Artelinate OZ277 OZ439	Fe (II) catalysed free radical alkylation of heme and proteins	<i>Artemisia annua</i> L.
 <b>Lapachol</b>	Atovaquone ELQ300 SL-2-25	Inhibition of the cytochrome <i>bc</i> 1 complex within mitochondrial electron transport chain	<i>Tabebuia avellanedae</i> Lorentz ex Griseb.
 <b>Cipargamin</b>	KAE609 (NITD609)	Inhibition of PfATP4	Natural product inspired library

 <p><b>Yingzhaosu</b></p>	Arteflene	Presumed to be similar to artemisinin	<i>Artabotrys uncinatus</i> (Lam.) Merr.
 <p><b>Febrifugine</b></p>	Halofuginone	Targeting the cytoplasmic prolyl-tRNA (transfer RNA) synthetase (PfcPRS)	<i>Dichroa febrifuga</i> Lour.

## 1.6. Aim of the present study

In Guinea, microbial infections including malaria appear among the main causes of morbidity and mortality of the population. For social, economic and cultural reasons an important fringe of the affected people use medicinal plants to face these pathologies. As part of a valorization program of these plant species, ethnopharmacological investigations have been carried out and plants species employed for the treatment of malaria, skin diseases, oral diseases and urinary disorders were inventoried (Figure 1.3). An extensive bibliographic review, followed by a preliminary biological screening resulted in the selection of some promising plant species including *Terminalia albida*, *Tetracera alnifolia*, *Combretum paniculatum* and *Pavetta crassipes*. In the present research, we propose to deepen the biological and phytochemical investigations on some promising plants extracts, through the evaluation of their potential antimicrobial and antiplasmodial properties, and the corresponding active constituents.



**Figure 1.3.** Plant selection strategy (map of Guinea showing the sampling area and selected plant species)

***Terminalia albida* Scott-Elliott. (Combretaceae),** distributed throughout the Guinean regions, is widely used in traditional medicine to treat a variety of ailments such as malaria, skin diseases, oral diseases and urinary disorders<sup>7,10,78</sup>. It has been demonstrated

that the methanolic extract of the stem bark of *T. albida* considerably increased the survival rate in mice infected with *Plasmodium berghei*<sup>79</sup>. Although promising *in vitro* activities against *Candida albicans*, *Staphylococcus aureus* and *Plasmodium falciparum* have been obtained for methanolic and dichloromethane extracts from the root of *T. albida*, none of the compounds responsible for these activities have been isolated or identified. For this reason, *T. albida* was selected for this study.

***Tetracera alnifolia* Wild. (Dilleniaceae)**, a forest liana up to 15 m high, is widely used in Guinean traditional medicine and other African countries for the management of several diseases including skin diseases, oral diseases, infectious diseases and malaria, cough, sexually transmitted infections and leprosy<sup>78,80,81</sup>. A preliminary pharmacological study revealed that the hydroethanolic leaf extracts of the *T. alnifolia* possessed significant anti-inflammatory and analgesic activities. Some metabolites including flavonoids, saponins, terpenoids and tannins have previously been detected in the leaves extracts<sup>82,80</sup>. Promising *in vitro* activities against *Candida albicans*, *Staphylococcus aureus* and *Plasmodium falciparum* were obtained for both methanolic and dichloromethane extracts of the leaves of *T. alnifolia*<sup>78</sup>. Based on these promising results, *T. alnifolia* was selected in this study.

***Combretum paniculatum* Vent. (Combretaceae)**, a scandent shrub or robust liana with vivid scarlet flowers attaining 15 m length is widespread in tropical Africa. This species is well known in Guinean traditional medicine for the treatment of several ailments including malaria, skin diseases and oral diseases<sup>78,10,7</sup>. Previous studies have reported the antimicrobial activities of *C. paniculatum* against ringworm and significant cytotoxicity against breast cancer cells<sup>83,84</sup>. Significant antiplasmodial activity was found for both the dichloromethane and methanolic extracts of *C. paniculatum*<sup>78</sup>. Based on these preliminary results, *C. paniculatum* was selected for further research in this study.

The research accomplished in this project may be divided in three main parts.

- a) The first part of this study concerns the selection of promising plants species by using an approach combining an ethnobotanical survey, bibliographic review and biological screening.

- b) The second part deals with the isolation and identification of chemical constituents from selected plant species by using the bioassay-guided fractionation approach.
- c) The third part of this research concerns dereplication and targeted isolation of promising compounds. Comprehensive LC-MS methodology dereplication combined with regression analysis and bioactivity were applied for the rapid isolation of target compounds

### **1.7. Thesis overview**

This thesis is divided into 7 chapters. The first part of the study covers a general introduction (**Chapter 1**), in which the role of ethnopharmacology and herbal medicines in drug discovery is described. In addition, the contribution of plants in the fight against malaria and bacterial and fungal infections has also been explained.

**Chapter 2** concerns the materials, reagents and methods used to carry out this study.

**Chapter 3** summarizes the plant selection strategy, and describes the ethnobotanical investigations followed by bibliographic review, and a preliminary biological screening of selected plants species.

In **Chapter 4** the bioassay-guided isolation of antiplasmodial and antimicrobial constituents from *Terminalia albida* is described. Fractionation and isolation of compounds was carried out by flash chromatography and semi-preparative HPLC-DAD-MS. Compounds were identified by NMR spectroscopic and HRMS analysis. In this section several compounds responsible as least in part for the antiplasmodial activity have been identified.

**Chapter 5** deals with the dereplication of compounds present in the *n*-butanol fraction of *Terminalia albida* and the isolation of some target compounds. The dereplication step was performed by using *in silico* MS/MS analysis and molecular networking. Promising compounds isolated by preparative HPLC-DAD-MS were evaluated against *Plasmodium falciparum*.

**Chapter 6** describes the bioassay-guided isolation of antiplasmodial and antimicrobial constituents from *Tetracera alnifolia*. Purification and isolation of compounds was carried

out by column chromatography, semi-preparative HPLC-DAD-MS and LC-SPE-NMR. Compounds were identified by NMR spectroscopic and HRMS analysis. The antiplasmodial and antimicrobial activities of fractions and isolated compounds are also described in this section.

**Chapter 7** is dedicated to the characterization of potential antiplasmodial compounds from the leaves of *Combretum paniculatum*. In this section, an approach integrating dereplication, regression analysis and bioactivity was carried out for the rapid characterization of promising antiplasmodial constituents. This step provided a better understanding on the metabolites responsible for the antiplasmodial activity of this plant species.

**In Chapter 8** the general conclusions and future perspectives are reported.

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## **CHAPTER 2**

# **General experimental procedures**



## **2.1. Solvents and reagents**

Dichloromethane, ethyl acetate, *n*-butanol, *n*-hexane, acetone, acetonitrile, acetonitrile far UV, isopropyl alcohol and methanol (all HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK). All reagents including ammonia (25%), dimethyl sulfoxide (DMSO), formic acid (eluent additive for HPLC) hydrochloric acid (25%) and sulphuric acid were purchased from Acros Organics (Geel, Belgium) or Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and formic acid (both for UPLC-MS) were obtained from Biosolve Chimie (Dieuze, France). Ultrapure water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA). NMR deuterated solvents ( $\text{CDCl}_3$ , acetonitrile- $d_3$ , methanol- $d_4$ , DMSO- $d_6$ , pyridine- $d_5$ , and  $\text{D}_2\text{O}$ ), as well as the reference compound ellagic acid, were obtained from Sigma-Aldrich.

## **2.2. Chromatographic methods**

### **2.2.1. Thin-layer chromatography**

Analytical and preparative plates for thin-layer chromatography (TLC) were purchased from Merck (Darmstadt, Germany). Silica gel 60 F254 plates (20 x 20 cm) were used for normal phase (NP) TLC. Samples were applied on TLC plates and developed in a chromatographic chambers previously saturated with mobile phase. Afterwards, plates were visualized using a ultraviolet (UV) lamp at 254 nm and 365 nm. Preparative thin layer chromatography was performed using TLC Silica gel 60 F254 (20 x 20 cm, layer thickness 1 mm). The fractions containing the products of interest are applied horizontally on the plates and placed in a developing chamber. Pure compounds are recovered by desorption using an appropriate solvent. Vanillin-sulphuric acid reagent used for spraying the TLC plates was prepared by mixing 1 g of vanillin, 5 mL of sulphuric acid and 95 mL of ethanol.

## **2.2.2. Flash chromatography**

Flash chromatography was carried out on a Reveleris® iES system from Grace (Columbia, MD, USA) using the Reveleris® Navigator™ software. The system is comprised of a binary pump with four solvent selections, an ultraviolet (UV) detector, an evaporating light scattering detector (ELSD) and a fraction collector. The ELSD carrier solvent was isopropyl alcohol. The columns used were pre-packed Flash Grace Reveleris® silica cartridges with a particle size of 40 µm and 25 µm. For polar fractions, GraceResolv (12 g and 40 g) Rp18 columns were used. The column size was chosen according to the weight of the sample to be purified. Samples were injected in dry or liquid form. Detection and collection were based on UV and ELSD. Eluate was collected in two trays of 96 tubes.

## **2.2.3. High-performance liquid chromatography**

High-performance liquid chromatography (HPLC) analyses were carried out on an Agilent® 1200 series HPLC system, with degasser, quaternary pump, autosampler, thermostatic column compartment and diode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA). A silica based Luna C18 (2) (250 x 4.6 mm, 5 µm) from Phenomenex (Torrence, CA, USA) and a Kinetex column C18 (100 mm × 2.10 mm, 2.6 µm) from Phenomenex (Torrance, CA, USA) were used to obtain separation.

## **2.2.4. Semi-preparative high-performance liquid chromatography**

A semi-preparative LC-MS system was used for the isolation of compounds, and was comprised of a binary sample manager, injector and collector, a quaternary gradient module, a system fluidics organizer, an HPLC-pump, a DAD and a Micromass Quattro mass spectrometer with triple quadrupole detector (TQD), all supplied by Waters® (Milford, MA, USA). The HPLC was equipped with a Kinetex® C18 or A Luna® C18 (2) 100 Å column (250 x 10 mm, 5 µm). The DAD spectrum was recorded from 200 to 450 nm, and mass spectra in ESI (+) and ESI (-) mode, MS scan range:  $m/z$  100 to 1000; the capillary voltage 3.00 kV, cone voltage 50 V, extractor voltage 3 V, source temperature 135 °C, desolvation temperature 400 °C, desolvation gas flow 750 L/h, cone gas flow 50 L/h. MassLynx version 4.1 was used to process the data.

## **2.2.5. High-performance liquid chromatography solid phase extraction nuclear magnetic resonance spectroscopy**

Liquid chromatography – solid-phase extraction – nuclear magnetic resonance spectroscopy (LC-SPE-NMR) is a hyphenated technique, which combines a chromatographic (HPLC) system with an SPE system and a spectroscopic NMR system. Our analyses has been carried out on a HPLCPDA-SPE-NMR system, consisting of an Agilent® 1200 series HPLC system with an in-line solvent degasser, quaternary pump, autosampler, column compartment, and a diode-array detector (Agilent Technologies, Santa Clara, CA, USA). The HPLC instrument is connected to a Bruker/Spark solid-phase extraction (SPE) system (Spark Holland, Emmen, The Netherlands) with HySphere Resin GP (general phase) cartridges (polydivinyl-benzene material with particle size 5–15 µm, dimensions 10 x 2 mm) for compound collection. After the detector, water was added to the eluent stream with a make-up pump using a flow rate of 3 mL/min (Knauer K 120, Berlin, Germany) in order to increase the retention of the analytes on the SPE cartridges using a Bruker / Spark SPE system, equipped with HySphere Resin General Phase (GP) cartridges. After collection, each cartridge was dried with pressurized nitrogen gas for 40 min and eluted with 60 µL of deuterated solvent (acetonitrile-d<sub>3</sub> or methanol-d<sub>4</sub>) into 3 mm NMR tubes with a Gilson Liquid Handler 125. Chromatographic separation and analyte trapping on SPE cartridges were controlled using Hystar ver. 3.2 software (Bruker Daltonik, Bremen, Germany), whilst the elution process was controlled by Prep Gilson ST ver. 1.2 software (Bruker Biospin, Karlsruhe, Germany).

## **2.3. Spectroscopic Methods**

### **2.3.1. Nuclear magnetic resonance spectroscopy**

NMR spectra were recorded on a Bruker DRX-400 instrument (Rheinstetten, Germany), equipped with either a 3 mm broadband inverse (BBI) probe or a 5 mm dual <sup>1</sup>H/<sup>13</sup>C probe, using standard Bruker pulse sequences and operating at 400 MHz for <sup>1</sup>H and at 100 MHz for <sup>13</sup>C. Distortionless enhancement by polarization transfer spectra (DEPT-135 and DEPT-90) were also recorded. The 2D NMR experiments including, homonuclear correlation (<sup>1</sup>H-<sup>1</sup>H) spectroscopy (COSY), heteronuclear (<sup>1</sup>H-<sup>13</sup>C) single quantum coherence (HSQC) and heteronuclear (<sup>1</sup>H-<sup>13</sup>C) multiple bond correlation (HMBC) NMR

experiments were recorded. Methanol-d<sub>4</sub> (99.8% D), and chloroform-d (99.8% D) acetonitrile-d<sub>3</sub>, methanol-d<sub>4</sub> (99.8% D), DMSO-d<sub>6</sub> (99.8% D), pyridine-d<sub>5</sub> (99.8% D), and D<sub>2</sub>O (99.8% D) were used as solvents. NMR data processing was performed with Topspin (version 4.0.6, Bruker Biospin).

### 2.3.2. Mass spectrometry

Mass measurements were done using a Xevo-G2XS-QTof mass spectrometer (Waters) coupled with an ACQUITY LC system equipped with MassLynx version 4.1 software. A Waters Acquity UHPLC BEH SHIELD RP18 column (2.1 mm x 100 mm, 1.7  $\mu$ m) was used with a mobile phase consisting of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), which were pumped at a rate of 0.4 mL/min. Three gradient systems have been used. Gradient system (1): 2% B (0-1 min), 2-100% B (1-5 min), 100% B (5-7 min), 100-2% B (7-8 min), 2% B (8-10 min). Gradient system (2): 2% B (0-1 min), 100% B (1-15 min), 100% B (15-16 min), 100-2% B (16-17 min), 2% B (17-20 min). Gradient system (1): 2% B (0-1 min), 2-26% B (1-14 min), 65 % B (14-24 min), 100% B (24-26 min), 100% B (26-29 min), 100-2% B (29-31 min), 2% B (31-36 min).

For all analyses, full scan data were recorded in ESI (-) and ESI (+) mode from *m/z* 50 to 1500 in sensitivity mode (approximate resolution: 22000 FWHM) using a spray voltage at either -1.0 kV and +1.5 kV, respectively. Cone gas flow and desolvation gas flow were set at 50.0 L/h and 1000.0 L/h, respectively; and source temperature and desolvation temperature at 120 °C and 550 °C, respectively. Leucine enkephalin was used as lock mass during the analysis.

### 2.3.3. Optical rotation

A Jasco P-2000 polarimeter equipped with a spectra Manager™ was used for the measurement of the optical rotation. Samples were dissolved in appropriate solvents including MeOH, DCM, DMSO. The optical rotation was measured at 589 nm with a path length of 50 mm and at 20°C (T).

## **2.4. Biological methods**

An integrated screening concept for anti-infective activity was applied, in which antibacterial, antifungal and antiparasitic ‘whole organism’ assays and cytotoxicity on MRC-5 cells (human lung fibroblasts) are performed in parallel. Standardization across the different bioassays maximizes efficiency, minimizes cost and allows easy and reproducible data acquisition. It included the use of standard (20 mM or 20 mg/ml) stock solutions in 100% DMSO; fixed concentrations in all screens (using 2- or 4-fold serial dilutions); standard lay-out of 96-well microplates to facilitate plate production and to minimize human errors during the bioassay, always including negative, positive and reference controls; spectrophotometric reading of endpoints, whenever possible; and standard templates (spreadsheet), allowing rapid result processing and reporting. Due to this standardised approach, in which serial dilutions are tested, reproducible results can be obtained, and for general large-scale screenings only single determinations are done.

### **2.4.1. Antiplasmodial activity**

Extracts, fractions and pure compounds were tested against *Plasmodium falciparum* K1 chloroquine resistant strain. Parasites were cultured in human erythrocytes at 37°C under a microaerophilic atmosphere maintained in continuous log phase growth in RPMI-1640 medium supplemented with 2% penicillin/streptomycin solution, 0.37 mM hypoxanthine, 25 mM HEPES (4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid), 25 mM NaHCO<sub>3</sub>, and 10% O+ human serum together with 4% human O+ erythrocytes. Cultures were maintained at 37 °C under a microaerophilic atmosphere (4% CO<sub>2</sub>, 3% O<sub>2</sub>, and 93% N<sub>2</sub>). Stock solutions of extracts and pure compounds were prepared in DMSO (20 mg/mL and 20 mM respectively) and diluted with culture medium before being added to asynchronous parasite cultures. The lactate dehydrogenase assay was used for the evaluation of the antiplasmodial activity. Indeed, the conversion of lactate into pyruvate, which is catalyzed by the parasitic lactate dehydrogenase (LDH) enzyme and the coenzyme 3-acetyl pyridine adenine dinucleotide (APAD), is measured. Assays were performed in 96-well tissue culture plates, each well containing 10 µL of the test solution (extracts or compounds), together with 190 µL of the parasite inoculum (1% parasitemia, 2% hematocrit). After 72 h of incubation at 37 °C, plates were stored at -20 °C until further processing. After thawing, 20 µL of hemolyzed parasite suspension from each well

was transferred into another plate together with 100 µL of Malstat reagent and 10 µL of a 1:1 mixture of phenazine ethosulfate (PES) (2 mg/mL) and nitroblue tetrazolium (NBT) (0.1 mg/mL). The plates were kept in the dark for 2 h, and the change in color was measured spectrophotometrically at 655 nm. The results were expressed as percentage reduction in parasitemia compared to control wells. IC<sub>50</sub> values were calculated from drug concentrationresponse curves. Chloroquine diphosphate was used as an antiplasmodial reference drug<sup>1,2,3</sup>.

#### **2.4.2. Cytotoxicity**

The cytotoxicity was determined on MRC-5 SV2 cells (human lung fibroblasts), which were cultured in MEM (minimum essential medium), supplemented with 20 mM L-glutamine, 16.5 mM NaHCO<sub>3</sub>, 5% fetal calf serum. Cultures were kept at 37 °C and 5% CO<sub>2</sub>. Assays were performed in sterile 96-well tissue culture plates, each well containing 10 µL of test solution containing the test compound, together with 190 µL of cell suspension ( $3 \times 10^4$  cells/well). After 72 hours of incubation, 50 µL resazurine was added to each well and 4 h later, proliferation/viability was assessed fluorimetrically ( $\lambda$  excitation 550 nm,  $\lambda$  emission 590 nm). The % reduction in cell viability compared to the untreated controls was calculated and IC<sub>50</sub> values were determined. Tamoxifen was used as reference drug<sup>1,2</sup>. The selectivity index (SI) corresponding to the ratio between the cytotoxic and antiparasitic activities of tested sample was calculated as follow: SI= CC<sub>50</sub> MRC-5 Cell/IC<sub>50</sub> *P. falciparum*.

#### **2.4.3. Antimicrobial activity**

Plant extracts, fractions and isolated compounds were tested against the following microorganisms: *Escherichia coli* ATCC8739 (Gram-negative), *Staphylococcus aureus* ATCC 6538 (Gram-positive).

Assays were performed in 96-well microtiter plates, each well containing 10 µL of test samples (extracts or pure compounds) together with 190 µL of inoculum ( $5 \times 10^3$  cfu/mL). After 17 h incubation, bacterial viability was evaluated fluorimetrically after addition of resazurin. The level of antimicrobial activity was arbitrarily ranked according to the following criteria: strong (IC<sub>50</sub> ≤ 10 µg/ml); good (10 µg/ml < IC<sub>50</sub> ≤ 20 µg/ml); moderate

(20 µg/ml < IC<sub>50</sub> ≤ 40 µg/ml); weak (40 µg/ml < IC<sub>50</sub> ≤ 64 µg/ml); inactive (IC<sub>50</sub> ≥ 64 µg/ml). The minimum inhibitory concentration (MIC) of the most active extracts has been determined. Doxycycline was used as reference drug for both *S. aureus* and *E. coli*<sup>1,4,5</sup>

#### 2.4.4. Antifungal activity

The antifungal activity of extracts and compounds was assessed against *Candida albicans* ATCC59630. The fungal strains were cultured in RPMI-1640 medium supplemented with Mops buffer and glucose at 37 °C. Assays were performed in 96-well microtiter plates, each well containing 10 µL of extract or pure compounds and 190 µL of fungal inoculum (5 x10<sup>3</sup> cfu/mL). Fungal viability was evaluated fluorimetrically after 24h incubation and addition addition of 10 µL resazurin per well ( $\lambda$  excitation 550 nm,  $\lambda$  emission 590 nm). The results are expressed as % reduction in fungal growth/viability compared to control wells and an IC<sub>50</sub> and MIC is determined. Flucytosine was used as reference drug<sup>1,4</sup>.

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## **CHAPTER 3**

# **Selection strategy of plant species: antimicrobial investigation of ethnobotanically selected Guinean plant species.**

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### **3.1. Introduction**

Infectious diseases are a significant burden to public health and economic stability of societies all over the world<sup>1</sup>. Worldwide, more than 400 million deaths caused by microbial pathologies are recorded every year, which is a higher burden than cancer or cardiovascular diseases<sup>2</sup>. Despite the considerable improvement in global health in the last century, millions of people still lack access to quality health services, including access to antimicrobial medicines, or are impoverished as a result of high health care expenses<sup>3</sup>. Furthermore, the occurrence of resistance has caused the currently available antimicrobial drugs to become less effective or even ineffective<sup>4</sup>. In general, it is observed that pathogenic viruses, bacteria, fungi, and protozoa are more and more difficult to treat with the existing drugs<sup>5</sup>. Indeed, many currently available antifungal and antibacterial agents have undesirable toxicity, and the widespread use of these drugs has led to a rapid development of drug-resistant strains, which are the leading cause of therapeutic failure<sup>6</sup>. To overcome the drawbacks of the current antimicrobial drugs and to obtain more efficacious drugs, an antimicrobial drug having a novel mode of action would ideally be developed<sup>7</sup>. Research on natural products has contributed significantly to the discovery of new lead compounds with antimicrobial activity. Indeed, in nature many different types of compounds with the potential to treat diseases, including infectious diseases, can be found. Among the different sources of natural compounds with valuable antimicrobial activity, medicinal plants have extensively been studied<sup>8</sup>

Many infectious diseases have emerged or reemerged in Africa in the 21st century, caused by known or newly discovered microorganisms<sup>9,10</sup>. In Guinea, microbial infections are one of the main causes of morbidity and mortality. Like in many developing countries, the Guinean population, particularly in rural areas, still relies on traditional medicines for the treatment of the most common pathologies such as skin diseases, urinary disorders and oral diseases. Despite the widespread use of medicinal plants, very little ethnobotanical, phytochemical and biological information is available on the plants traditionally used for the management of these diseases.

This present study was carried out in order to collect information on which medicinal plants are used in Guinea for the management of infectious diseases including urinary

disorders, skin diseases and oral diseases and to investigate their *in vitro* antimicrobial activity.

### **3.2. Materials and methods**

#### **3.2.1. Ethnobotanical investigation**

An ethnobotanical investigation was carried out in three Guinean regions (Lower Guinea, Middle Guinea and Upper Guinea) in the period 2015-2016. The first step of our survey was conducted between November and February 2015 and the second step between June and September 2016. These regions are quite distinct and heterogeneous from a geo-ecological point of view: Low Guinea, area of littoral plains; Middle Guinea, with mountainous solid masses and lateritic high plateaus and Upper Guinea, a vast plateau. The global population was estimated at 12,771,246 habitants in 2019, with an average density of 51.91 inhabitants per km<sup>2</sup>. The investigated areas are characterized by a diversity of ethnic groups and languages. The main ethnic groups are Soussous (Low Guinea), Peuhls (Middle Guinea) and Malinkees (Upper Guinea). One hundred and two participants, including 74 traditional healers (50 males and 24 females) and 28 herbalists (20 males and 8 females) were interviewed in the study viz. 37 (36.3%) from Lower Guinea (22 in Kindia, 15 in Télimélé), 36 (35.3%) from Middle Guinea (19 in Labé, 17 in Pita), 29 (28.4%) from Upper Guinea (16 in Kankan and 13 in Siguiri). Each interview was conducted according to a semi-structured questionnaire (in French; added as annex) designed to obtain the following information: local names of the plant species, causes and symptoms of diseases, plant parts used in the preparation of remedies, mode of preparation and mode of administration. Plant materials, also including specimens of the cited plants, were collected during the survey. The samples from each plant species were pressed, dried and mounted on herbarium sheets for identification. The plant species were botanically identified by the botanists from the Research and Valorization Center on Medicinal Plants of Dubreka, "CRVPM – Dubreka". The most cited plants were subjected to a complete bibliographic review, searching scientific databases (Scifinder, PubMed, Web of Science), thus covering international peer-reviewed journals. As search terms, the scientific plant name was used, as such and in combination with "antimicrobial" or "antimalarial".



**Figure 3.1.** map of Guinea showing the investigated areas

### 3.2.2. Preparation of plant extracts

The plant parts (leaves, stem bark and root) were collected separately during the survey. The collected samples were shade dried, powdered and successively extracted with dichloromethane and 80% methanol at room temperature. To 20 g of powdered dried plant material 150 ml of dichloromethane was added; the mixture was stirred for 24 h, and this action was carried out 3 times. After filtration, 150 ml of 80% methanol was added to the residual plant material and stirred for 24 h; again this action was carried out 3 times. The extracts were filtered and dried under reduced pressure, at 40 °C. A total of 17 leaf, 6 stem bark and 2 root samples were extracted in this way, each time yielding an apolar (dichloromethane) and a polar (80% methanol) extract, i.e. a total of 50 samples (Table 2). Aliquots of 10 mg were weighed and submitted for biological testing.

### **3.2.3. Biological evaluation: antimicrobial activity and cytotoxicity**

The antimicrobial activity of extracts from the most cited plant species ( $\geq 10$  mentions) was evaluated according to Cos et al. (2006) and Baldé et al. (2010). Briefly, an integrated screening concept for anti-infective activity was applied, in which antibacterial, antifungal and antiparasitic ‘whole organism’ assays and cytotoxicity on MRC-5 cells (human lung fibroblasts) are performed in parallel. Standardization across the different bioassays maximizes efficiency, minimizes cost and allows easy and reproducible data acquisition. It included the use of standard (20 mM or 20 mg/ml) stock solutions in 100% DMSO; fixed concentrations in all screens (using 2- or 4-fold serial dilutions); standard lay-out of 96-well microplates to facilitate plate production and to minimize human errors during the bioassay, always including negative, positive and reference controls; spectrophotometric reading of endpoints, whenever possible; and standard templates (spreadsheet), allowing rapid result processing and reporting. Due to this standardised approach, in which serial dilutions are tested, reproducible results can be obtained, and for general large-scale screenings only single determinations are done. Plant extracts were tested against the following microorganisms: *Escherichia coli* ATCC8739 (Gram-negative), *Staphylococcus aureus* ATCC 6538 (Gram-positive) and *Candida albicans* ATCC59630 (yeast)<sup>11,12</sup>. The level of antimicrobial activity was arbitrarily ranked according to the following criteria: strong ( $IC_{50} \leq 10 \mu\text{g}/\text{ml}$ ); good ( $10 \mu\text{g}/\text{ml} < IC_{50} \leq 20 \mu\text{g}/\text{ml}$ ); moderate ( $20 \mu\text{g}/\text{ml} < IC_{50} \leq 40 \mu\text{g}/\text{ml}$ ); weak ( $40 \mu\text{g}/\text{ml} < IC_{50} \leq 64 \mu\text{g}/\text{ml}$ ); inactive ( $IC_{50} \geq 64 \mu\text{g}/\text{ml}$ )<sup>13</sup>. The minimum inhibitory concentration (MIC) of the most active extracts has been determined. The following positive controls were used: flucytosine for *C. albicans* ( $IC_{50} 0.52 \mu\text{M}$ ) and doxycycline for *S. aureus* ( $IC_{50} 0.09 \mu\text{M}$ ) and *E. coli* ( $IC_{50} 0.61 \mu\text{M}$ ). Antiplasmodial activity and cytotoxicity were assessed as previously described by Cos et al. (2006) and Tuenter et al. (2016). Extracts were tested *in vitro* against the chloroquine-resistant strain *Plasmodium falciparum* K1 using the lactate dehydrogenase assay. Tamoxifen was used as the positive control for cytotoxicity on MRC-5 cells ( $IC_{50} 10.50 \mu\text{M}$ ), and chloroquine ( $IC_{50} 0.20 \mu\text{M}$ ) for *P. falciparum*. All reference compounds are routinely used in the screening platform and their activities were in the range that is usually observed<sup>11,14</sup>.

### 3.3. Results and discussion

#### 3.3.1. Ethnobotanical survey

One hundred and twelve plant species belonging to 102 genera distributed in 41 botanical families were inventoried in this study (Table 1). The most represented families were Leguminosae with (18 species), Apocynaceae, Combretaceae, Euphorbiaceae, Malvaceae and Rubiaceae (7 species each), Annonaceae (5 species), Lamiaceae, Meliaceae and Moraceae (4 species each), and Anacardiaceae, Compositea and Phyllantaceae (3 species each). The most frequently used plants were *Terminalia albida* (16 mentions), *Combretum paniculatum* (15), *Pavetta crassipes* (15), *Landolphia heudelotii* (14), *Swartzia madagascariensis* (14), *Daniellia oliveri* (13), *Lannea velutina* (13), *Tetracera alnifolia* (13), *Entada africana* (13), *Hannoia undulata* (12), *Opilia celtidifolia* (12), *Combretum glutinosum* (11), *Leucas martinicensis* (11), *Gardenia ternifolia* (11), *Landolphia dulcis* (11), *Morinda morindoides* (10), *Pericopsis laxiflora* (10) and *Sterculia tragacantha* (10). Some of these plants, such as *D. oliveri*, *E. africana*, *L. heudelotii*, *L. dulcis*, *G. ternifolia*, *S. madagascariensis*, *S. tragacantha*, *T. alnifolia*, *P. crassipes*, *G. ternifolia* and *T. albida* have already been reported to treat infectious diseases, including sexually transmitted diseases in Guinean traditional medicine<sup>15,16</sup>, and skin diseases<sup>17</sup>.

Apart from Guinea, the most-cited plant species in this study are also used in the traditional medicine in other countries for the management of skin disease, oral diseases, urinary disorders and/or symptoms of infectious diseases, like fever. Indeed, in some West-African countries, *Combretum paniculatum* and *Swartzia madagascariensis* have been used over the years to treat bacterial diseases<sup>18</sup>. *Sterculia tragacantha* is used to treat syphilis, gonorrhea, fever, boils, gout and as anti-inflammatory agent<sup>19</sup>. The whole plant of *L. martinicensis* is used in Burkina Faso to treat wounds, fever and insect bites<sup>20</sup>. The decoction of the leaves is also used to treat fevers, kidney disorders and inflammatory diseases<sup>21</sup>. The leaves of *P. crassipes* are used in Tanzania in the treatment of gonorrhea<sup>22</sup>. The boiled leaf powder is used to treat hematuria and fevers<sup>23</sup>. In Mali, the leaves of *O. celtidifolia* are used for the management of skin diseases (dermatitis) and malaria. The decoction of the leaves is also used against fever in Ivory Coast and against dental abscesses in Senegal. Moreover, the plant is used orally and externally against leprosy<sup>24</sup>. *L. velutina* is used by Malian traditional healers to treat numerous ailments such as

wounds, sexually transmitted diseases, insect bites and snake bites<sup>25</sup>. This high consensus among users from different countries proves that these medicinal plants play an important role in the management of infectious pathologies and could possibly be an indication of their effectiveness.

The plant parts used for the preparation of the herbal medicinal drugs consisted of leaves, stem bark, root, fruit, seeds, gum and the whole plant. The leaves were the most frequently used part (74 species), followed by stem bark (35 species), roots (33 species), seed (7 species), whole plant (4 species), gum (3 species) and rhizome (1 species). The main methods used for preparing the recipes were decoction (66.0%), followed by maceration (14.7%), ointment (10.5%), infusion (8.9%) and oil (1.6%). The diagnosis of diseases by traditional healers was mainly based on interviewing patients and visual observation of the symptoms of each disease. Causes of skin diseases were attributed to poor hygiene, blood impurity, witchcraft or spells. Most of the traditional healers have stated that urinary problems are caused by adultery, aging, or blood impurity.

A total of 56 (56/112) plant species were used to treat skin diseases, including scabies, boils, impetigo, wounds, chicken pox and eczema. Five skin diseases were not clearly identified. Herbal preparations are administered orally, topically, or by having a bath. In most cases, the oral route is combined with external application of the remedies. Various ingredients, including palm oil, palm kernel oil, shea butter, jathropha gum, carapa oil and lophira oil were used for the preparation of the ointments.

A total 58 (58/112) plant species were used to treat urinary disorders, including gonorrhea, "souhaï souhaï" (urinary incontinence), "dambou" (urinary retention), or inflammation of the penis. Most of the preparations used for the management of urinary disorders were administered orally. The dose and the duration of treatment depended mainly on the severity of the diseases and could last from 3 days to one month.

Thirty eight (38/112) plant species were employed to treat oral diseases including "fanikron" (oral candidiasis), "moussou gnighè" (toothache), "Dawouran" (stomatitis), "Dassouma" (bad breath), "Tamadi" (gingivitis), or "Baa" (inflammation of the gum),

which is characterized by the appearance of fever, headache, pain and sores on the affected parts.

Fifty plant species cited in our study were previously reported to be used in the management of malaria and fever in Guinean traditional medicine. Among these plants species, nine were the most cited in our study. It has been reported that the same plant species or recipe could be frequently and indistinctly used for the traditional treatment of various diseases such as malaria, bacterial and viral infections<sup>13</sup>. Based on this observation, the antiplasmodial activity of our plant extracts was evaluated as well.

### **3.3.2. Antimicrobial activity**

#### **3.3.2.1. Antibacterial activity**

Fifty extracts from 18 plant species belonging to 10 botanical families were tested for antimicrobial activity (Table 2). Eight of the tested plant species showed antibacterial activity against *S. aureus* ( $IC_{50} < 64 \mu\text{g/ml}$ ). The highest activities were obtained for the dichloromethane extracts of the leaves of *P. crassipes* ( $IC_{50} : 8.5 \mu\text{g/ml}$ ), the root bark of *S. madagascariensis* ( $IC_{50} : 12.8 \mu\text{g/ml}$ ) and the methanolic extract of the root bark of *T. albida* ( $16.8 \mu\text{g/ml}$ ). The dichloromethane extracts of the leaves of *C. paniculatum*, *C. glutinosum* and *T. alnifolia* and the methanolic extract of the stem bark of *T. albida* demonstrated moderate activity against *S. aureus* ( $IC_{50} : 24.2, 24.5, 31.2$  and  $33.4 \mu\text{g/ml}$ , respectively), while the antibacterial activities of the methanolic extract of the stem bark of *E. africana*, the leaves of *L. heudelotii* and the dichloromethane extract of the stem bark of *T. albida* ( $IC_{50} : 48.9, 51.8$  and  $57.7 \mu\text{g/ml}$  respectively) were relatively weak. None of the tested extracts were active against *E. coli*.

Our results are in agreement with various other studies carried out on medicinal plants demonstrating greater activity of the plant extracts towards Gram-positive bacteria, like *S. aureus*, compared to Gram-negative bacteria, like *E. coli*<sup>13,26,27</sup>. Gram-negative bacteria are generally more difficult to inhibit, due to the presence of an outer membrane that tends to prevent the entry of inhibitors<sup>28</sup>.

The antibacterial activity of some of these plant species has previously been studied. Among these, *C. glutinosum* leaves extracts demonstrated antibacterial activity against several microorganisms such as *S. aureus* (MIC: 125 µg/ml), *Enterococcus faecalis* (MIC: 250 µg/ml), *P. aeruginosa* and *E. coli* ( MIC: 500 µg/ml)<sup>29,30</sup>.

Four triterpenoids (betulinol, betulonal, betulonic acid and cabraleone) were isolated from the hexane and dichloromethane extracts of the leaves<sup>31</sup>. Two of these compounds (betulonic acid and cabraleone) demonstrated significant analgesic and anti-inflammatory activity<sup>32</sup>. Moreover, betulin and its semisynthetic derivatives such as 28-O-(*N*-acetylanthraniloyl) betulin have been found to possess considerable antibacterial activity against , *S. aureus* and *E. faecalis* (MIC<sub>90</sub> of 6.25 µM)<sup>33</sup>.

In our study, *S. madagascariensis* has mainly been cited for the treatment of oral diseases. Previous reports indicated that the leaves of *S. madagascariensis* are widely used in northern Nigeria for the treatment of infectious diseases, like cutaneous wounds, scabies and venereal diseases<sup>34,15</sup>. The root of *S. madagascariensis* is used in combination with *Isoberlinia doka*, *Annona senegalensis*, *Gardenia ternifolia*, *Terminalia glaucescens* and *Erythrina senegalensis* in the management of sexually transmitted diseases in Guinean traditional medicine. Moreover, this association has shown significant antibacterial activity against *S. aureus*<sup>15</sup>. On the other hand, the aqueous extracts of the leaves of *S. madagascariensis* exhibited antibacterial activity against many bacteria such as *Shigella* spp., *Salmonella typhi* and *E. coli*<sup>35</sup>. Tannins may have contributed in part to the antimicrobial activities of the extracts against *E. coli*, *Salmonella* spp and *Shigella* spp. as noted in previous studies<sup>36,37</sup>. Several compounds such as swartziadione, isolated from the root, showed good antimicrobial activity against *Aspergillus* and *Staphylococcus* <sup>38</sup>.

Among the extracts tested in our study, the dichloromethane extract from *Pavetta crassipes* leaves was the most active against *S. aureus* (MIC 64 µg/ml). Nonetheless, this activity is lower than that previously found for the alkaloid fraction of the leaves which showed moderate activity against *S. aureus* (MIC range of 4.7 to 35.0 µg/ml) and *E. coli* (MIC of 26.3 µg/ml)<sup>12</sup>. A precipitate obtained from the aqueous extact of *P. crassipes* leaves inhibited the growths of *Escherichia. coli*, *Corynebacterium ulcerans*, and *Pseudomonas aeruginosa* at 6.2 mg/mL. It also inhibited *Streptococcus pyogenes*, *Klebsiella pneumoniae*

and *Neisseria gonorrhoea* at 12.5 mg/mL<sup>22</sup>. The antimicrobial activity of *P. crassipes* could in all likelihood be attributed to the presence of secondary metabolites such as alkaloids, flavonoids and tannins. Indeed, the polyphenolic compounds 5-O-caffeoylequinic acid methyl ester and quercetin-3-O-rutinoside (rutin) were isolated from the leaves<sup>22,39</sup>. Although rutin tested alone did not inhibit the growth of a wide range of bacteria such as *S. aureus*, *E. coli*, *S. pyogenes*, *C. ulcerans*, *K. pneumoniae*, *N. gonorrhoeae* and *P. aeruginosa*<sup>40,39,41</sup>, it synergistically enhanced the antibacterial activity of other flavonoids (quercetin, morin, galangin, kaempferol and myricetin) against *Bacillus cereus* and *Salmonella enteritidis*<sup>42,43</sup>.

### 3.3.2.2. Antifungal activity

*C. albicans* is the *Candida* species most often responsible for symptomatic skin infections. Moreover, most of what is known about *C. albicans* pathogenesis on epithelial surfaces has been studied for the oral and vaginal mucosa<sup>44</sup>. In our study, ten of the tested extracts, mainly polar, showed antifungal activity against *C. albicans* ( $IC_{50} < 64 \mu\text{g/ml}$ ). Methanolic extracts of the stem and root bark of *T. albida* ( $IC_{50}$  1.2 and 7.9  $\mu\text{g/ml}$ , respectively), the leaves of *T. alnifolia* ( $IC_{50}$  1.6  $\mu\text{g/ml}$ ), the root bark of *S. madagascariensis* ( $IC_{50}$  7.8  $\mu\text{g/ml}$ ) and the leaves of *L. heudoletii* ( $IC_{50}$  8.2  $\mu\text{g/ml}$ ) demonstrated strong antifungal activity. Only two apolar extracts showed pronounced antifungal activities: the extracts from the leaves of *T. alnifolia* ( $IC_{50}$  3.7  $\mu\text{g/ml}$ ) and the root bark of *S. madagascariensis* ( $IC_{50}$  7.8  $\mu\text{g/ml}$ ). The methanolic extract of the stem bark of *L. heudoletii* exhibited a good antifungal activity ( $IC_{50}$  13.3  $\mu\text{g/ml}$ ), while the methanolic extracts of the leaves of *C. glutinosum* and *S. madagascariensis* were weakly active (33.6 and 42.9  $\mu\text{g/ml}$ , respectively).

The pronounced antifungal activity of *T. alnifolia* found in our study contrasted with that previously reported in Nigeria by Lawal et al., in 2014 which mentioned resistance of *C. albicans* to all the tested extracts<sup>45</sup>. Although *Landolphia* species have been well studied for their antifungal activity<sup>46,47</sup>, to best of our knowledge, this is the first report of *L. heudoletii* against *Candida albicans*.

The potent antifungal activity of *Terminalia albida* found in our study was much higher than that previously found by Traoré et al., in 2015 ( $IC_{50}$  34.55  $\mu\text{g/ml}$ ), in a study carried

out on the methanolic extract of the root bark<sup>13</sup>. The antifungal activities of *Terminalia* species are related to the presence of tannins and saponins<sup>48</sup>. Several compounds, such as swartziadione, isolated from the root of *S. madagascariensis*, showed good antifungal activity against *Candida* spp<sup>38</sup>. Furthermore, two quinone methide diterpenes isolated from the root bark were reported to possess moderate antifungal properties against *C. albicans*<sup>49</sup>. Although the antifungal activity of *C. glutinosum* found in our study was weak ( $IC_{50}$  33.6 µg/ml), two compounds (betulinic acid and betulin) previously isolated from its leaves have been reported for their selective antifungal activity<sup>50</sup>. Furthermore, betulin has demonstrated moderate antifungal activities against *Candida krusei* and *C. albicans* with MIC 125 and 250 µg/mL, respectively<sup>51</sup>.

### **3.3.2.3. Antiplasmodial activity**

Twenty one extracts from twelve plant species were strongly active against *P. falciparum*, including the dichloromethane and methanolic extracts of *T. albida* root (0.6 and 0.8 µg/ml) and stem bark ( $IC_{50}$  0.8 and 2.4 µg/ml, respectively), the dichloromethane and methanolic extracts of *L. heudelotii* leaves ( $IC_{50}$  0.5 and 3.5 µg/ml, respectively), the dichloromethane extract of *Gardenia ternifolia* ( $IC_{50}$  1.3 µg/ml), the dichloromethane and methanolic extracts of the stem bark of *C. paniculatum* ( $IC_{50}$  0.4 and 2.9 µg/ml, repectively), as well as the dichloromethane extract of the leaves ( $IC_{50}$  2.0 µg/ml), the dichloromethane and methanolic extracts of *C. glutinosum* leaves ( $IC_{50}$  1.3 and 3.6 µg/ml, respectively), the dichloromethane extracts of *T. alnifolia* leaves ( $IC_{50}$  1.4 µg/ml, respectively), the dichloromethane extract of *E. africana* ( $IC_{50}$  1.8 µg/mL), the dichloromethane extracts of the leaves of *H. undullata*, *M. morindoides* and *P. crassipes* leaves ( $IC_{50}$  3.1, 3.8 and 4.1 µg/ml, respectively), the dichloromethane and methanolic extracts of *L. matinicensis* ( $IC_{50}$  3.1 and 2.8 µg/ml, respectively) (Table 2).

Although the antimalarial potential of some of these plants have already been reported, it is important to note that the antiplasmodial activities found in our study are more pronounced than those found by Traore et al., in 2014 for *Tetracera alnifolia* ( $IC_{50}$  20.2 µg/ml) and *Combretum glutinosum* ( $IC_{50}$  8.1 µg/ml) harvested in different regions of Guinea. This variability in the antiplasmodial activity observed between species could in all likelihood be attributed to the period and the places of the samples collection. Indeed,

previous studies have demonstrated that medicinal plants that grow in various environments produce different active substance contents because of their wide distribution in different geological zones<sup>53</sup>. Therefore, it is well known that the accumulation and synthesis of secondary metabolites are closely related to the environmental factors in growing locations, such as temperature, illumination, and moisture<sup>54</sup>.

On the other hand, the antiplasmodial activity found in our study for some plant species was in accordance with previous reports on *Pavetta crassipes*, *Terminalia albida*<sup>12,52,55</sup>, *Landolphia heudelotii*<sup>56</sup> and *Morinda morindoides* (3.5 µg/mL)<sup>57</sup>. The petroleum ether soluble fractions of *M. morindoides* exhibited a pronounced antiplasmodial activity with IC<sub>50</sub> < 3.0 µg/ml<sup>58</sup>. The dichloromethane extract of *M. morindoides* administrated orally at a dose of 200 mg/kg displayed significant antiplasmodial activity with 74% chemosuppression of parasiteamia *in vivo* against *Plasmodium berghei* ANKA in mice<sup>59</sup>. The phytochemical study of the leaves has led to the isolation of several secondary metabolites, such as flavonoids, iridoids, anthraquinones, tannins, saponins and terpenes<sup>60,57</sup>. Potent antiplasmodial activity was found for a phenylpropanoid conjugated iridoid and its three congeners isolated from *Morinda morindoides* (IC<sub>50</sub> < 1 µM)<sup>61</sup>. Furthermore, quercetin, isolated from the methanolic extract of the leaves, exhibited good antiplasmodial activity (IC<sub>50</sub> 5.5 µg/ml), while alizarin and chrysarin showed moderate activities with IC<sub>50</sub> values ranging from 14 to 26 µg/mL, respectively<sup>57</sup>. The methanolic extract and fractions from the root bark of *Gardenia ternifolia* demonstrated promising antimarial activity with a chemosuppressive effect ranging between 30-59% and 14-51%, respectively, *in vivo* against *Plasmodium berghei* ANKA in Swiss albino mice<sup>62</sup>. The strong antiplasmodial activity of *Landolphia heudelotii* found in our study is in agreement with previously reported results. Indeed, the study carried out by Rolland et al. in 2018, showed that the aqueous and ethanolic extract of *L. heudelotii* demonstrated promising activity against *P. falciparum* NF54 and K1 strains. The antiplasmodial activity of *Leucas martinicensis* was previously reported (IC<sub>50</sub> 13.3 µg/ml)<sup>63</sup>, although it was considerably less active than the extracts tested in this study (IC<sub>50</sub> < 5 µg/ml).

The antiplasmodial activity of *P. crassipes* is well documented: one study carried out in Togo showed that the leaves are effective against *P. falciparum*<sup>64</sup>. Studies carried out in

Burkina Faso revealed that the alkaloid extract had significant activity against both chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum*<sup>65,66,67</sup>. Moreover a bioguided fractionation of the leaf extract has shown that the alkaloid extract and two of its fractions possessed a strong antiplasmodial activity ( $IC_{50}$  0.71 and 2.2  $\mu$ g/ml). The antiplasmodial activity found for this plant is most likely related to the presence of elaeocarpidin and hydroxy-elaeocarpidin, which are two alkaloids that exhibit significant antiplasmodial activity ( $IC_{50}$  range 4-10 and 1.8  $\mu$ g /ml respectively)<sup>12,68</sup>.

In Nigeria *Hannoia* species are ethnobotanically used for the treatment of febrile conditions. The *in vivo* antiplasmodial activities of *Hannoia* species were reported previously<sup>69</sup>. The strong antiplasmodial activity of *Hannoia undulata* ( $IC_{50}$  3.1  $\mu$ g/ml) found in our study is in line with previous studies carried out on other *Hannoia* species. In fact, the methanolic and aqueous extracts from the stem bark of *H. klaineana* displayed good antiplasmodial activity ( $IC_{50}$  0.67 and 1.35  $\mu$ g/ml, respectively). Four quassinoids isolated from *Hannoia chlorantha* were highly active of which two of them, chaparrinone and 15-desacetylundulatone were the most active ( $IC_{50}$  0.11 and 0.023  $\mu$ g/ml, repectively)<sup>70</sup>.

Extracts from *S. madagascariensis* and *T. alnifolia* leaves showed good antiplasmodial activity ( $IC_{50}$  7.4 and 9.9  $\mu$ g/ml, respectively). Extracts from four plant species demonstrated moderate antiplasmodial activity: *Entada africana* leaves (methanolic extract:  $IC_{50}$  10.8  $\mu$ g/ml), *S. madagascariensis* leaves (methanolic extract:  $IC_{50}$  17.7  $\mu$ g/ml), *Pericopsis laxiflora* leaves (dichlomethane extract:  $IC_{50}$  12.0  $\mu$ g/ml) and *Opilia celtidifolia* stem bark (dichlomethane extract:  $IC_{50}$  12.7  $\mu$ g/ml).

Although, most of the selected plants in this study have undergone preliminary antiplasmodial screening, it is important to point out that the metabolites involved in these activities have not been isolated and identified for *Combretum paniculatum*, *Combretum glutinosum*, *Landolphia heudoletii*, *Leucas matinicensis*, *Terminalia albida* and *Tetracera alnifolia*. This finding could be of interest for future investigations since most of these plants have demonstrated promising antiplasmodial activities.

### **3.4. Conclusion**

The present study provides a comprehensive overview of Guinean medicinal plants employed in traditional medicine for the management of various microbial diseases, including urinary disorders, skin diseases and oral diseases, which still nowadays are commonly occurring health issues in Guinea. Although the present biological investigation concerned a limited number of the pathogens responsible for these microbial diseases, some of the studied plant species showed promising antimicrobial activities and these results may partially justify the traditional use of certain selected plant species. These promising plant extracts could be considered as a potential source for the development of new antifungal and/ or antimalarial agents. Further research, focusing on the isolation of the actives constituents of some promising plant extracts including *T. albida*, *T. alnifolia* and *C. paniculatum* is well detailed in chapter 4, 5, 6 and 7.

**Table I : Ethnomedicinal uses of collected medicinal plants**

Family (number of species within family)	Botanical name	Herbarium number	Local Name	Pathological condition	Plant part	Preparation form	Citation per part	Citation per species
Amaranthaceae (1)	<i>Cyathula prostrata</i> (L.) Blume	D2HK1	Blapapa (G)	Urinary disorders	L	Dc	2	2
Anacardiaceae (3)	<i>Lannea velutina</i> A. Rich.	D3HK5	Bémbe wagna (M)	Oral diseases	Sb	Dc	5	13
				Urinary disorders	L	Dc/Mc	5/3	
	<i>Pseudospondias microcarpa</i> (A.Rich.) Engl.	D3HK6	Dhologa (P)	Skin diseases	Sb	Dc	3	9
				Oral diseases	Sb	If	6	
Annonaceae (5)	<i>Rhus natalensis</i> Bernh. Ex C. Krauss	D3HK7	Woddiagutel (P)	Urinary disorders	R	Pd	1	1
	<i>Annona senegalensis</i> Pers.	D4KH5	Sounouningbé (M) Souhinyi (S) Doukoumè (P)	Oral diseases	L	Dc	3	7
				Urinary disorders	R	If	4	
	<i>Sorindeia juglandifolia</i> (A. Rich.) Planch. ex Oliv.	D4KH6	Kansi bomba (S) Sandji bombo (P)	Skin diseases	L	Dc	3	3
	<i>Uvariopsis guineensis</i> Keay	D4KH7	Kinkirissi (S) Porékinkirissa (P)	Skin diseases	L	Dc/If	6/1	8
					Wp	If	1	
	<i>Uvaria chamae</i> P. Beauv.	D4KH8	Moronda (s) Boilè (P)	Urinary disorders	Sb	Dc	2	11

				Skin diseases	L	Dc/Mc	3/2	
					R	Dc	4	
<i>Xylopia aethiopica</i> (Dunal) A.Rich.	D4KH9	Kanin (M)		Urinary disorders	Seed	Dc	2	7
				Skin diseases	Fr	Dc	5	
Apocynaceae (7)	<i>Adenium obesum</i> (Forsk.) Roem. & Schult.	D7HK7	Loukhouré (S) Djindji pètè (P) Boulou kourané (M)	Urinary disorders	R	Dc	1	4
				Skin diseases	L	Mc	3	
	<i>Calotropis procera</i> (Aiton) Dryand	D7HK8	Badyouba (M)	Urinary disorders	L	Dc	1	1
	<i>Carissa edulis</i> (Forssk.)Vahl	D7HK9	Camboro (P)	Urinary disorders	R	Dc	2	3
						Pd	1	
	<i>Holarrhena floribunda</i> (G. Don) TDurand & Schinz	D7HK10	Yètè (S) Eindhanma (P) Kouna sana (M)	Skin diseases	L	Dc	2	2
	<i>Landolphia dulcis</i> (Sabine ex G.don) Pichon	D7HK11	Howenyi (S) Poorè kodoukou (P)	Skin diseases	L	Dc/Mc	6/2	11
				Oral diseases	Sb	If	3	
	<i>Landolphia heudelotii</i> A.DC.	D7HK12	Foré (S) Poorè(PGbayi(M)	Urinary disorders	Sb	Dc/If	7/2	14
				Skin diseases	L	Dc	3	
					Sb	Pd	2	
	<i>Strophanthus sarmentosus</i> DC.	D7HK13	Kindè (P)	Skin diseases	R	Dc	1	1

Bignoniaceae (1)	<i>Newbouldia laevis</i> (P.Beauv.) Seem.	D18HK4	Kinki(S) Soukounden (P) Kindin Kanya (M)	Skin diseases	L	Dc	3	3
Compositea (3)	<i>Acanthospermum hispidum</i> DC.	D13HK2	Tatouwho gnalé (S)	Urinary disorders	L	Mc	2	5
	<i>Aspilia africana</i> (Pers.) C.D. Adams		Wowoné	Urinary disorders	L	Dc	3	
	<i>Mikania cordata</i> (Burm.f.) B.L.Rob.	D13HK4	Khoffo (S) Noré (P)	Skin diseases	L	Dc	1	1
Celastraceae (2)	<i>Maytenus senegalensis</i> (Lam.) Exell	D32HK1	Gbéké ou gbée	Oral diseases	Sb	Dc	1	1
			Djiel gootel	Urinary disorders	R	Dc	1	1
	<i>Salacia senegalensis</i> (Lam.) DC.	D25HK2	Kinkirissi (S), Porémahouni (P)	Skin diseases	R	Dc	5	5
Chrysobalanaceae (1)	<i>Parinari curatellifolia</i> Planch. Ex Benth	D33HK9	Coura tebhé	Urinary disorders	Seed	Pd	1	1
Cochlospermaceae (1)	<i>Cochlospermum tinctorium</i> Perrier ex A. Rich.	D35HK3	Trouban (M)	Urinary disorders	R	Dc	2	2
Cannabaceae (1)	<i>Trema guineensis</i> (Schum. & Thonn.) Ficalho	D27HK2		Skin diseases	L	Dc	7	7
Combretaceae (7)	<i>Combretum glutinosum</i> Perr. Ex DC.	D36HK7	Saataga (P)	Skin diseases	L	Dc	11	11
	<i>Combretum molle</i> R.Br. ex G.don	D36HK8	Wagnaka (M)	Oral diseases	L	Dc	1	1

	<i>Combretum nigricans</i> Lepr.ex Guill. & Perr.	D36HK9	Sembabali (M) Dhoki (P)	Urinary disorders	L	Dc	3	3
	<i>Combretum paniculatum</i> Vent.	D36HK10	Toubanombo (M)	Oral diseases	Sb	Dc	4	15
			Tantafiri (S)			If	2	
			Yayé safari (P)	Skin diseases	L	Dc	9	
	<i>Guiera senegalensis</i> J.F. Gmel.	D36HK11	Koungbènin (M)	Oral diseases	L	Dc	1	1
	<i>Terminalia albida</i> Scott-Elliot	D36HK12	Kobérafighè (S)	Urinary disorders	R	Dc	1	16
			Höröningbè (M)			Dc	7	
			Bori billel (P)	Oral diseases	R	If	2	
						Dc	4	
				Skin diseases	Sb	Dc	2	
	<i>Terminalia macroptera</i> Guill. & Perr.	D36HK13	Horo ningbè (M)	Oral diseases	R	Dc	1	1
Connaraceae (1)	<i>Santaloides afzelii</i> (R.Br ex Planch) G. Schellenb	D37HK1	Kononinsori (M)	Oral diseases	L	Dc	1	1
Dilleniaceae (1)	<i>Tetracera alnifolia</i> Willd.	D42HK2	Nintè (S)	Oral diseases	L	Dc	8	13
			Göröguel (P)	Skin diseases	L	If	5	
Euphorbiaceae (6)	<i>Alchornea cordifolia</i> (Schumach.& Thonn.) Müll. Arg.	D50HK5	Bolonta (S)	Oral diseases	L	Dc	3	9
			Gharkassaki (P)		L	Dc	1	
			Köyiran (M)	Urinary disorders	Sb	Dc	2	
					R	Dc	3	
	<i>Anthostema senegalense</i> A. Juss.	D50HK6	Wanni (S)	Skin diseases	Sb	Dc	1	1
	Manoninyiri (M)							

	<i>Chrozophora senegalensis</i> (Lam.) A. Juss. Ex Speng	D51HK8	Sangba yirifolo (M)	Oral diseases	R	Dc	1	1
	<i>Euphorbia hirta</i> L.	D50HK9	Demba sindji (M)	Oral diseases	L	Dc	1	4
				Urinary disorders	Wp	Dc	3	
	<i>Jatropha curcas</i> L.	D50HK10	Baanin (M)	Oral diseases	Gum	Pd	4	4
	<i>Macaranga heudelotii</i> Baill.	D50HK11	Lilèmon nah (G)	Urinary disorders	R	Pr	1	1
Hypericaceae (2)	<i>Harungana madagascariensis</i> Lam. ex Poir.	D62HK4	Soungala (P)	Skin diseases	Seed	Mc	2	2
	<i>Psorospermum senegalense</i> Spach	D62HK5	Keti diankouma (P)	Skin diseases	Sb/L	Dc	3	3
Gentianaceae (2)	<i>Anthocleista nobilis</i> G. Don	D73HK2	Fartanin (M)	Oral diseases	R	Dc	1	4
				Skin diseases	Sb	Dc	3	
	<i>Anthocleista procera</i> Leprieur ex Bureau	D73HK3	Fartani (M)	Urinary disorders	R	Mc/Dc	4	7
					R	Dc	3	
Lamiaceae (4)	<i>Clerodendrum capitatum</i> (Willd.) Schumach. & Thonn.	D66HK2	Firiforêt (S)	Skin diseases	L	Dc	3	3
	<i>Leucas martinicensis</i> (Jacq.) R.Br.	D66HK3	Dassi dadala (M)	Oral diseases	L	Dc	6	11
				Urinary disorders	L	Dc	2	
					Wp	Dc	3	
	<i>Ocimum gratissimum</i> L.	D66HK4	Barkeri (S) Soukoran (P) Soukouran (M)	Skin diseases	L	Dc	6	6
	<i>Vitex doniana</i> Sweet	D66HK5	Koukoui (S)	Skin diseases	L	Dc	9	9

			Boummè (P) Kodo (M)						
Lecythidaceae (1)	<i>Napoleonaea leonensis</i> Hutch. & Dalziel	D68HK1	Koumbaboya (S)	Skin diseases	L	Dc	8	8	
Leguminosae (18)	<i>Anthonotha crassifolia</i> (Baill.) J.Leonard	D51HK5	Bambouri (S) Boubè (P) Fouroumon (M)	Skin diseases	L	Mc	1	6	
					Sb	DC	5		
				Oral diseases	Sb	Dc	1	1	
	<i>Burkea africana</i> Hook.	D51HK6	Gbélenba (M)	Oral diseases Skin diseases Urinary disorders	Sb	Dc	4	13	
					R	Mc	3		
					L	Dc	6		
	<i>Daniellia oliveri</i> (Rolfe) Hutch.& Dalziel	D51HK7	Houloungni (S) Teiwè (P) Sandan (M)	Skin diseases	Sb/L	Dc	3	8	
					L	If	5		
	<i>Detarium senegalense</i> J.F.Gmel.	D51HK8		Skin diseases Oral diseases	L	Dc	3	6	
					Mc	Dc	1		
					L	Dc	2		
	<i>Dialium guineense</i> Willd.	D51HK9	Mokè (S) Mèko (P) Kofina (M)	Skin diseases	Sb	Mc	4	7	
					L	Dc	3		
	<i>Dichrostachys cinerea</i> (L.) Wight & Arn.	D84HK10		Urinary disorders Skin diseases	Mc	Dc	2	13	
					L	Dc	1		
	<i>Entada africana</i> Guill.& Perr.	D51HK11	Dialan-kamba (M)	Oral diseases	Sb	Dc	5	13	
					Mc	Dc	4		
				Urinary disorders	L	Dc	2		
					R	Mc	2		

	<i>Erythrina senegalensis</i> DC.	D51HK12	Lérou (M)	Oral diseases	L	Dc	2	2
	<i>Erythrophleum suaveolens</i> (Guill. & Perr.) Brenan	D51HK13	Méli (S) Téli (P) Tali (M)	Skin diseases	L	Pd	2	2
	<i>Isoberlinia doka</i> Craib.& Stapf.	D51HK14	So (M)	Oral diseases	L	Dc	1	1
	<i>Lonchocarpus laxiflorus</i> Guill. & Perr.	D51HK15	Sackri (S) Mogo kolo (M)	Skin diseases	Wp	Dc	1	1
	<i>Parkia biglobosa</i> (Jacq.) G. Don.	D84HK16	Néri (S) Nètè (P) Néré (M)	Urinary disorders	L	Dc	3	7
				Skin diseases	Sb	Dc/ Paste	2	
					Seed	Pd	2	
	<i>Prosopis africana</i> (Guill.&Perr.) Taub.	D84HK17	Gbélé (M)	Oral diseases	Sb	Dc	2	6
						Pd	2	
					L	Mc	2	
	<i>Pericopsis laxiflora</i> (Baker) Meeuwen	D51HK18	Kolokolo	Urinary disorders	R	Dc	4	10
					L	Dc	6	
	<i>Piliostigma thonningii</i> (Schum.) Milne-Redh.	D51HK19	Gnanam (M)	Urinary disorders	L	Dc	4	9
					Sb	Dc	1	
				Skin diseases	R	Dc	4	
	<i>Pterocarpus erinaceus</i> Poir.	D51HK20	Khari (S) Bani danè (P) Gbèn (M)	Oral diseases	Sb	Mc	2	7
						Dc	2	
				Urinary disorders	L	Dc	3	
	<i>Swartzia madagascariensis</i> Desv.	D51HK21	Samakara (M)	Oral diseases	R	Dc	4	14
					Sb	Dc/If	2/3	

					L	Dc	5	
	<i>Xeroderris stuhlmannii</i> (Taub.) Mendonca. & Susa	D51HK22	Moussolamana (M)	Oral diseases	Sb	Dc	1	1
Lythraceae (1)	<i>Lawsonia inermis</i> L.	D75HK2	Diabé (M)	Urinary disorders	L	Dc	4	4
Malvaceae (7)	<i>Bombax costatum</i> Pellegr. & Vuillet	D20HK1	Boumboun (M)	Oral diseases	Gum	Pr	4	4
	<i>Ceiba pentandra</i> (L.) Gaertn.	D20HK2	Banan (M)	Oral diseases	Sb	Mc	1	1
	<i>Grewia villosa</i> Willd.	D131HK3	Billi bèbi (P)	Urinary disorders	R	Dc	7	7
	<i>Gossypium barbadense</i> L.	D77HK4	Hottollohi	Urinary disorders	Seed	Dc/Pd	2/1	3
	<i>Hibiscus sabdariffa</i> L.	D77HK3	Folèrè (P)	Skin diseases	L	Dc	2	2
	<i>Sida rhombifolia</i> L.	D77HK5	Bassabassa (M)	Oral diseases	L	Paste	1	1
	<i>Sterculia tragacantha</i> (Lindl.)	D127HK4	Forkè (M)	Urinary disorders	L	Dc	10	10
Meliaceae (4)	<i>Azadirachta indica</i> A. Juss.	D80HK3	Cassia thioukal (P)	Skin diseases	L	Pr	1	1
	<i>Carapa procera</i> DC.	D80HK4	Koubi (S)	Oral diseases	Seed	Oil	1	3
			Gobie (P)	Skin diseases	L	Mc	2	
			Kobi (M)					
	<i>Khaya senegalensis</i> (Desv.) A. Juss.	D80HK5	Kahi (P)	Oral diseases	Sb	Dc	1	4
			Diala (M)	Urinary disorders	L	Dc	1	
			Yalando (Kissi)	Skin diseases	Gum	If	2	
	<i>Trichilia emetica</i> Vahl.	D80HK6	Soulafinsan (M)	Skin diseases	L	Dc	7	7

Moraceae (4)	<i>Ficus capensis</i> Thunb.	D86HK1	Toro (M)	Urinary disorders	R	Dc	1	1
	<i>Ficus exasperata</i> Vahl	D86HK2	Gnogni (S) Gniègnè (P) Founda (K)	Skin diseases	L	Mc	6	8
				Skin diseases	Sb	Pd	2	
	<i>Ficus umbellata</i> Vahl	D86HK3	Andakkè	Urinary disorders	R	Mc	2	2
<i>Ficus vallis-choudae</i> Delile	D86HK4	Tyibbé (P)		Skin diseases	L	Dc	2	6
					Sb	Dc	4	
Myrtaceae (1)	<i>Syzygium guineense</i> (Willd.) DC.	D90HK3	Khayo (S) Kadio (P) Kissa (M)	Urinary disorders	L	Dc	3	3
Nymphaeaceae (1)	<i>Nymphaea lotus</i> L.	D92HK1	Kokoun (M)	Urinary disorders	L	Dc	1	1
Ochnaceae (1)	<i>Lophira lanceolata</i> Tiegh. ex Keay.	D93HK2	Mana (M)	Urinary disorders	L	Mc	2	4
						Oil	2	
Olacaceae (1)	<i>Ximenia americana</i> L.	D95HK2	Gbanin (M)	Urinary disorders	L	Dc	1	7
				Skin diseases	Seed	Oil	5	
				Urinary disorders	R	Dc	1	
Oleaceae (1)	<i>Schrebera arborea</i> A. Chev.	D95HK3	Coulatetagan (P)	Urinary disorders	R	Dc	1	1
Opiliaceae (1)	<i>Opilia celtidifolia</i> (Guill.Perr.) Endl. ex walp.	D97HK1	Kourangbei (M)	Urinary disorders	L	Dc	8	12

				Oral diseases	Sb	If	4	
Pandaceae (1)	<i>Microdesmis keayana</i> J.Léonard	D99HK1	Hely (G)	Urinary disorders	L	Pd	1	1
Poaceae (2)	<i>Cynodon dactylon</i> (L.) Pers.	D162HK3	Doumbourou (P)	Urinary disorders	Fr	Dc	1	1
	<i>Cymbopogon giganteus</i> Chiov.	D162HK4	Kêkaara (M)	Urinary disorders	R	Dc	4	4
Phyllanthaceae (4)	<i>Bridelia micrantha</i> (Hochst.) Baill.	D50HK7	Tolingni (S) Daafi (P) Dafin sagba (M)	Urinary disorders	Sb	Mc	5	8
	<i>Hymenocardia acida</i> Tul.	D103HK3	Barambara (S) Pélitoro (P) Bran- bran (M)	Skin diseases	Sb	If	3	
				Oral diseases	L	If	2	
						Dc	4	
	<i>Margaritaria discoidea</i> (Baill.) G.L. Webster	D103HK4	Mètè (S) Kéeri (P) Baakoönkön (M)	Skin diseases	L	Dc	2	2
Polygalaceae (1)	<i>Securidaca longipedunculata</i> Fresen.	D108HK2	Djodo (M)	Oral diseases	Sb	Dc	1	4
					L	Dc	2	
					R	Mc	1	
				Urinary disorders	L	Mc	1	6
					R	Mc	2	
						Dc	1	

Rubiaceae (7)	<i>Bertiera spicata</i> (C.F. Gaertn.) K. Schum.	D117HK8	Zowotoumo (G)	Urinary disorders	L	Mc	1	1
<i>Gardenia ternifolia</i> Schumach. & Thonn.	D117HK9	Teinghè (SBossè (P), Bourén (M)	Skin diseases	L	Pd	6	11	
			Urinary disorders	R	Dc	5		
<i>Leptactina senegambica</i> Hook.f.	D117HK10	Caroucaroundin (P)	Urinary disorders	Sb	Dc	2	3	
			Skin diseases	L	Dc	1		
<i>Mitragyna inermis</i> (Willd.) O. Kuntze	D117HK11	Fofo (S) Popo (P)	Skin diseases	Sb	Mc	1	1	
<i>Morinda geminata</i> DC.	D117HK12	Wanda (P) Wanda (M)	Skin diseases	R/L	Dc	2	5	
				L	Dc	3		
<i>Morinda morindoides</i> (Baker) Milne-Redh.	D117HK13	Wanda (P)	Skin diseases	L	Dc	8	10	
			Oral diseases	L	If	2		
<i>Pavetta crassipes</i> K. Schum.	D117HK14	Pinperemagni (M)	Urinay disorders	L	Dc	15	15	
Rhizophoraceae (1)	<i>Anisophyllea laurina</i> R.Br. ex Sabine	D115HK2	Kantinyi (S) Kanssi (P) Djaudi (M)	Skin diseases	L	Dc	3	3
Sapotaceae (1)	<i>Manilkara multinervis</i> (Barker) Dubard	D122HK2	Karatouré (P)	Skin diseases	Sb	Dc	8	8
Solanaceae (1)	<i>Physalis angulata</i> L.	D125HK4	Ponpontianin (S) Pompom digga (P)	Skin diseases	L	Dc	3	3
			Mc			1	1	

Simaroubaceae (1)	<i>Hannoa undulata</i> (Guill. & Perr.) Planch.	D124HK2	Diafrékété (M)	Urinary disorders	L	Dc	7	12
				Oral diseases	L	If	5	
Vitaceae (2)	<i>Cissus aralioïdes</i> (Welw. ex. Baker) Planch.	D137HK2	Fafarou (P)	Urinary disorders	R	Dc/Mc	1/2	8
					R	Mc	3	
				Skin diseases	R	Pd	2	
	<i>Cissus populnea</i> Guill. & Perr.	D137HK3	Lakhassè foret (S)	Skin diseases	R	Pd	1	1
Zingiberaceae (2)	<i>Aframomum sulcatum</i> (Oliv. & D.Hanb. ex Baker) K.Schum	D171HK2	Yaya (S)	Urinary disorders	L	Dc	9	9
	<i>Zingiber officinale</i> Roscoe	D171HK3	Gnamacouleidi (P)	Urinary disorders	Rh	Pd	2	2

Dc: decoction; If: Infusion; Mc: maceration; Pd: pomade; L: leaves; Rb: root bark; Rh: rhizome; Sb: stem bark; Wp: whole plant; P: pular; M: malinké; S: sussu; G: Guerzé;

**Table 2: *In vitro* antimicrobial, antiplasmodial and cytotoxic activities of plant extracts.**

Botanical names	Plant part (solvent)	Antimicrobial activity (IC <sub>50</sub> µg/ml)			Antimicrobial activity (MIC µg/ml)		Antiplasmo- dial activity (IC <sub>50</sub> µg/ml)	Cytotoxicity (CC <sub>50</sub> µg/ml)	Selectivity index
		<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>S. aureus</i>	<i>C. albicans</i>			
<i>Combretum paniculatum</i> Vent.	Sb (CH <sub>2</sub> Cl <sub>2</sub> )	>64	>64	>64			0.4	7.8	19.5
	Sb (MeOH)	>64	>64	>64			2.9	24.1	8.3
	L (CH <sub>2</sub> Cl <sub>2</sub> )	24.5	>64	>64	>64	nd	1.4	7.6	5.4
	L (MeOH)	>64	>64	>64			16.0	17.2	1.0
<i>Combretum glutinosum</i> Perr. Ex DC.	L (CH <sub>2</sub> Cl <sub>2</sub> )	24.2	>64	>64			1.3	6.9	5.3
	L (MeOH)	47.80	>64	33.6			3.6	4.3	1.2
<i>Daniellia oliveri</i> (Rolfe) Hutch.& Dalziel	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	>64.0			>64.0	>64.0	Nd
	L (MeOH)	>64.0	>64.0	>64.0			53.5	>64.0	>1.2
<i>Entada africana</i> Guill. & Perr.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64	Nd	>64			1.8	7.7	4.2
	L (MeOH)	>64	Nd	>64			10.8	33.1	3.0
	Sb (CH <sub>2</sub> Cl <sub>2</sub> )	>64	Nd	>64			64.0	39.3	0.6
	Sb (MeOH)	>64	Nd	48.9			35.1	33.4	0.9
<i>Hannoia undulata</i> (Guill. & Perr.) Planch.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	>64.0			3.1	15.5	5.0
	L (MeOH)	>64.0	>64.0	>64.0			>64.0	>64.0	Nd

<i>Gardenia ternifolia</i> Schumach. & Thonn.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.00	Nd	>64.0			1.3	33.7	25.9
	L (MeOH)	>64.00	Nd	>64.0			21.9	>64.0	2.9
<i>Landolphia heudelotii</i> A.DC.	Sb (CH <sub>2</sub> Cl <sub>2</sub> )	>64	>64	>64			2.5	>64.0	>25
	Sb (MeOH)	>64	>64	13.3	nd	>64.0	3.0	25.9	8.6
	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64	>64	>64			0.5	8.0	16.
	L (MeOH)	51.8	>64	8.2	nd	16.0	7.4	>64	>8.6
<i>Landolphia dulcis</i> ( Sabine ex G. Don) Pichon	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	>64.0			3.5	27.3	7.8
	L (MeOH)	>64.0	>64.0	>64.0			>64.0	>64.0	nd
<i>Lannea velutina</i> A. Rich.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	nd	>64.0			nd	7.53	nd
	L (MeOH)	>64.0	nd	4.2	nd	16.0	8.7	>64.0	>7.3
<i>Leucas martinicensis</i> (Jacq.) R.Br.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	nd	>64			1.0	30.8	30.8
	L (MeOH)	>64.0	nd	>64			2.8	30.3	10.8
<i>Morinda morindoides</i> (Baker) Milne-Redh.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	nd	>64			4.1	15.5	3.7
	L (MeOH)	>64.0	nd	>64			34.4	32.5	<0.9
<i>Opilia celtidifolia</i> (Guill.Perr.) Endl. Ex walp.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	>64.0			26.1	>64.0	>2.4
	L (MeOH)	>64.0	>64.0	>64.0			>64.0	>64.0	Nd
	Sb (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	>64.0			12.7	>64.0	>5.0
	Sb (MeOH)	>64.0	>64.0	>64.0			>64.0	>64.0	Nd
<i>Pavetta crassipes</i> K. Schum.	L (CH <sub>2</sub> Cl <sub>2</sub> )	8.5	>64	>64.0	64.0	nd	3.8	6.7	1.7
	L (MeOH)	>64	>64	>64			22.7	>64	>2.8
<i>Pericopsis laxiflora</i> (Baker) Meeuwen	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	>64.0			12.0	>64.0	>5.3
	L (MeOH)	>64.0	>64.0	>64.0			43.0	>64.0	>1.4

<i>Sterculia tragacantha</i> Lindl.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	nd	>64.0			43.0	16.0	<0.3
	L (MeOH)	>64.0	nd	>64.0			35,5	64.0	>1.8
<i>Swartzia madagascariensis</i> Desv.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	>64.0			21.0	>64.0	>3.0
	L (MeOH)	>64.0	>64.0	42.9			>64.0	>64.0	Nd
	Sb (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	>64.0			17.7	>64.0	>3.6
	Sb (MeOH)	>64.0	>64.0	>64.0			>64.0	>64.0	Nd
	Rb (CH <sub>2</sub> Cl <sub>2</sub> )	16.8	>64.0	7.8	nd	16.0	7.4	>64.0	>8.6
	Rb (MeOH)	>64.0	>64.0	>64.0			>64.0	>64.0	Nd
<i>Terminalia albida</i> Scott-Elliott	Sb (CH <sub>2</sub> Cl <sub>2</sub> )	57.7	>64.0	>64.0			2.4	30.5	12.7
	Sb (MeOH)	33.4	>64.0	1.2	nd	16.0	0.8	22.9	28.6
	Rb (CH <sub>2</sub> Cl <sub>2</sub> )	>64	>64.0	>64.0			0.6	25.8	43.0
	Rb (MeOH)	12.8	>64.0	2.4	>64.0	16.0	0.8	>64.0	26.8
<i>Tetracera alnifolia</i> Willd.	L (CH <sub>2</sub> Cl <sub>2</sub> )	>64.0	>64.0	5.22	>64.0	32.0	1.4	6.6	3.9
	L (MeOH)	>64.0	>64.0	1.6	nd	4.0	9.9	35.0	3.5
Chloroquine							0.20 μM		
Doxycycline		0.09 μM	0.61 μM		0.25 μM				
Flucytosine				0.52 μM		1.00 μM			
Tamoxifen								10.50 μM	

L: leaves; Rb: root bark; Sb: stem bark; Nd: not determined; Pf: *Plasmodium falciparum*

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## CHAPTER 4

***Terminalia albida* part I:** bioassay-guided isolation of antiplasmodial and antimicrobial constituents from the roots of *Terminalia albida*

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#### **4.1. Introduction**

Medicinal plants have been serving for many years as sources of therapeutic agents and have shown beneficial uses in multiple areas of application<sup>1</sup>. Indeed, many bioactive products discovered from natural sources so far have played a pivotal role in improving human health, in spite of facing a tough competition with synthetic compounds<sup>2</sup>. Of the 1,562 drugs approved between 1981 and 2014 in USA by the Food and Drug Administration, 64 (4%) were unaltered natural products, 141 (9%) were botanical drugs (mixtures), 320 (21%) were natural derivatives and 61 (4%) were synthetic drugs but containing natural pharmacophores<sup>3</sup>. The increased interest in herbal medicine by the general public has stimulated a greater scientific awareness in exploring and understanding the pharmacologically active constituents of medicinal plants<sup>4,5</sup>. Nowadays medicinal plants still represent an important pool for the identification of novel drug leads<sup>6</sup>. However, the potential of many of them as new antimicrobial scaffolds has not sufficiently been explored<sup>7</sup>. Furthermore, the isolation and purification of plant compounds in an adequate yield remains a major concern<sup>8</sup>.

In Guinea, medicinal plants play an important role in the management of the most common diseases, especially in rural areas. *Terminalia albida* Sc. Elliot (Combretaceae), distributed throughout the Guinean regions, is widely used in traditional medicine to treat a variety of ailments such as malaria, skin diseases, oral diseases and urinary disorders<sup>9,10,11,12</sup>. It has been demonstrated that the methanolic extract of the stem bark of *T. albida* considerably increased the survival rate in mice infected with *Plasmodium berghei*<sup>13</sup>. A preliminary biological screening carried out as part of our research program confirmed the previously reported antiplasmodial activity and gave a possible explanation for the use of extracts in case of oral and skin diseases. Promising *in vitro* activities against *Candida albicans*, *Staphylococcus aureus* and *Plasmodium falciparum* were obtained for both methanolic and dichloromethane extracts of the root of *T. albida*<sup>9</sup>. However, hitherto most of the compounds responsible for such activities have not been isolated or identified yet. Therefore, the bioassay-guided isolation of *Terminalia albida* root extracts has been carried out.



**Clade** : Angiosperms

**Order** : Myrales

**Family** : Combretaceae

**Genus** : *Terminalia*

**Species** : *albida*

**Figure 4.1.** *Terminalia albida* Scott-Elliott. Picture CRVPM-Dubreka

## 4.2. Materials and methods

### 4.2.1. Solvent and reagents

Dichloromethane, ethyl acetate, *n*-butanol, *n*-hexane, acetone, acetonitrile, acetonitrile far UV and methanol (all HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK). All reagents, such as sulphuric acid and formic acid (eluent additive for HPLC) were purchased from Acros Organics (Geel, Belgium) or Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and formic acid (both for UPLC-MS) were obtained from Biosolve Chimie (Dieuze, France). Ultrapure water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA). NMR deuterated solvents ( $\text{CDCl}_3$ , acetonitrile- $d_3$ , methanol- $d_4$ , DMSO- $d_6$ , pyridine- $d_5$ ) were obtained from Sigma-Aldrich.

### 4.2.2. General experimental methods

Purification of extracts and fractions was carried out using a Grace Reveleris X2 flash chromatographic system (Lokeren, Belgium) equipped with an evaporative light scattering detector (ELSD), a diode array detector (DAD), and a fraction collector. The chromatographic profiles of fractions collected during different isolation steps were

obtained on an Agilent HPLC system (1200 series) and/or by thin layer chromatography (TLC) on NP F254 plates (20 cm × 20 cm) from Merck (Darmstadt, Germany). The TLC plates were observed under UV light (254 and 366 nm) and under visible light after spraying with a vanillin-sulphuric acid reagent (prepared by mixing 5 g vanillin with 475 mL ethanol and 25 mL sulphuric acid).

A semipreparative HPLC system equipped with DAD and ESI-MS detectors was used for the isolation of the pure compounds. The system was composed of a sample manager, injector, and collector (2767), a quaternary gradient module (2545), a System Fluidics Organizer, an HPLC pump (515), a diode array detector (2998), and a Micromass Quattro TQD mass spectrometer, all supplied by Waters (Milford, MA, USA). For data processing MassLynx version 4.1 was used. Optical rotations were measured on a JASCO P-2000 spectropolarimeter (Easton, MD, USA).

1D and 2D Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DRX-400 NMR spectrometer (Rheinstetten, Germany) equipped with either a 3 mm inverse broadband (BBI) probe or a 5 mm dual  $^1\text{H}/^{13}\text{C}$  probe using standard Bruker pulse sequences and operating at 400 MHz for  $^1\text{H}$  and at 100 MHz for  $^{13}\text{C}$  NMR spectra. The spectra were processed with Topspin (version 4.0.6).

Accurate mass measurements were carried out on a Xevo-G2XS-QToF mass spectrometer (Waters) coupled with an Acquity LC system equipped with MassLynx version 4.1 software. A Waters Acquity UHPLC BEH Shield RP18 column (2.1 mm x 100 mm, 1.7 $\mu\text{m}$ ) was used with a mobile phase consisting of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), which were pumped at a rate of 0.4 ml/min. The gradient system was set as follows: 2% B (0-1 min), 2-100% B (1-5 min), 100% B (5-7 min), 100-2% B (7-8 min), 2% B (8-10 min). For all analyses, full scan data were recorded in ESI (-) and ESI (+) mode from  $m/z$  50 to 1500 in sensitivity mode (approximate resolution: 22000 FWHM) using a spray voltage at either - 0.8 kV and +1.0 kV, respectively. Cone gas flow and desolvation gas flow were set at 50.0 L/h and 1000.0 L/h, respectively; and source temperature and desolvation temperature at 120 °C and 550 °C, respectively. Leucine enkephalin was used as lock mass during the analysis.

#### **4.2.3. Plant Material**

Roots of *Terminalia albida* were harvested in Dubréka, Republic of Guinea in June 2016. The plant was identified by the botanists from the Research and Valorization Center on Medicinal Plants, Dubréka, where a voucher specimen (D36HK13) is kept. The collected root samples were dried at room temperature and milled.

#### **4.2.4. Extraction and isolation**

The dried and milled root of *Terminalia albida* (470.9 g), defatted four times with *n*-hexane (2.0 L each 24 h) was extracted five times (2.5 L each 24 h) with 80% MeOH. The filtrate was concentrated under reduced pressure and freeze-dried to obtain the crude methanolic extract (110 g), which was redissolved in water and successively partitioned with CH<sub>2</sub>Cl<sub>2</sub>, EtOAc, and *n*-BuOH to yield a dichloromethane soluble fraction (A<sub>D</sub>) (6.0 g), an ethyl acetate soluble fraction (A<sub>E</sub>) (15.1 g), an *n*-butanol soluble fraction (A<sub>N</sub>) (24.2 g) and a residual water fraction. The dichloromethane fraction was further separated by flash chromatography using a silicagel column (Grace Resolv 80 g), eluted with a gradient of dichloromethane (A), ethyl acetate (B) and methanol (C) at a flow rate of 60 mL/min. The gradient was set as follows: 100% A (0-10 min), followed by an increasing concentration of B (10-60 min) till 100% B; then an increasing concentration of C (60-85 min) till 100% C; finally, this condition was maintained for 10 min (85-100mn). The collected tubes were pooled in 10 fractions (A<sub>D1</sub>-A<sub>D10</sub>) based on similarity of their TLC profiles (mobile phase: dichloromethane / ethyl acetate: 7/3 v/v).

The purification of the most active fractions A<sub>D1</sub> (0.8 g) and A<sub>D3</sub> (1.2 g) was performed using flash chromatography with a silicagel column (GraceResolv 40 g) eluted with a mixture of *n*-hexane (A), dichloromethane (B) and ethyl acetate (C) at a flow rate of 40 mL/min resulting in the isolation of compounds **1** (4.2 mg), **2** (6.5 mg) and **3** (9.6 mg). The gradient was set as follows: 0-5 min: 100% A, followed by an increasing concentration of B in 30 min (5-35 min: A - 100% B), which was maintained for 10 min (35-45 min: 100% B); then the amount of C was increased during 20 min (45-65 min: B - 100% C); finally, this condition was maintained for 10 min. The isolation and purification of compounds **4** (45.3 mg) from fraction A<sub>D7</sub> (0.53 g) and **5** (7.4 mg) from fraction A<sub>D8</sub> (0.62 g) (sample concentration 20 mg/mL; injection volume 400 µL) was achieved on the

semipreparative HPLC-MS using a C18 Kinetex column (250 mm × 10.0 mm, particle size, 5 µm) from Phenomenex (Utrecht, The Netherlands) and eluted with a gradient of H<sub>2</sub>O + 0.1% formic acid (A) and acetonitrile far UV (B) at a flow rate of 3 mL/min. The DAD spectrum was recorded from 200 to 450 nm, and mass spectra in the ESI (+) and ESI (-) modes, MS scan range: *m/z* 100 to 1000; capillary voltage 3.00 kV, cone voltage 50 V, extractor voltage 3 V, source temperature 135 °C, desolvation temperature 400 °C, desolvation gas flow 750 L/h, cone gas flow 50 L/h.

The ethyl acetate fraction (5.0 g) was subjected to flash chromatography using a silicagel column (GraceResolv 80 g), eluted with a gradient of dichloromethane (A), ethyl acetate (B) and methanol (C) at a flow rate of 60 mL/min. The gradient was set as follows: 100% A (0-8 min), followed by an increasing concentration of B (8-40 min) till 100% B, which was maintained for 10 min (40-50 min); then the amount of C was increased till 100% (50-80 min) and this condition was maintained for 10 min (80-90 min). Fractions were pooled in 5 sub-fractions (A<sub>E1</sub>-A<sub>E5</sub>) based on the similarity of their TLC and HPLC profiles. A Kinetex C18 (100 mm × 2.10 mm, 2.6 µm) (Phenomenex, Torrance, CA, USA) column was used with H<sub>2</sub>O + 0.1 % formic acid (A) and acetonitrile (B) as the mobile phase. For all fractions a flow rate of 1 mL/mn of the following gradient (min/%B) was used: 0.0/5, 5.0/5, 50.0/95, 55.0/95, 57.0/5, 62.0/5.

Several fractions were subjected to semipreparative HPLC-MS analysis resulting in the purification of fraction A<sub>E1</sub> (0.6 g) with the following gradient (0-5 min) 30% B, (5-35 min) 50% B, (35–40 min) 50% B, (40–50 min) 95% B, affording compounds **6** (5.0 mg) and **7** (24.1 mg). The purification of fraction A<sub>E3</sub> (0.81g) with the gradient (0-5 min) 30% B, (5-35mn) 35% B, (35–40 min) 35% B, (40–50 min) 95% B yielded compounds **8** (9.3 mg) and **9** (5.2 mg). Fraction A<sub>E4</sub> (1.3 g), using the gradient (0-5 min) 20% B, (5-35mn) 30% B, (35–45 min) 95 % B, resulted in the isolation of compounds **10** (8.4 mg), **11** (4.7 mg), **12** (64 mg) **13** (43.4 mg) and **14** (4.7 mg). During the whole purification process, the flow rate was set at 3.0 mL/min and the sample concentration for all fractions was 20 mg/mL The injection volume for fraction A<sub>E1</sub> and A<sub>E3</sub> was 400 µL, while for fraction A<sub>E4</sub>, 300 µL was injected.

#### **4.2.5. LC-ESI-MS**

The 80% ethanol extract was prepared by dissolving 5 g of *Terminalia albida* root powder in 30 mL of solvent and stirred at room temperature for 24 h. The extract was evaporated under reduced pressure at 35 °C. For the LC-MS analysis the extract was dissolved in 80% MeOH (v/v) at a concentration of 1 mg/mL, which was diluted with water in order to obtain a final concentration of 0.1 mg/mL.

#### **4.2.6. Biological evaluation**

##### **4.2.6.1. Antibacterial and antifungal activity**

The antimicrobial activity of all fractions and pure compounds was evaluated according to Cos et al. (2006) and Baldé et al. (2010). Fractions and pure compounds were tested against the following microorganisms: *Escherichia coli* ATCC8739 (Gram-negative), *Staphylococcus aureus* ATCC 6538 (Gram-positive) and *Candida albicans* ATCC59630 (yeast). The following positive controls were used: flucytosine for *C. albicans* ( $IC_{50}$  0.7 ± 0.01 µM) and doxycycline for *S. aureus* ( $IC_{50}$  0.3 ± 0.2 µM) and *E. coli* ( $IC_{50}$  0.6 ± 0.3 µM). These reference compounds are routinely used in the screening platform and their activities were in the range that is usually observed<sup>14,16</sup>.

##### **4.2.6.2. Antiplasmodial and cytotoxicity assays**

Antiplasmodial activity and cytotoxicity were assessed as previously described by Cos et al. (2006) and Tuenter et al. (2016). Fractions and pure compounds were tested *in vitro* against the chloroquine-resistant strain *Plasmodium falciparum* K1 using the lactate dehydrogenase assay. The most active compounds were tested in triplicate; mean and standard deviation (SD) were calculated. Tamoxifen was used as the positive control for cytotoxicity on MRC-5 cells ( $IC_{50}$  10.0 ± 1.5 µM), and chloroquine ( $IC_{50}$  0.15 ± 0.1 µM) for *P. falciparum*. These reference compounds are routinely used in the screening platform and their activities were in the range that is usually observed<sup>14,16</sup>.

#### 4.2.6.3. Physicochemical and spectral data of isolated compounds

*Pantolactone (3-hydroxy-4,4-dimethyldihydrofuran-2(3H)-one) (1).* Yellow powder;  $^1\text{H}$ -NMR ( $\text{CD}_3\text{CN}$ ):  $\delta_{\text{H}}$  0.97 (3H, s) and 1.13 (3H, s) (C-Me<sub>2</sub>), 4.09 (1H, d,  $J$  = 5.8 Hz, CH), 3.93 (2H, m, CH<sub>2</sub>), 3.78 (1H, d,  $J$  = 5.8 Hz, OH);  $^{13}\text{C}$ -NMR ( $\text{CD}_3\text{CN}$ ):  $\delta_{\text{C}}$  19.2 and 22.7 (2x Me), 41.2 (C), 75.9 (CH<sub>2</sub>), 76.3 (CH) 177.6 (C=O).

*Friedelin (2).* Colorless needles; HR-ESI-MS  $m/z$  425.2361 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>49</sub>O, 425.3783)  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ ):  $\delta_{\text{H}}$  0.68 (3H, s, Me-24), 0.83 (3H, s, Me-25), 0.88 (3H, s, Me-29), 0.91 (3H, s, Me-23), 0.96 (3H, s, Me-26), 1.01 (3H, s, Me-30), 1.14 (3H, s, Me-27), 1.21 (3H, s, Me-28), 2.25 (1H, m, H-4);  $^{13}\text{C}$ -NMR: see Table 4.1.

*3,4,3'-Tri-O-methylellagic acid (3).* Pale yellow needles; HR-ESI-MS  $m/z$  343.0450 [M-H]<sup>-</sup> (calculated for C<sub>17</sub>H<sub>11</sub>O<sub>8</sub>, 343.0454,  $\Delta$  -1.16 ppm);  $^1\text{H}$ -NMR (DMSO-d<sub>6</sub>):  $\delta_{\text{H}}$  7.58 (1H, s, H-5), 7.67 (1H, s, H-5'), 4.09 (-OMe), 4.08 (-OMe) 4.04 (-OMe);  $^{13}\text{C}$ -NMR (DMSO-d<sub>6</sub>):  $\delta_{\text{C}}$  111.3 (s, C-1), 141.5 (s, C-2), 141.0 (d, C-3), 153.8 (s, C-4), 111.7 (d, C-5), 112.0 (s, C-6), 158.4 (s, C-7), 112.6 (s, C-1'), 141.5 (s, C-2'), 141.0 (s, C-3'), 153.8 (s, C-4'), 107.4 (d, C-5'), 113.4 (d, C-6'), 158.5 (d, C-7'), 56.7 (q, OCH<sub>3</sub>), 61.0 (q, OCH<sub>3</sub>), 61.3 (q, OCH<sub>3</sub>).

*Sericic acid (4).* Amorphous powder;  $[\alpha]_D^{20}$  30.5 ( $c$  0.0087, MeOH); HR-ESI-MS  $m/z$  503.3374 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>47</sub>O<sub>6</sub>, 503.3373,  $\Delta$  1.9 ppm),  $m/z$  549.3435 [M-H+FA]<sup>-</sup>, FA (formic acid) adduct ion ;  $^1\text{H}$ -NMR (DMSO-d<sub>6</sub>):  $\delta_{\text{H}}$  0.64 (3H, s, Me-26), 0.83 (3H, s, Me-30), 0.87 (3H, s, Me-29), 0.90 (3H, s, Me-25), 1.08 (3H, s, Me-24), 1.23 (3H, s, Me-27) 5.22 (1H, brs, H-12), 2.86 (1H, d,  $J$ =9.4 Hz, H-3), 3.57 (1H, m, H-2) 3.74 (1H, d,  $J$  = 11.0 Hz) and 3.29 (1H, d,  $J$  = 11.0 Hz) (H-23), 3.11, (1H, brs, H-19), 2.92 (1H, brs, H-18);  $^{13}\text{C}$ -NMR: see Table 4.1.

*Arjunolic acid (5).* Amorphous white powder;  $[\alpha]_D^{20}$  53.7 ( $c$  0.0017, MeOH); HR-ESI-MS:  $m/z$  489.3564 [M+H]<sup>+</sup> (calculated for C<sub>30</sub>H<sub>49</sub>O<sub>5</sub>, 489.3580  $\Delta$  -3.26 ppm), Na<sup>+</sup> adduct ion at  $m/z$  511.3410 [M+Na]<sup>+</sup>;  $^1\text{H}$ -NMR ( $\text{CD}_3\text{OD}$ ):  $\delta_{\text{H}}$  0.68 (3H, s, Me-23), 0.81 (3H, s, Me-25), 0.89 (3H, s, Me-29), 0.93 (3H, s, Me-30), 1.02 (3H, s, Me-26), 1.16 (3H, s, Me-27) 5.27 (1H, brs, H-12), 3.68 (1H, m, H-2), 3.33 (1H, d,  $J$  = 9.7 Hz, H-3), 3.49 (1H, d,  $J$  = 11.0 Hz) and 3.25 (1H, d,  $J$  = 11.0 Hz) (H-23), 2.83 (1H, dd,  $J$  = 13.6, 4.15 Hz, H-18);  $^{13}\text{C}$ -NMR: see Table 4.1.

*Arjungenin (6)*. Amorphous white powder;  $[\alpha]_{D}^{20}$  30.2 (*c* 0.004, MeOH); HR-ESI-MS: *m/z* 503.3364 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>47</sub>O<sub>6</sub>, 503.3373, Δ -1.78 ppm); FA adduct ion at *m/z* 549.3417 [M-H+FA]<sup>-</sup>; <sup>1</sup>H-NMR (CD<sub>3</sub>OD): δ<sub>H</sub> 0.69 (3H, s, Me-23), 0.76 (3H, s, Me-26), 0.93 (3H, s, Me-29), 0.95 (3H, s, Me-30), 1.01 (3H, s, Me-25), 1.30 (3H, s, Me-27) 5.31 (1H, brs, H-12), 3.68 (1H, m, H-2), 3.34 (1H, d, *J* = 9.7 Hz, H-3), 3.50 (1H, d, *J* = 11.0 Hz) and 3.27 (1H, d, *J* = 11.0 Hz, H-23), 3.24 (1H, brs, H-19), 3.05 (1H, brs, s, H-18); <sup>13</sup>C-NMR: see Table 4.1.

*Arjunic acid (7)*. White powder;  $[\alpha]_{D}^{20}$  = 30.2 (*c* 0.004, MeOH); HR-ESI-MS: *m/z* 489.3576 [M+H]<sup>+</sup> (calculated for C<sub>30</sub>H<sub>49</sub>O<sub>5</sub>, 489.3580 Δ -0.81 ppm); <sup>1</sup>H-NMR (CD<sub>3</sub>OD): δ<sub>H</sub> 0.76 (3H, s, Me-26), 0.80 (3H, s, Me-24), 0.93 (3H, s, Me-29), 0.95 (3H, s, Me-30), 0.99 (3H, s, Me-25), 1.00 (3H, s, Me-23), 1.29 (3H, s, Me-27) 5.31 (1H, brs, H-12), 3.61 (1H, dt, *J* = 4.4, 11.2 Hz, H-2), 2.90 (1H, d, *J*=9.6 Hz, H-3), 3.24 (1H, d, *J* = 3.5 Hz, H-19), 3.05 (1H, brs, s, H-18); <sup>13</sup>C-NMR: see Table 4.1.

*2α,3β,21β,23-tetrahydroxyolean-12-en-28-oic acid (8)*. Amorphous white powder;  $[\alpha]_{D}^{20}$  31.9 (*c* 0.0066, MeOH); HR-ESI-MS: *m/z* 503.3375 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>47</sub>O<sub>6</sub>, 503.3373, Δ -0.39 ppm), FA adduct ion at *m/z* 549.3431 [M-H+FA]<sup>-</sup>; <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>): δ<sub>H</sub> 0.54 (3H, s, Me-23), 0.66 (3H, s, Me-26), 0.84 (3H, s, Me-29), 0.88 (3H, s, Me-30), 0.91(3H, s, Me-25), 1.25 (3H, s, Me-27) 5.23 (1H, brs, H-12), 3.49 (1H, m, H-2), 3.31 (1H, d, *J* = 10.6 Hz, H-3), 3.50 (1H, d, *J* = 11.0 Hz) and 3.27 (2H, d, *J* = 11.0 Hz) (H-23), 3.24 (1H, brd, *J* = 10.8 Hz, H-19), 3.05 (1H, brd, *J* = 10.8 Hz, H-18); <sup>13</sup>C-NMR: see Table 4.1.

*Terminolic acid (9)*. Amorphous white powder;  $[\alpha]_{D}^{20}$  26.6 (*c* 0.0024, MeOH); HR-ESI-MS: *m/z* 503.3368 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>47</sub>O<sub>6</sub>, 503.3373, Δ -0.99 ppm), FA adduct ion at *m/z* 549.3423 [M-H+FA]<sup>-</sup>; <sup>1</sup>H-NMR (Pyridin-d<sub>5</sub>): δ<sub>H</sub> 0.90 (3H, s, Me-30), 0.98 (3H, s, Me-29), 1.21 (3H, s, Me-27), 1.62 (3H, s, Me-26), 1.74 (3H, s, Me-24), 1.77 (3H, s, Me-25), 5.55 (1H, brs, H-12), 5.09 (1H, s, H-6), 4.40 (1H, d, *J* = 9.3 Hz, H-2), 4.24 (1H, d, *J* = 9.3 Hz, H-3), 4.40 (1H, d, *J* = 10.4 Hz) and 4.04 (1H, d, *J* = 10.4 Hz) (H-23), 3.31 (1H, d, *J* = 14.4 Hz, H-18); <sup>13</sup>C-NMR: see Table 4.1.

*Arjunic acid 28-O-β-D-glucopyranoside (10)*. Amorphous white needles;  $[\alpha]_{D}^{20}$  36.2 (*c* 0.0026, MeOH) HR-ESI-MS *m/z* 649.3943 [M-H]<sup>-</sup> (calculated for C<sub>36</sub>H<sub>57</sub>O<sub>10</sub>, 649.3952, Δ -

1.38 ppm), FA adduct ion at  $m/z$  695.4002 [M-H+ FA] $^-$ ;  $^1\text{H-NMR}$  ( $\text{CD}_3\text{OD}$ ):  $\delta_{\text{H}}$  0.68 (3H, s, Me-23), 0.79 (3H, s, Me-26), 0.90 (3H, s, Me-29), 0.92 (3H, s, Me-30), 1.02 (3H, s, Me-25), 1.18 (3H, s, Me-27), 5.25 (1H, brs, H-12), 3.70 (1H, m, H-2), 3.50 (1H, d,  $J$  = 11.0 Hz) and 3.26 (H, d,  $J$  = 11.0 Hz, H-23), 3.37 (1H, m, H-3), 2.86 (1H, dd,  $J$  = 13.4Hz, 4.17 Hz, H-18); sugar moiety: 5.37 (1H, d,  $J$  = 8.1 Hz, H-1'), 3.26 (1H, m, H-2'), 3.41 (1H, m, H-3'), 3.36 (1H, m, H-4'), 3.36 (1H, m, H-5'), 3.82 and 3.68 (2H, d,  $J$  = 11.3 Hz, H-6');  $^{13}\text{C-NMR}$ : see Table 4.1.

*Chebuloside II (11)*. Amorphous with powder;  $[\alpha]_{D}^{20}$  10.5 ( $c$  0.0086, MeOH); HR-ESI-MS:  $m/z$  665.3879 [M-H] $^-$  (calculated for  $\text{C}_{36}\text{H}_{57}\text{O}_{11}$ , 665.3901,  $\Delta$  -3.3 ppm), FA adduct ion at  $m/z$  711.3950 [M-H+ FA] $^-$ ;  $^1\text{H-NMR}$  (Pyridin-d<sub>5</sub>):  $\delta_{\text{H}}$  0.85 (3H, s, Me-29), 0.85 (3H, s, Me-30), 1.16 (3H, s, Me-27), 1.73 (3H, s, Me-26), 1.76 (3H, s, Me-24), 1.80 (3H, s, Me-25), 5.49 (1H, brs, H-12), 5.09 (1H, d, H-6), 4.41 (1H, m, H-2), 4.26 (1H, m, H-3), 4.42 (1H, d,  $J$  = 11.0 Hz) and 4.06 (2H, d,  $J$  = 11.0 Hz, H-23), 3.19 (1H, d,  $J$  = 9.7 Hz, H-18); sugar moiety: 6.30 (1H, d,  $J$  = 8.0 Hz, H-1'), 4.19 (1H, m, H-2'), 4.26 (1H, m, H-3'), 4.37 (1H, m, H-4'), 3.99 (1H, m, H-5'), 4.42 (2H, m, H-6');  $^{13}\text{C-NMR}$ : see Table 4.1.

*Sericoside (12)*. Amorphous with powder;  $[\alpha]_{D}^{20}$  11.9 ( $c$  0.0083, MeOH); HR-ESI-MS:  $m/z$  665.3896 [M-H] $^-$  (calculated for  $\text{C}_{36}\text{H}_{57}\text{O}_{11}$ , 665.3901,  $\Delta$  0.75 ppm), FA adduct ion at 711.3953 [M-H+ FA] $^-$ ;  $^1\text{H-NMR}$  (Pyridin-d<sub>5</sub>):  $\delta_{\text{H}}$  0.95 (3H, s, Me-29), 1.02 (3H, s, Me-25), 1.12 (3H, s, Me-30), 1.13 (3H, s, Me-26), 1.56 (3H, s, Me-24), 1.58 (3H, s, Me-27), 5.47 (1H, brs, H-12), 4.29 (1H, m, H-2), 4.46 (1H, d,  $J$  = 10.8 Hz) and 3.73 (2H,,d,  $J$  = 10.8Hz) (H-23), 3.55 (1H, d,  $J$ =9.36 Hz, H-19), 3.54 (1H, m, H-3), 3.51 (1H, brd,  $J$  = 10.8 Hz, H-18); sugar moiety: 6.37 (1H, d,  $J$ =8.05 Hz, H-1'), 4.22 (1H, m, H-2'), 4.29 (1H, m, H-3'), 4.38 (1H, m, H-4'), 4.03 (1H, m, H-5'), 4.43 (2H, m, H-6');  $^{13}\text{C-NMR}$ : see Table 4.1.

*Arjunglucoside I (13)*. Amorphous with powder;  $[\alpha]_{D}^{20}$  20.2 ( $c$  0.0047, MeOH); HR-ESI-MS:  $m/z$  665.3892 [M-H] $^-$  (calculated for  $\text{C}_{36}\text{H}_{57}\text{O}_{11}$ , 665.3901,  $\Delta$  -1.35 ppm), , FA adduct ion  $m/z$  711.3953 [M-H+ FA] $^-$ ;  $^1\text{H-NMR}$  ( $\text{CD}_3\text{OD}$ ):  $\delta_{\text{H}}$  0.69 (3H, s, Me-23), 0.74 (3H, s, Me-26), 0.93 (3H, s, Me-30), 0.94 (3H, s, Me-29), 1.02 (3H, s, Me-25), 1.29 (3H, s, Me-27), 5.32 (1H, brs, H-12), 3.34 and 3.68 (1H,m, H-2), 3.35 (1H, m, H-3), 3.49 (1H, d,  $J$  = 11.03 Hz) and 3.25 (2H, d,  $J$  = 11.0 Hz, H-23), 3.26 (1H, d,  $J$  = 9.7 Hz, H-19), 3.04 (1H, brs, H-18); sugar

moiety: 5.36 (1H, d,  $J = 8.1$  Hz, H-1'), 3.3 (1H, m, H-2'), 3.34 (1H, m, H-3'), 3.34 (1H, m, H-4'), 3.34 (1H, m, H-5'), 3.81 and 3.67 (2H, d,  $J = 12.3$  Hz, H-6');  $^{13}\text{C}$ -NMR: see Table 4.1.

*Calophy whole membrane B (14)*.  $^1\text{H}$ -NMR data ( $\text{CD}_3\text{OD}$ ):  $\delta_{\text{H}}$  6.76 (1H, m, H-3), 6.76 (1H, m, H-4), 7.03 (1H, s, H-6), 2.72 (2H, m, H-7), 3.69 (2H, m, H-8), 4.7 (1H, d,  $J = 7.2$  Hz, H-1'), 3.46 (1H, m, H-2'), 3.46 (1H, m, H-3'), 3.34 (1H, m, H-4'), 3.56 (1H, d,  $J = 9.2$  Hz, H-5'), 4.06 (2H, dd,  $J = 8.9$  Hz, 9.17 Hz, H-6') (2H, dd,  $J = 8.9$  Hz; 9.2 Hz, H-6'), 4.73 (1H, m, H-1''), 3.86 (1H, m, H-2''), 3.71 (1H, m, H-3''), 3.37 (1H, m, H-4''), 3.65 (1H, m, H-5''), 1.23 (3H, d,  $J = 6.36$  Hz, H-6'').  $^{13}\text{C}$ -NMR ( $\text{CD}_3\text{CN}$ )  $\delta_{\text{C}}$  146.7 (C, C-1), 146.6 (C, C-2), 117.0 (CH, C-3), 125.1 (CH, C-4), 131.8 (C, C-5), 119.3 (CH, C-6), 39.8 (CH<sub>2</sub>, C-7), 64.5 (CH<sub>2</sub>, C-8), 104.4 (CH, C-1'), 74.9 (CH, C-2'), 77.5 (CH, C-3'), 71.6 (CH, C-4'), 77.1 (CH, C-5'), 68.0 (CH, C-6'), 102.1 (CH, C1''), 72.2 (CH, C-2''), 72.4 (CH, C-3''), 73.9 (CH, C-4''), 69.6 (CH, C-5''), 18.0 (CH<sub>3</sub>, C-6'');  $^{13}\text{C}$ -NMR: see Table 4.1.

### 4.3. Results and Discussion

The present study reports the isolation and identification of the active principles of *Terminalia albida* used in Guinean traditional medicine for the management of microbial diseases including malaria. The dichloromethane and ethyl acetate fractions with an IC<sub>50</sub> of 11.9  $\mu\text{g}/\text{mL}$  and 5.9  $\mu\text{g}/\text{ml}$ , respectively, against *P. falciparum* (Table 4.2) were subjected to normal-phase and reverse-phase flash chromatography and semipreparative HPLC with DAD and ESIMS detection to afford 14 compounds. Five compounds were isolated and identified from the dichloromethane extract and nine from the ethyl acetate extract. Chemical structures of the isolated compounds were established by HR-ESI-MS combined with 1D NMR ( $^1\text{H}$ -,  $^{13}\text{C}$ -NMR, DEPT 135, DEPT 90) and 2D NMR (COSY, HSQC, HMBC). The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectroscopic as well as mass spectrometric data, of these compounds, available as Supplementary Material, were in agreement with previously published data, allowing the identification of pantolactone (3-hydroxy-4,4-dimethylidihydrofuran-2(3H)-one) (1)<sup>17</sup>, friedelin (2)<sup>18</sup>, 3,4,3'-tri-O-methyl-ellagic-acid (3)<sup>19</sup>, sericic acid (4)<sup>20</sup>, arjunolic acid (5)<sup>21</sup>, arjungenin (6)<sup>22</sup>, arjunic acid (7), 2 $\alpha$ ,3 $\beta$ ,21 $\beta$ ,23-tetrahydroxyolean-12-en-28-oic-acid (8)<sup>23</sup>, terminolic acid (9)<sup>24</sup>, arjunolic acid 28-O- $\beta$ -D-glucopyranoside (arjunglucoside II) (10)<sup>25</sup>, chebuloside II (11)<sup>26</sup>, sericoside (12)<sup>27</sup>, arjunglycoside I (13)<sup>28</sup>, and calophy whole membrane B (14)<sup>29</sup> (Figure 4.2). Apart from pantolactone (1), an ellagic acid derivative (3) and calophy whole membrane B

**(14)**, which is a hydroxytyrosol-diglycoside, all isolated compounds were triterpenes, most of them typical for the genus *Terminalia*. It can be noted that sericoside **(12)** is the C-28 glycosyl ester of sericic acid **(4)**, that arjunglucoside II **(10)** is the C-28 glycosyl ester of arjunolic acid **(5)**, that arjunglycoside I **(13)** is the C-28 glycosyl ester of arjungenin **(6)**, and that chebuloside II **(11)** is the C-28 glycosyl ester of terminolic acid **(9)**.

None of the isolated compounds showed cytotoxicity up to the highest test concentration of 64  $\mu\text{M}$ . The antiplasmodial activity of all these compounds has been determined and the highest activity was obtained for pantolactone **(1)** ( $0.6 \pm 0.03 \mu\text{M}$ ) (Table 4.2). A range of other constituents, including 3,4,3'-tri-O-methyl-ellagic acid **(3)**; the triterpenes arjunolic acid **(5)**, arjungenin **(6)**, arjunic acid **(7)** and arjunic acid 28-O- $\beta$ -D-glucopyranoside **(10)**; and the phenol glycoside calophymembranside-B **(14)**, showed IC<sub>50</sub> values in the range 5 – 15  $\mu\text{M}$ . Apart from friedelin **(2)** (IC<sub>50</sub> 31.19  $\mu\text{M}$ ) and sericoside **(12)** (IC<sub>50</sub> 44.4  $\mu\text{M}$ ), all other compounds were completely inactive. To best of our knowledge, this is the first report on the *in vitro* antiplasmodial activity of pantolactone **(1)**, a  $\gamma$ -butyrolactone derivative. Pantolactone was obtained before from *Pandanus simplex*, an endemic species in the Philippines <sup>30</sup>; glycosides were found in *Lygopodium japonicum* <sup>31</sup> and *Oryza sativa* (rice) <sup>32,33</sup>.

Most of the isolated compounds in our study were oleanane-type triterpenoids. The structural differences between these 10 oleanane triterpenoids occur at positions C-6, C-19, C-21, C-23 and C-28. As it appeared from the results of the biological assay, the substitution pattern of the triterpenoids, mainly consisting of hydroxyl groups and glucopyranosyl ester substitution at C-28, had an important influence on the antiplasmodial activity. Also the  $\alpha$ - or  $\beta$ - configuration of hydroxyls linked to asymmetric carbons had a strong impact on the bioactivity of certain triterpenes. Amongst the tested oleanane terpenoids, arjungenin **(6)** was the most active against *P.falciparum* with an IC<sub>50</sub> value of  $5.9 \pm 2.2 \mu\text{M}$ . This activity is much higher compared to that recently reported by Oluyemi et al. (2020) against both *P.falciparum* chloroquine sensitive (D10) and resistant (W2) strains ( $81 \pm 17$  and  $127 \pm 9 \mu\text{M}$ , respectively).

Based on the activity of arjungenin (**6**), an attempt was made to establish some structure-activity relationships (SAR). It seems that removal of the  $\alpha$ -hydroxyl group in position C-19 as in arjunolic acid (**5**) and the addition of a glucosyl moiety in position C-28 to yield arjunglucoside II (**10**), only has a small influence on the antiplasmodial activity. However, when arjungenin (**6**) is only glycosylated as in arjunglycoside I (**13**), the activity is completely lost. Compounds **5**, **6** and **10** all have a hydroxymethyl group (C-23) with  $\alpha$ -orientation, but when C-23 is a methyl, as in arjunic acid (**7**), the IC<sub>50</sub> value remains in the same range, i.e. between 5 and 15  $\mu$ M. However, both compounds in which C-24 was the hydroxymethyl group ( $\beta$ -orientation), i.e. sericic acid (**4**) (IC<sub>50</sub> >64  $\mu$ M) and sericoside (**12**) (IC<sub>50</sub> 44.4  $\mu$ M) were completely or almost completely inactive, respectively. Also the C-4 isomer of sericoside (**12**), i.e. arjunglycoside I (**13**), only different in the orientation of the hydroxymethyl group, was inactive. The same is true for several other compounds with a C-23 hydroxymethyl ( $\alpha$ -orientation) functionality, such as 2 $\alpha$ ,3 $\beta$ ,21 $\beta$ ,23-tetrahydroxyolean-12-en-28-oic-acid (**8**) and terminolic acid (**9**): apparently, the presence of a 21  $\beta$ -OH or a 6  $\beta$ -OH, respectively, has a negative influence on the activity. The latter observation is in agreement with previous studies which have shown that the introduction of a hydroxyl group in position C-6 of oleanane-type triterpenoids could decrease their antifungal<sup>35</sup> or antiplasmodial activity<sup>34</sup>. Remarkably, glycosylation at C-28 results in a loss of activity of arjungenin (**6**) compared to arjunglycoside I (**13**); whereas arjunolic acid (**5**) and arjunglucoside II (**10**) are equally (moderately) active; and terminolic acid (**9**) / chebuloside II (**11**), as well as sericic acid (**4**) / sericoside (**12**) all are inactive.

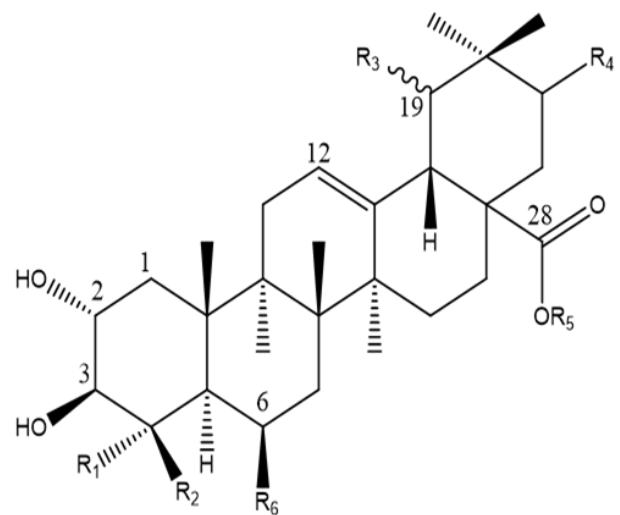
In contrast to these polyhydroxylated oleanane-type triterpenes, friedelin is a friedelane-type triterpene with only one hydroxyl group. Friedelin has been previously isolated from several *Terminalia* species including *T. glaucescens* *T. mollis* and *T. avicennioides*<sup>36</sup>. The activity of friedelin against *P. falciparum* found in our study (31.2  $\mu$ M) is lower than previously reported (IC<sub>50</sub> 7.7 and 7.2  $\pm$  0.5  $\mu$ M)<sup>37,7</sup>.

The phenol glycoside calophyembranaside-B (**14**) has previously been isolated from *Calophyllum membranaceum*<sup>29</sup> and *Clematis mandshurica*<sup>38</sup>, but its antiplasmodial activity (IC<sub>50</sub> 7.6  $\pm$  0.05) is reported here for the first time.

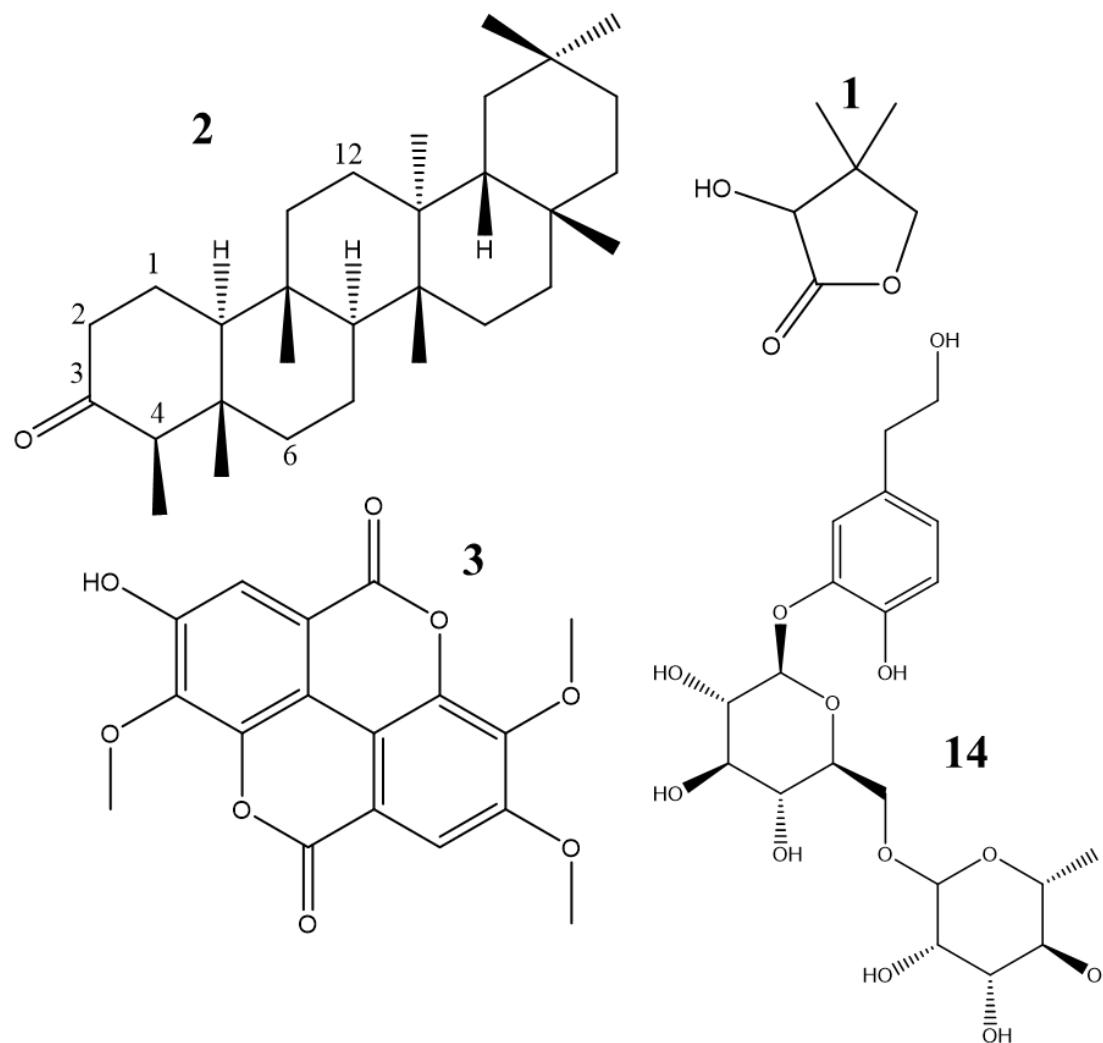
Apart from pantolactone (**1**), calophyminembranide-B (**14**) and some of the triterpenes, also 3,4,3'-tri-*O*-methyl-ellagic-acid (**3**) was obtained as one of the active constituents of *T. albida*. Since it is well known that the use of extraction solvents like methanol may contribute to the formation of artefacts such as methyl ethers<sup>39,40,41</sup>, it was checked if compound **3** was present in the genuine plant material. Therefore, an ethanolic extract was prepared from the root and analysed by LC-MS. The ethanolic extract displayed a pseudomolecular ion at *m/z* 343.045 [M-H]<sup>-</sup> at the same retention time as the isolated compound 3,4,3'-tri-*O*-methyl-ellagic acid (Figure 4.3), confirming that 3,4,3'-tri-*O*-methyl-ellagic acid was not an artefact. Although polymethoxylated derivatives of ellagic acid have been reported in other plant species such as *Terminalia macroptera*<sup>42,43</sup>, *Syzygium aromaticum*<sup>44</sup> and *Irvingia malayana*<sup>45</sup>, to the best of our knowledge, this is the first time that this compound was isolated from *Terminalia albida*, and that antiplasmodial activity was reported for a trimethyl ether of ellagic acid.

Based on the antibacterial and antifungal activity found during our preliminary biological screening<sup>9</sup>, the fractions and compounds isolated from the methanolic extract were tested against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Although the methanolic extract was active against *S. aureus* (IC<sub>50</sub> 12.8 µg/ml) and *C. albicans* (IC<sub>50</sub> 7.9 µg/ml), none of its fractions and isolated compounds showed antibacterial or antifungal activity. It appears that the initial fractionation of the methanolic extract has led to the loss of the antibacterial and the antifungal activities. In contrast to our result, a previous study carried out by Runyoro et al. (2013) revealed that terminolic acid (**9**) isolated from *Combretum zeyheri* demonstrated activity against *C. albicans* strains MTCC1637 (MIC 125 µg/mL) and ATCC90028 (MIC 62.50 µg/mL)<sup>35</sup>. Furthermore, a study carried out by Gossan et al. (2016) demonstrated that terminolic acid exhibited moderate antibacterial activity against *S. aureus* and *E. coli* (minimum inhibitory concentration (MIC) within a range of 64 and 256 µg/mL)<sup>46</sup>. Eldeen et al. (2008) have reported the effectiveness of arjunic acid (**7**) against *B. subtilis* (IC<sub>50</sub> 2.1 µg/mL) *S. aureus* (3.0 µg/mL), *E. coli* (3.5 µg/mL), and *K. pneumoniae* (5.9 µg/mL)<sup>47</sup>. Ghosh and Sil (2013) have observed moderate antifungal activity against *Candida albicans*, *C. krusei* and *C. parapsilosis* for a mixture of arjunolic acid and asiatic acid with MIC values between 50 and 200 mg/mL<sup>48</sup>. It is important to point out that, despite the historical efficacy of bioassay-guided fractionation of plant extracts, loss of activity and failure in isolation of active compounds during the

fractionation process could occur<sup>49,50</sup>. The main reasons of these pitfalls may be the degradation of compounds during the purification process, a low concentration of bioactive compounds making their isolation difficult, and/or potential synergistic effects<sup>50</sup>. These may be causes for the loss of activity during our fractionation process. This may be avoided in “synergy-directed fractionation”<sup>51</sup> which combines chromatographic separation and synergy testing in combination with a known active constituent in the original extract.



Compounds	R1	R2	R3	R4	R5	R6
4	CH <sub>3</sub>	CH <sub>2</sub> OH	αOH	H	H	H
5	CH <sub>2</sub> OH	CH <sub>3</sub>	H	H	H	H
6	CH <sub>2</sub> OH	CH <sub>3</sub>	αOH	H	H	H
7	CH <sub>3</sub>	CH <sub>3</sub>	αOH	H	H	H
8	CH <sub>2</sub> OH	CH <sub>3</sub>	H	OH	H	H
9	CH <sub>2</sub> OH	CH <sub>3</sub>	H	H	H	OH
10	CH <sub>2</sub> OH	CH <sub>3</sub>	H	H	1-β-D-GlcP	H
11	CH <sub>2</sub> OH	CH <sub>3</sub>	H	H	1-β-D-GlcP	OH
12	CH <sub>3</sub>	CH <sub>2</sub> OH	αOH	H	1-β-D-GlcP	H
13	CH <sub>2</sub> OH	CH <sub>3</sub>	αOH	H	1-β-D-GlcP	H



**Figure 4.2.** Compounds **1–14** isolated from *Terminalia albida*

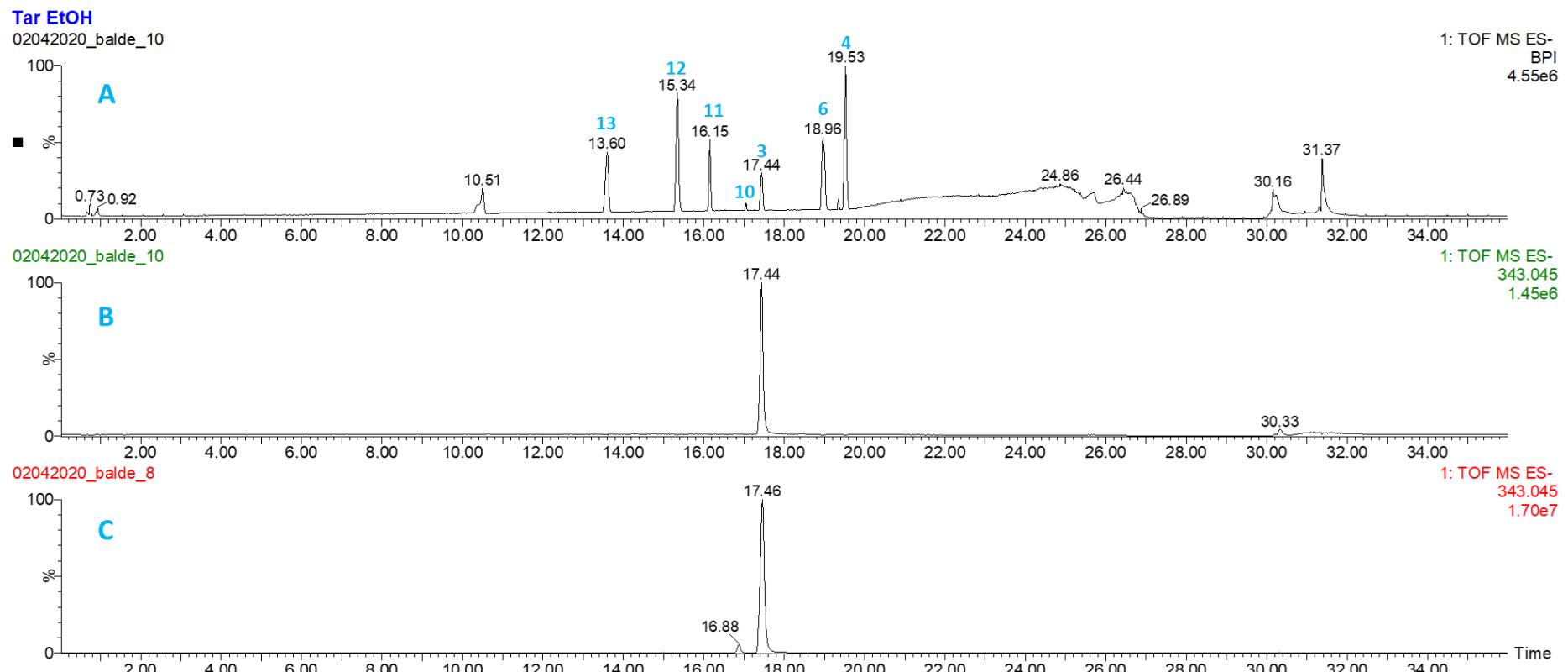
**Table 4.1.**  $^{13}\text{C}$  NMR assignments of the isolated triterpenes (compounds **2**, **4-13**) in  $\text{CDCl}_3$ ,  $\text{CD}_3\text{OD}$ ,  $\text{DMSO-d}_6$  and pyridine- $\text{d}_5$  (ppm)

	Friedeli n (2) <sup>a</sup>	Seric Acid (4) <sup>c</sup>	Arjunolic acid (5) <sup>b</sup>	Arjun genin (6) <sup>b</sup>	Arjunic acid (7) <sup>b</sup>	2 $\alpha$ ,3 $\beta$ ,21 $\beta$ ,23- tetrahydroxyo lean-12-en- 28-oic-acid (8) <sup>c</sup>	Arjunic acid 28- <i>O</i> - $\beta$ -D- gluco- pyranosi de (10) <sup>b</sup>	Termi nolic acid (9) <sup>d</sup>	Chebulo side II (11) <sup>d</sup>	Sericosi de (12) <sup>d</sup>	Arjungly coside (13) <sup>b</sup>
Position	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$	$\delta\text{C}$
1	22.3	46.8	47.9	47.7	47.9	46.6	50.1	47.9	50.3	47.5	47.8
2	41.5	67	69.7	69.7	69.5	67.3	68.9	69.7	69.1	68.7	69.8
3	213.2	83.8	78.1	78.3	84.5	75.4	78.1	78.2	78.3	85.8	78.3
4	58.2	42.9	44.1	44.1	40.5	42.5	44.3	44.1	44.6	44.0	44.1
5	42.1	55.5	48.1	48.3	56.8	46.1	48.6	48.0	48.8	56.6	48.2
6	41.3	18.9	19.1	28.6	19.7	17.6	67.4	19.1	67.6	19.5	19.2
7	18.2	32.8	33.3	33.3	33.9	32.4	41.0	33.3	41.1	33.5	33.3
8	53.1	37.5	40.5	40.7	40.8	41.2	39.5	40.8	39.5	40.3	40.8
9	37.4	47.4	49.0	49.2	49.2	47.3	49.3	49.0	48.8	48.6	49.0
10	59.5	41.0	39.1	39.2	39.4	37.5	38.6	39.1	38.2	38.5	39.1
11	35.6	27.9	24.6	24.8	24.6	23.3	24.6	24.6	24.1	24.5	24.9
12	30.5	122.0	123.3	124.7	124.7	122.2	122.5	123.5	123.1	123.0	124.8

13	39.7	143.5	145.6	144.8	144.7	143.5	143.9	145.2	143.5	144.4	144.4	
14	38.3	42.9	43.0	42.7	42.7	42.5	42.6	43.1	42.8	42.1	42.7	
15	32.8	28.3	28.8	19.3	34.0	27.2	27.9	28.9	28.3	29.0	29.4	
16	35.9	27.1	33.9	29.4	29.4	23.3	24.1	23.9	23.5	28.0	28.4	
17	29.9	44.6	49.1	46.7	46.7	44.6	48.9	49.0	47.0	46.5	47.1	
18	42.8	43.1	42.8	45.2	45.2	43.2	41.8	42.6	41.8	44.6	45.0	
19	35.3	80.1	47.3	82.4	82.4	27.9	46.2	47.2	46.2	81.0	82.4	
20	28.2	34.9	31.6	36	36.0	34.9	30.7	31.5	30.8	35.3	35.9	
21	32.4	23.4	34.9	29.5	29.5	80.1	34.9	34.9	34.0	28.9	29.5	
22	39.2	32.3	24.1	34.1	28.6	32.0	33.9	33.1	32.5	33.0	33.2	
23	6.8	63.8	66.2	66.3	29.3	63.8	65.8	66.3	66.1	65.7	66.3	
24	14.6	24.5	13.9	13.8	17.4	13.6	15.8	13.8	16.1	24.2	13.8	
25	17.9	13.4	17.8	17.4	17.0	16.6	19.0	17.5	19.1	17.2	17.5	
26	20.2	16.6	17.5	17.8	17.8	17.9	18.4	17.8	18.9	17.5	17.8	
27	18.6	24.0	26.5	25.1	25.0	24.2	26.1	26.3	26.1	24.9	25.1	
28	32.1	179.3	176.1	176.1	180.2	179.2	180.2	178.2	176.4	177.3	178.6	
29	35.0	23.4	33.6	25.1	28.7	28.2	33.0	23.9	23.6	28.8	25.2	
30	31.8	28.1	24.0	28.7	25.1	24.5	23.6	33.5	33.1	24.7	28.6	
1'									95.7	95.8	95.9	95.8
2'									73.9	74.1	74.2	73.8
3'									78.3	78.8	78.9	78.7

4'		71.1	71.1	71.1	71.1
5'		78.7	79.3	79.4	78.3
6'		62.4	62.1	62.2	62.4

Deuterated solvents: <sup>a</sup> CDCl<sub>3</sub>; <sup>b</sup> CD<sub>3</sub>OD; <sup>c</sup> DMSO-d<sub>6</sub>; <sup>d</sup> Pyridine-d<sub>5</sub>



**Figure 4.3.** base peak intensity chromatogram (BIP) of the ethanolic extract with major peaks labelled (compound no.) (A); Extracted Ion Chromatogram (EIC) of  $m/z$  343.045 (B) and of 3,4,3'-tri-O-methyl-ellagic acid (3) (C)

**Table 4.2.** *In vitro* antimicrobial, antiplasmodial and cytotoxic activity of extracts, fractions and isolated compounds from *Terminalia albida* root

Fraction and Compound names	Antibacterial and antifungal activity ( $IC_{50}$ $\mu M$ )			Antiprotozoal activity ( $IC_{50}$ $\mu M$ )	Antiprotozoal activity ( $IC_{50}$ $\mu g/ml$ )	Cytotoxicity ( $CC_{50}$ $\mu M$ )	Selectivity index
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>Pf-K1</i>	<i>Pf-K1</i>	<i>MRC-5</i>	<i>MRC-5/PfK1</i>
Methanol 80% extract	12.8	>64.0	7.9	0.8	Nd	21.5	26.8
Dichloromethane fraction	>64.0	>64.0	>64.0	11.9	Nd	28,6	2.3
Ethyl acetate fraction	>64.0	>64.0	>64.0	5.9	Nd	>64,0	>10.8
<i>n</i> -Butanol fraction	>64.0	>64.0	>64.0	>64.0	Nd	>64,0	Nd
Aqueous residue	>64.0	>64.0	>64.0	39.5	Nd	>64.0	>1.6
Pantolactone (1)	>64.0	Nd	>64.0	0.60 ± 0.03*	0.08	>64.0	>106.6
Friedelin (2)	>64.0	Nd	>64.0	31.2	13.3	>64.0	>106.6
3,4,3'-tri- <i>O</i> -methyl-ellagic acid (3)	>64.0	Nd	>64.0	5.9 ± 1.0*	2.0	>64.0	>12.3
Sericic acid (4)	>64.0	Nd	>64.0	>64.0	Nd	>64.0	Nd
Arjunolic acid (5)	>64.0	Nd	>64.0	9.9±5.6*	4.8	>32.0	>3.2
Arjungenin (6)	>64.0	Nd	>64.0	5.9 ± 2.2*	2.9	>64.0	>13.4
Arjunic acid (7)	>64.0	Nd	>64.0	9.7	4.7	>32.0	>3.3
2 $\alpha$ , 3 $\beta$ , 21 $\beta$ , 23 tetra hydroxy-olean-12-en-28 oic-acid (8)	>64.0	Nd	>64.0	>64.0	Nd	>64.0	Nd
Terminolic acid (9)	>64.0	Nd	>64.0	>64.0	Nd	>64.0	Nd

Arjunglucoside II ( <b>10</b> )	>64.0	Nd	>64.0	12.2	7.9	>64.0	>5.2
Chebuloside-II ( <b>11</b> )	>64.0	Nd	>64.0	>64.0		>64.0	Nd
Sericoside ( <b>12</b> )	>64.0	Nd	>64.0	44.4	29.6	>64.0	Nd
Arjunglucoside I ( <b>13</b> )	>64.0	Nd	>64.0	>64.0	Nd	>64.0	Nd
Calophymembranside-B ( <b>14</b> )	>64.0	Nd	>64.0	7.6 ± 0.05*	3.5	>64.0	>8.3
Chloroquine				0.15 ± 0.1 μM*			
Doxycycline	0.3 ± 0.2 μM*	0.6 ± 0.3 μM*					
Flucytosine			0.7 ± 0.01 μM**				
Tamoxifen					10.0 ± 1.5 μM*		

\*n = 3

#### **4.4. Conclusion**

The results of this study support, at least in part, the traditional use of *T. albida* against microbial diseases, more in particular malaria. This study reported the bioassay-guided isolation of constituents from the roots of *T. albida*, which are active against microbial diseases including malaria, from the dichloromethane and the ethyl acetate extract. Compounds from different phytochemical classes, including pantolactone, 3,4,3'-tri-*O*-methyl-ellagic-acid, the phenol glycoside calophyminbranside-B, and a range of hydroxylated oleanane-type triterpenes, were found to contribute to the activity against *P. falciparum*. In addition, a series of less active or inactive hydroxylated triterpenes was obtained. Most of the isolated compounds are reported for the first time in this species. Although less active than the reference control (chloroquine), some of these compounds could constitute a good antiplasmoidal scaffold for the discovery of new potential antimalarial drugs.

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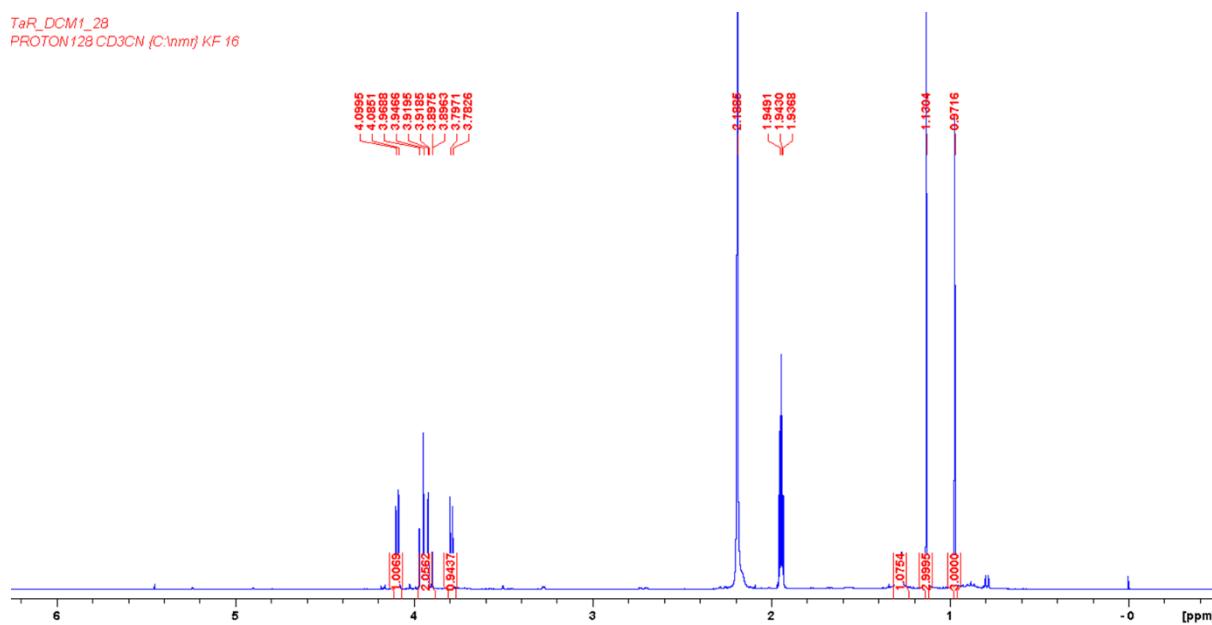
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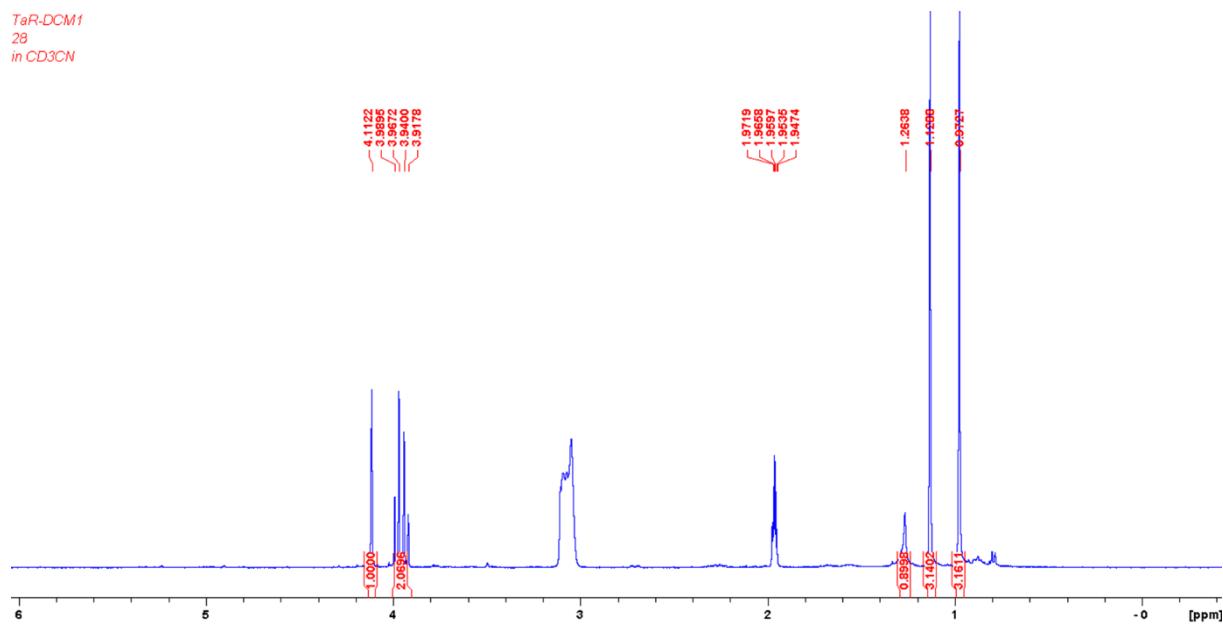
## Supplementary information

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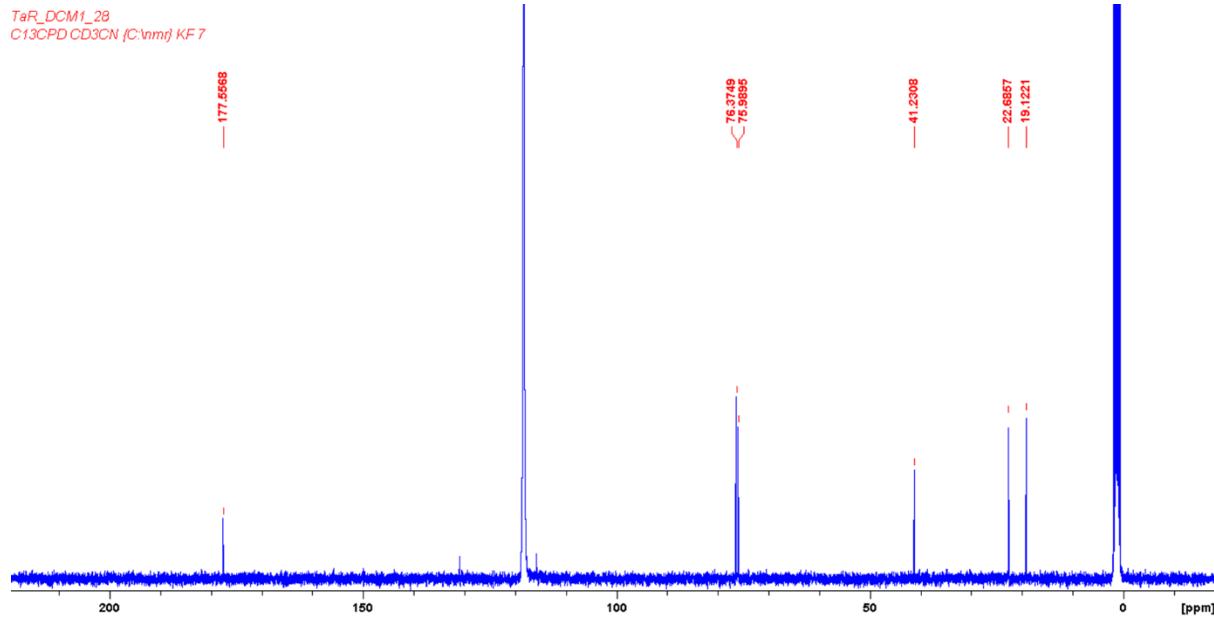


**Figure S 4.1.** <sup>1</sup>H NMR spectrum (400 MHz, CD<sub>3</sub>CN) of pentolactone (**1**)

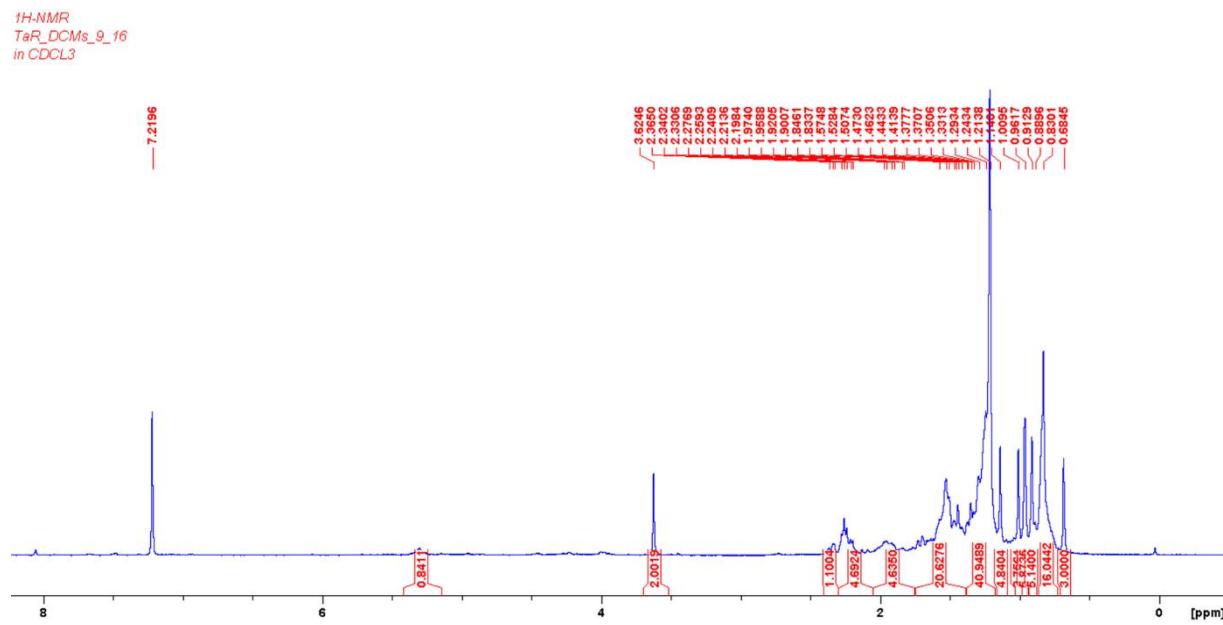
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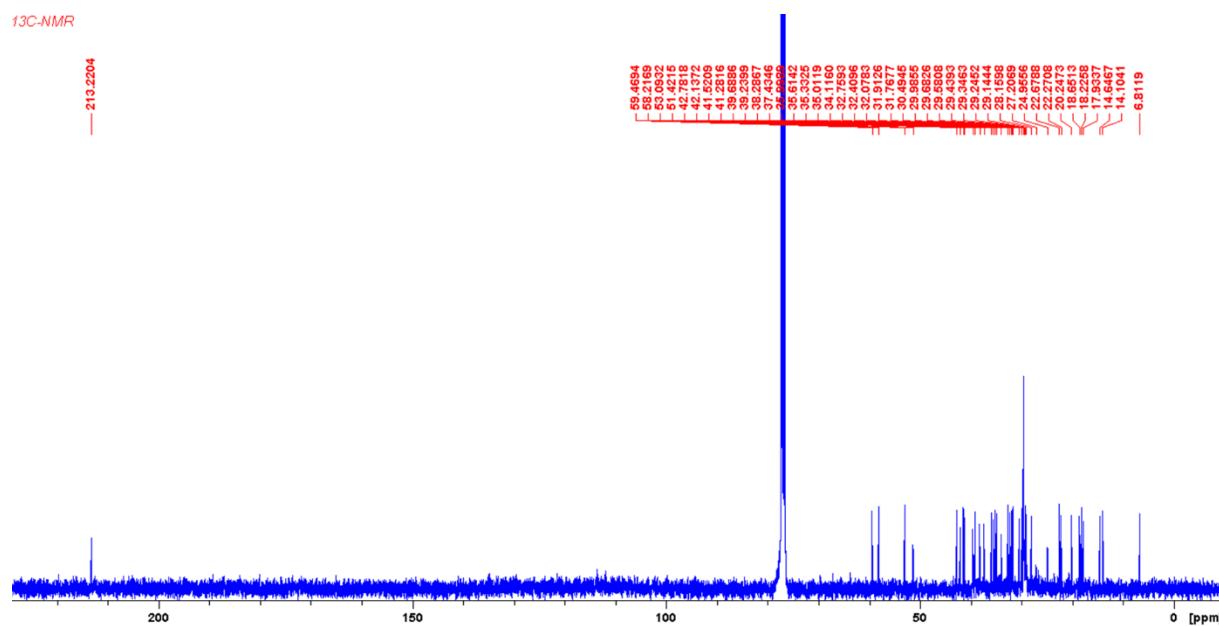
**Figure S 4.2.** <sup>1</sup>H NMR spectrum (400 MHz, CD<sub>3</sub>CN) of pentolactone where D<sub>2</sub>O was added (**1**)



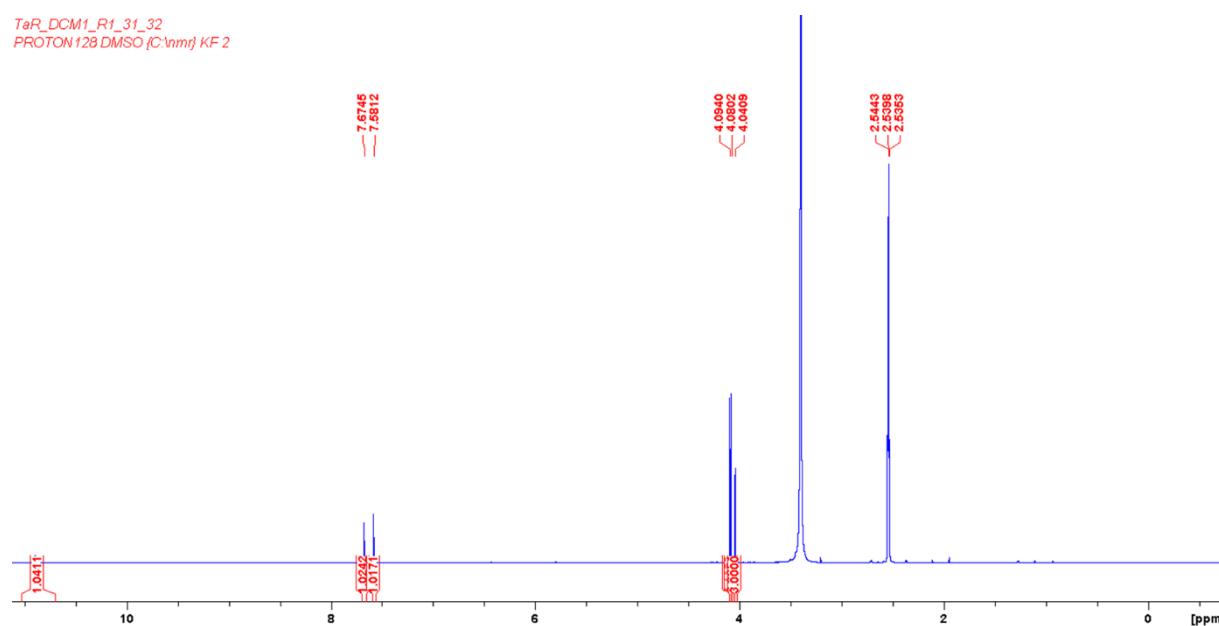
**Figure S 4.3.** <sup>13</sup>C NMR spectrum (100 MHz, CD<sub>3</sub>CN) of pentolactone (**1**)



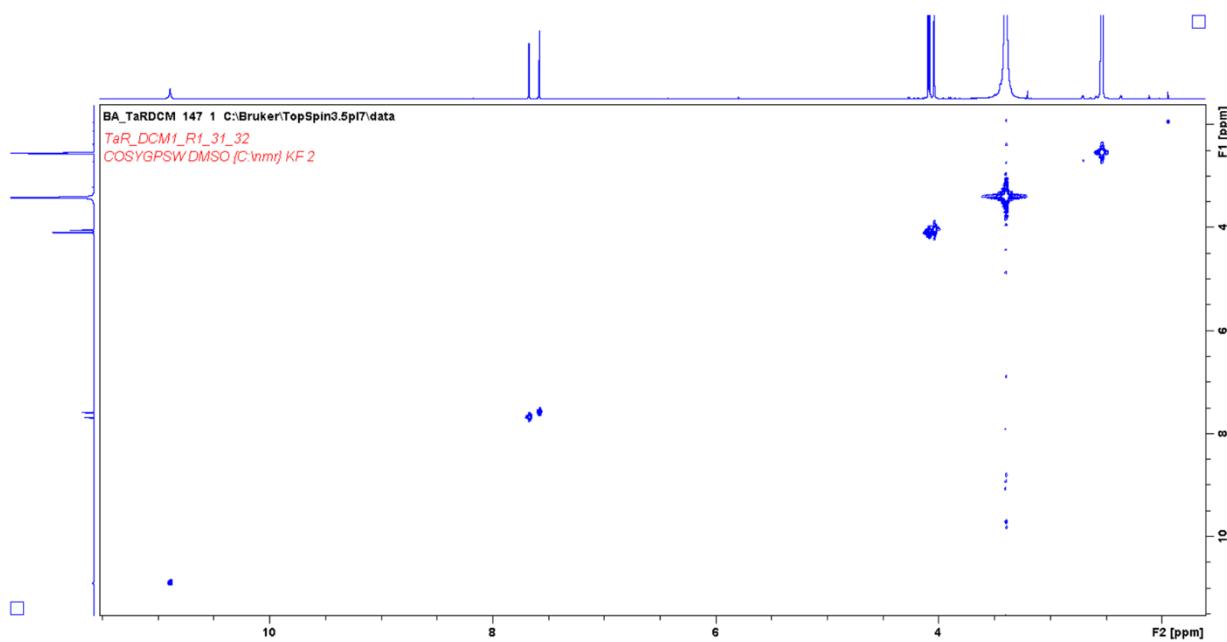
**Figure S 4.4.** <sup>1</sup>H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of Friedelin (**2**)



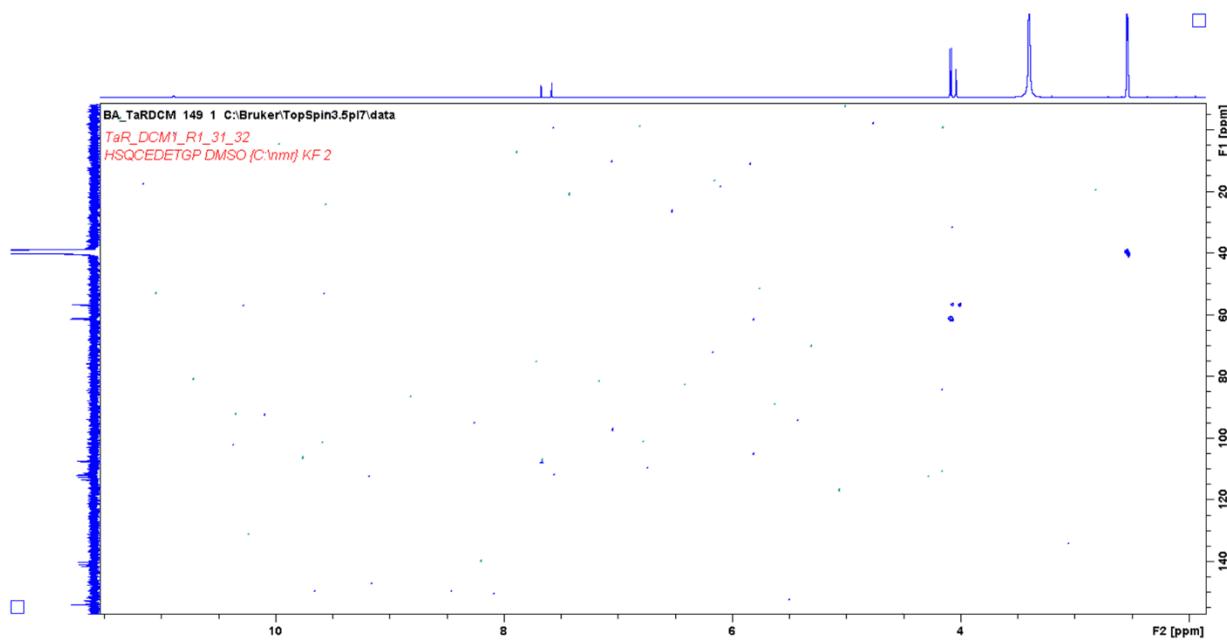
**Figure S 4.5.**  $^{13}\text{C}$  NMR spectrum (100 MHz,  $\text{CDCl}_3$ ) of Friedelin (**2**)



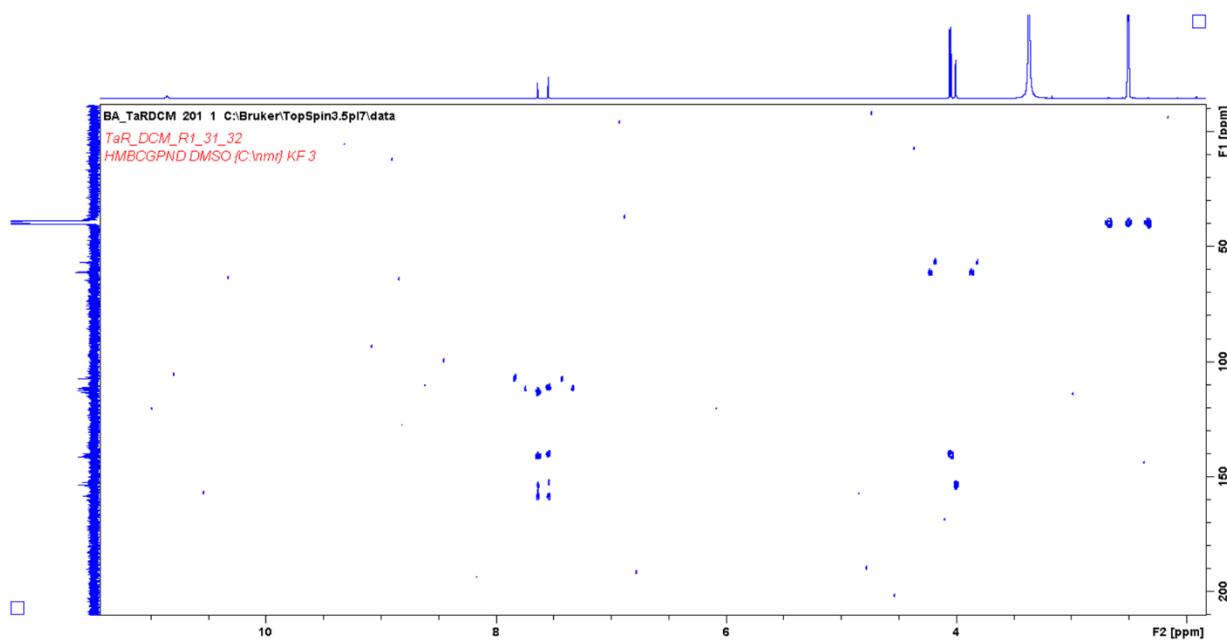
**Figure S 4.6.**  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{DMSO-d}_6$ ) of 3,4,3'-Tri-*O*-methylellagic acid (**3**)



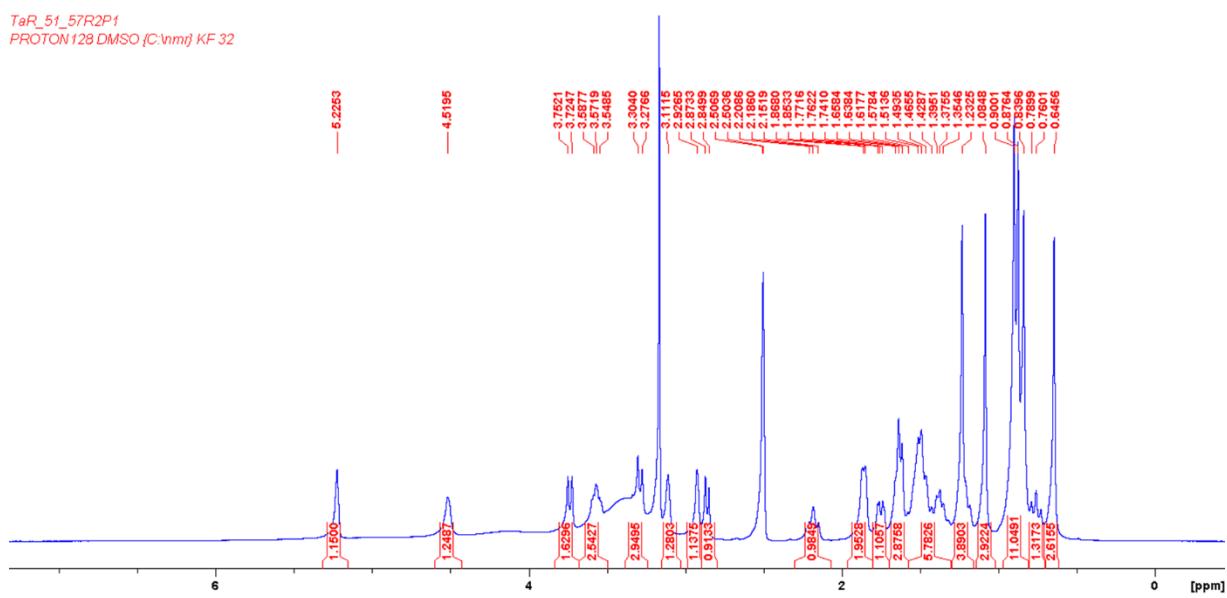
**Figure S 4.7.** COSY spectrum (DMSO-*d*<sub>6</sub>) of 3,4,3'-Tri-*O*-methylellagic acid (**3**)



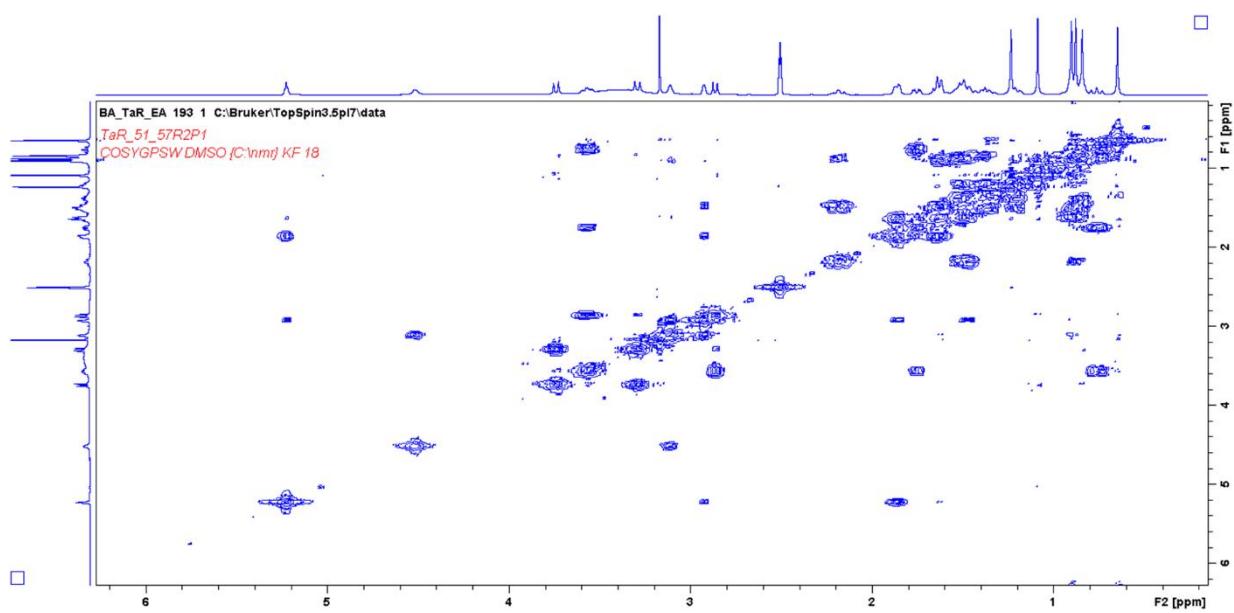
**Figure S 4.8.** HSQC spectrum (DMSO-*d*<sub>6</sub>) of 3,4,3'-Tri-*O*-methylellagic acid (**3**)



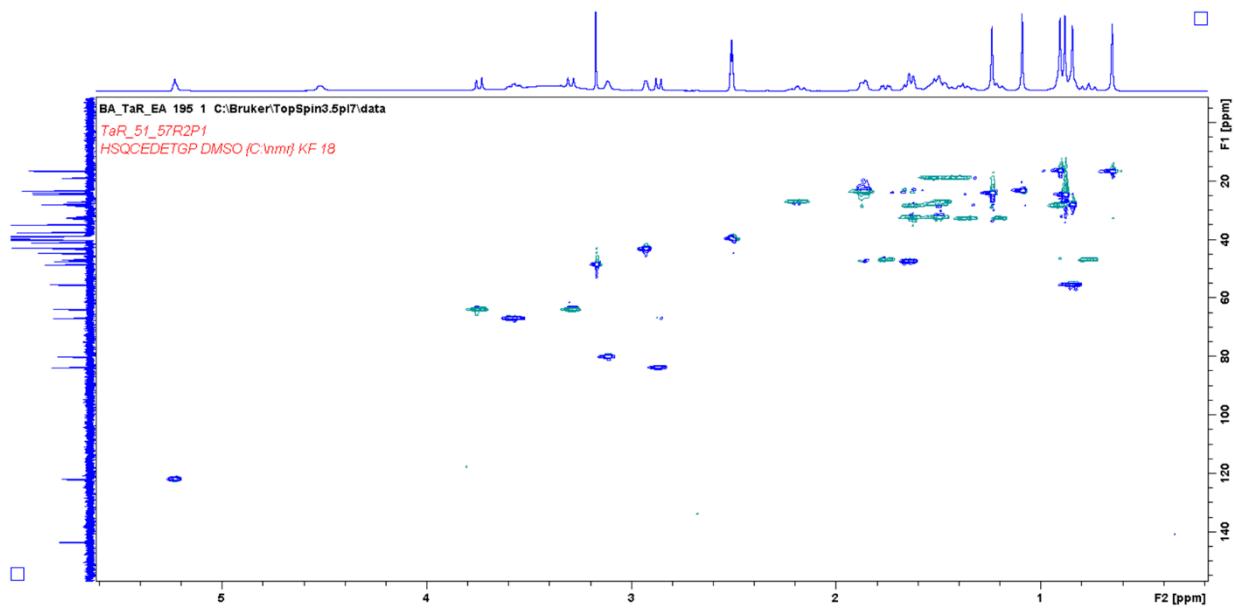
**Figure S 4.9.** HMBC spectrum (DMSO- $d_6$ ) of 3,4,3'-Tri-O-methylellagic acid (**3**)



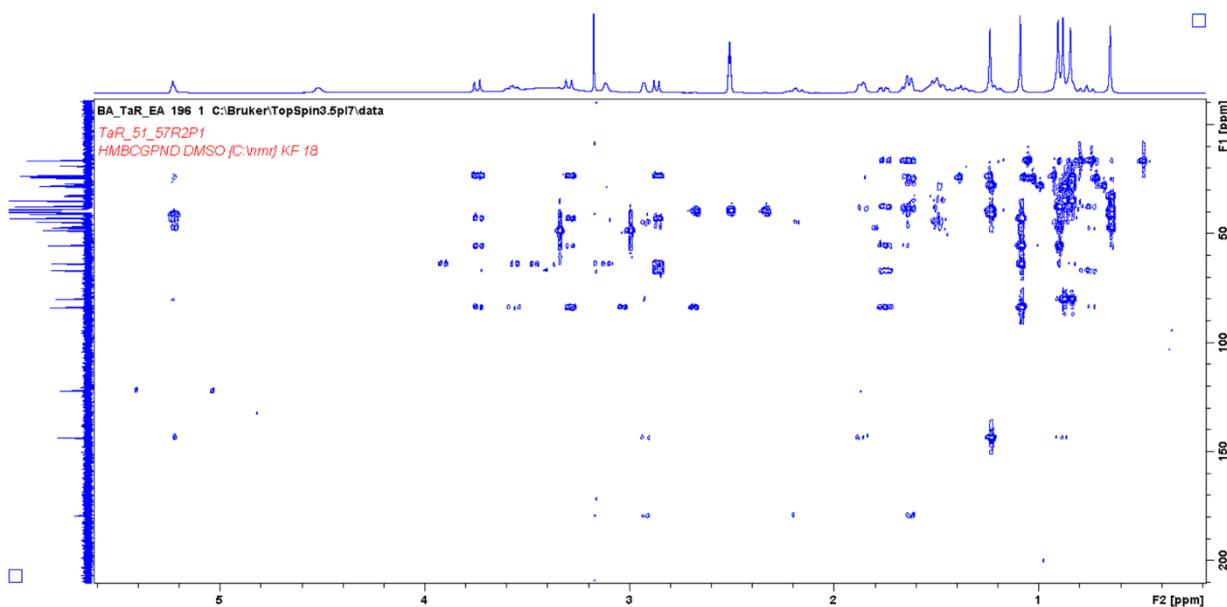
**Figure S 4.10.**  $^1\text{H}$  NMR spectrum (400 MHz, DMSO- $d_6$ ) of Sericic acid (**4**)



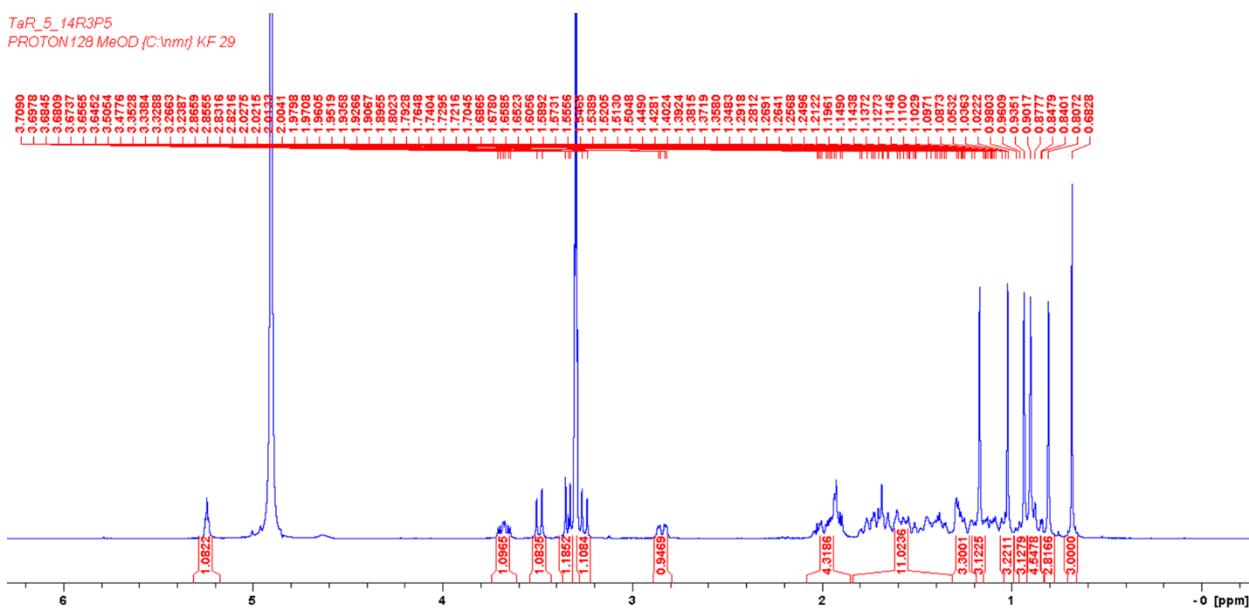
**Figure S 4.11.** COSY spectrum (DMSO-*d*<sub>6</sub>) of Sericic acid (**4**)



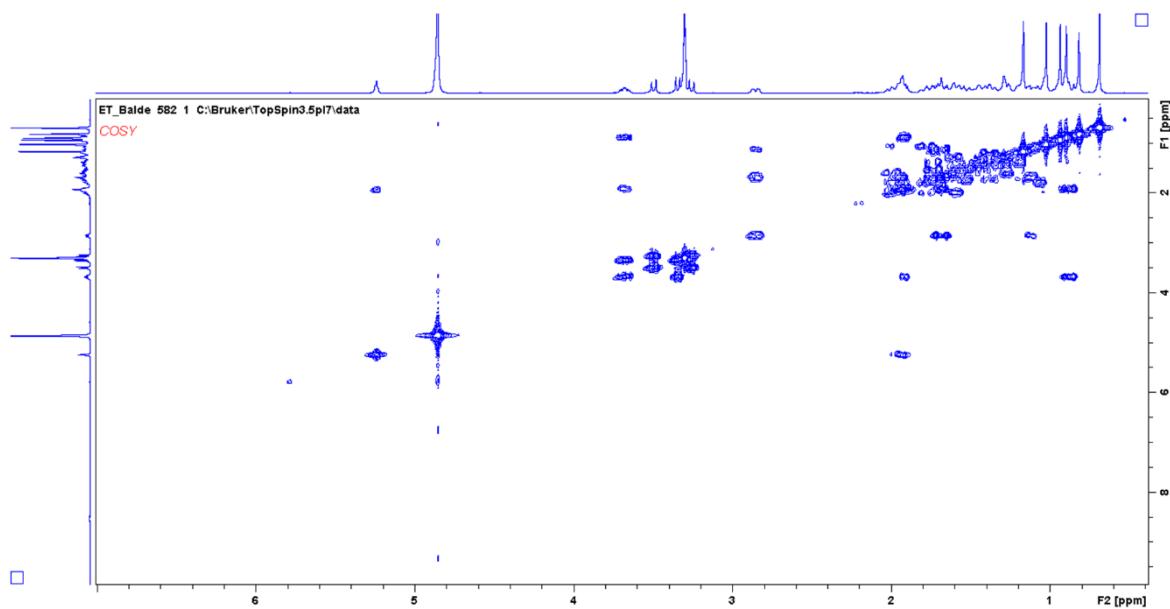
**Figure S 4.12.** HSQC spectrum (DMSO-*d*<sub>6</sub>) of Sericic acid (**4**)



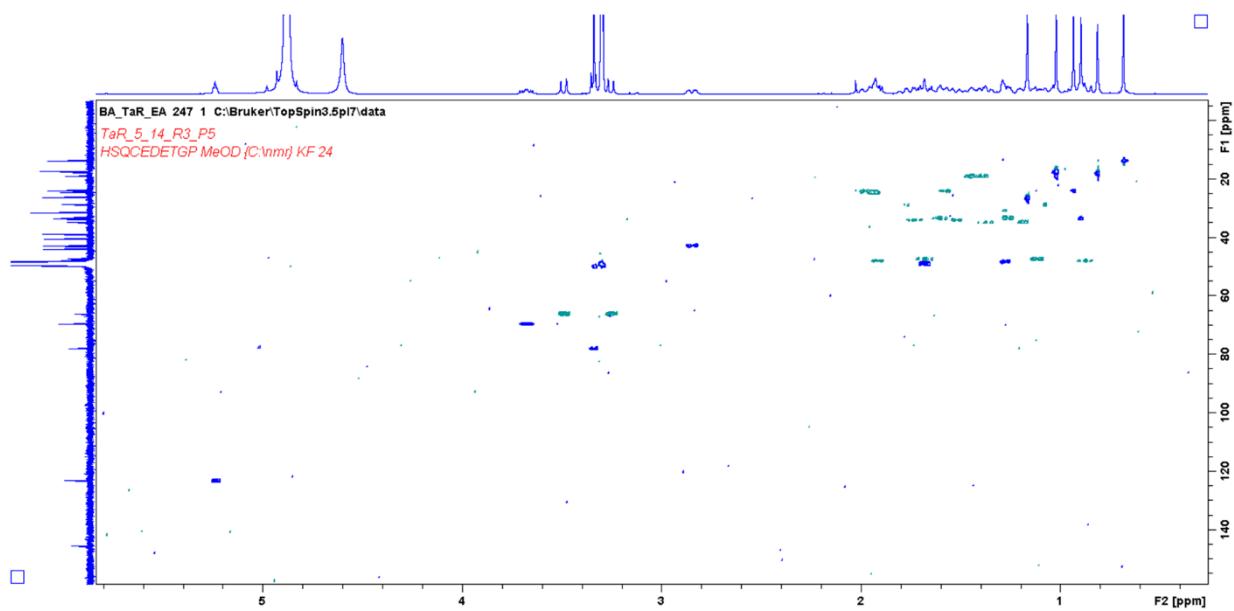
**Figure S 4.13.** HMBC spectrum (DMSO-*d*<sub>6</sub>) of Sericic acid (**4**)



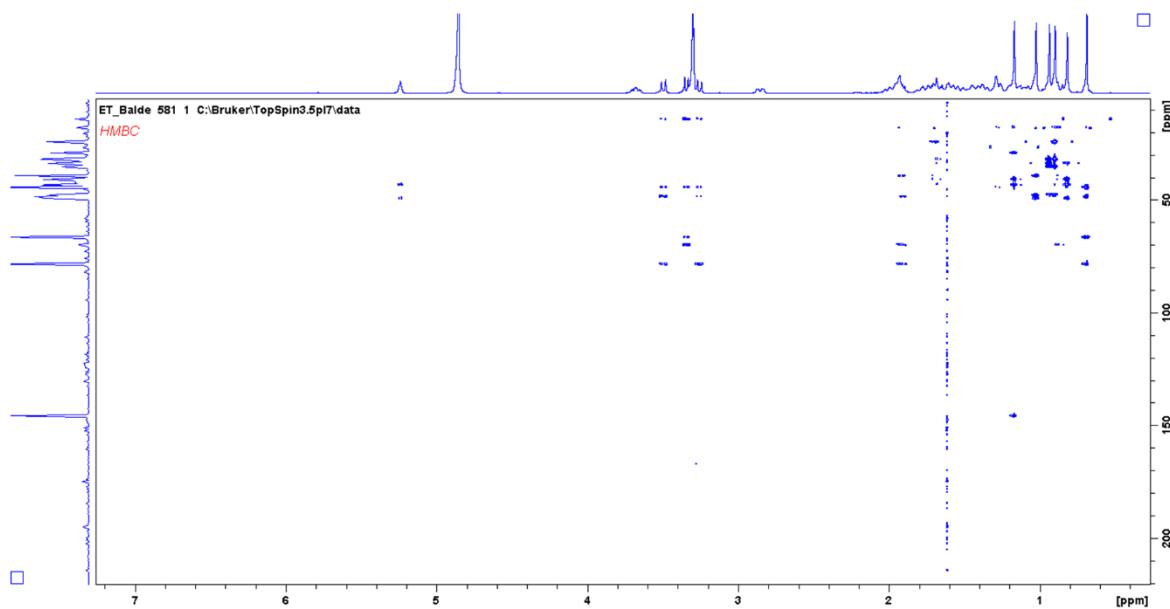
**Figure S 4.14.**  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CD}_3\text{OD}$ ) of Arjunolic acid (5)



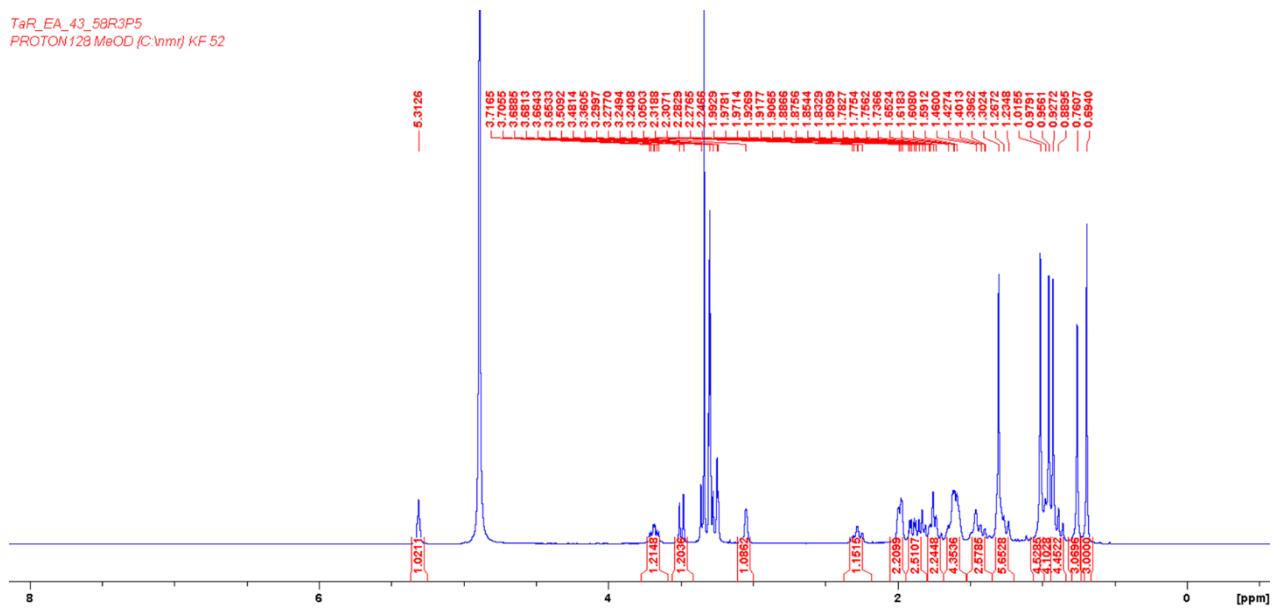
**Figure S 4.15.** COSY spectrum (CD<sub>3</sub>OD) of Arjunolic acid (5)



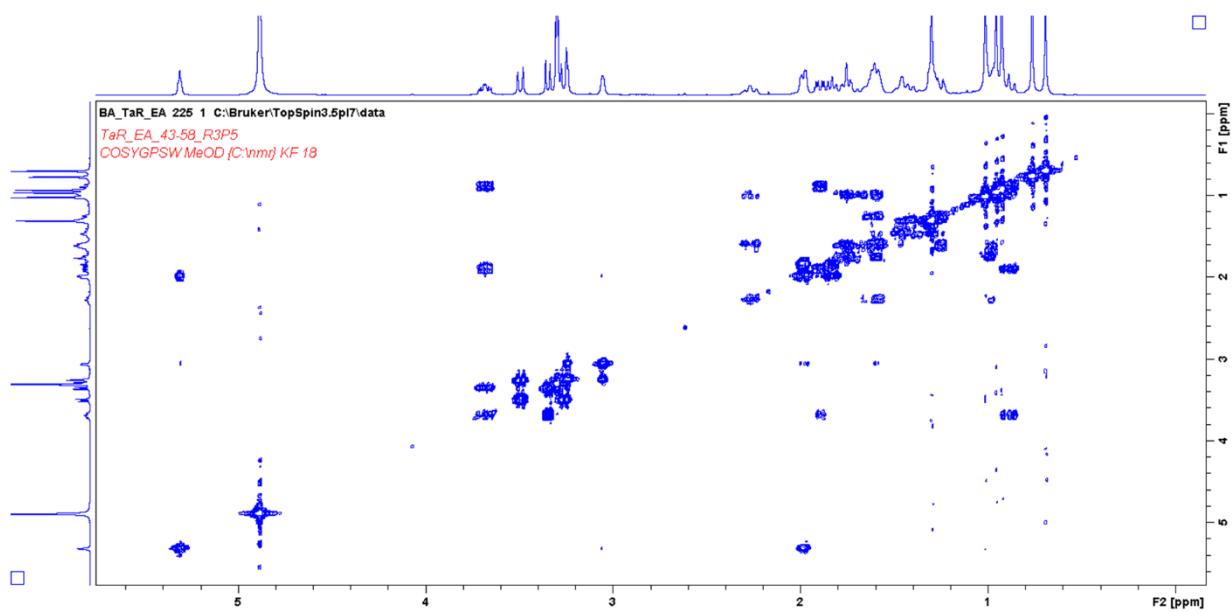
**Figure S 4.16.** HSQC spectrum (CD<sub>3</sub>OD) of Arjunolic acid (5)



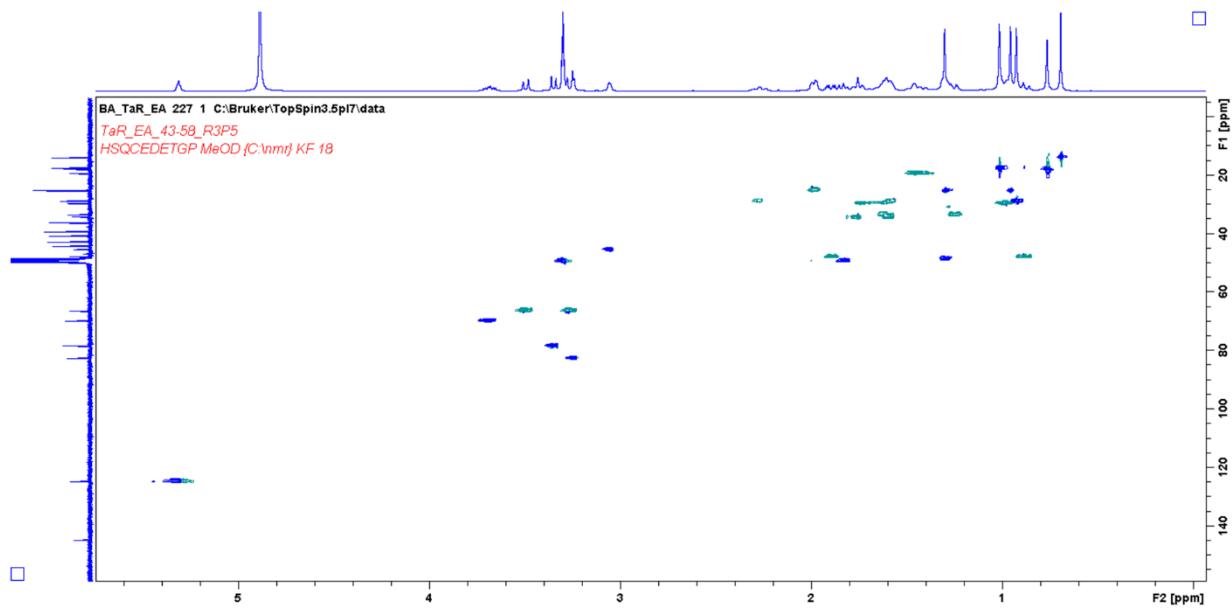
**Figure S 4.17.** HMBC spectrum (CD<sub>3</sub>OD) of Arjunolic acid (**5**)



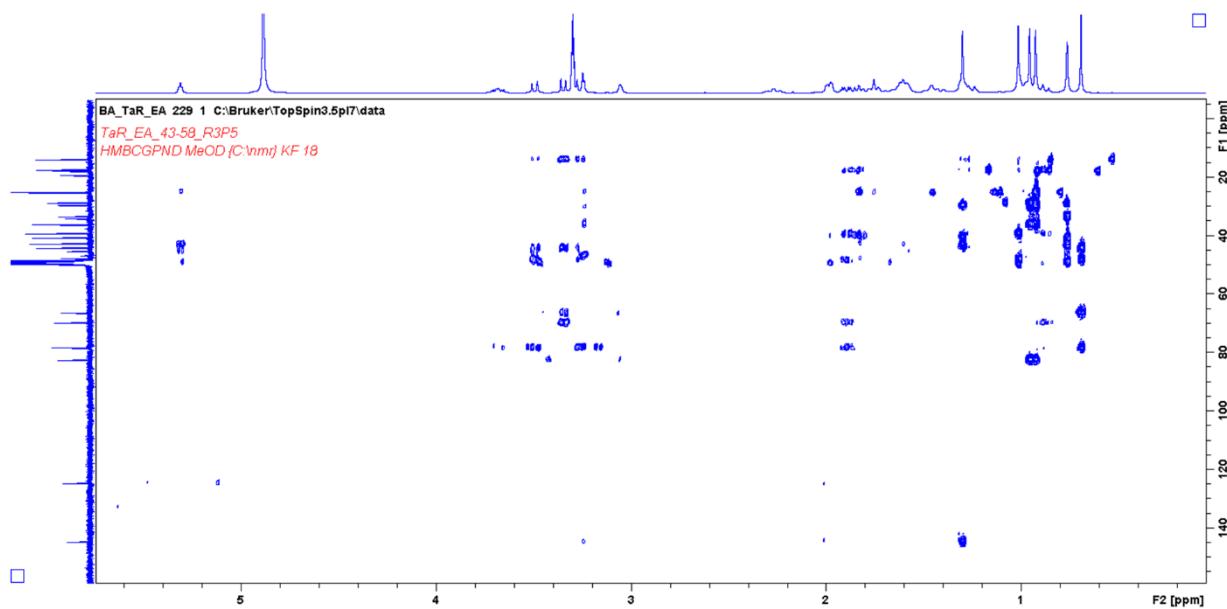
**Figure S 4.18.** <sup>1</sup>H NMR spectrum (400 MHz, CD<sub>3</sub>OD) of Arjungenenin (**6**)



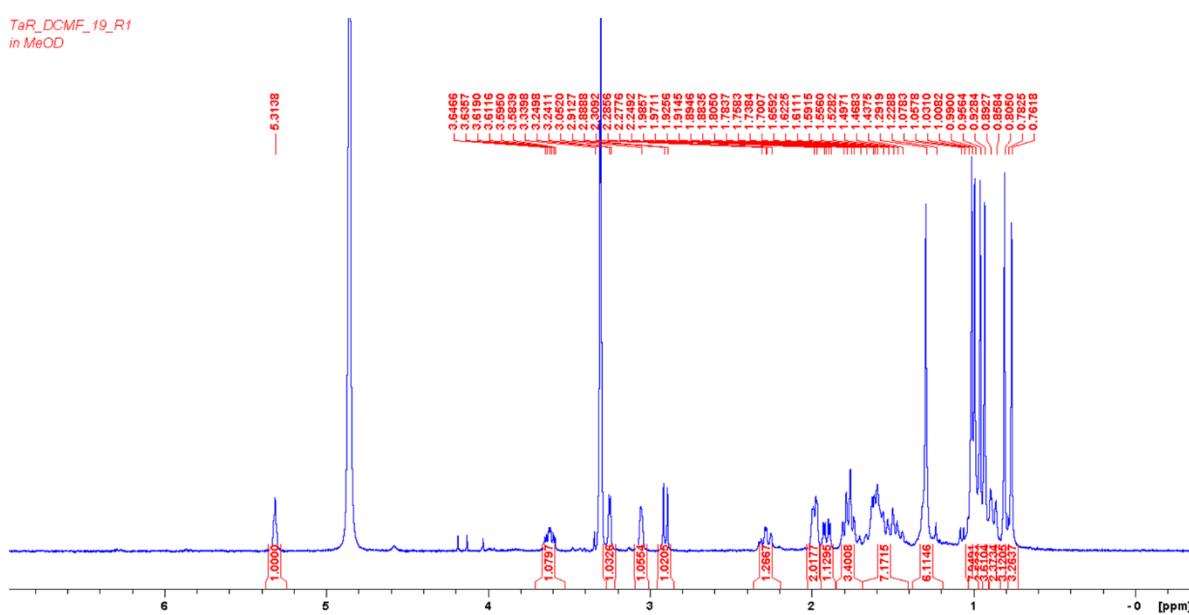
**Figure S 4.19.** COSY spectrum ( $\text{CD}_3\text{OD}$ ) of Arjungenin (6)



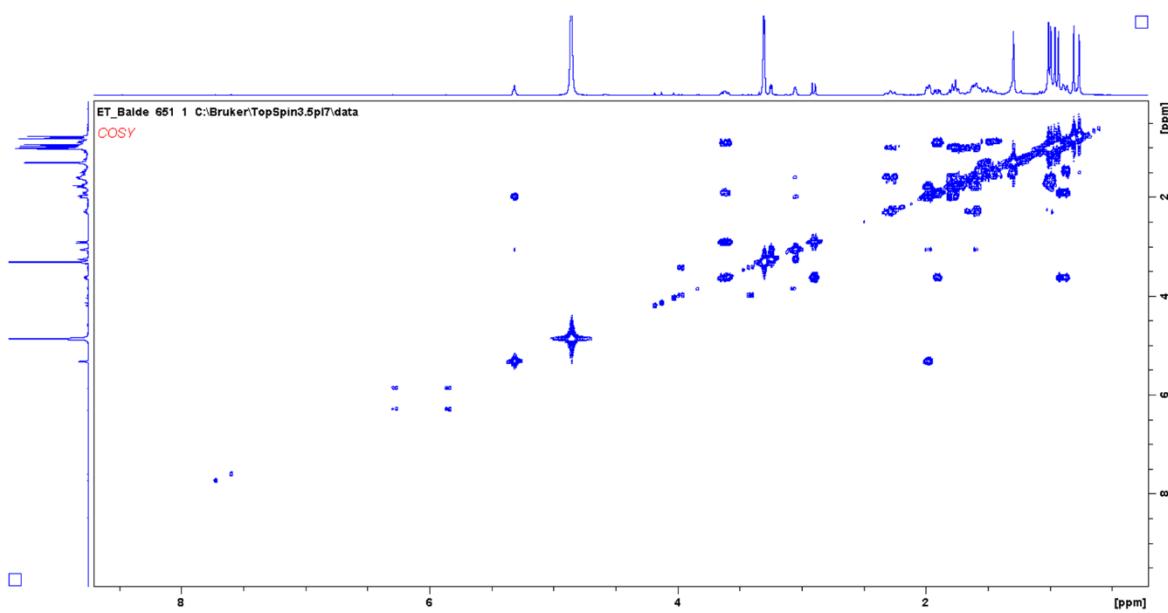
**Figure S 4.20.** HSQC spectrum ( $\text{CD}_3\text{OD}$ ) of Arjungenine (6)



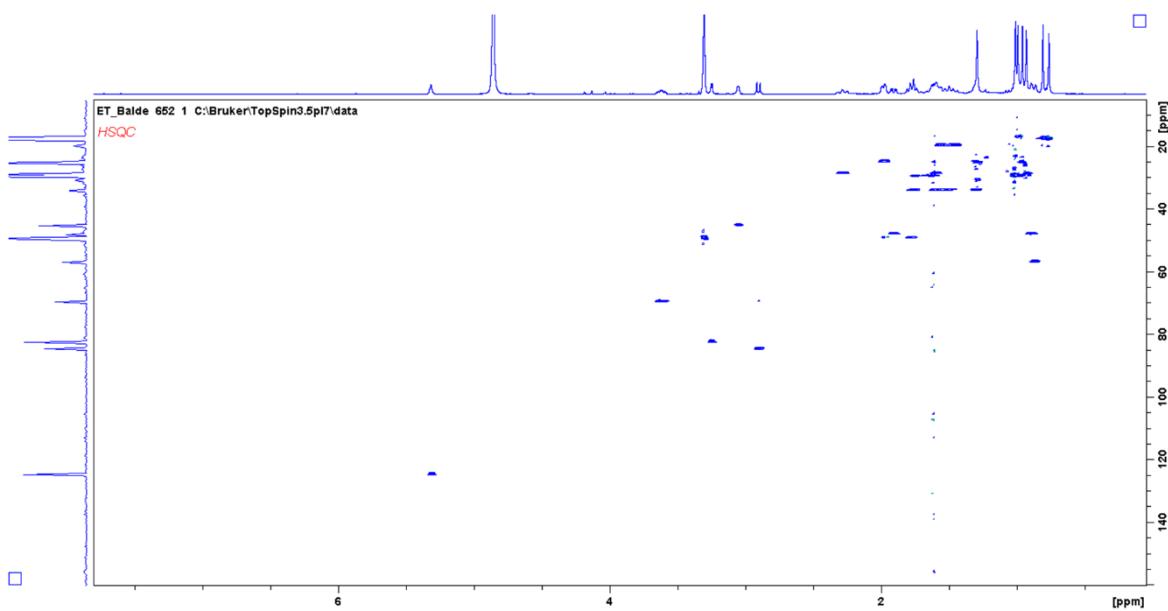
**Figure S 4.21.** HSQC spectrum ( $\text{CD}_3\text{OD}$ ) of Arjungenine (6)



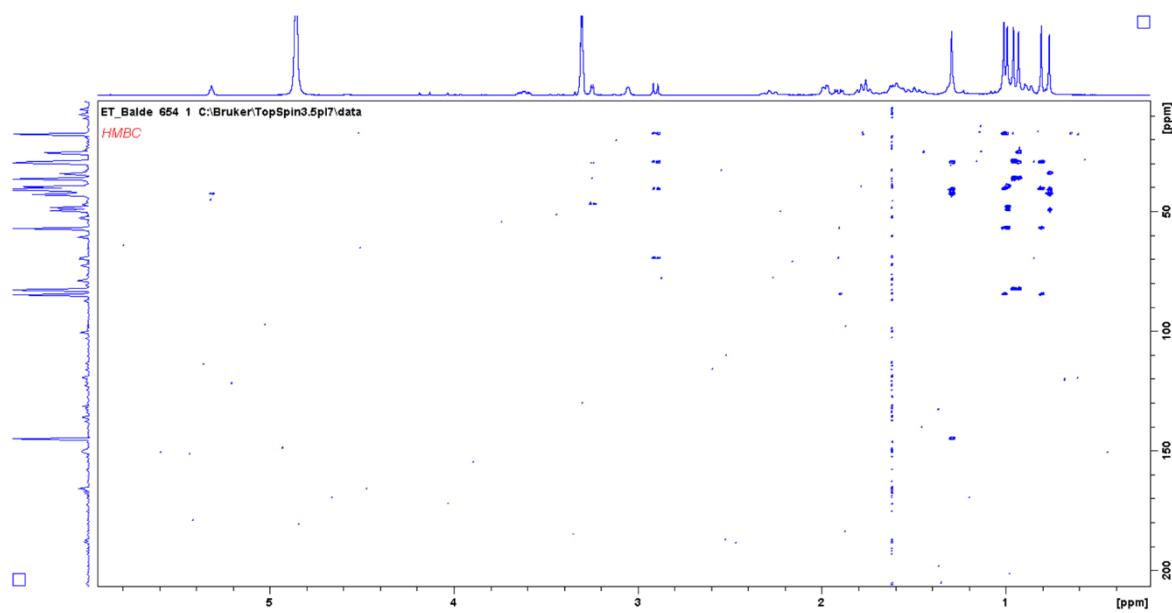
**Figure S 4.22.**  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CD}_3\text{OD}$ ) of Arjunic acid (7)



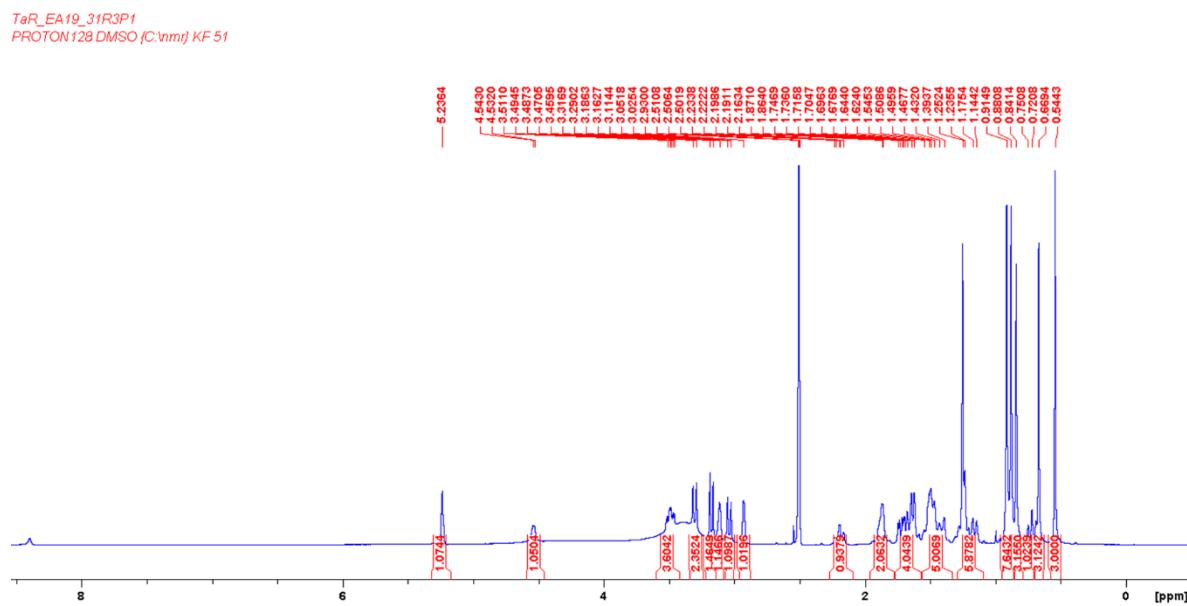
**Figure S 4.23.** COSY spectrum ( $\text{CD}_3\text{OD}$ ) of Arjunic acid (7)



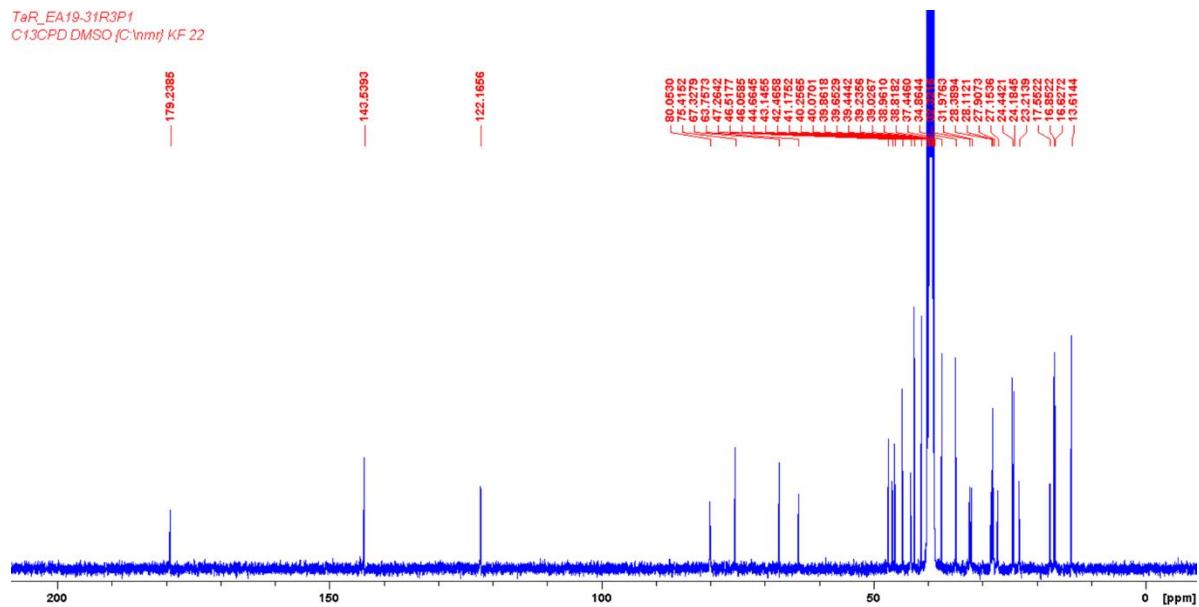
**Figure S 4.24.** HSQC spectrum ( $\text{CD}_3\text{OD}$ ) of Arjunic acid (7)



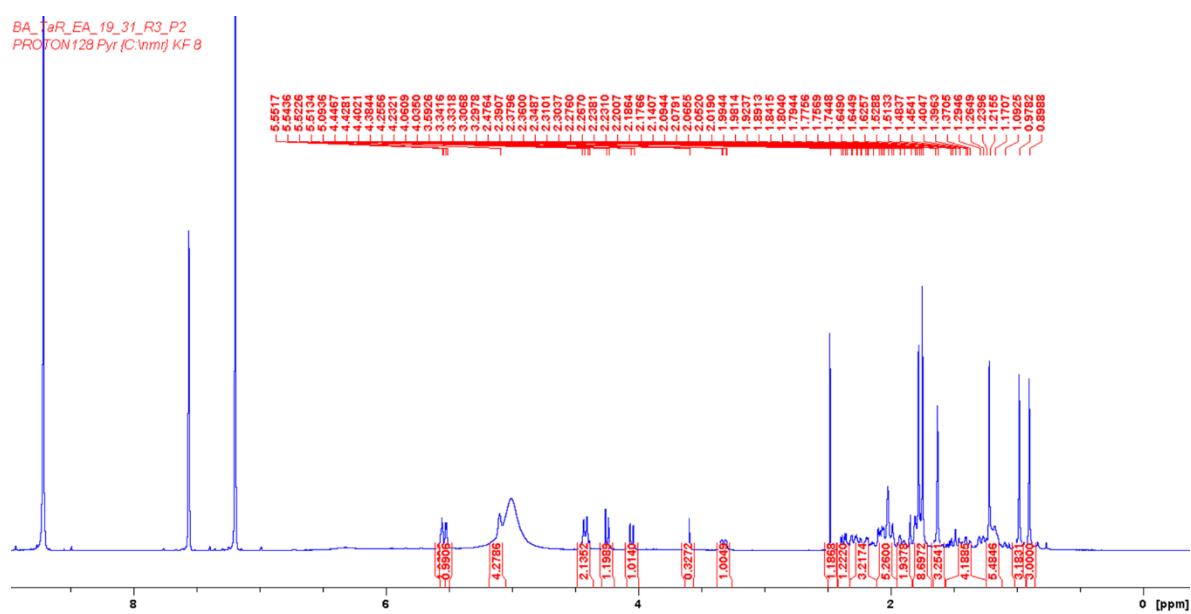
**Figure S 4.25.** HMBC spectrum ( $\text{CD}_3\text{OD}$ ) of Arjunic acid (7)



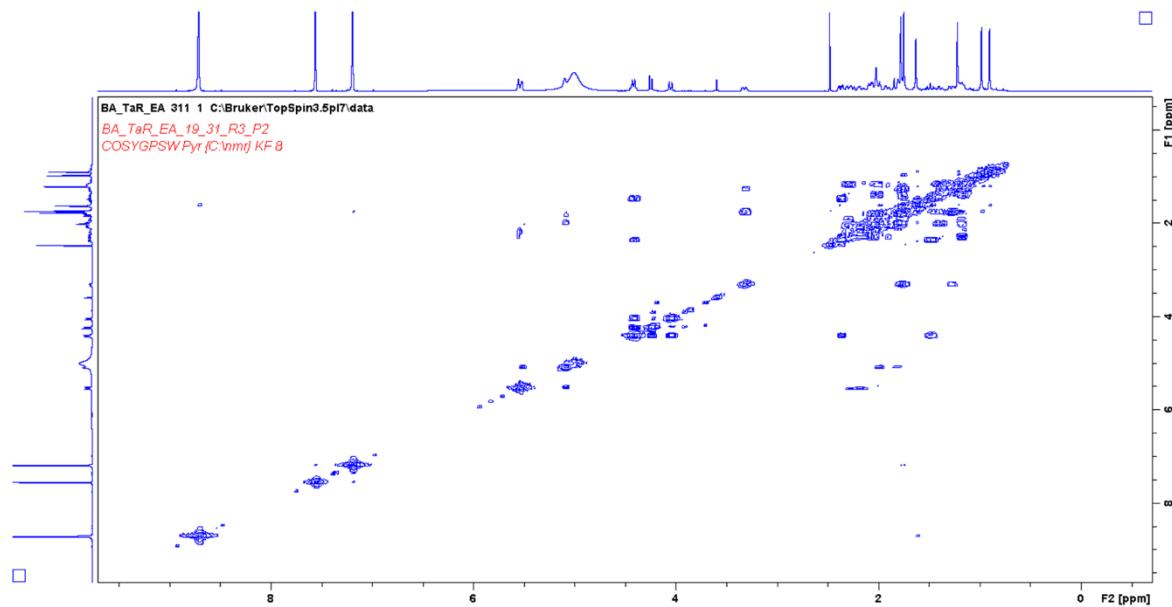
**Figure S 4.26.**  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{DMSO-d}_6$ ) of  $2\alpha,3\beta,21\beta,23$ -tetrahydroxyolean-12-en-28-oic acid (8).



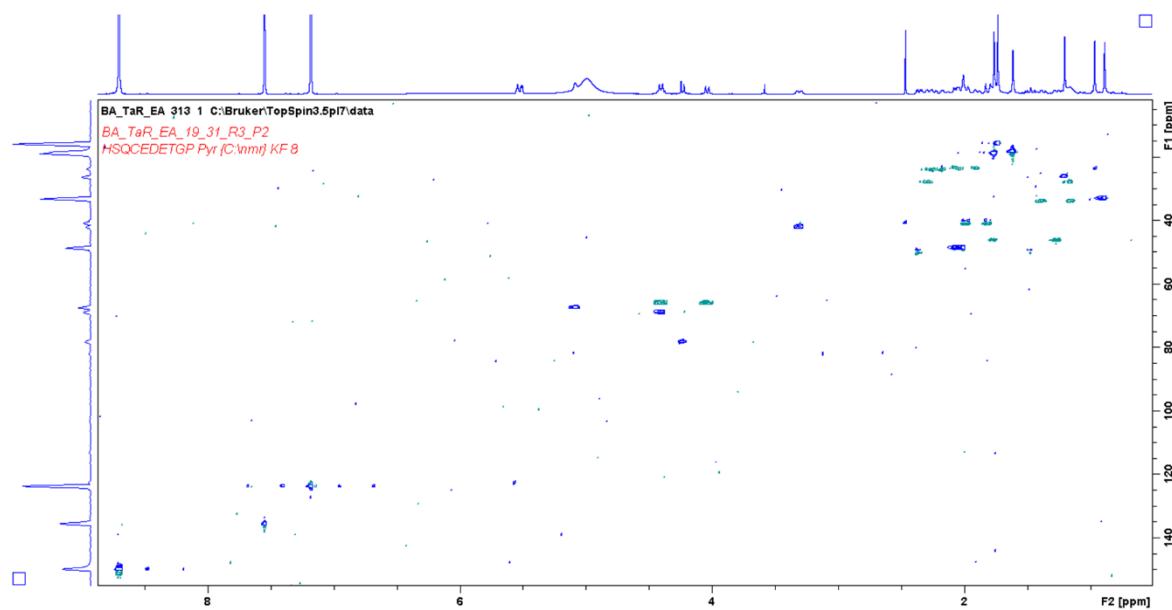
**Figure S 4.27.**  $^{13}\text{C}$  NMR spectrum (100 MHz, DMSO-d<sub>6</sub>) 2 $\alpha$ ,3 $\beta$ ,21 $\beta$ ,23-tetrahydroxyolean-12-en-28-oic acid (**8**).



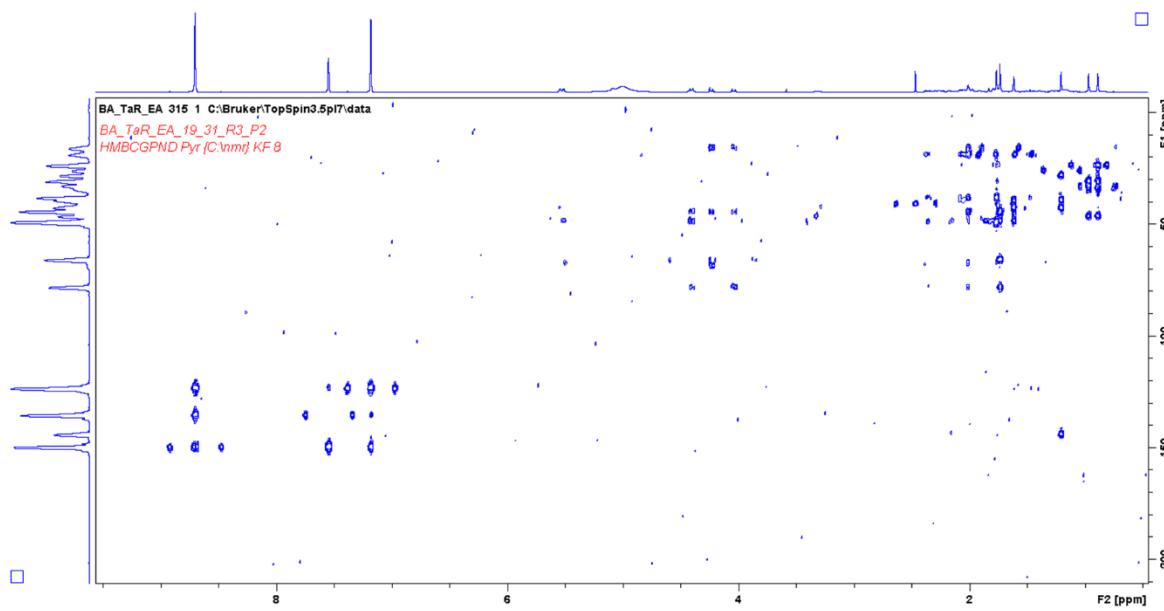
**Figure S 4.28.**  $^1\text{H}$  NMR spectrum (400 MHz, Pyridin-d<sub>5</sub>) of Terminolic acid (**9**)



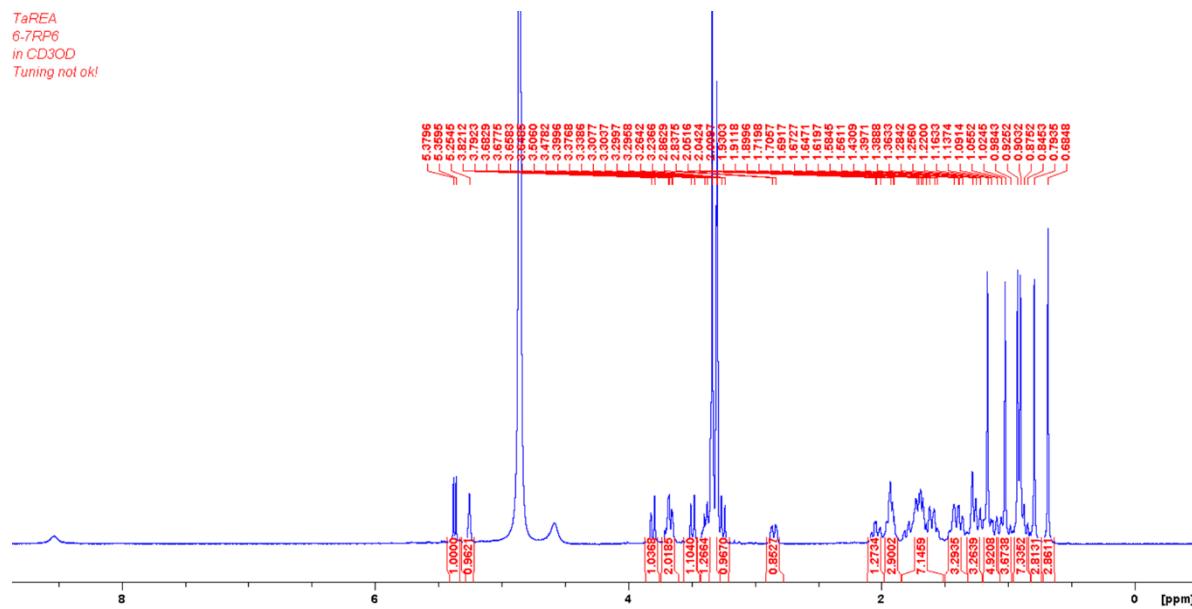
**Figure S 4.29.** COSY spectrum (Pyridin-*d*5) of Terminolic acid (**9**)



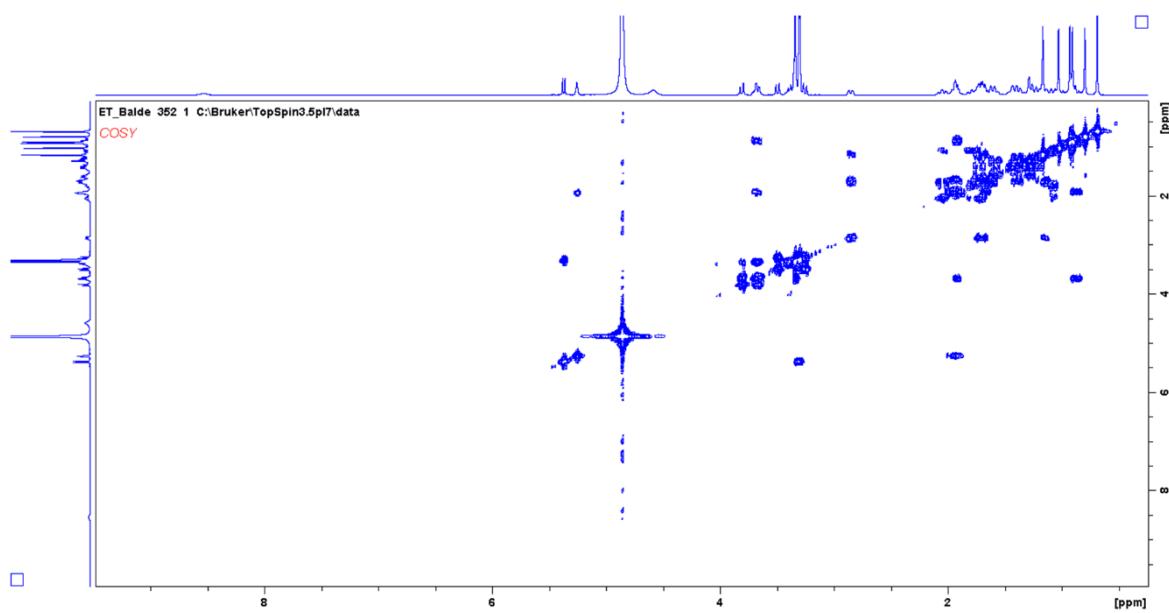
**Figure S 4.30.** HSQC spectrum (Pyridin-*d*<sub>5</sub>) of Terminolic acid (**9**)



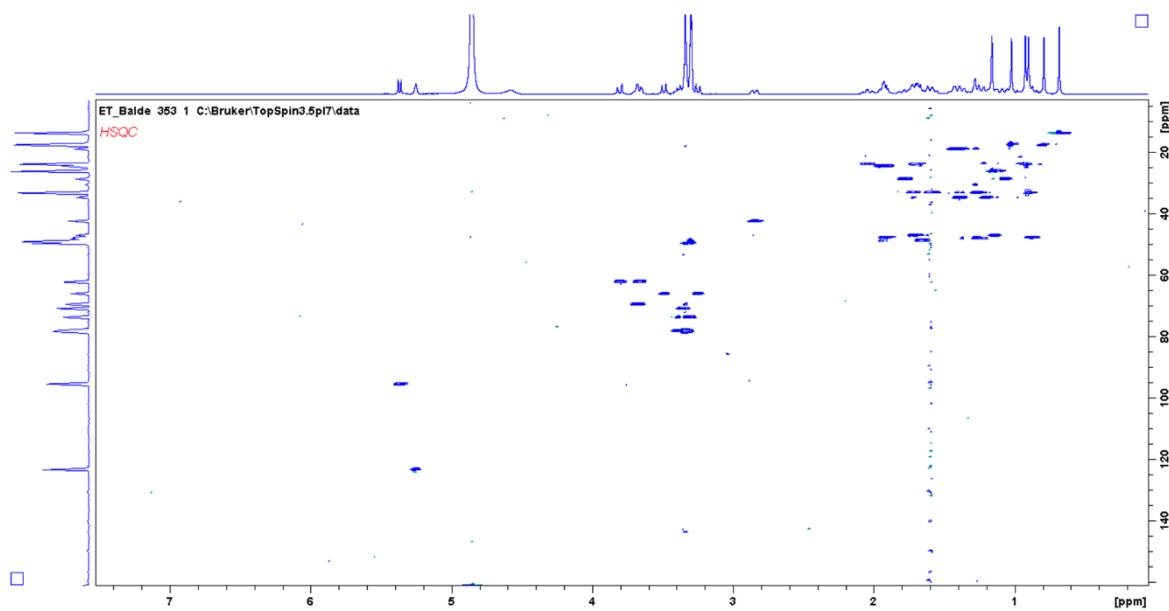
**Figure S 4.31.** HMBC spectrum (Pyridin-*d*<sub>5</sub>) of Terminolic acid (**9**)



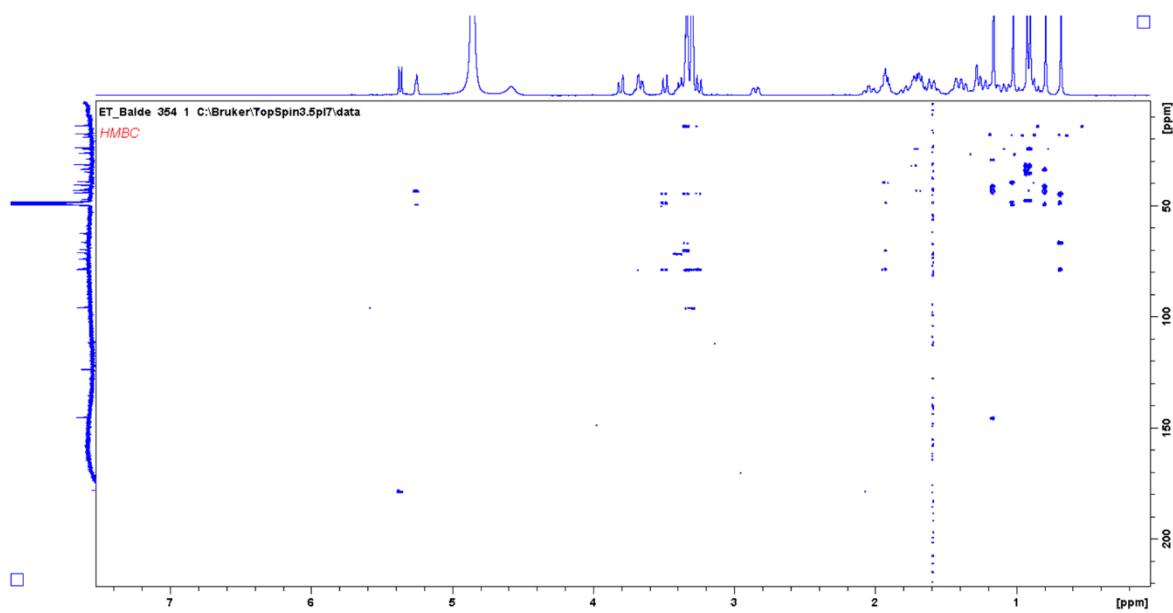
**Figure S 4.32.** <sup>1</sup>H NMR spectrum (400 MHz, CD<sub>3</sub>OD) of Arjunic acid 28-*O*- $\beta$ -D-glucopyranoside (**10**)



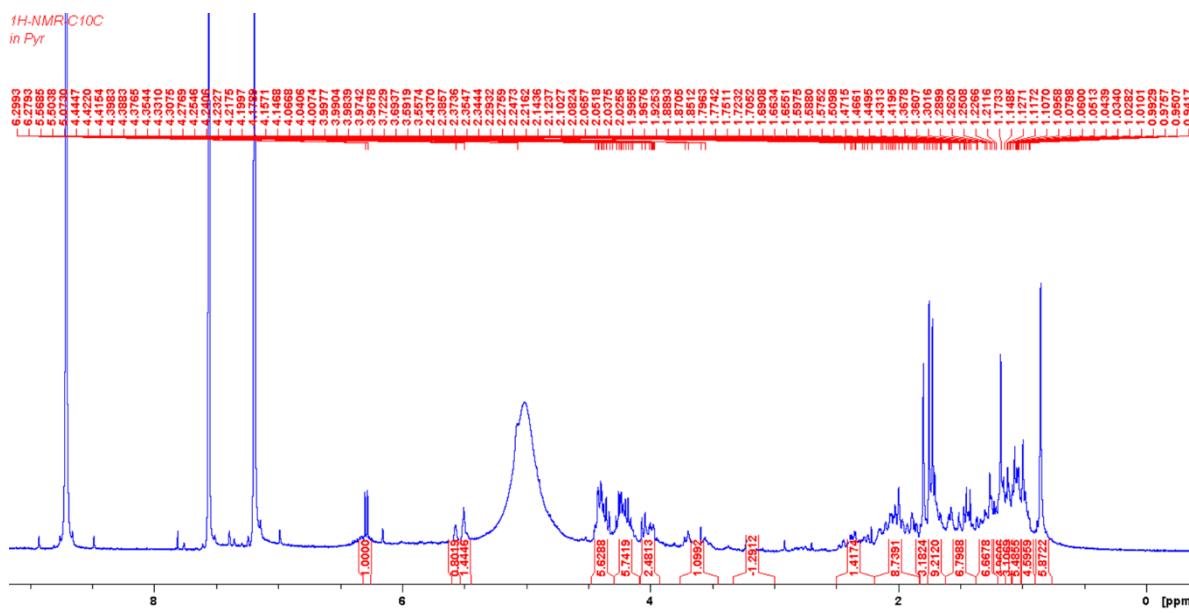
**Figure S 4.33.** COSY spectrum ( $\text{CD}_3\text{OD}$ ) of Arjunic acid 28- $O$ - $\beta$ -D-glucopyranoside (**10**)



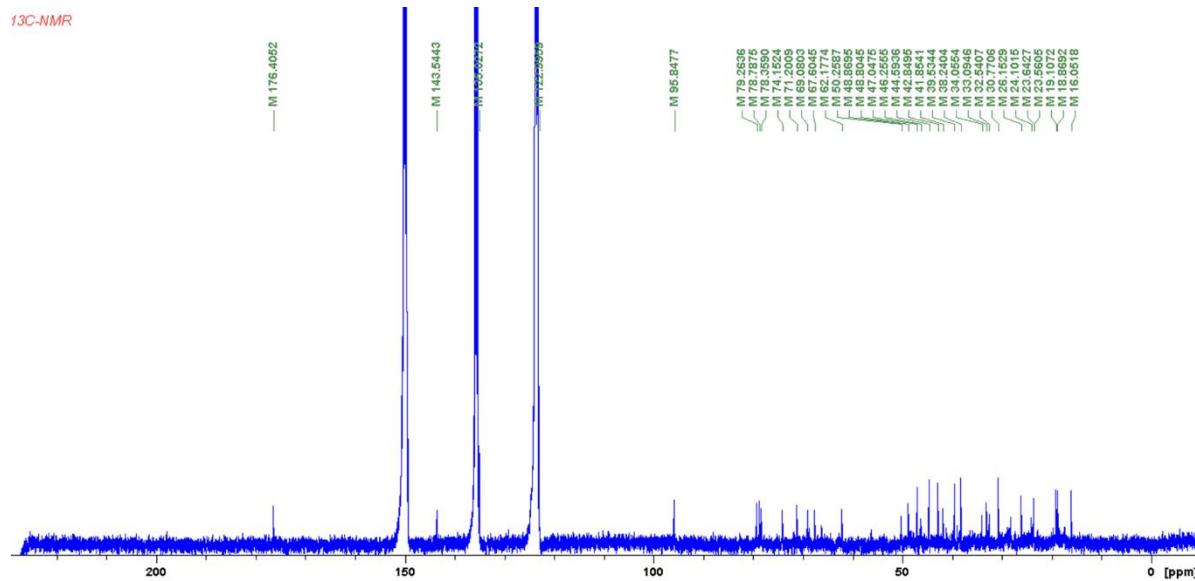
**Figure S 4.34.** HSQC spectrum ( $\text{CD}_3\text{OD}$ ) of Arjunic acid 28- $O$ - $\beta$ -D-glucopyranoside (**10**)



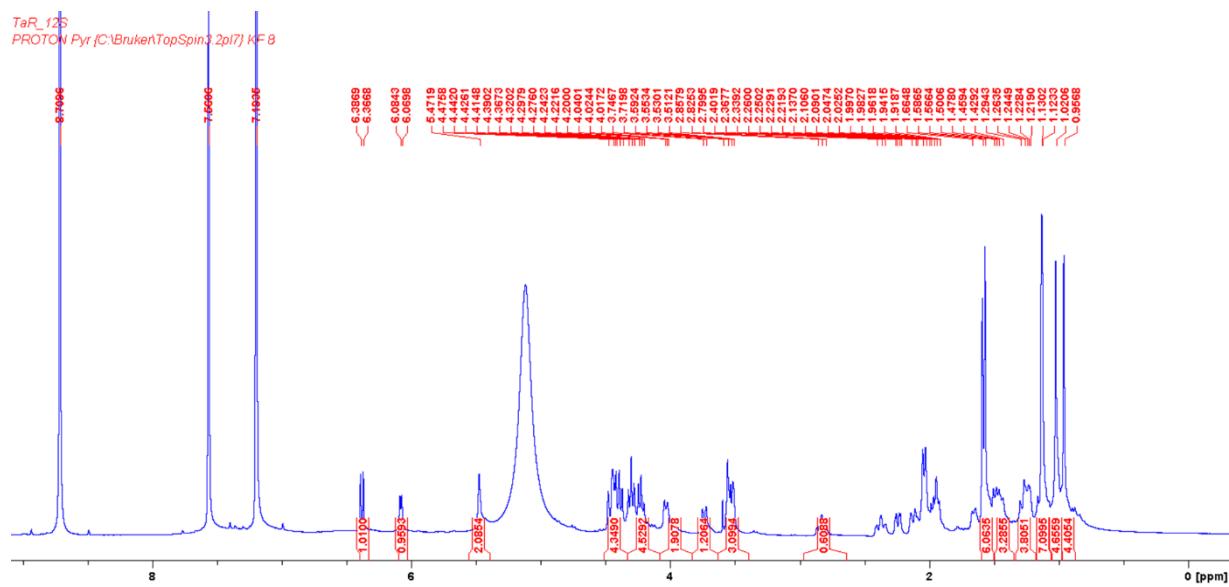
**Figure S 4.35.** HMBC spectrum (CD<sub>3</sub>OD) of Arjunic acid 28-O-β-D-glucopyranoside (**10**)



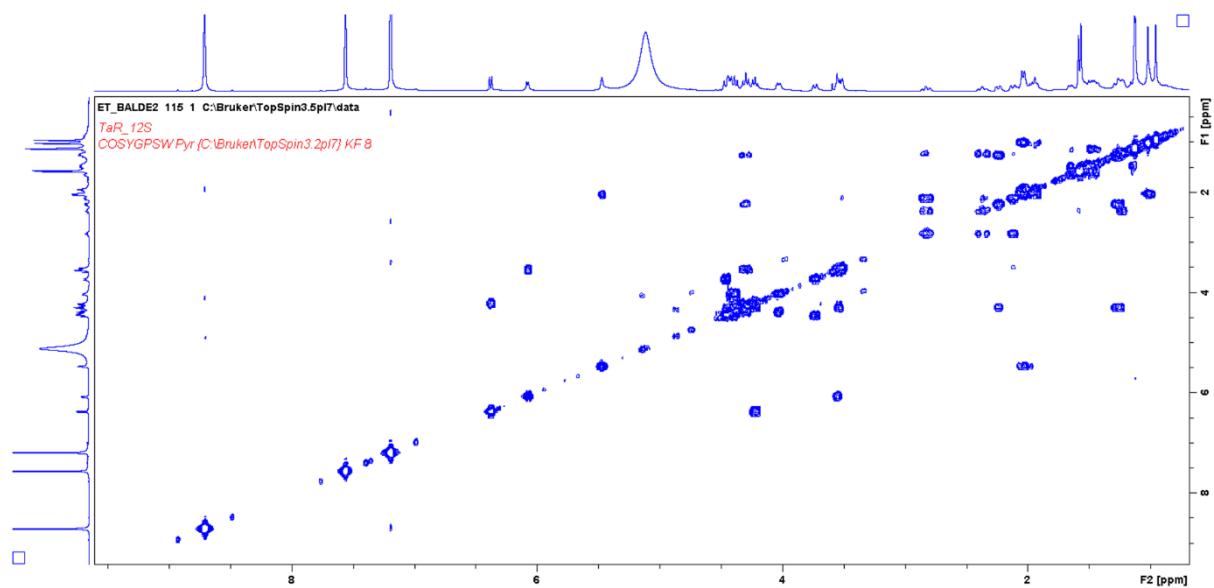
**Figure S 4.36.** <sup>1</sup>H NMR spectrum (400 MHz, CD<sub>3</sub>OD) of Chebuloside II (**11**)



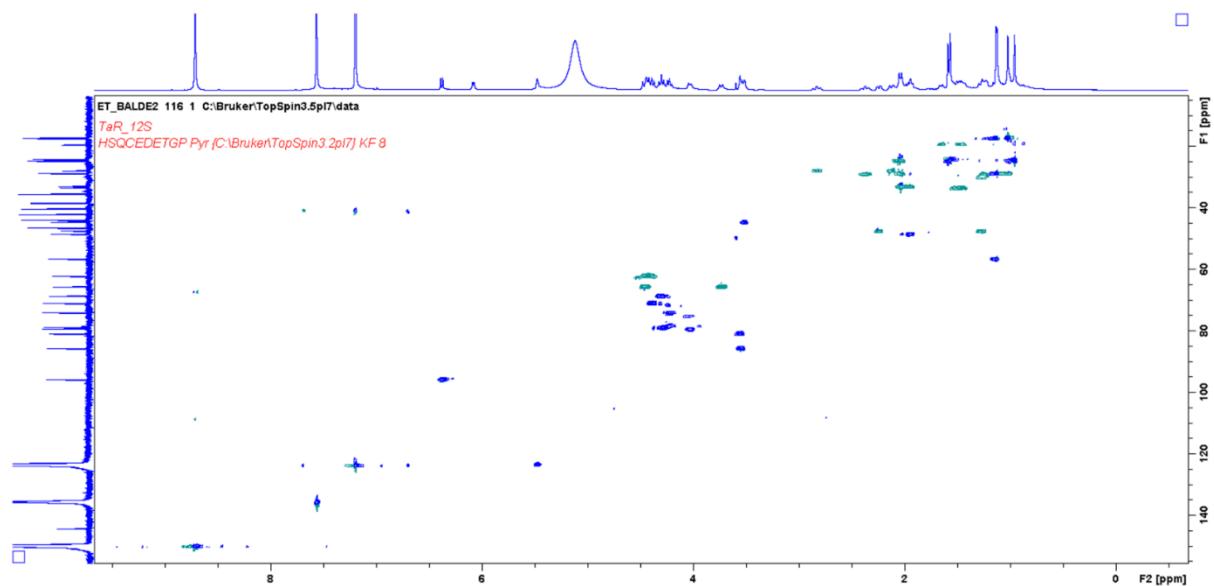
**Figure S 4.37.**  $^{13}\text{C}$  NMR (100 MHz, Pyridine-*d*<sub>5</sub>) spectrum of Chebuloside II (**11**)



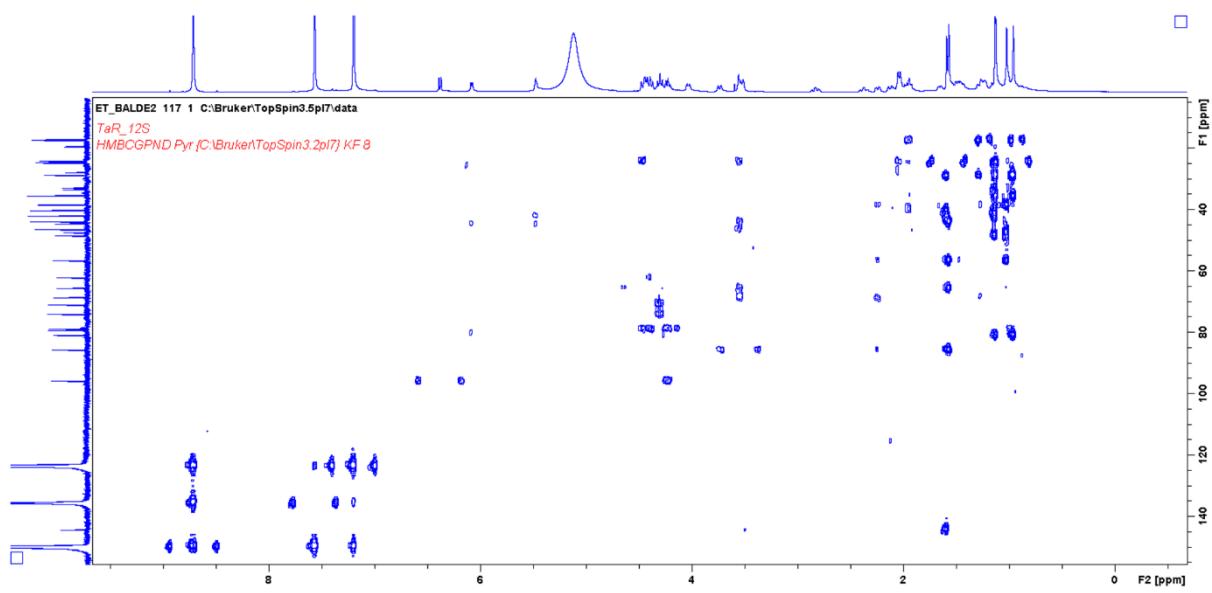
**Figure S 4.38.**  $^1\text{H}$  NMR spectrum (400 MHz, Pyridin-*d*5) Sericoside (**12**)



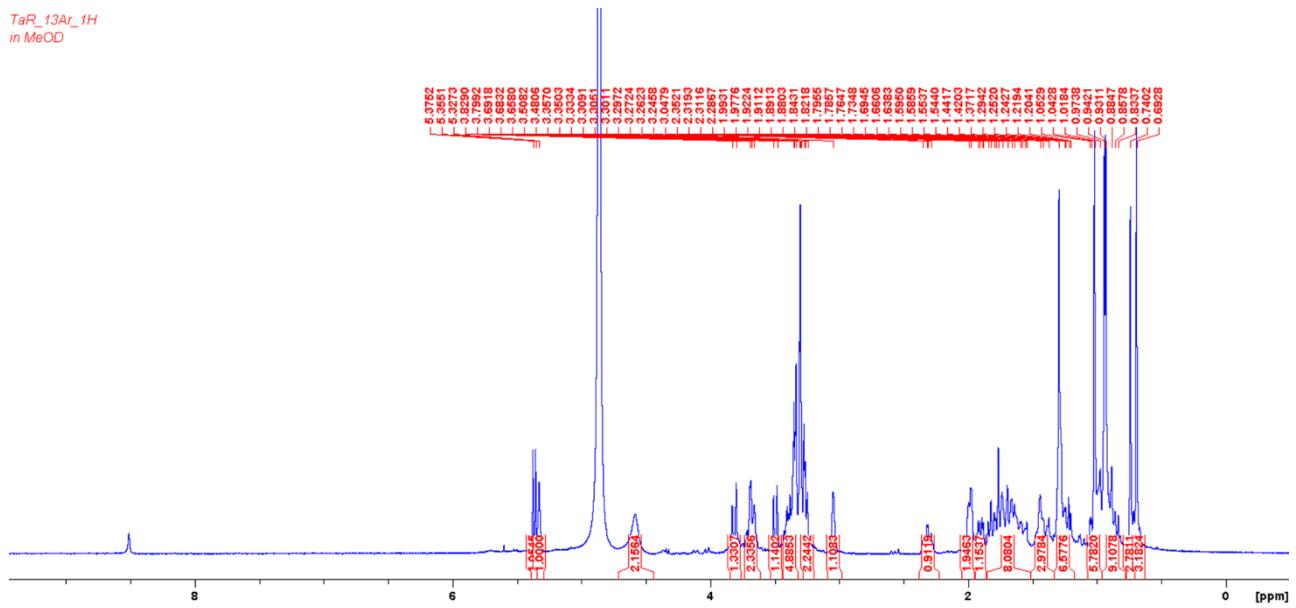
**Figure S 4.39.** COSY spectrum (Pyridin-*d*<sub>5</sub>) of Sericoside (**12**)

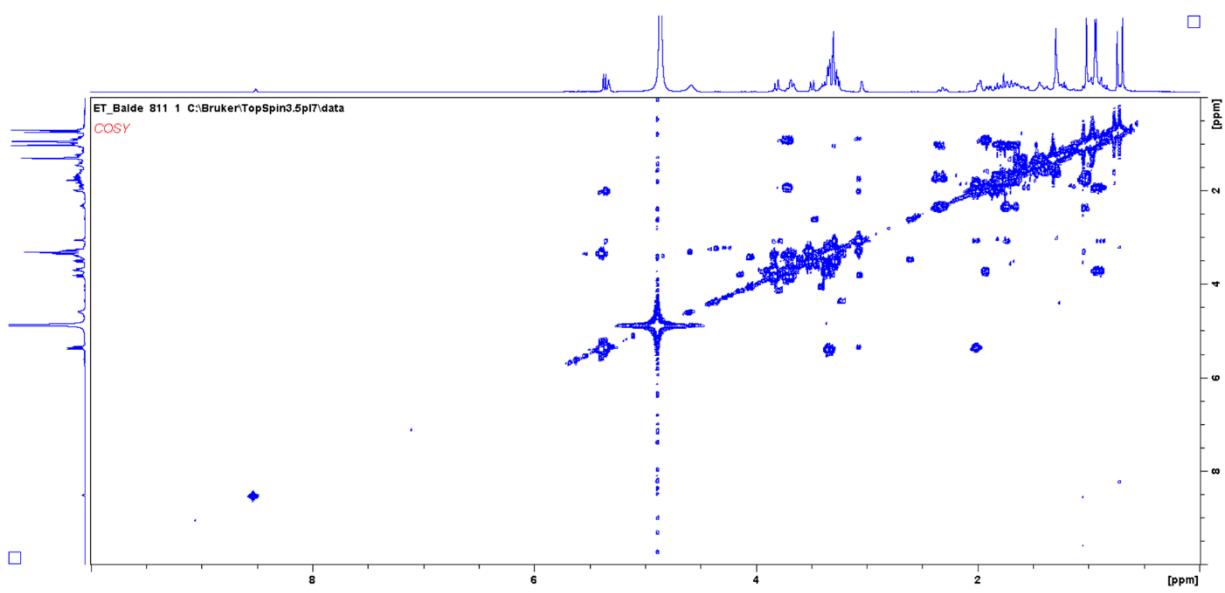


**Figure S 4.40.** HSQC spectrum (Pyridin-*d*<sub>5</sub>) of Sericoside (**12**)

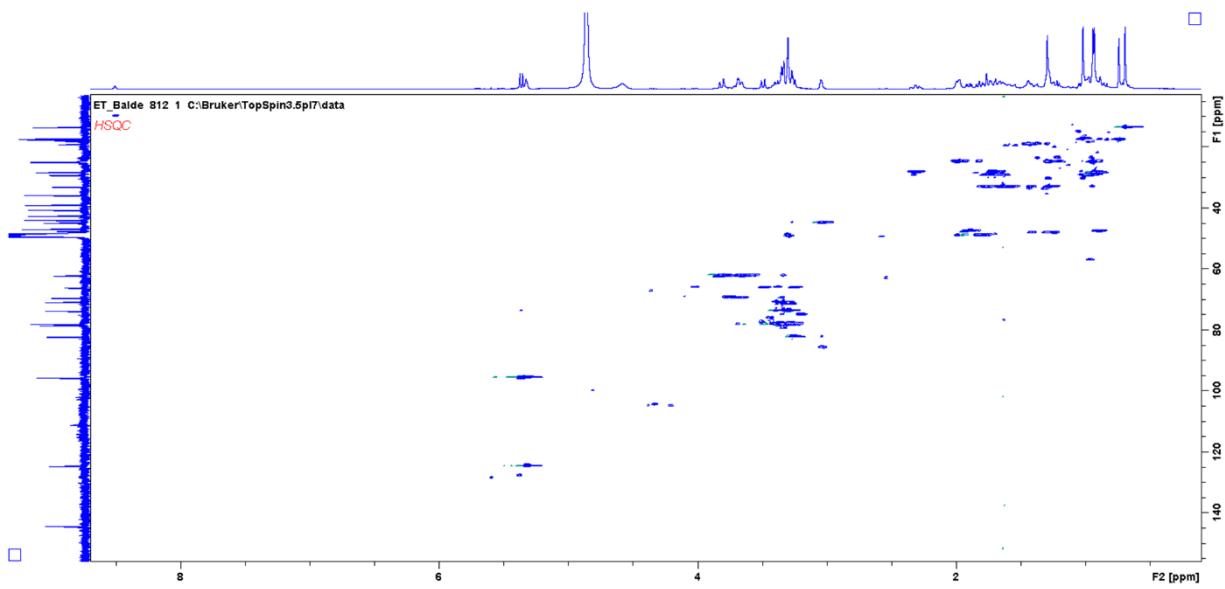


**Figure S 4.41.** HMBC spectrum (Pyridin-*d*<sub>5</sub>) of Sericoside (**12**)

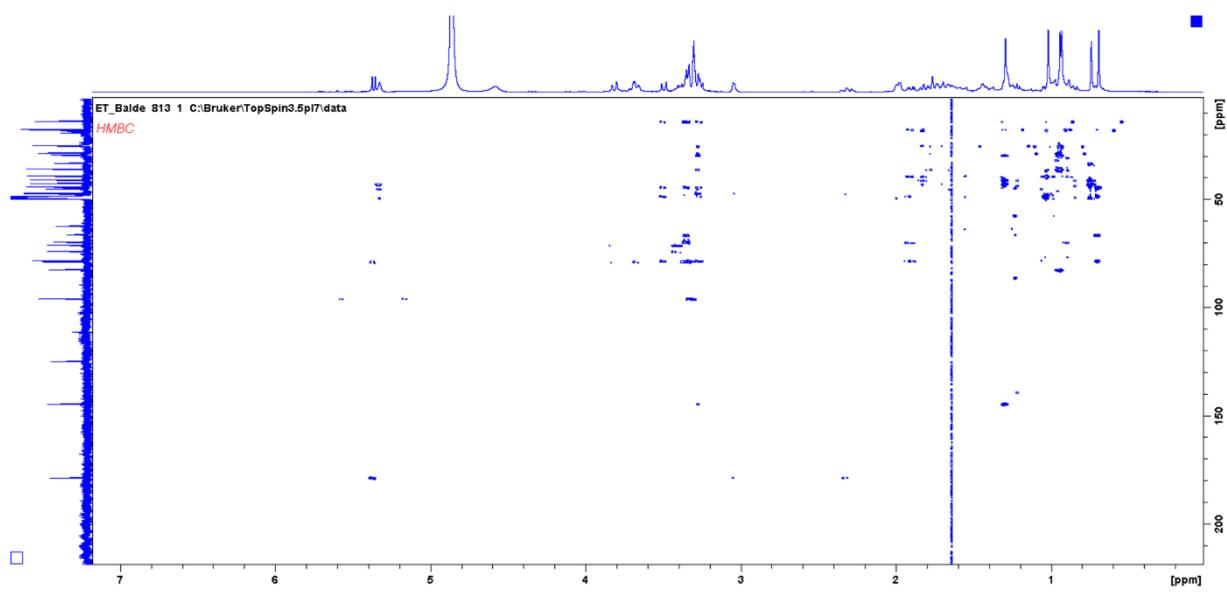




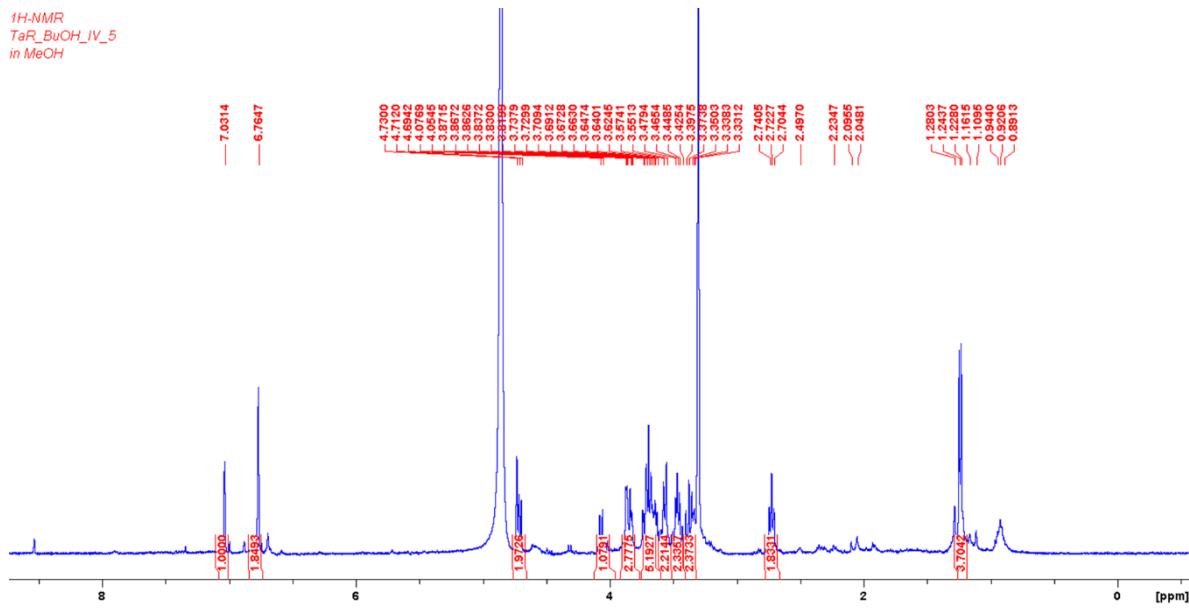
**Figure S 4.43.** COSY spectrum ( $\text{CD}_3\text{OD}$ ) of Arjunglucoside I (**13**)

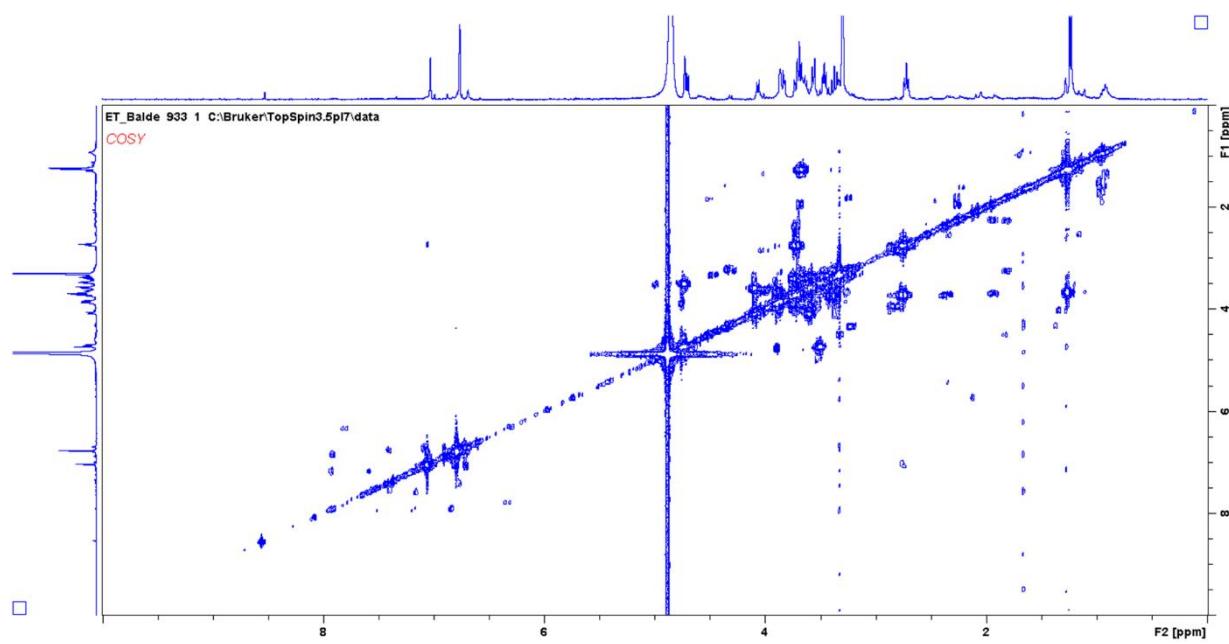


**Figure S 4.44.** HSQC spectrum ( $\text{CD}_3\text{OD}$ ) of Arjunglucoside I (**13**)

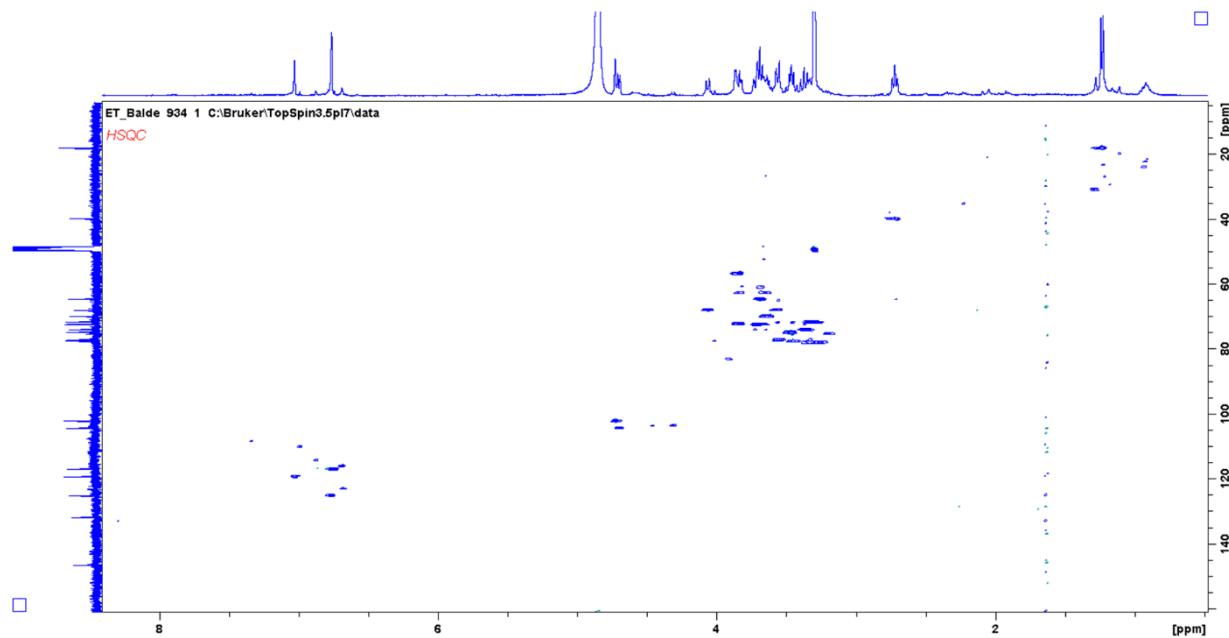


**Figure S 4.45.** HMBC spectrum ( $\text{CD}_3\text{OD}$ ) of Arjunglucoside I (**13**)

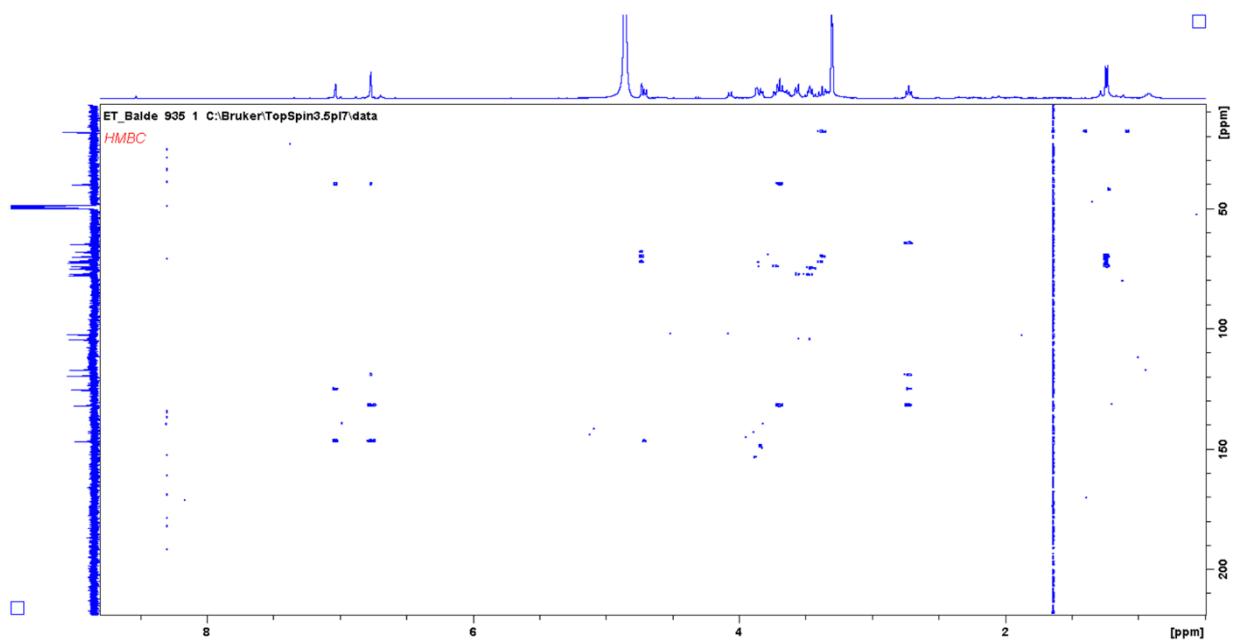




**Figure S 4.47.** COSY spectrum (CD<sub>3</sub>OD) of Calophytmembranside B (**14**)



**Figure S 4.48.** HSQC spectrum (CD<sub>3</sub>OD) of Calophytmembranside B (**14**)



**Figure S 4.49.** HMBC spectrum ( $\text{CD}_3\text{OD}$ ) of Calophytemembranside B (**14**)

## CHAPTER 5

***Terminalia albida* part II:** antiplasmodial  
oleanane triterpenoids from *Terminalia albida* root  
bark

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## 5.1. Introduction

The genus *Terminalia* comprises about 250 species, mostly medium-sized or large trees. It is the second largest genus in the Combretaceae family<sup>1</sup>. The name “*Terminalia*” is derived from the Latin word “terminus”, since the leaves are located at the tip of the branch<sup>2</sup>. Species of this genus are widely distributed throughout the tropical and subtropical regions of Asia, Australia, and Africa, and are frequently used in traditional medicine in many countries for the treatment of both communicable and non-communicable diseases<sup>3</sup>. Several *Terminalia* species have been investigated for their phytochemical constituents, which resulted in the identification of around 368 compounds, including terpenoids, tannins, flavonoids, lignans, and simple phenols, amongst others. Some of the isolated compounds have demonstrated various bioactivities, *in vitro* or *in vivo*, such as antimalarial, antifungal, antibacterial, antitumor, anti-HIV-1, antioxidant, antidiarrheal, and analgesic<sup>1</sup>. In the Guinean flora, around 11 *Terminalia* species have been inventoried and most of them are widely used for their medicinal properties. Among these, *Terminalia albida* Sc. Elliot, a savannah shrub up to 12 m high, is distributed in all Guinean regions, where it is widely used in traditional medicine for the management of several diseases<sup>4,5,6,7</sup>. Previous pharmacological studies suggested that *T. albida* extracts were active against *Plasmodium falciparum*, *Candida albicans*, and *Staphylococcus aureus* and increased the survival rate of mice infected with *Plasmodium berghei*<sup>8,9</sup>. Among the current research strategies for new biologically active natural products, dereplication is vital to effectively distinguish known from new compounds. The rapid development of modern hyphenated techniques and various novel bioinformatics approaches such as molecular networking (MN) and *in silico* tools to predict fragmentation during mass spectrometric analysis have emerged recently and provide new perspectives for early metabolite identification in natural products (NPs) research<sup>10,11</sup>.

During our continuing research towards the discovery of novel antiplasmodial and antimicrobial agents, we have previously reported on the bioassay-guided isolation of antiplasmodial constituents from the roots of *Terminalia albida*, resulting in the isolation of the triterpenoids arjunolic acid, arjungenin, arjunic acid and arjunglucoside II, showing IC<sub>50</sub> values in the range of 5 – 15 µM against *P. falciparum* strain K1, among other active constituents. These compounds were isolated from the CH<sub>2</sub>Cl<sub>2</sub> and EtOAc fractions of the

total 80% MeOH extract<sup>12</sup>. Although the *n*-BuOH fraction was not active, the possibility cannot be excluded that this polar fraction contains inactive glycosides, which may release active aglycones after removal of the glycosidic moieties in the gastrointestinal tract, more in particular in the colon<sup>13</sup>. Therefore, in the present study the *n*-BuOH fraction of the total root extract of *T. albida* has been subjected to extensive dereplication studies. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) combined with molecular networking and *in silico* MS/MS analysis was performed prior to subsequent isolation of the target compounds. Several molecular masses and their respective fragmentations patterns did not correlate with known compounds, thus revealing potentially new natural products which could be studied in more detail. Reported herein are the targeted isolation guided by mass spectrometry and the structure elucidation of oleanane-type triterpenes, as well as their antiplasmodial and antimicrobial properties.

## 5.2. Materials and methods

### 5.2.1. General experimental procedures

Purification of extracts and fractions was carried out using a MCI column and a Grace Reveleris X2 flash chromatographic system (Lokeren, Belgium) equipped with an evaporative light scattering detector (ELSD), a diode array detector (DAD) and a fraction collector. The ELSD carrier solvent was isopropyl alcohol and the column used was a pre-packed Flash Pure BUCHI C<sub>18</sub> (40g) with a particle size of 40 µm. A semi-preparative HPLC system equipped with DAD and ESI-MS detectors was used for the isolation of compounds. The system was composed of a sample manager, injector, and collector (2767), a quaternary gradient module (2545), a System Fluidics Organizer, a HPLC pump (515), a diode array detector (2998), and a Micromass Quattro TQD mass spectrometer, all supplied by Waters (Milford, MA, USA). For data processing MassLynx version 4.1 was used. Optical rotations were measured on a JASCO P-2000 spectropolarimeter (Easton, MD, USA). 1D and 2D Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DRX-400 NMR spectrometer (Rheinstetten, Germany) equipped with either a 3 mm inverse broadband (BBI) probe or a 5 mm dual <sup>1</sup>H/<sup>13</sup>C probe using standard Bruker pulse sequences and operating at 400 MHz for <sup>1</sup>H and at 100 MHz for <sup>13</sup>C NMR spectra.

The spectra were processed with Topspin version 4.0.6). Chemical shifts are expressed in  $\delta$  (ppm) and referenced to the residual solvent signals.

MeCN and MeOH (all HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK). All reagents, such as TFA and formic acid (eluent additive for HPLC) were purchased from Acros Organics (Geel, Belgium) or Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and formic acid (both for UPLC-MS) were obtained from Biosolve Chimie (Dieuze, France). Ultrapure water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA). NMR solvents (methanol- $d_4$ , pyridine- $d_5$ ) were obtained from Sigma-Aldrich.

### **5.2.2. Plant Material**

Roots of *T. albida* Sc. Elliot (Combretaceae) were harvested in Dubréka, Republic of Guinea in June 2016. The plant was identified by the botanists from the Research and Valorization Center on Medicinal Plants, Dubréka, where a voucher specimen (D36HK13) is kept. The collected root samples were dried at room temperature and milled. A "Material Transfer Agreement" has been established between the University of Antwerp and the Department of Pharmacy, University Gamal Abdel Nasser of Conakry / Research and Valorization Center on Medicinal Plants.

### **5.2.3. LC-ESI-MS analysis.**

Fractions were dissolved in UPLC/MS grade MeOH at a concentration of 1 mg/mL, which was diluted with water in order to obtain a final concentration of 0.1 mg/mL. Analyses were performed on a Xevo-G2-XS-QTof mass spectrometer (Waters) coupled to an Acquity LC system equipped with MassLynx version 4.1 software. A Waters Acquity UHPLC BEH Shield RP18 column (2.1 mm x 100 mm, 1.7 $\mu$ m) was used with a mobile phase consisting of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), which were pumped at a rate of 0.4 mL/min. The gradient system was set as follows: 2% B (0-1 min), 2-100% B (1-5 min), 100% B (5-7 min), 100-2% B (7-8 min), 2% B (8-10 min). For all analyses, full scan data were recorded in ESI (-) and ESI (+) mode from  $m/z$  50 to 1500 in sensitivity mode (approximate resolution: 22000 FWHM) using a spray voltage at either - 0.8 kV and +1.0 kV, respectively. Cone gas flow and desolvation gas flow were set at 50.0 L/h and 1000.0 L/h, respectively; and source temperature and desolvation

temperature at 120 °C and 550 °C, respectively. Leucine encephalin was used as lock mass during the analysis.

#### **5.2.4. Molecular networking and In-silico MS/MS derePLICATION of the butanolic fraction.**

##### **5.2.4.1. In-Silico Ms/Ms derePLICATION**

The UHPLC–HR-MS raw data were firstly converted to Abf files (Reifycs Abf Converter) and processed with MS-DIAL version 4.16<sup>19</sup> for mass signal extraction between 50 and 1500 Da from 0 to 25 min. Respective MS1 and MS2 tolerance were set to 0.01 and 0.05 Da in centroid mode. The optimised detection threshold was set to  $3 \times 10^4$  for MS1. The peaks were finally aligned on a quality control (QC) reference file with a retention time tolerance of 0.1 min and a mass tolerance of 0.025 Da. MS-FINDER software<sup>20</sup> was used for *in silico* fragmentation predictions. Compounds were tentatively identified according to their similarity score, which was based on comparison between experimental MS/MS fragments and *in silico* spectra. To find the potential candidates, only compounds consisting essentially of C, H and O have been considered. The MS1 and MS2 tolerances were set at 0.01 and 0.25 Da, respectively. An isotopic ratio tolerance of 20% has been considered. The natural product databases integrated in MSFINDER (PlantCyc, UNPD, KNApSAcK and NANPDB) have been used for compound identification. The molecular formulas determined by using MS-FINDER software have been queried in different natural product databases such as Dictionary of Natural Products, ChemSpider and SciFinder in order to obtain the molecular structures of the compounds.

##### **5.2.4.2. GNPS molecular networking**

After MS-DIAL processing, two files (the feature quantification table “TXT file” and the MS/MS spectral summary “MGF file” have been exported and uploaded to the GNPS website (<http://gnps.ucsd.edu>). The molecular network was created using the GNPS web platform<sup>21</sup>. All MS/MS signals within 17 Da of the precursor *m/z* were removed, and only the top six fragment peaks were compared for analysis. The data were then clustered with MS-Cluster with a precursor ion masse tolerance of 0.05 Da and an MS/MS fragment ion tolerance of 0.5 Da. Next, consensus spectra that contained less than 2 spectra were

discarded. A network was then created where edges were filtered to have a cosine score above 0.65 and more than one matched peak. Edges between two nodes were kept in the network if, and only if, each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against the spectral libraries of GNPS. The library spectra were filtered in the same manner as the input data. The data were then imported into Cytoscape v3.7.2 (The Cytoscape Consortium, New York, NY, USA) for visualisation. All matches kept between network spectra and library spectra were required to have a score above 0.6 and at least 1 matched peak. Analogue search was enabled against the library with a maximum mass shift of 100.0 Da.

### 5.2.5. Extraction and isolation

The dried and milled root bark of *T. albida* (470.9 g) defatted with *n*-hexane (4 x 2.5 L) was extracted 4 times with 80% MeOH (4 x 2L; each for 24 h). The filtrate was concentrated under reduced pressure and freeze-dried to obtain a crude 80% MeOH extract (110 g), which was redissolved in water (1 L) and successively partitioned with EtOAc (2 x 8 L) and *n*-BuOH (2 x 8 L) to give an EtOAc soluble fraction (15.1 g), an *n*-BuOH soluble fraction (24.2 g) and a residual H<sub>2</sub>O phase. The *n*-BuOH fraction (15 g) (Figure 3) was subjected to MCI column chromatography and eluted with 5, 10, 20, 30, 40, 50, 60, 70, 80 and 100% MeOH-H<sub>2</sub>O to give 10 pooled fractions (fractions A to J).

The LC-MS profile of all the fractions was recorded and the fractions F and H containing the products of interest were selected and further purified. Indeed, the purification of fraction F (0.3 g) by semi-preparative HPLC-MS operated with a C<sub>18</sub> Kinetex column and eluted with H<sub>2</sub>O + 0.1% formic acid (A) and MeCN (B) resulted in the isolation of compounds **1** (9.4 mg), **2** (4.2 mg) and **3** (3.5 mg). Mass-based fraction collection was used to enable highly specific peak triggering with the fragment masses of *m/z* 499 [M-H]<sup>-</sup> (compounds **1**, **2** and **3**) set as the target masses with a threshold of 6.58e<sup>5</sup>. The flow rate was set at 3 mL/min and the gradient was programmed as follows: (0-5 min) 30% B, (5-35mn) 50% B, (35-40 min) 50% B, (40-50 min) 95% B. The sample concentration was 25 mg/mL and the injection volume was 400 µL. The DAD spectrum was recorded from 200 to 450 nm, and mass spectra in the ESI modes, MS scan range: *m/z* 100 to 1000; capillary voltage 3.00 kV, cone voltage 50 V, extractor voltage 3 V, source temperature 135 °C, desolvation temperature 400 °C, desolvation gas flow 750 L/h, and cone gas flow 50 L/h. The fraction H (1.9 g) was subjected to a reverse phase flash

chromatography using a C-18 pre-packed column (Flash Pure BUCHI-40 µm) eluted with a gradient of H<sub>2</sub>O + 0.1% formic acid (A) and acetonitrile (B) at the flow rate of 40mL/min. This separation yielded 5 sub-fractions H<sub>A</sub> (0.28 g), H<sub>B</sub> (0.34 g), H<sub>C</sub> (0.37 g), H<sub>D</sub> (0.25 g), H<sub>E</sub> (0.19 g). The gradient was set as follow (0-5 min) 5%B, (6-46 min) 50%B, (46-59 min) 50% B, (59-80 min) 100% B, (80-90 min) 100% B. Subfractions selected for further purification based on their LC-MS profile were subjected to a semi-preparative HPLC-MS system operated with a C<sub>18</sub> Kinetex column and eluted with a gradient of H<sub>2</sub>O + 0.1% formic acid (A) and MeCN (B). The purification of H<sub>A</sub> was performed with the following gradient (0-5 min) 30% B, (5-35mn) 50% B, (35–40 min) 50% B and (40–50 min) 95% B and resulted in the isolation of compounds **4** (3.5mg) and **5** (3.3 mg).

The purification of fraction H<sub>B</sub> by using the following gradient: (0-5 min) 30% B, (5-35 min) 35% B, (35–40 min) 35% B, and (40–50 min) 95% B afforded compounds **6** (4.6 mg), **7** (5.2 mg) and **8** (2.7mg). Fraction H<sub>C</sub> fractionated by using the following gradient (0-5 min) 20% B, (5-35min) 30% B, and (35–45 min) 95 % B, resulted in the isolation of compounds **9** (2.3 mg) and **10** (4.5 mg). The flow rate was set at 3.0 mL/min and samples were dissolved at the concentration of 20 mg/mL. For all fractions, 400 µL was injected. The automatic collection was triggered by *m/z* 455 [M-H]<sup>-</sup> (compound **4**, **6** and **7**); *m/z* 519 (compound **5**); *m/z* 499 [M-H]<sup>-</sup> (compound **8**) and *m/z* 503 [M-H]<sup>-</sup> (compound **9** and **10**) reaching a threshold of  $4.94 \times 10^5$ . The MS method described above was used during the whole purification process.

*2α,3β,19α,23-tetrahydroxy-11-oxo-olean-12-en-28-oic acid 28-O-β-D-glucopyranosyl ester (1)*: amorphous white solid;  $[\alpha]_D^{20} = + 4.2$  (*c* 0.01, MeOH); <sup>1</sup>H NMR (pyridine-*d*<sub>5</sub>, 400 MHz) and <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 100MHz), see Table S 5.1; HR-ESIMS *m/z* 679.3710 [M-H]<sup>-</sup> (calculated for C<sub>36</sub>H<sub>55</sub>O<sub>12</sub>, 679.3694), major peak formic acid adduct ion at *m/z* 725.3723 [M-H+HCOOH]<sup>-</sup> (calculated for C<sub>37</sub>H<sub>57</sub>O<sub>14</sub>, 725.3748).

*Albidanoside A (2)*: Amorphous white solid;  $[\alpha]_D^{20} = + 9.1$  (*c* 0.3, MeOH); <sup>1</sup>H NMR (pyridine-*d*<sub>5</sub>, 400 MHz) and <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 100MHz), see Tables 1 and 2, respectively; HR-ESIMS *m/z* 679.3690 [M-H]<sup>-</sup> (calculated for C<sub>36</sub>H<sub>55</sub>O<sub>12</sub>, 679.3694), major peak formic acid adduct ion at *m/z* 725.3739 [M-H+HCOOH]<sup>-</sup> (calculated for C<sub>37</sub>H<sub>57</sub>O<sub>14</sub>, 725.3748).

*Ivorenoside C (3)*: amorphous white powder;  $[\alpha]_D^{20} = + 23$  (c 0.2, MeOH);  $^1\text{H}$  NMR (pyridine-*d*<sub>5</sub>, 400 MHz) and  $^{13}\text{C}$  NMR (pyridine-*d*<sub>5</sub>, 100MHz), see Table S 5.1; HR-ESIMS *m/z* 679.3690 [M-H]<sup>-</sup> (calculated for C<sub>36</sub>H<sub>55</sub>O<sub>12</sub>, 679.3694), major peak formic acid adduct ion at *m/z* 725.3741 [M-H+HCOOH]<sup>-</sup> (calculated for C<sub>37</sub>H<sub>57</sub>O<sub>14</sub>, 725.3748).

*Albidic acid A (4)*: amorphous white powder;  $[\alpha]_D^{20} = + 39.5$  (c 0.3, MeOH);  $^1\text{H}$  NMR (pyridine-*d*<sub>5</sub>, 400 MHz) and  $^{13}\text{C}$  NMR (pyridine-*d*<sub>5</sub>, 100MHz), see Tables 1 and 2, respectively; HR-ESI-MS: *m/z* 517.3156 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>45</sub>O<sub>7</sub>, 517.3165), fragments at *m/z* 499.3062 [M-H-18]<sup>-</sup>, 455.3158 [M-H-18-44]<sup>-</sup>.

*Albidinolic acid (5)*: colourless powder;  $[\alpha]_D^{20} = + 6.9$  (c 0.2, MeOH);  $^1\text{H}$  NMR (methanol-*d*<sub>4</sub>, 400 MHz) and  $^{13}\text{C}$  NMR (methanol-*d*<sub>4</sub>, 100 MHz), see Tables 1 and 2; HR-ESIMS *m/z* 519.3328 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>47</sub>O<sub>7</sub>, 519.3322), formic acid adduct ion at *m/z* 565.3376 [M-H+HCOOH]<sup>-</sup> calculated for C<sub>31</sub>H<sub>49</sub>O<sub>9</sub>, 565.3377).

*Albidic acid B (6)*: amorphous white powder;  $[\alpha]_D^{20} = + 14.7$  (c 0.4, MeOH);  $^1\text{H}$  NMR (pyridine-*d*<sub>5</sub>, 400 MHz) and  $^{13}\text{C}$  NMR (pyridine-*d*<sub>5</sub>, 100MHz), see Tables 1 and 2; HR-ESIMS *m/z* 517.3170 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>45</sub>O<sub>7</sub>, 517.3165), fragments at *m/z* 499.3062 [M-H-18]<sup>-</sup>, *m/z* 455.3162 [M-H-18-44]<sup>-</sup>.

*Albidic acid C (7)*: amorphous white powder;  $[\alpha]_D^{20} = + 6.8$  (c 0.4, MeOH);  $^1\text{H}$  NMR (pyridine-*d*<sub>5</sub>, 400 MHz) and  $^{13}\text{C}$  NMR (pyridine-*d*<sub>5</sub>, 100MHz), see Tables 1 and 2; HR-ESIMS *m/z* 517.3187 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>45</sub>O<sub>7</sub>, 517.3165), fragments at *m/z* 499.3072 [M-H-18]<sup>-</sup>, *m/z* 455.3169 [M-H-18-44]<sup>-</sup>.

*Albidienic acid (8)*: amorphous white solid;  $[\alpha]_D^{20} = +109.7$  (c 0.2, MeOH);  $^1\text{H}$  NMR (pyridine-*d*<sub>5</sub>, 400 MHz) and  $^{13}\text{C}$  NMR (pyridine-*d*<sub>5</sub>, 100MHz), see Tables 1 and 2; HR-ESIMS *m/z* 499.3067 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>43</sub>O<sub>6</sub>, 499.3060), fragments at *m/z* 455.3162 [M-H-44]<sup>-</sup>.

*Albidolic acid (9)*: amorphous white powder;  $[\alpha]_D^{20} = +17.1$  (c 0.3, MeOH);  $^1\text{H}$  NMR (methanol-*d*<sub>4</sub>, 400 MHz) and  $^{13}\text{C}$  NMR (methanol-*d*<sub>4</sub>, 100 MHz), see Tables 1 and 2; HR-

ESIMS *m/z* 503.3373 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>47</sub>O<sub>6</sub>, 503.3373), formic acid adduct ion at *m/z* 549.3420 [M-H+HCOOH]<sup>-</sup> calculated for C<sub>31</sub>H<sub>49</sub>O<sub>8</sub>, 549.3427).

*Albidolic acid (10)*: colourless solid powder;  $[\alpha]_D^{20} = +23.5$  (c 0.2, MeOH); <sup>1</sup>H NMR (pyridine-*d*<sub>5</sub>, 400 MHz) and <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 100MHz), see Tables 1 and 2; HR-ESIMS *m/z* 503.3370 [M-H]<sup>-</sup> (calculated for C<sub>30</sub>H<sub>47</sub>O<sub>6</sub>, 503.3373), fragments at *m/z* 549.3429 [M-H+HCOOH]<sup>-</sup> calculated for C<sub>31</sub>H<sub>49</sub>O<sub>8</sub>, 549.3427).

## 5.2.6. Biological evaluation

### 5.2.6.1. Antibacterial and antifungal activity

The antimicrobial activity of all fractions and pure compounds was evaluated according to Cos et al. (2006) and Baldé et al. (2010)<sup>20,21</sup>. Fractions and pure compounds were tested against the following microorganisms: *E. coli* ATCC8739 (Gram-negative), *S. aureus* ATCC 6538 (Gram-positive) and *C. albicans* ATCC59630 (yeast). The following positive controls were used: flucytosine for *C. albicans* (IC<sub>50</sub> 0.7 ± 0.01 μM) and doxycycline for *S. aureus* (IC<sub>50</sub> 0.28 ± 0.2 μM).

### 5.2.6.2. Antiplasmodial and cytotoxicity assays.

Antiplasmodial activity and cytotoxicity were assessed as previously described<sup>22,24</sup>. Fractions and pure compounds were tested *in vitro* against the chloroquine-resistant strain *Plasmodium falciparum* K1 using the lactate dehydrogenase assay. Tamoxifen was used as the positive control for cytotoxicity on MRC-5 cells (IC<sub>50</sub> 10.0 ± 1.5 μM), and chloroquine (IC<sub>50</sub> 0.15±0.10 μM) for *P. falciparum*. These reference compounds are routinely used in the screening platform and their activities were in the range that is usually observed.

### 5.3. Results and discussion

In order to obtain a complete overview of the phytochemical composition of the *n*BuOH extract of the root of *T. albida*, its fractions were subjected to extensive dereplicative studies by LC-MS/MS combined with *in silico* MS/MS analysis using MS-FINDER and molecular networking. The potential candidates obtained for each constituent by consulting the natural product databases integrated in MS-FINDER were ranked according to their similarity score, which was based on comparison between experimental MS/MS fragments and *in silico* spectra of candidates. Next, the molecular structures of compounds which could not be identified by using MS-FINDER were manually searched in different databases such as the Dictionary of Natural Products, ChemSpider and SciFinder. The dereplicative analysis led to the characterization of 45 constituents (Table 5.1). Compounds which did not have a match in the databases during the dereplication process were considered to be unknown. The establishment of the molecular network (MN) of MS/MS data revealed correlations of these compounds with already known compounds indicating their possible similarity to oleanane triterpenoids. Detailed analysis of the MN allowed to highlight a cluster consisting essentially of oleanane triterpenoids (Figure 5.1). They were tentatively identified by comparison with *in silico* fragmentation data using MS-FINDER. Although these compounds showed correlations with known compounds within the network, it is important to stress that in this way it is not possible to fully identify them. Nowadays, it is well known that MN can provide more structural information by assigning similar chemical scaffolds to their related neighbours and eventually give a potential chemical scaffolds for unknown nodes, but MN cannot provide insights into the deep annotation of similar structures<sup>14</sup>. Therefore, a complementary identification strategy using preparative LC-MS and 1D (<sup>1</sup>H-NMR, <sup>13</sup>C-NMR, DEPT-135, DEPT-90) and 2D (COSY, HSQC, HMBC) NMR data analysis has been applied, resulting in the isolation and complete identification of 10 target compounds. <sup>1</sup>H- and <sup>13</sup>C-NMR assignments are listed in Table 5.2; 5.3 and Table S 5.1.

**Table 5.1. Summary of dereplicated compounds from the *n*-BuOH fraction of *T. albida*.**

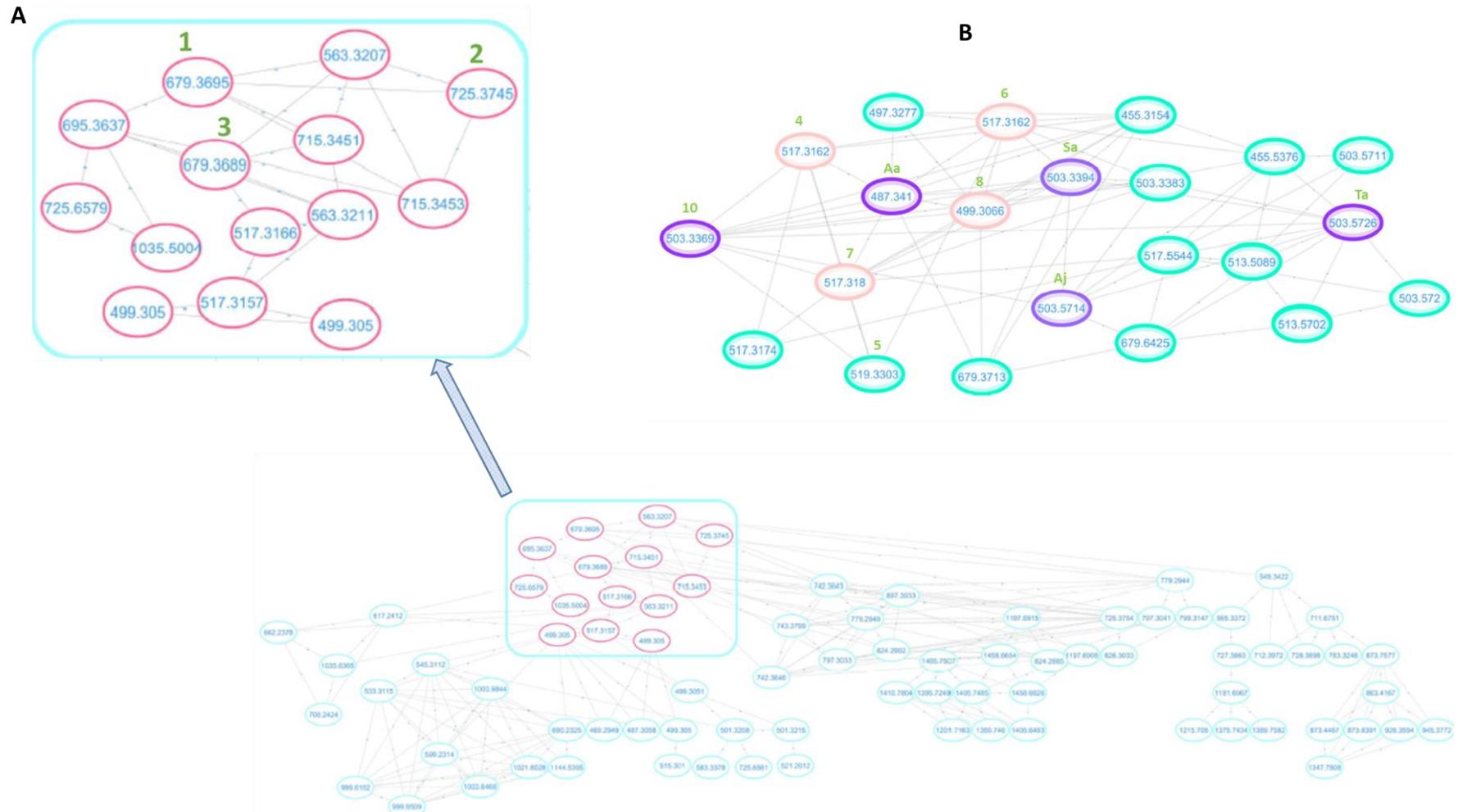
N°	RT (min)	Compound identification	Molecular Formula	ESI negative full		ESI negative mode MS/MS	Δ ppm	MS level
1	4.98	Inerminoside A1 <sup>c</sup>	C <sub>21</sub> H <sub>31</sub> O <sub>14</sub>	507.1714	[M-H]-	297.7334, 161.8494,	0.00	L3
2	5.13	Inerminoside A1 isomer <sup>c</sup>	C <sub>21</sub> H <sub>31</sub> O <sub>14</sub>	507.1721	[M-H]-	476.0734, 342.8264,	1.38	L3
3	5.74	Rehmannioside A <sup>c</sup>	C <sub>21</sub> H <sub>32</sub> O <sub>15</sub>	523.1667	[M-H]-	361.1405	0.76	L3
4	6.54	Dicaffeoylacteoside <sup>b</sup>	C <sub>20</sub> H <sub>30</sub> O <sub>12</sub>	461.1661	[M-H]-	315.2970, 153.0575	0.43	L3
5	10.51	Ducheside A <sup>b</sup>	C <sub>20</sub> H <sub>15</sub> O <sub>12</sub>	447.0562	[M-H]-	300.9984	-0.4	L3
6	10.69	Ellagic acid <sup>a</sup>	C <sub>14</sub> H <sub>8</sub> O <sub>8</sub>	300.998	[M-H]-	283.991	-1.32	L1
7	10.89	Compound 1 <sup>a</sup>	C <sub>37</sub> H <sub>57</sub> O <sub>14</sub>	725.3723	[M-H+FA]-	679.3710, 517.3156, 455.3158	-3.44	L1
8	10.94	Compound 2 <sup>a</sup>	C <sub>36</sub> H <sub>55</sub> O <sub>12</sub>	679.3690	[M-H]-	517.3660, 455.3156	0.58	L1
9	11.04	Triterpenoid saponin- <i>O</i> -hexoside <sup>c</sup>	C <sub>37</sub> H <sub>59</sub> O <sub>14</sub>	727.3911	[M-H]-	565.3389	-0.82	L2
10	11.76	Centellasaponin B <sup>d</sup>	C <sub>43</sub> H <sub>69</sub> O <sub>18</sub>	873.4453	[M-H+FA]-	827.4402, 711.3956, 503.3370, 485.9660	-3.54	L3

11	12.01	Triterpenoid saponin- <i>O</i> -hexoside <sup>c</sup>	C <sub>37</sub> H <sub>59</sub> O <sub>14</sub>	727.3891	[M-H]-	565.3373	-1.92	L2
12	12.13	Centellasaponin B isomer <sup>c</sup>	C <sub>43</sub> H <sub>69</sub> O <sub>18</sub>	873.4459	[M-H+FA]-	827.4435, 711.3937, 665.3922, 503.3365	-2.86	L3
13	12.58	Triterpenoid saponin- <i>O</i> -hexoside <sup>c</sup>	C <sub>37</sub> H <sub>59</sub> O <sub>14</sub>	727.3925	[M-H]-	565.3372	2.74	L2
14	12.72	3 $\alpha$ , 21 $\beta$ , 29-trihydroxy-16-oxoserrat-14-en-24-oic acid <sup>b</sup>	C <sub>30</sub> H <sub>45</sub> O <sub>6</sub>	501.3214	[M-H]-	483.3156, 439.2803	0.39	L3
15	12.73	Componud 3 <sup>a</sup>	C <sub>37</sub> H <sub>57</sub> O <sub>14</sub>	725.3753	[M-H+FA]-	679.3690, 517.3157, 499.3056, 455.3156	0.68	L1
16	13.08	Ellagic Acid methylester <sup>b</sup>	C <sub>15</sub> H <sub>7</sub> O <sub>8</sub>	315.0138	[M-H]-	299.9905	-0.95	L2
17	13.16	Arjunglucoside <sup>a,c</sup>	C <sub>37</sub> H <sub>59</sub> O <sub>13</sub>	711.3956	[M-H+FA]-	665.3917, 503.3381	0.00	L1
18	13.56	Sericoside <sup>a,c</sup>	C <sub>37</sub> H <sub>59</sub> O <sub>13</sub>	711.3988	[M-H+FA]-	665.3896, 503.3371	4.4	L1
19	14.03	Centellasaponin B isomer <sup>c</sup>	C <sub>43</sub> H <sub>69</sub> O <sub>18</sub>	873.4476	[M-H+FA]-	827.4431, 711.3954, 665.2607, 503.3378	-0.91	L3
20	14.32	2,3,8-Tri- <i>O</i> -Methylellagic acid <sup>a</sup>	C <sub>17</sub> H <sub>11</sub> O <sub>8</sub>	343.0452	[M-H]-	297.9746, 268.8221	-0.58	L1
21	14.75	Quercotriterpenoside <sup>d</sup>	C <sub>43</sub> H <sub>61</sub> O <sub>15</sub>	817.3991	[M-H]-	799.3206, 781.3249, 448.7066	-2.32	L3
22	14.98	Quercotriterpenoside isomer <sup>d</sup>	C <sub>43</sub> H <sub>61</sub> O <sub>15</sub>	817.3979	[M-H]-	799.3895, 781.3054, 648.2248	-3.79	L3

23	15.08	Kermesic Acid <sup>c,d</sup>	C <sub>16</sub> H <sub>9</sub> O <sub>8</sub>	329.0292	[M-H] <sup>-</sup>	285.003	-1.51	L3
24	15.28	Chebuloside II <sup>a</sup>	C <sub>37</sub> H <sub>59</sub> O <sub>13</sub>	711.3939	[M-H+FA] <sup>-</sup>	665.3924, 503.3366, 485.3336	-2.38	L1
25	15.71	1-Methyl-2-carboxy-3,5,6,8-tetrahydroxyanthraquinone <sup>b</sup>	C <sub>16</sub> H <sub>9</sub> O <sub>8</sub>	329.0289	[M-H] <sup>-</sup>	312.9984	-2.4	L2
26	15.92	Galiosin <sup>b</sup>	C <sub>26</sub> H <sub>25</sub> O <sub>16</sub>	593.1134	[M-H] <sup>-</sup>	549.3071	-1.51	L1
27	15.97	Compound <b>5</b> <sup>a</sup>	C <sub>30</sub> H <sub>47</sub> O <sub>7</sub>	519.3328	[M-H] <sup>-</sup>	501.3214	1.15	L1
28	16.25	Compound <b>4</b> <sup>a</sup>	C <sub>30</sub> H <sub>45</sub> O <sub>7</sub>	517.3156	[M-H] <sup>-</sup>	499.3062, 455.3158	-1.73	L1
29	16.61	Arjunglucoside II <sup>a,b</sup>	C <sub>36</sub> H <sub>57</sub> O <sub>10</sub>	695.4003	[M-H] <sup>-</sup>	633.2308	-1.84	L1
30	16.63	Paradoxoside C <sup>d</sup>	C <sub>37</sub> H <sub>58</sub> O <sub>12</sub>	695.3998	[M-H] <sup>-</sup>	469.7827	0.4	L2
31	16.79	Compound <b>9</b> <sup>a</sup>	C <sub>30</sub> H <sub>47</sub> O <sub>6</sub>	503.3373	[M-H] <sup>-</sup>	487.3083, 469.7904	0.00	L1
32	16.80	Compound <b>6</b> <sup>a</sup>	C <sub>30</sub> H <sub>45</sub> O <sub>7</sub>	517.3170	[M-H] <sup>-</sup>	499.3062, 455.3162	0.96	L1
33	17.29	2 $\alpha$ ,3 $\beta$ ,21 $\beta$ , 23 tetra hydroxy-olean-12-en-28 oic-acid <sup>a,b</sup>	C <sub>30</sub> H <sub>47</sub> O <sub>6</sub>	503.3394	[M-H] <sup>-</sup>	485.3279,	1.78	L1
34	17.57	Compound <b>7</b> <sup>a</sup>	C <sub>30</sub> H <sub>45</sub> O <sub>7</sub>	517.3187	[M-H] <sup>-</sup>	499.3072, 455.3169	4.25	L1
35	18.22	Arjungenin <sup>a,b</sup>	C <sub>30</sub> H <sub>47</sub> O <sub>6</sub>	503.3370	[M-H] <sup>-</sup>	489.3279, 469,7824	0.79	L1
36	18.80	Compound <b>10</b> <sup>a</sup>	C <sub>30</sub> H <sub>47</sub> O <sub>6</sub>	503.3370	[M-H] <sup>-</sup>	499.3057, 455.3159	-0.59	L2
37	18.84	Compound <b>8</b> <sup>a</sup>	C <sub>30</sub> H <sub>43</sub> O <sub>6</sub>	499.3067	[M-H] <sup>-</sup>	487.3062, 455.3162	1.40	L1
38	18.93	Terminolic acid <sup>a</sup>	C <sub>30</sub> H <sub>47</sub> O <sub>6</sub>	503.3364	[M-H] <sup>-</sup>	485.3279, 467.7102	-1.78	L1

39	18.96	Tetrahydroxyolean-12-en-28-oic acid <sup>a</sup>	C <sub>31</sub> H <sub>49</sub> O <sub>8</sub>	549.3433	[M-H+FA]-	503.3377, 485.3283	1.09	L1
40	18.97	Arjunglucoside II <sup>a</sup>	C <sub>36</sub> H <sub>57</sub> O <sub>10</sub>	649.3941	[M-H]-	487.3080	1.69	L3
41	19.50	Seric acid <sup>a</sup>	C <sub>30</sub> H <sub>47</sub> O <sub>6</sub>	503.3384	[M-H]-	485.3270, 441.3448	-2.18	L1
42	20.86	Gallyl arjunolic acid isomer <sup>d</sup>	C <sub>37</sub> H <sub>51</sub> O <sub>9</sub>	639.3523	[M-H]-	594.3665	-1.56	L2
43	20.89	Arjunic acid <sup>a</sup>	C <sub>30</sub> H <sub>47</sub> O <sub>5</sub>	487.3415	[M-H]-	469.3319	-1.64	L1
44	21.80	Gallyl arjunolic acid isomer <sup>d</sup>	C <sub>37</sub> H <sub>51</sub> O <sub>9</sub>	639.3553	[M-H]-	594.7043	3.12	L2
45	21.86	Gallyl arjunolic acid isomer <sup>d</sup>	C <sub>37</sub> H <sub>51</sub> O <sub>9</sub>	639.3525	[M-H]-	594.6952	-1.25	L2

<sup>a</sup>Structure confirmed by NMR spectroscopy; <sup>b</sup>Compounds determined by in silico MS/MS fragmentation with MS-FINDER; <sup>c</sup>Compounds identified by molecular networking; <sup>d</sup>Compounds tentative identified based on MS, MS2 experimental data



**Figure 5.1. A (heterosides) and B (aglycones):** Clusters corresponding to the oleanane triterpenoid type of the *n*-BuOH fraction with nodes labelled (compound 1-10) and previously isolated compounds: Ar (arjunglucoside I), Aj (arjunglucoside II), Ar (arjungenine), C (Chebuloside II), s (sericoside), sa (sericic acid).

**Table 5.2.  $^1\text{H-NMR}$  assignments ( $\delta_{\text{H}}$  in ppm) for compounds 2, 4-10 (400 MHz, pyridine- $d_5$  and methanol- $d_4$ )**

	<b>2<sup>b</sup></b>	<b>4<sup>b</sup></b>	<b>5<sup>b</sup></b>	<b>6<sup>b</sup></b>	<b>7<sup>b</sup></b>	<b>8<sup>b</sup></b>	<b>9<sup>b</sup></b>	<b>10<sup>c</sup></b>
Position	$\delta_{\text{H}}$ ( $J$ in Hz)							
			0.89, m				0.89, m	
1a	1.38 <sup>a</sup>	1.38, m		1.29 <sup>a</sup>	1.46, m	1.52, d (12.9)		1.37, m
1b	3.76, m	3.78, m	1.91, m	3.72, m	3.90, m	3.81, m	1.92, m	2.27, m
2	4.34, m	4.35, m	3.68, m	4.39, m	4.41, m	4.46, m	3.68, m	4.22, m
3	4.18, m	4.18, m	3.35, m	3.54, d (8.7)	3.6, m	3.62, d (9.5)	3.34, m	4.19, m
4								
5	1.79, d (11.9)	1.79, m	1.29 <sup>a</sup>	1.08 <sup>a</sup>	1.17 <sup>a</sup>	1.18 <sup>a</sup>	1.27, br s	2.43, m
6a	1.17 <sup>a</sup>	1.44 <sup>a</sup>	1.45, m	0.91 <sup>a</sup>	0.91, m	1.55, m		1.43, s
6b	1.73, m	1.76, m	1.60, m	1.68, m	1.68, m	1.70, m		
7a	1.40, m	1.84, m	1.28, m	1.34, m	1.34, m	1.43, m	1.28, br s	1.28, m
7b	1.79, m	2.09, m	1.61, m	1.58, m	1.58, m	1.54, m		1.61, m
8								
9	2.67, s	2.68, s	1.99 <sup>a</sup>	2.6, s	2.83, s	2.65, s	1.68, m	1.78, m
10								
11			1.28 <sup>a</sup> , 1.77 br s					2.0, m
12	6.36, s	6.39, s	5.33, s	6.4, s	6.16, s	6.21, s	5.25, s	5.48, s
13								
14								
15a	1.30, m	1.24, m	1.0, m	1.32, m	1.32, m	1.29, m	1.06 <sup>a</sup>	1.12, m
15b	2.43, m	2.17, m	1.62, m	2.09, m	2.09, m	2.11, t (13.2)	1.80, m	
16a	1.52, m	2.09, m	0.92, m	1.34, m	1.34, m		0.90, m	1.98, m
16b	0.86, d (11.1)	2.17, m	1.7, m	1.58, m	1.58, m		1.27, m	2.16, m
17								
18	3.23, d (10.8)	3.37, d (10.6)	1.99 <sup>a</sup>	3.34, br d (10.2)	3.22, m		2.88, d (14.4)	3.38, dd (11.4, 11.4)

19	3.76, m	3.81, d (10.6)	3.55, m	3.83, br d (10.2)	3.66, d (5.1)	5.74, s	1.07, m	1.61, m	1.49, m	2.07, m
20										
21a	1.27 <sup>a</sup>	1.36, m	3.41, m	1.38 m	n.o	1.41, m	3.18, brs		1.39, m	
21b	1.63 <sup>a</sup>	1.72, m		1.71 m	n.o	1.64, m			1.78, m	
22a	1.75, m	1.84, m	n.o	1.71 m	1.5, m	1.67, m	1.14 m		1.91, m	
22b	1.88, m	2.09, m	n.o	1.67 m	1.71, m	2.49, m	1.46 m		2.13, m	
23a	3.69, m	3.68, d (10.6)	3.26, d (10.6)	1.55 s	1.60, s	1.60 s	3.25 d (11.02)		3.70, m	
23b	4.25, m	4.21, d (10.6)	3.49, d (10.6)	n.o	n.o	n.o	3.48 d (11.02)		4.18, m	
24a	1.04, s	1.04, s	0.69, s	3.71 m	3.74, m	3.77, d (10.6)		0.68, s	1.04, s	
24b				4.44 m	4.45, m	4.49, d (10.6)				
25	1.45, s	1.43, s	1.02, s	1.34 s	1.38, s	1.46, s	1.02, s		1.35, s	
26	1.37, s	1.24, s	0.79, s	1.19 s	1.20, s	1.21, s	0.82, s		1.03, s	
27	1.39, s	1.43, s	1.27, s	1.50 s	1.78, s	1.24, s	1.18, s		1.20, s	
28										
29	1.17, s	1.21, s	1.12, s	1.24, s	0.99, s	0.96, s	0.92, s		1.19,s	
30	1.0, s	1.12, s	0.97, s	1.13, s	1.12, s	1.01, s	0.92, s		3.56, s	
1'	6.32, d (8.3)									
2'	4.19 <sup>a</sup>									
3'	2.28, m									
4'	4.36 <sup>a</sup>									
5'	4.04, m									
6'	4.42 m, 4.46, m									

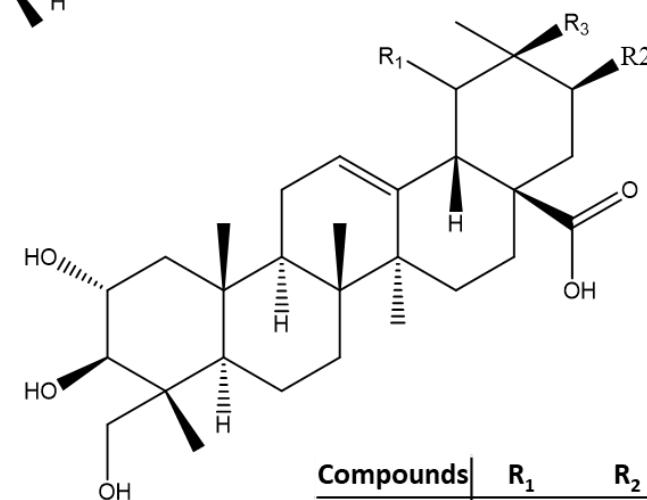
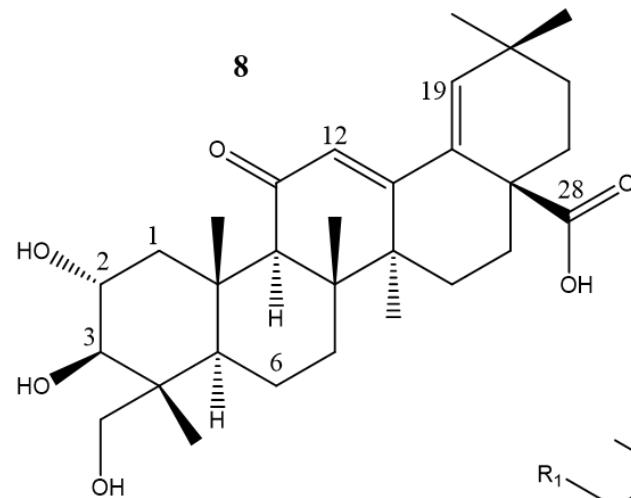
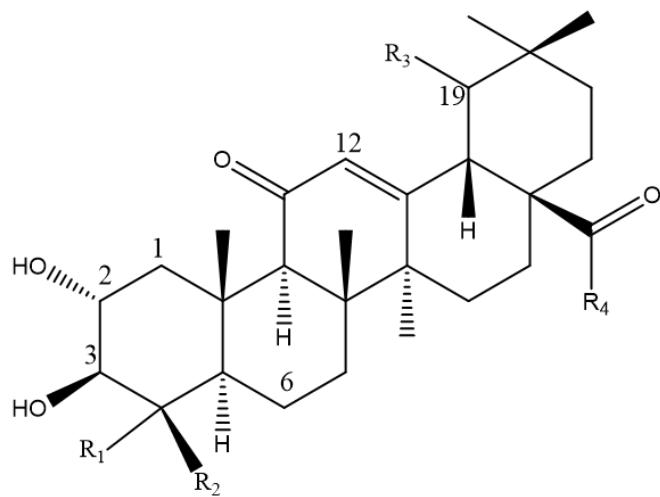
<sup>a</sup>Overlapping signals, <sup>b</sup>pyridine-d<sub>5</sub>, <sup>c</sup>methanol-d<sub>4</sub> n.o: not observed.

**Table 5.3.  $^{13}\text{C}$ -NMR assignments ( $\delta_c$  in ppm) for compounds 2, 4-10 (100 MHz, pyridine-*d*5 and methanol-*d*4)**

	<b>2<sup>b</sup></b>	<b>4<sup>b</sup></b>	<b>5<sup>c</sup></b>	<b>6<sup>b</sup></b>	<b>7<sup>b</sup></b>	<b>8<sup>b</sup></b>	<b>9<sup>c</sup></b>	<b>10<sup>b</sup></b>
Position	$\delta_c$ , Type							
			47.5, CH <sub>2</sub>				47.9, CH <sub>2</sub>	
1	48.7, CH <sub>2</sub>	48.7, CH <sub>2</sub>		48.6, CH <sub>2</sub>	48.5, CH <sub>2</sub>	48.3, CH <sub>2</sub>		47.7, CH <sub>2</sub>
2	68.6, CH	68.7, CH	69.4, CH	68.4, CH	68.5, CH	68.1, CH	69.4, CH	68.9, CH
3	77.8, CH	77.8, CH	78.0, CH	85.4, CH	85.4, CH	85.2, CH	77.9, CH	78.2, CH
4	43.8, C	43.7, C	44.0, C	44.1, C	44.2, C	43.9, C	44.2, C	43.7, C
5	47.6, CH	47.7, CH	48.2, CH	56.1, CH	56.4, CH	56.1, CH	48.0, CH	48.2, CH
6	18.0, CH <sub>2</sub>	17.6, CH <sub>2</sub>	18.9, CH <sub>2</sub>	18.5, CH <sub>2</sub>	18.5, CH	18.4, CH <sub>2</sub>	18.3, CH <sub>2</sub>	18.5, CH <sub>2</sub>
7	33.0, CH <sub>2</sub>	33.2, CH <sub>2</sub>	33.2, CH <sub>2</sub>	33.8, CH <sub>2</sub>	33.8, CH	34.6, CH <sub>2</sub>	30.4, CH <sub>2</sub>	32.9, CH <sub>2</sub>
8	45.5, C	45.3, C	40.4, C	45.3, C	44.6, C	43.8, C	43.1, C	39.8, C
9	62.7, CH	62.2, CH	48.8, CH	62.1, CH	62.6, CH	61.1, CH	48.6, CH	48.0, CH
10	38.6, C	38.6, C	38.8, C	38.5, C	38.8, C	38.2, C	39.0, C	38.6, C
11	199.8, C	199.9, C	24.6, CH <sub>2</sub>	199.6, C	200.3, C	199.7, C	24.0, CH <sub>2</sub>	24.0, CH <sub>2</sub>
12	131.8, CH	131.5, CH	124.5, CH	131, CH	129.3, CH	123.0, CH	123.3, CH	122.5, CH
13	162.3, C	163.0, C	144.0, C	163.4, C	171.8, C	163.8, C	145.2, C	145.1, C
14	44.2, C	44.3, C	42.5, C	44.3, C	44.5, C	45.1, C	40.5, C	42.2, C
15	28.4, CH <sub>2</sub>	28.6, CH <sub>2</sub>	29.2, CH <sub>2</sub>	28.5, CH <sub>2</sub>	28.3, CH <sub>2</sub>	27.6, CH <sub>2</sub>	28.8, CH <sub>2</sub>	28.4, CH <sub>2</sub>
16	24.2, CH <sub>2</sub>	25.3, CH <sub>2</sub>	28.3, CH <sub>2</sub>	33.8, CH <sub>2</sub>	33.6, CH <sub>2</sub>	33.1, CH <sub>2</sub>	33.2, CH <sub>2</sub>	23.8, CH <sub>2</sub>
17	46.7, C	45.3, C	n.o	45.2, C	46.1, C	49.0, C	n.o	47.2, C
18	49.7, CH	50.2, CH	40.6, CH	50.1, CH	49.3, CH	136.9, C	41.5, CH	41.4, CH
19	74.7, CH	75.1, CH	76.4, CH	75.0, CH	80.5, CH	138.9, CH	42.1, CH <sub>2</sub>	41.2, CH
20	36.3, C	36.5, C	39.2, C	36.5, C	35.8, CH	n.o	36.5, C	36.6, C
21	34.5, CH <sub>2</sub>	34.9, CH <sub>2</sub>	85.4, CH	34.8, CH <sub>2</sub>	29.0, CH <sub>2</sub>	34.1, CH <sub>2</sub>	74.4, CH	29.2, CH <sub>2</sub>
22	31.4, CH <sub>2</sub>	32.2, CH <sub>2</sub>	25.9, CH <sub>2</sub>	31.2, CH <sub>2</sub>	31.4, CH <sub>2</sub>	33.1, CH <sub>2</sub>	29.4, CH <sub>2</sub>	32.7, CH <sub>2</sub>

23	66.0, CH <sub>2</sub>	66.0, CH <sub>2</sub>	66.0, CH <sub>2</sub>	24.1, CH <sub>3</sub>	24.2, CH <sub>3</sub>	23.9, CH <sub>3</sub>	66.2, CH <sub>2</sub>	66.5, CH <sub>2</sub>
24	14.5, CH <sub>3</sub>	14.5, CH <sub>3</sub>	13.5, CH <sub>3</sub>	65.5, CH <sub>2</sub>	65.6, CH <sub>2</sub>	65.4, CH <sub>2</sub>	13.8, CH <sub>3</sub>	14.4, CH <sub>3</sub>
25	18.5, CH <sub>3</sub>	18.4, CH <sub>3</sub>	17.2, CH <sub>3</sub>	18.3, CH <sub>3</sub>	18.4, CH <sub>3</sub>	18.6, CH <sub>3</sub>	17.5, CH <sub>3</sub>	17.6, CH <sub>3</sub>
26	19.6, CH <sub>3</sub>	19.7, CH <sub>3</sub>	17.6, CH <sub>3</sub>	19.4, CH <sub>3</sub>	19.4, CH <sub>3</sub>	18.6, CH <sub>3</sub>	17.9, CH <sub>3</sub>	17.4, CH <sub>3</sub>
27	21.7, CH <sub>3</sub>	66.0 CH <sub>2</sub>	24.1, CH <sub>3</sub>	21.8, CH <sub>3</sub>	22.8, CH <sub>3</sub>	19.9, CH <sub>3</sub>	26.5, CH <sub>3</sub>	26.2, CH <sub>3</sub>
28	175.6, C	175.3,C	n.o	179.2, C	179.1, C	n.o	n.o	180.0, C
29	30.3, CH <sub>3</sub>	30.5, CH <sub>3</sub>	23.4, CH <sub>3</sub>	30.5, CH <sub>3</sub>	28.5, CH <sub>3</sub>	30.0 , CH <sub>3</sub>	28.4, CH <sub>3</sub>	19.8, CH <sub>3</sub>
30	17.8, CH <sub>3</sub>	17.9, CH <sub>3</sub>	18.3, CH <sub>3</sub>	17.9, CH <sub>3</sub>	24.5, CH <sub>3</sub>	27.9, CH <sub>3</sub>	19.5, CH <sub>3</sub>	73.9, CH <sub>2</sub>
1'	96.1, CH							
2'	74.2, CH							
3'	78.8, CH							
4'	71.1, CH							
5'	79.5, CH							
6'	62.2 CH <sub>2</sub>							

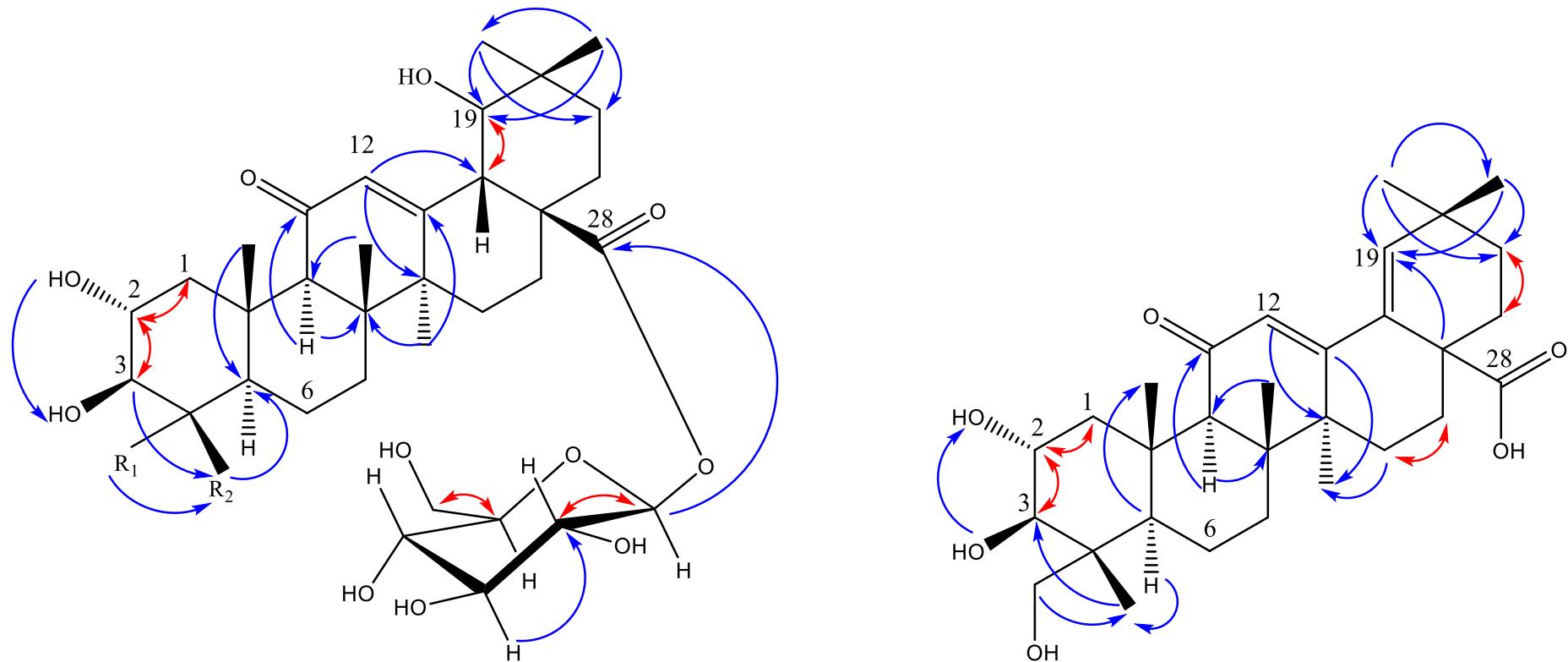
<sup>a</sup> Overlapping signals, <sup>b</sup>pyridine-d<sub>5</sub>, <sup>c</sup>methanol-d<sub>4</sub>, n.o: not observed.



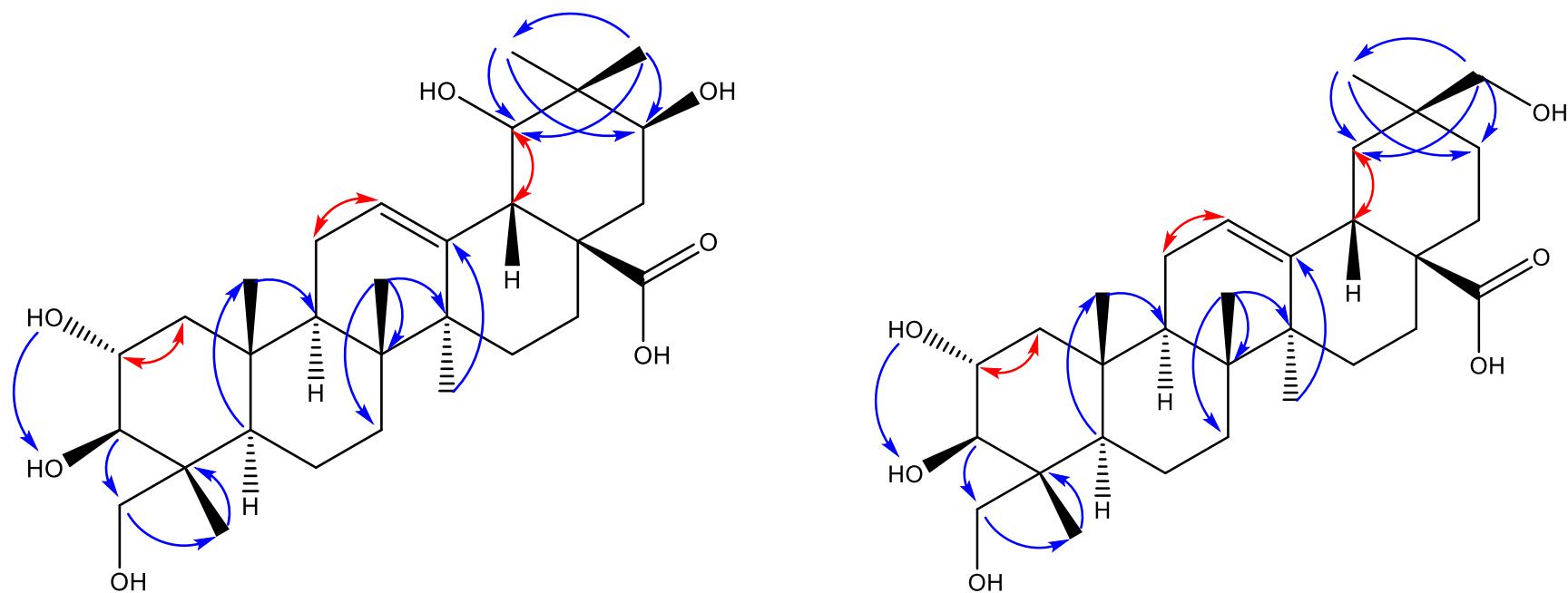
Compounds	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
<b>1</b>	CH <sub>2</sub> OH	CH <sub>3</sub>	α-OH	β-D-glucopyranoside
<b>2</b>	CH <sub>2</sub> OH	CH <sub>3</sub>	β-OH	β-D-glucopyranoside
<b>3</b>	CH <sub>3</sub>	CH <sub>2</sub> OH	β-OH	β-D-glucopyranoside
<b>4</b>	CH <sub>2</sub> OH	CH <sub>3</sub>	β-OH	OH
<b>6</b>	CH <sub>3</sub>	CH <sub>2</sub> OH	β-OH	OH
<b>7</b>	CH <sub>3</sub>	CH <sub>2</sub> OH	α-OH	OH

Compounds	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<b>5</b>	α-OH	β-OH	CH <sub>3</sub>
<b>9</b>	H	α-OH	CH <sub>3</sub>
<b>10</b>	H	H	CH <sub>2</sub> OH

**Figure 5.2.** Chemical structures of compounds **1-10** isolated from he *n*-BuOH extract of *Terminalia albida*

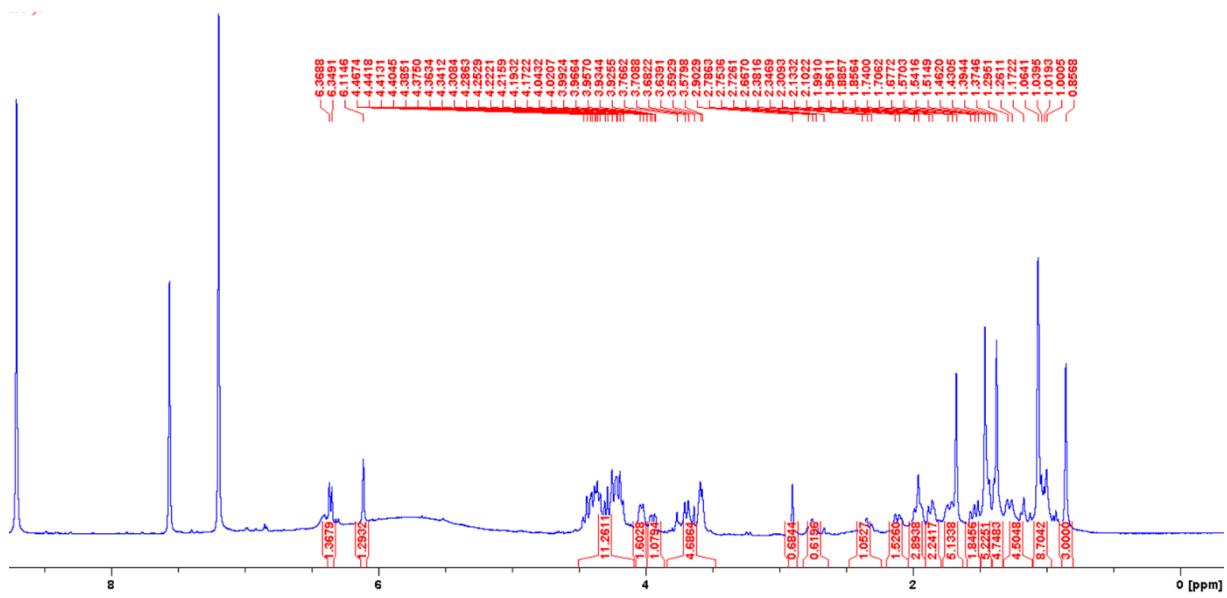


**Figure 5.3.**  $^1\text{H} - ^1\text{H}$  COSY (red arrows), key HMBC (blue arrows) correlation of compound **2** and **8**

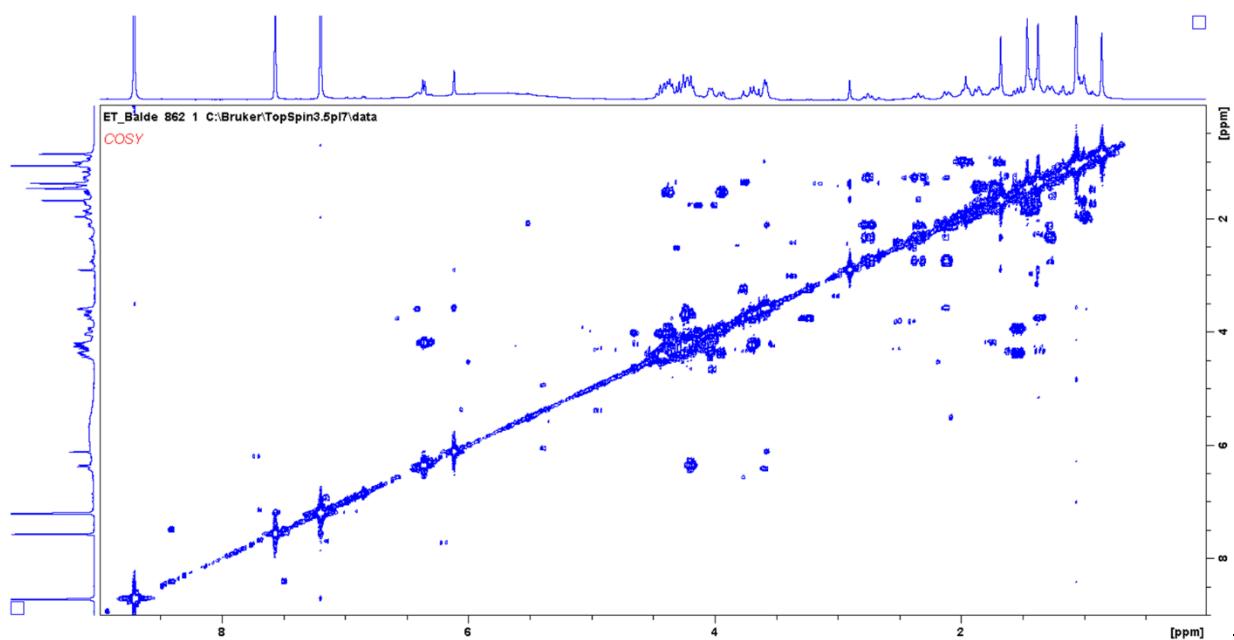


**Figure 5.4.**  $^1\text{H} - ^1\text{H}$  COSY (red arrows), key HMBC (blue arrows) correlation of compound 5 and 10

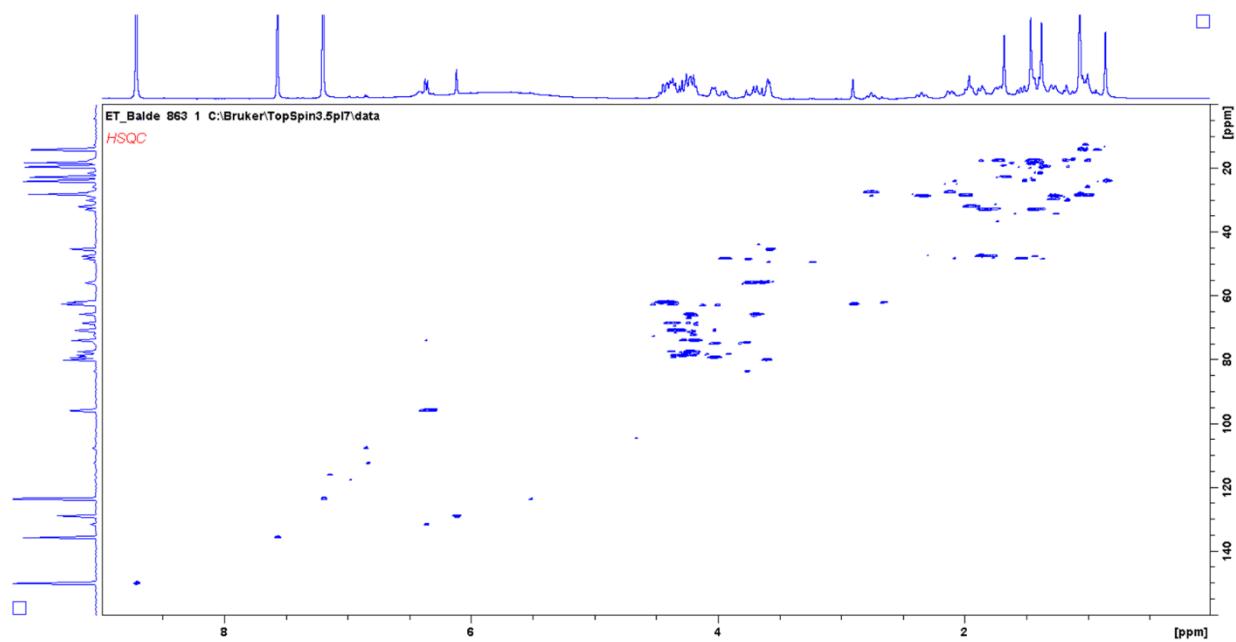
Compound **1** was obtained as a white powder and the molecular formula ( $C_{36}H_{56}O_{12}$ ) was determined by high resolution - electrospray ionization - mass spectrometry (HR-ESIMS), yielding deprotonated ion at  $m/z$  679.3710 [M-H] $^-$ . The  $^1H$ -NMR spectrum recorded in pyridine- $d_5$  showed six methyl singlets at  $\delta_H$  1.67, 1.46, 1.37, 1.06, 1.06 and 0.85, which were attributed to methyl groups at C-27, C-25, C-26, C-24, C-29 and C-30, respectively (Table 5.2). Additional proton resonances observed included those attributed to an olefinic proton at  $\delta_H$  6.11 (1H, br s), three oxymethine protons at  $\delta_H$  4.38 (1H, m), 4.23 (1H, m) and 3.59 (1H, d,  $J = 5.2$  Hz), two oxymethylene protons at  $\delta_H$  4.22 (1H, m) and 3.69 (1H, d,  $J = 10.9$  Hz) (H-23a and H-23b, respectively), which showed HMBC correlations with C-3 ( $\delta_C$  77.5) and C-24 ( $\delta_C$  14.6). In addition, two oxymethylene protons at  $\delta_H$  4.40 (1H, m) and 4.44 (1H, m) were attributed to C-6' of the sugar moiety. An anomeric proton signal at  $\delta_H$  6.35 (1H, d,  $J = 8.0$  Hz) correlated in the HSQC spectrum with an anomeric carbon at  $\delta_C$  95.8. The  $^{13}C$ -NMR spectrum displayed 36 carbon signals, among which six were attributed to a glucopyranosyl moiety and the remaining 30 to the triterpenoid skeleton. Signals observed at  $\delta$  200.2 (C-11), 128.9 (C-12) and 170.6 (C-13) were indicative of the presence of an  $\alpha,\beta$ -unsaturated carbonyl functionality in the C-ring, which was supported by the HMBC correlations observed between H-12, C-13, C-14, and C-18 and also between H-9 and C-11, C-13 and C-14. The C-19 assignment was also confirmed by its HMBC correlations with H-29, H-30 and its COSY correlation with H-18. The linkage of the sugar to C-28 was confirmed by the HMBC correlation between the anomeric proton H-1' ( $\delta$  6.35,  $J = 8.0$  Hz), indicating a  $\beta$ -configuration, and the signal at  $\delta$  176.6 (C-28). The structure of compound **1** was established as  $2\alpha,3\beta,19\alpha,23$ -tetrahydroxy-11-oxo-olean-12-en-28-oic acid 28-O- $\beta$ -D-glucopyranosyl ester, previously isolated from *Pteleopsis suberosa*<sup>16</sup>. The NMR spectrum of albidanoside A are displayed in Figures 5.4, 5.5, 5.6 and 5.7.



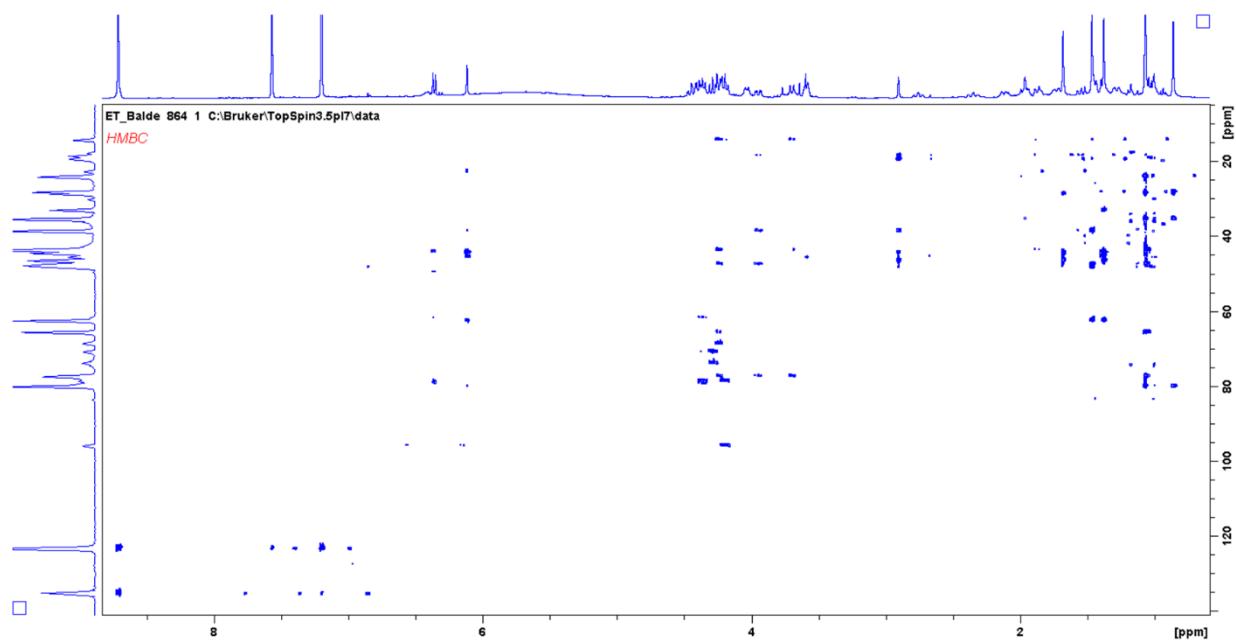
**Figure 5.4.**  $^1\text{H}$  NMR spectrum (pyridine-*d*<sub>5</sub>, 400 MHz) of compound (1)



**Figure 5.5.** COSY spectrum (pyridine-*d*<sub>5</sub>) of compound (1)



**Figure 5.6.** HSQC spectrum of (pyridine-*d*<sub>5</sub>) of compound (**1**)



**Figure 5.7.** HMBC spectrum of (pyridine-*d*<sub>5</sub>) of compound (**1**)

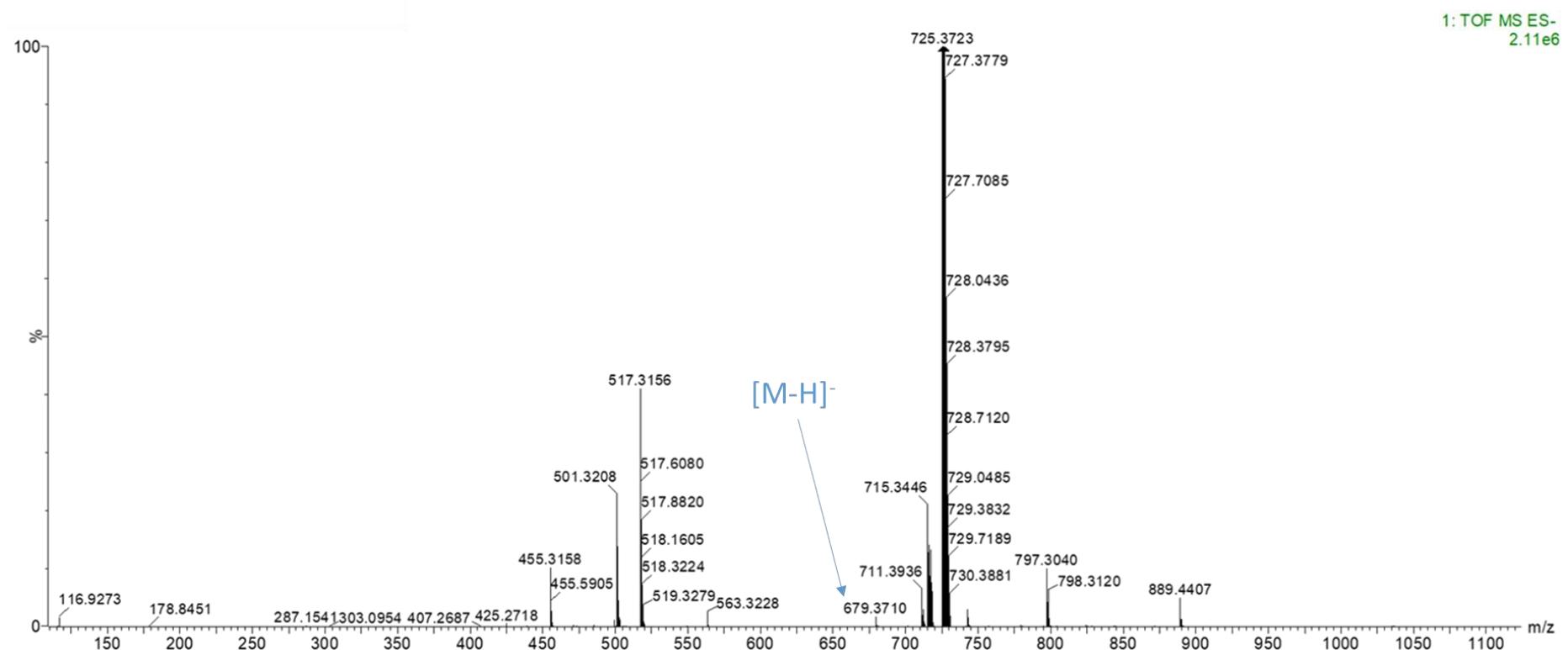
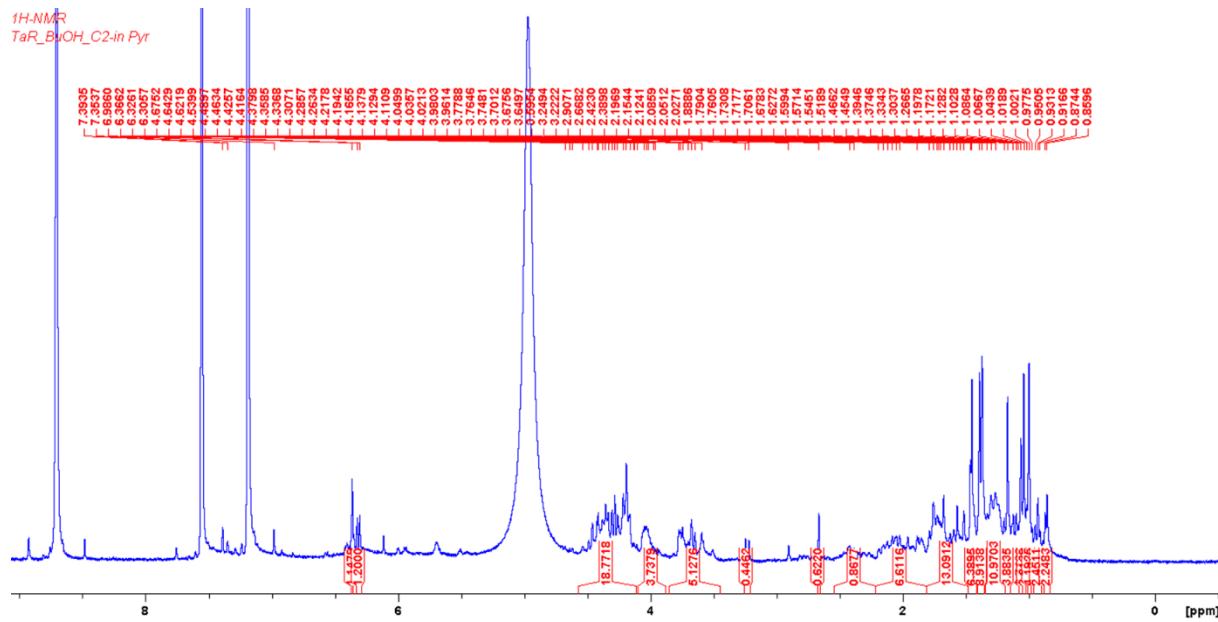


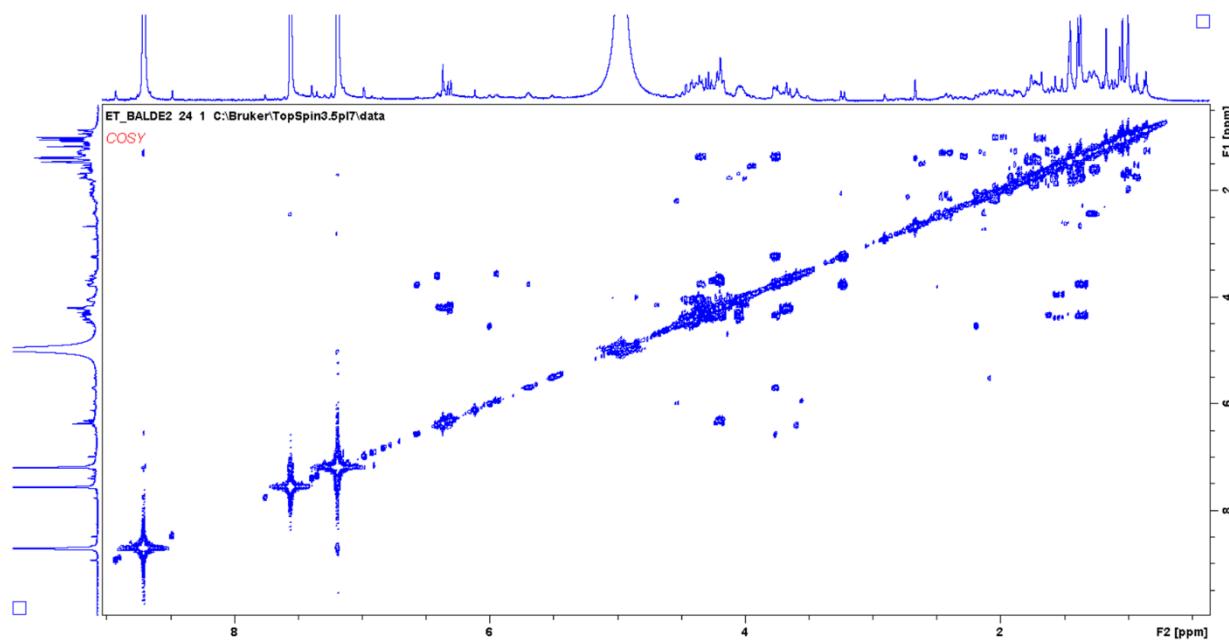
Figure 5.8. HR-ESI-MS spectrum of compound (1)

The HR-ESI-MS spectrum of compound **2** (molecular formula C<sub>36</sub>H<sub>56</sub>O<sub>12</sub>) showed a molecular ion at *m/z* 725.3723 corresponding to the formic acid adduct [M+HCOOH]<sup>-</sup> and a fragment at *m/z* 517.3159 resulting from the loss of a hexose unit [M+HCOOH-162]<sup>-</sup>, suggesting that compound **2** was an isomer of **1**. The <sup>1</sup>H and <sup>13</sup>C-NMR spectra (Table 5.2 and 5.3) in pyridine-*d*<sub>5</sub> were similar to those of compound **1**. The <sup>1</sup>H-NMR spectrum of compound **2** showed six methyl singlets at  $\delta_{\text{H}}$  1.00, 1.04, 1.17, 1.37, 1.39 and 1.45. An olefinic proton at  $\delta_{\text{H}}$  6.36 (1H, br s), three oxymethine protons at  $\delta_{\text{H}}$  4.34 (1H, m), 4.18 (1H, m) and 3.76 (1H, m), two oxymethylene protons at  $\delta_{\text{H}}$  3.69 (1H, m) and 4.25 (1H, m) linked to C-23, which showed HMBC correlations with C-3 ( $\delta_{\text{C}}$  77.8 ppm) and C-24 ( $\delta_{\text{C}}$  14.5 ppm). In addition, two oxymethylene protons at  $\delta_{\text{H}}$  4.42 (1H, m) and  $\delta_{\text{H}}$  4.46 (1H, m) were attributed to C-6' of the glucosyl moiety. An anomeric proton signal at  $\delta_{\text{H}}$  6.32 (1H d, *J* = 8.3 Hz), indicating a  $\beta$ -configuration, correlated in the HSQC spectrum with an anomeric carbon at  $\delta_{\text{C}}$  96.1. The <sup>13</sup>C-NMR spectrum displayed 36 carbon signals, among which six were attributed to a glucopyranosyl moiety and the remaining 30 to the triterpenoid skeleton. Signals observed at  $\delta_{\text{C}}$  199.8 (C-11), 131.8 (C-12) and 162.3 (C-13) were indicative of the presence of an  $\alpha,\beta$ -unsaturated carbonyl functionality in the C-ring, which was supported by the HMBC correlations observed between H-12 and C-9, C-13, C-14 and C-18, and also between H-9 and C-11<sup>15,16</sup>. The C-19 assignment was also confirmed by its HMBC correlations with H-29, H-30 and its COSY correlation with H-18 (figure 5.3). The linkage of the sugar moiety to C-28 was confirmed by the HMBC correlation between the anomeric proton H-1' ( $\delta_{\text{H}}$  6.32, *J* = 8.3 Hz), indicating a  $\beta$ -configuration, and the signal at  $\delta_{\text{C}}$  175.6 (C-28). Comparison of the <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of this compound with those of **1** revealed some differences in the C- and E-rings of their aglycones moieties. Indeed, the doublet at  $\delta_{\text{H}}$  3.23 (H-18, *J* = 10.8 Hz) in **2** was shifted compared to  $\delta_{\text{H}}$  3.57 (*J* = 5.2 Hz) in **1**, while the resonance of H-19 was observed at  $\delta$  3.76, compared to  $\delta_{\text{H}}$  3.59 (*J* = 5.2 Hz) for **1**. Furthermore, the difference between compounds **2** and **1** could be explained in terms of configurational dissimilarity at C-19. Indeed, the chemical shift of C-19 ( $\delta$  74.7) in compound **2** displayed a difference of -5.3 ppm to that of compound **1** ( $\delta_{\text{C}}$  80.0). Moreover, the chemical shift values of C-12, C-13 and the methyl groups C-29 and C-30 were different both in the <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of compound **2** in comparison with those of **1**. The coupling constant of H-18 (d, *J* = 10.8 Hz) and H-19 confirmed the  $\beta$ -orientation of the hydroxy group at C-19 of compound **2**. Therefore, compound **2** (albidanoside A) could be identified as a new epimer of **1**, i.e. 2 $\alpha$ ,3 $\beta$ ,19 $\beta$ ,23-tetrahydroxy-

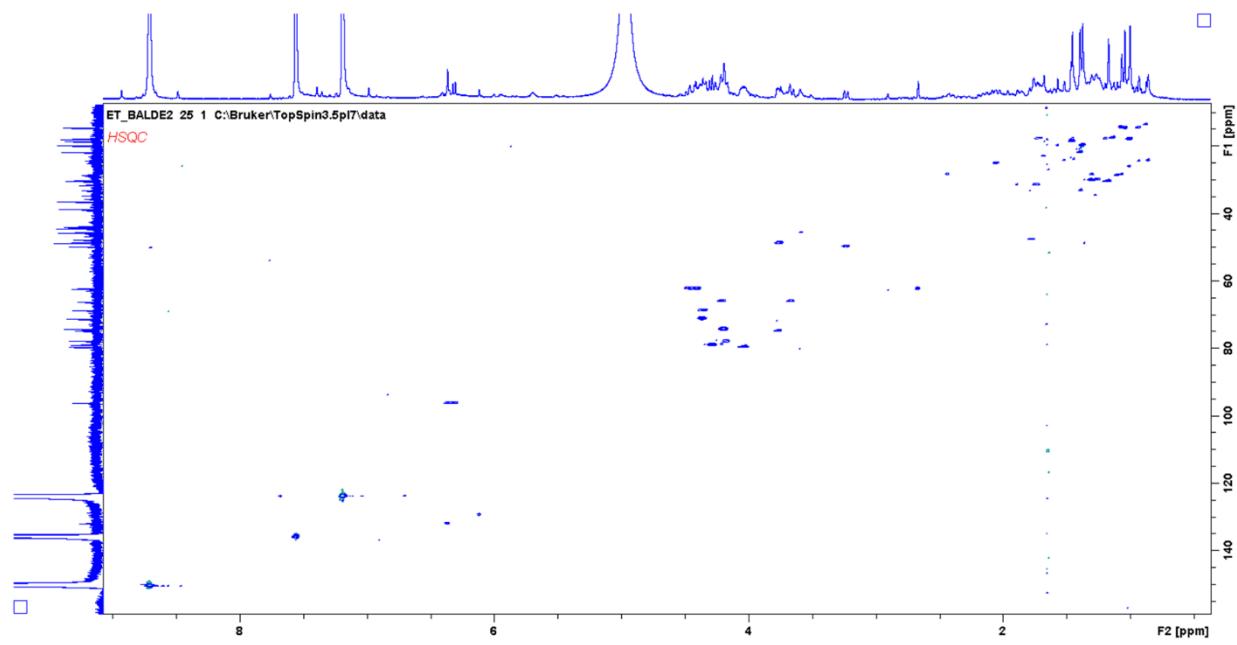
11-oxo-olean-12-en-28-oic acid 28-*O*- $\beta$ -D-glucopyranosyl ester. The NMR spectra of albidanoside A are displayed in Figures 5.9, 5.10, 5.11 and 5.12



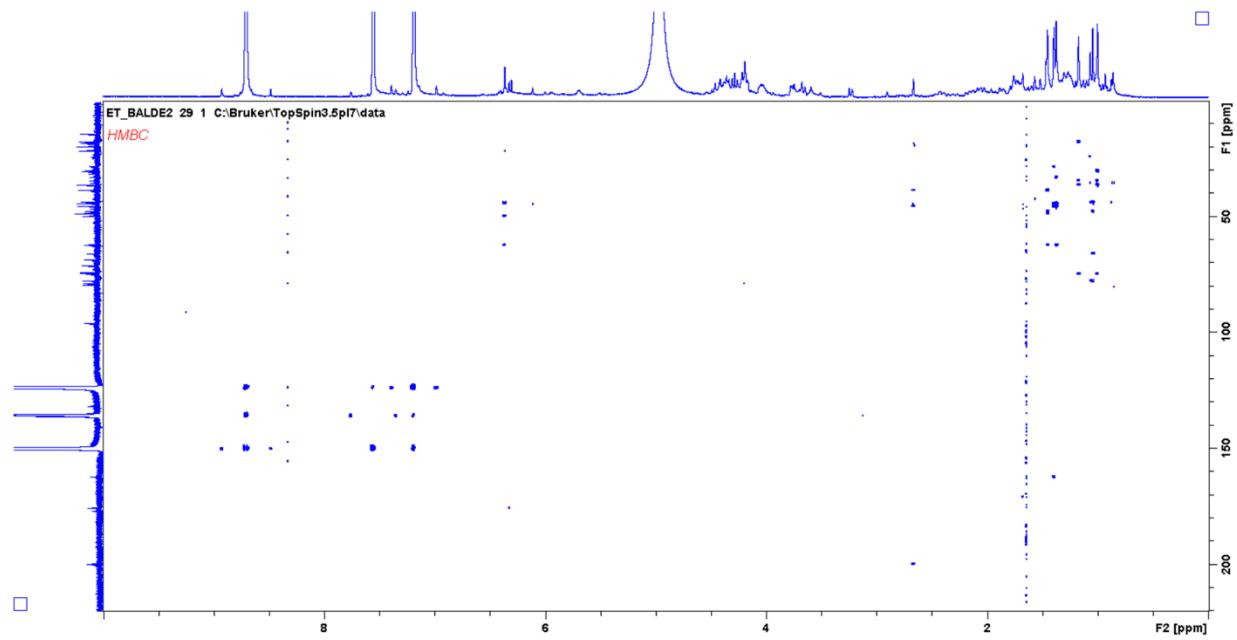
**Figure 5.9.**  $^1\text{H}$  NMR spectrum (pyridine- $d_5$ , 400 MHz) of compound (2)



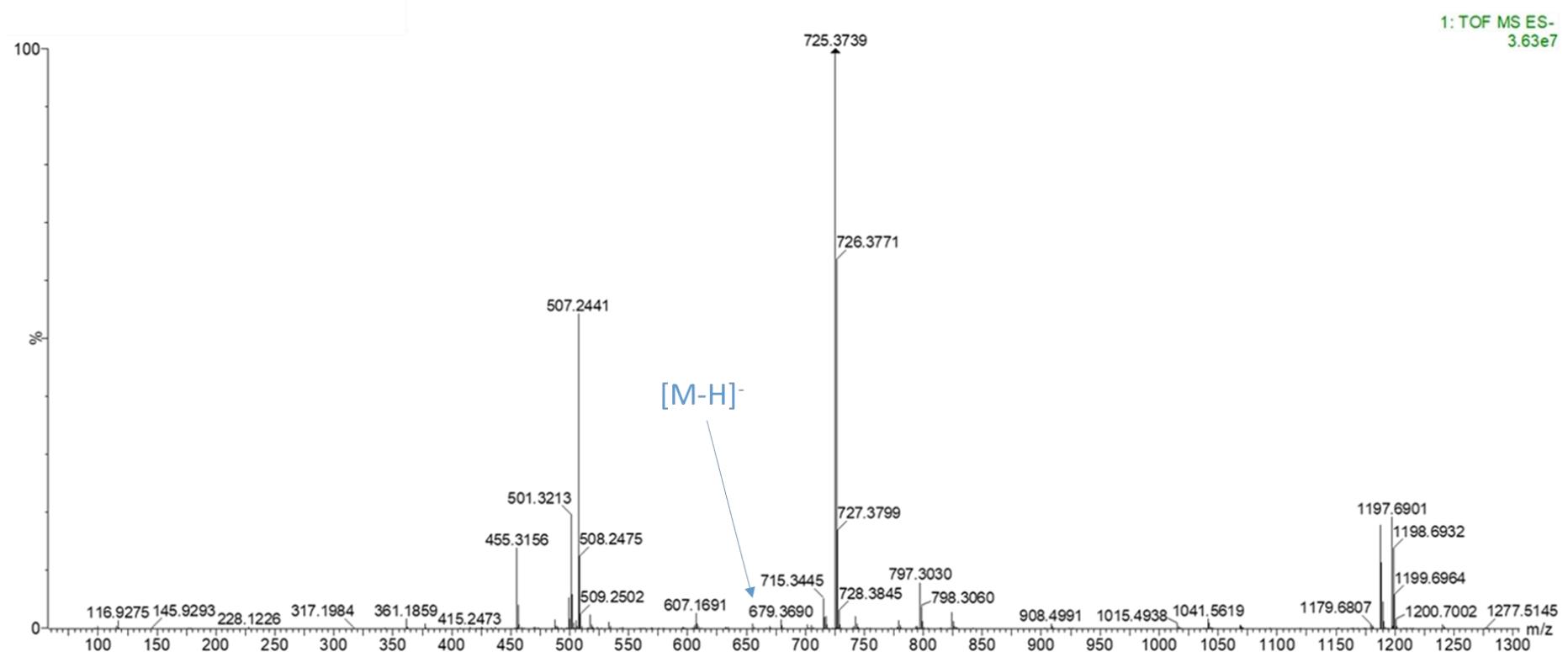
**Figure 5.10.** COSY spectrum (pyridine-*d*<sub>5</sub>) of compound (2)



**Figure 5.11.** HSQC spectrum (pyridine-*d*<sub>5</sub>) of compound (2)

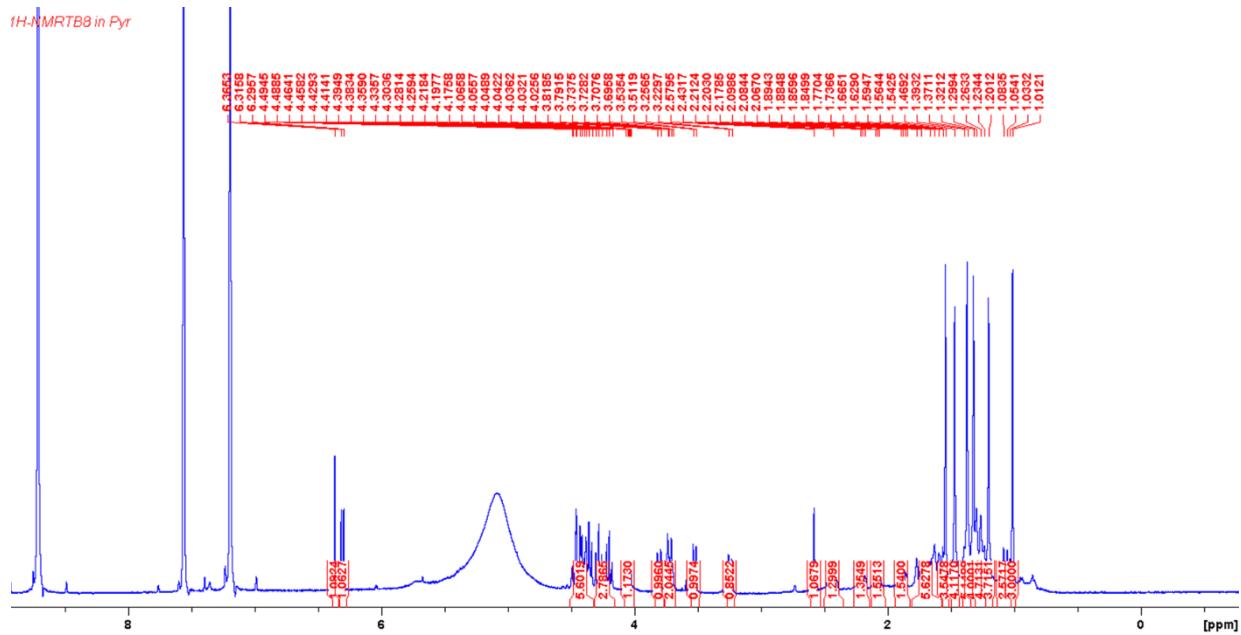


**Figure 5.12.** HMBC spectrum of (pyridine-*d*<sub>5</sub>) of compound (2)

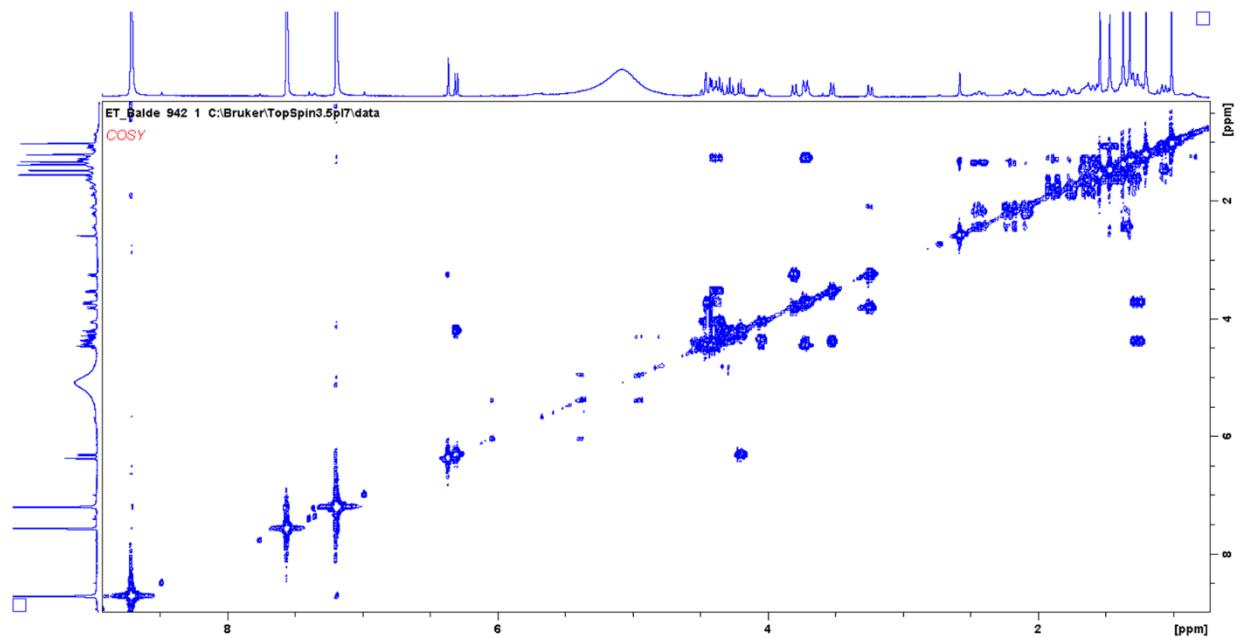


**Figure 5.13.** HR-ESI-MS spectrum of compound (2)

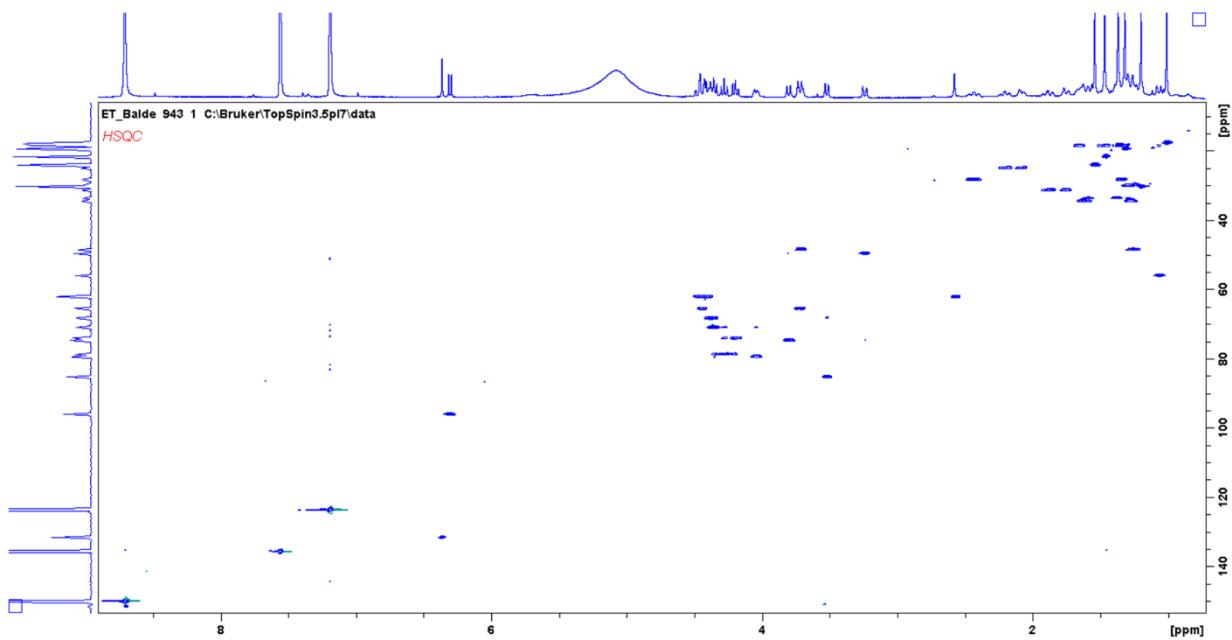
The molecular formula of compound **3** was deduced as C<sub>36</sub>H<sub>56</sub>O<sub>12</sub> based on the HR-ESIMS ion (*m/z* 679.3724 [M-H]<sup>-</sup>). The <sup>1</sup>H and <sup>13</sup>C-NMR spectra of **3** were highly similar to those of **2**, except for the assignments of the methyl- and hydroxymethyl substituents at C-4. Indeed, the chemical shift of the methyl group ( $\delta_c$  14.5 for C-24 in compound **2**,  $\beta$ -orientation) was shifted to  $\delta_c$  23.8, assigned to the C-23 methyl in compound **3** ( $\alpha$ -orientation). Moreover, the chemical shift values of carbons C-3 ( $\delta_c$  85.1) and C-5 ( $\delta_c$  55.9) of the A-ring in compound **3** were deshielded compared to  $\delta$  77.8 and 47.6, respectively, in compound **2**. Identically to compound **2**, the <sup>13</sup>C-NMR spectrum of **3** displayed 36 carbon signals, among which 6 were attributed to the sugar moiety and the remaining 30 to the triterpenoid aglycone. Signals observed at  $\delta$  199.4 (C-11), 131.4 (C-12) and 162.2 (C-13) were indicative of the presence of an  $\alpha,\beta$ -unsaturated carbonyl functionality in the C-ring which was supported by the HMBC correlations observed between H-12 and C-14, C-13 and C-18, and also between H-9 and C-11. The structure of **3** was established as  $\beta$ -D-glucopyranosyl-2 $\alpha$ ,3 $\beta$ ,19 $\beta$ ,24-tetrahydroxy-11-oxo-olean-12-en-28-oic acid (ivorenoside C), previously isolated from *Terminalia ivorensis*<sup>16</sup>.



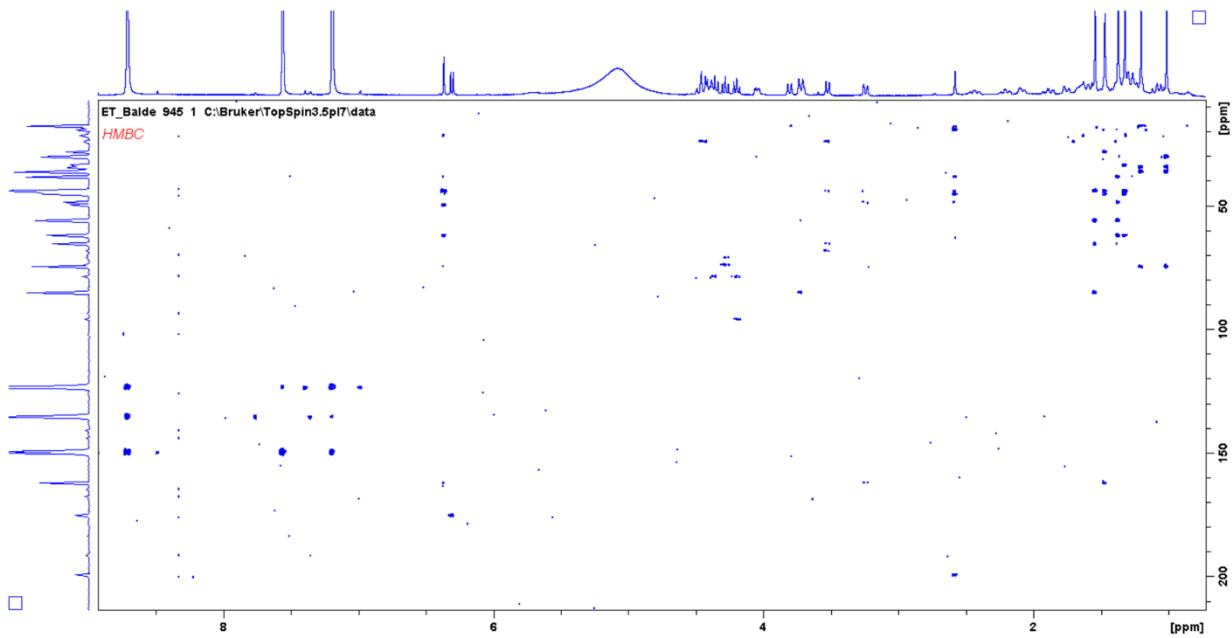
**Figure 5.14.**  $^1\text{H}$  NMR spectrum (pyridine-*d*5, 400 MHz) of compound (3)



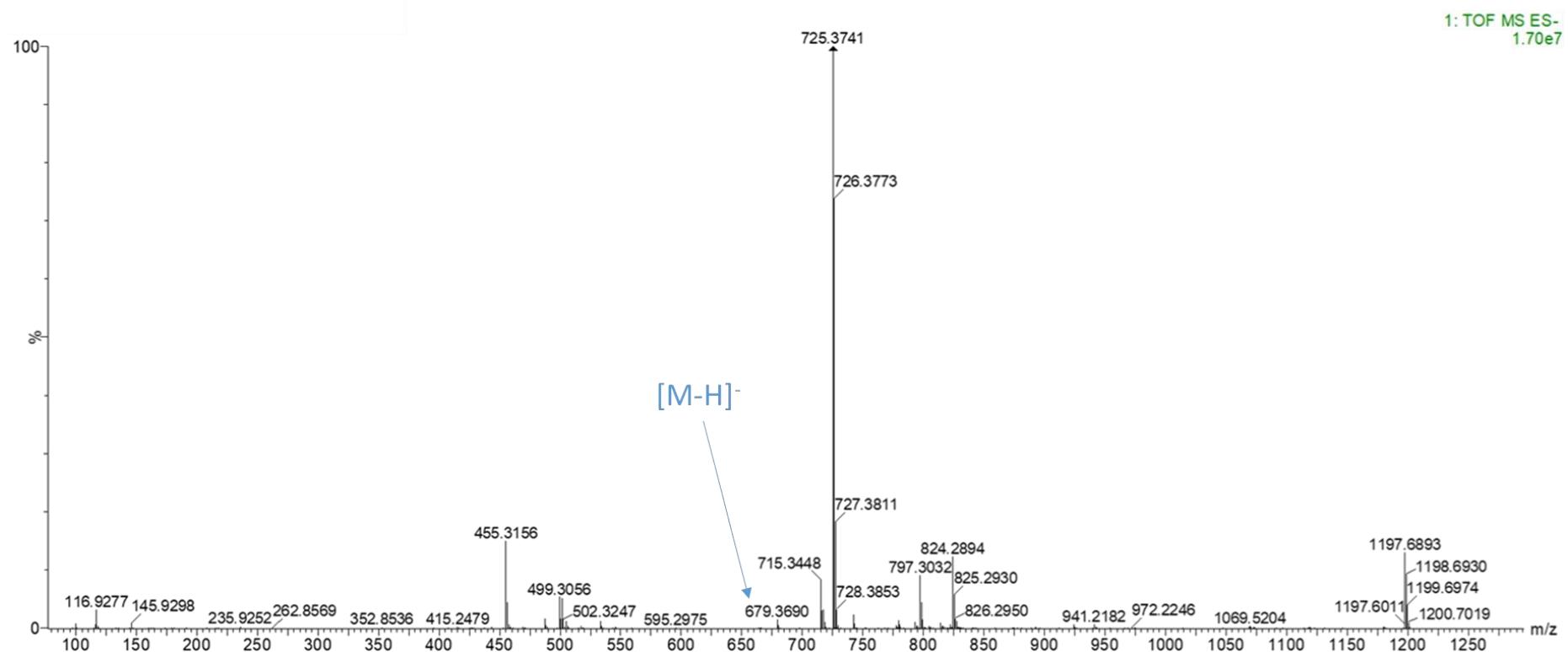
**Figure 5.15.** COSY spectrum (pyridine-*d*<sub>5</sub>) of compound (3)



**Figure 5.16.** HSQC spectrum of (pyridine- $d_5$ ) of compound (3)

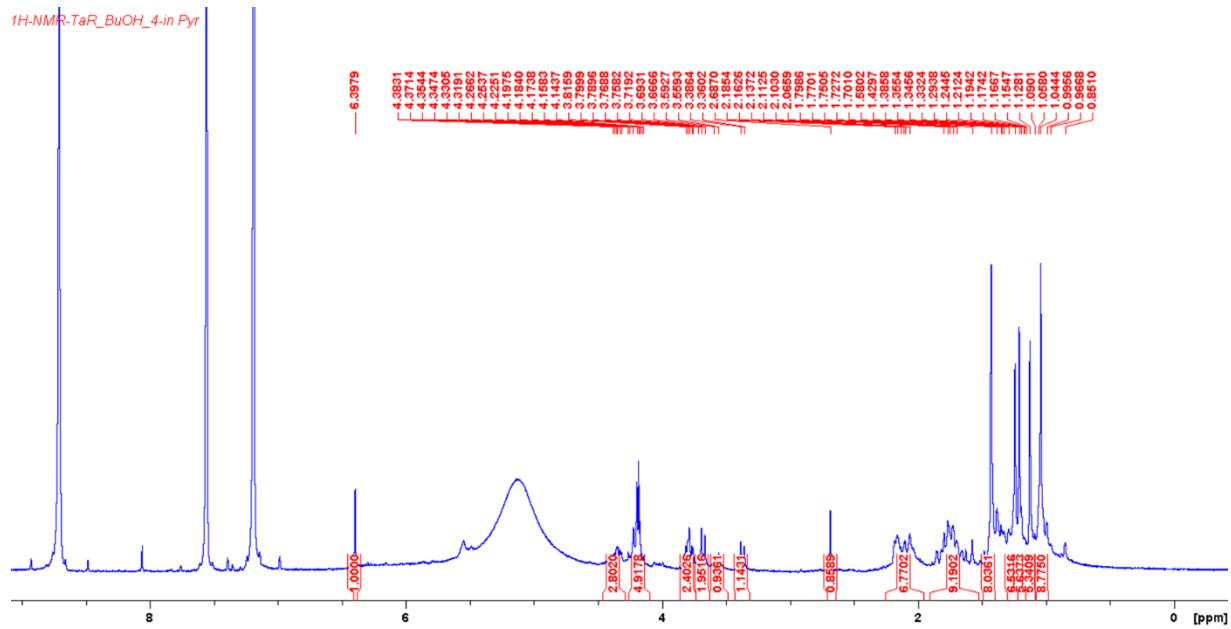


**Figure 5.17.** HMBC spectrum of (pyridine- $d_5$ ) of compound (3)

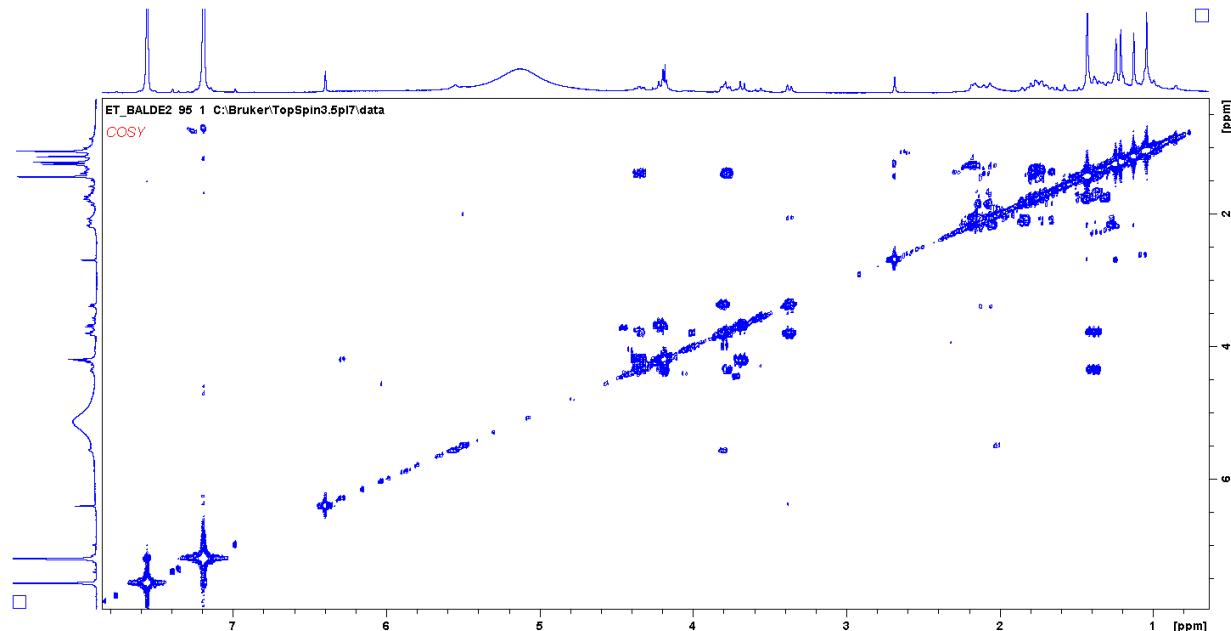


**Figure 5.18.** HR-ESI-MS spectrum of compound (3)

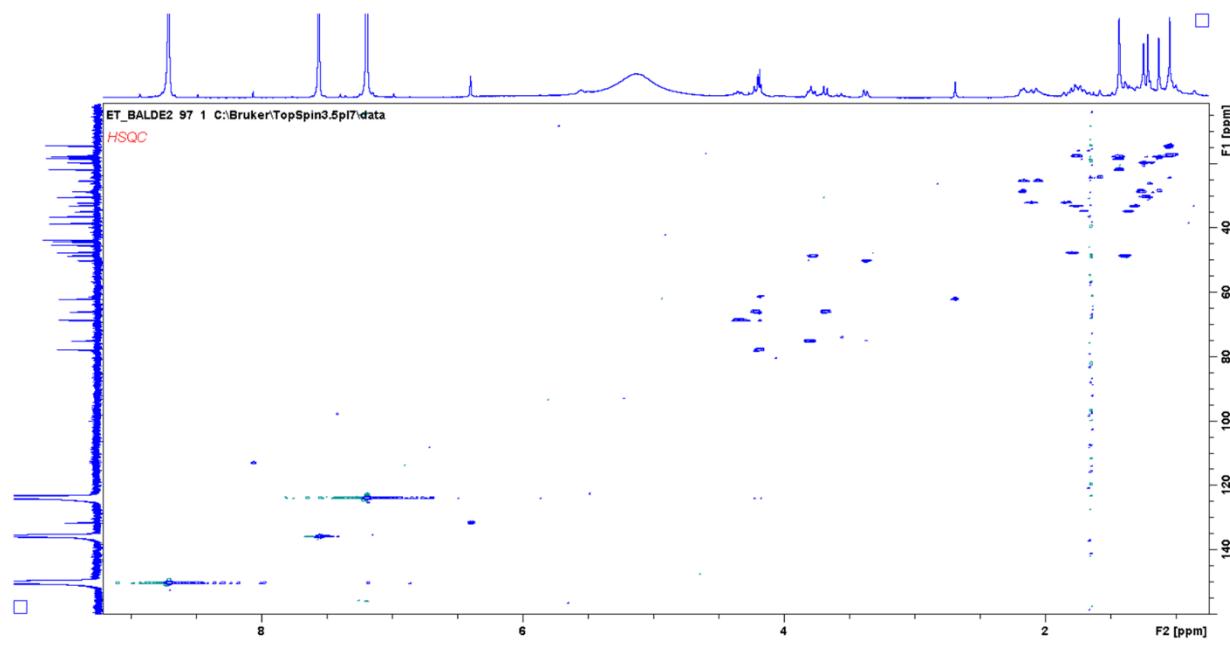
Compound **4** displayed a molecular ion at  $m/z$  517.3154 [M-H] $^-$  in its HR-ESIMS, revealing a difference of 162 mass units in comparison with the above mentioned compounds, which displayed a deprotonated ion at  $m/z$  679.3724 [M-H] $^-$  indicating that compound **4** is an aglycone. The  $^1\text{H}$ -NMR data (Table 5.2) showed six methyl singlets ( $\delta_{\text{H}}$  1.04, 1.12, 1.21, 1.24, 1.43 and 1.43 x 2), one trisubstituted olefinic proton at  $\delta_{\text{H}}$  6.39 (1H, br s), three hydroxymethylene protons at  $\delta_{\text{H}}$  4.35 (1H, m), 4.18 (1H, m) and 3.81 (1H, d,  $J$  = 10.6 Hz) and two oxymethylene protons at  $\delta_{\text{H}}$  3.68 (1H, d,  $J$  = 10.6 Hz) and 4.21 (1H, d,  $J$  = 10.6 Hz) linked to C-23, which showed HMBC correlations with C-3 ( $\delta$  77.8) and C-24 ( $\delta$  14.5). The  $^{13}\text{C}$ -NMR data (Figure 5.2) displayed 30 carbon signals which were assigned to a triterpenoid moiety. Signals observed at  $\delta_{\text{C}}$  199.9 ppm (C-11),  $\delta_{\text{C}}$  131.5 ppm (C-12) and  $\delta_{\text{C}}$  163.0 ppm (C-13) were indicative of the presence of an  $\alpha,\beta$ -unsaturated carbonyl functionality in the C-ring, which was supported by HMBC correlations observed between H-12 and C-14, C-18, between H-27 and C-14, and also between H-9 and C-11. The C-19 assignment has been confirmed by its HMBC correlations with H-29 and H-30 and its COSY correlation with H-18. Comparison of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of compound **4** with those of the above mentioned compounds indicated that albidic acid A (**4**) is the aglycone of compound **2** and identified as  $2\alpha,3\beta,19\beta,23$ -tetrahydoxy-11-oxo-olean-12-en-28-oic acid.



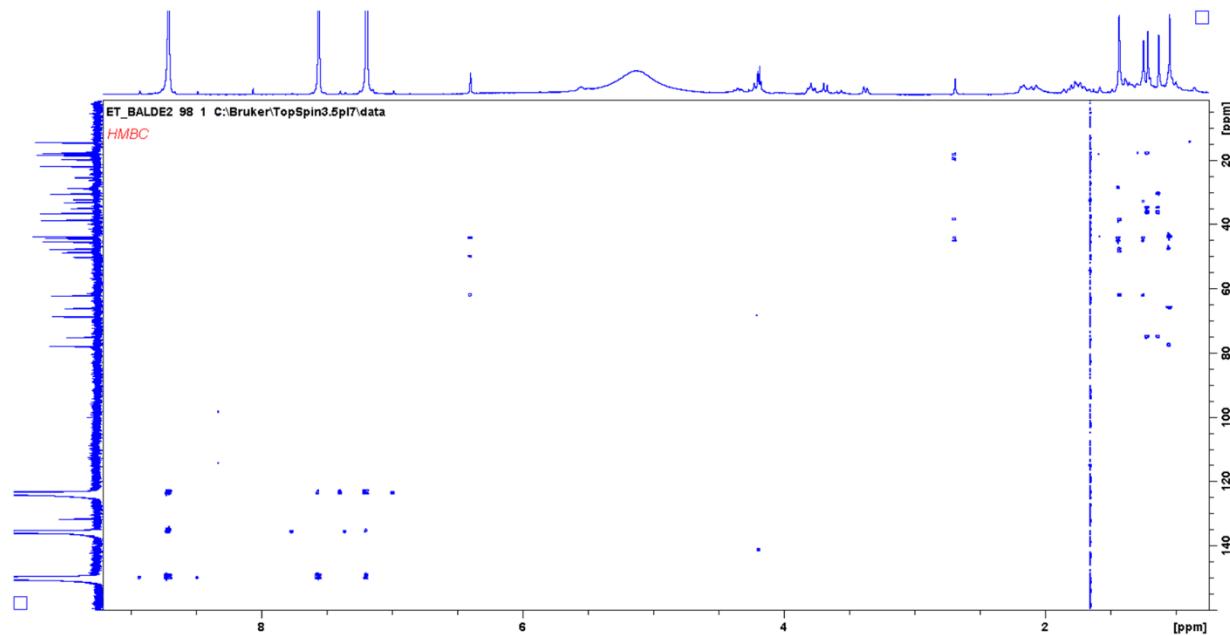
**Figure 5.19.**  $^1\text{H}$  NMR spectrum (pyridine- $d_5$ , 400 MHz) of compound (4)



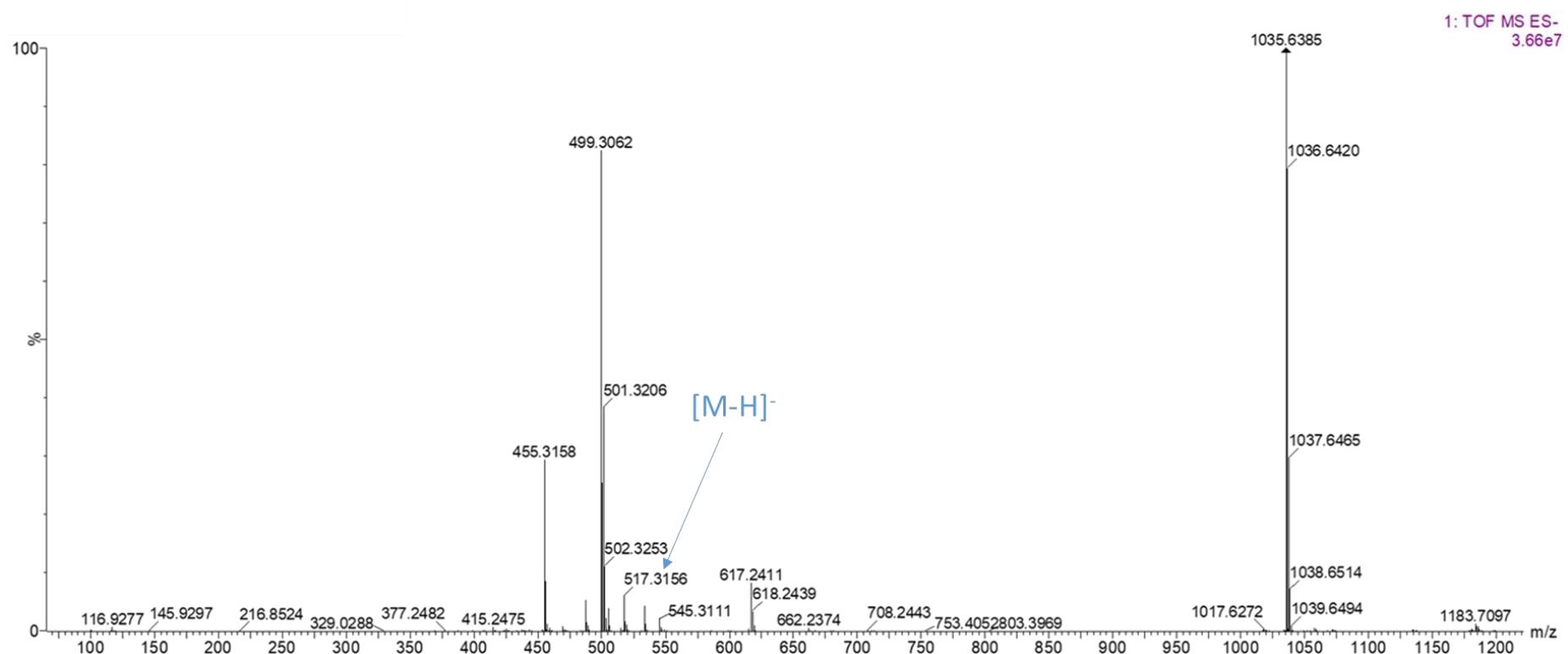
**Figure 5.20.** COSY spectrum (pyridine-*d*5) of compound (4)



**Figure 5.21.** HSQC spectrum of (pyridine- $d_5$ ) of compound (4)

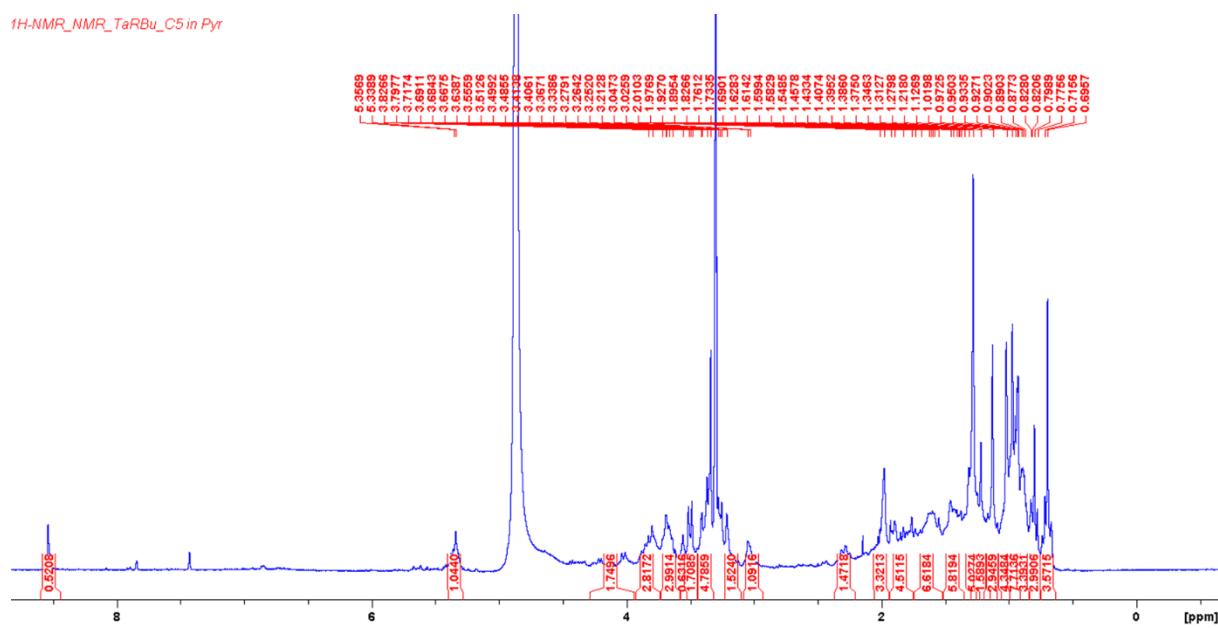


**Figure 5.22.** HMBC spectrum of (pyridine- $d_5$ ) of compound (4)

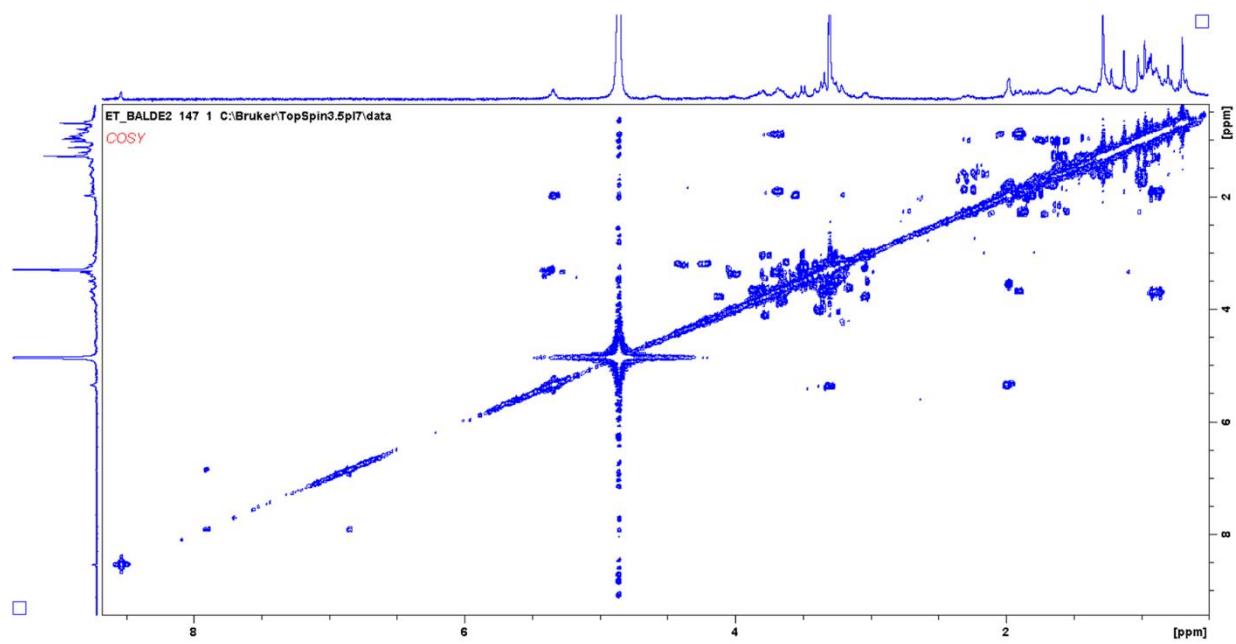


**Figure 5.23.** HR-ESI-MS spectrum of compound (4)

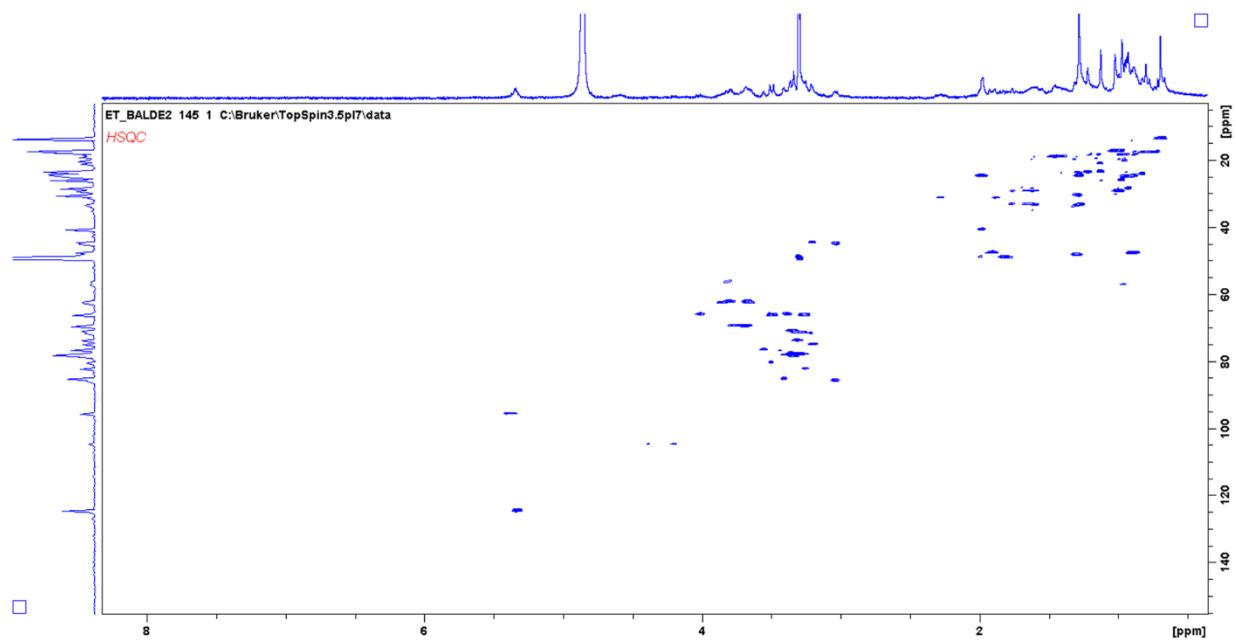
Compound **5** displayed a deprotonated ion at  $m/z$  519.3328 [M-H]<sup>-</sup> in HR-ESI-MS. The <sup>1</sup>H-NMR data (Table 5.2) showed six methyl singlets ( $\delta_H$  0.69, 0.79, 0.97, 1.02, 1.12 and 1.27), one olefinic proton at  $\delta_H$  5.33 (1H, br s), four hydroxymethylene protons at  $\delta_H$  3.68 (1H, m), 3.55 (1H, m), 3.41 (1H, m) and 3.34 (1H, m), two oxymethylene protons at  $\delta_H$  3.49 (1H, d,  $J = 10.8$  Hz) and 3.26 (1H, m) linked to C-23. These oxymethylene protons showed HMBC correlations with C-3 ( $\delta_C$  77.5) and C-24 ( $\delta_C$  14.6), C4 ( $\delta_C$  44.0) and C5 ( $\delta_C$  48.2) (figure 5.4). The <sup>13</sup>C-NMR spectrum of compound **5** displayed 30 carbon resonances, which were assigned to a triterpenoid skeleton (Table 5.3). Examination of the spectrum revealed that compound **5** lacks the  $\alpha,\beta$ -unsaturated carbonyl functionality in the C-ring present in compounds **1 - 4**. The presence of hydroxy groups at C-19 and C-21 of the E-ring was supported by HMBC correlations of H-19 and H-21 with C-20 ( $\delta_C$  39.2), C-29 ( $\delta_C$  19.5) and C-30 ( $\delta_C$  28.4). This was also supported by the COSY correlation observed between H-18 ( $\delta_H$  1.99) and H-19 ( $\delta_H$  3.55) (figure 5.4). Therefore the structure of albidinolic acid (**5**) was defined as  $2\alpha,3\beta,19\alpha,21\beta,23$ -pentahydroxyolean-12-en-28-oic acid.



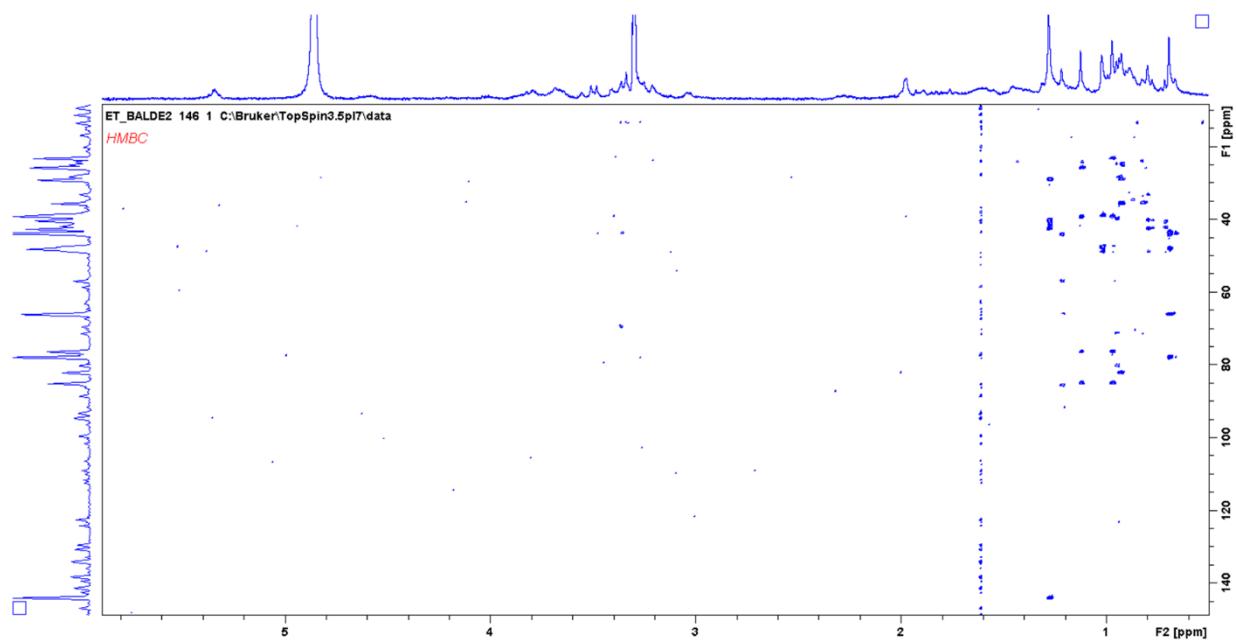
**Figure 5.24.**  $^1\text{H}$  NMR spectrum (methanol- $d_4$ , 400 MHz) of compound (5)



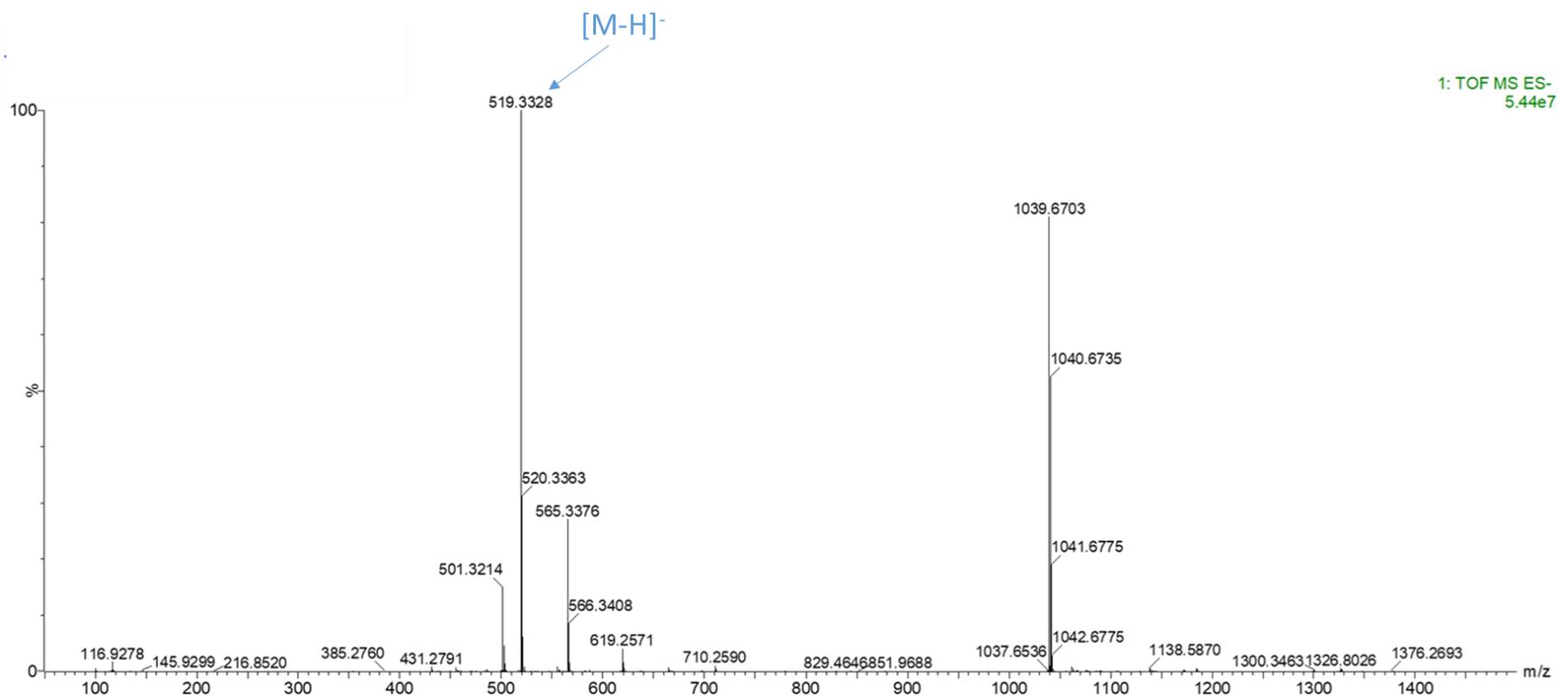
**Figure 5.25.** COSY spectrum (methanol-*d*<sub>4</sub>) of compound (5)



**Figure 5.26.** HSQC spectrum (methanol-*d*<sub>4</sub>) of compound (5)

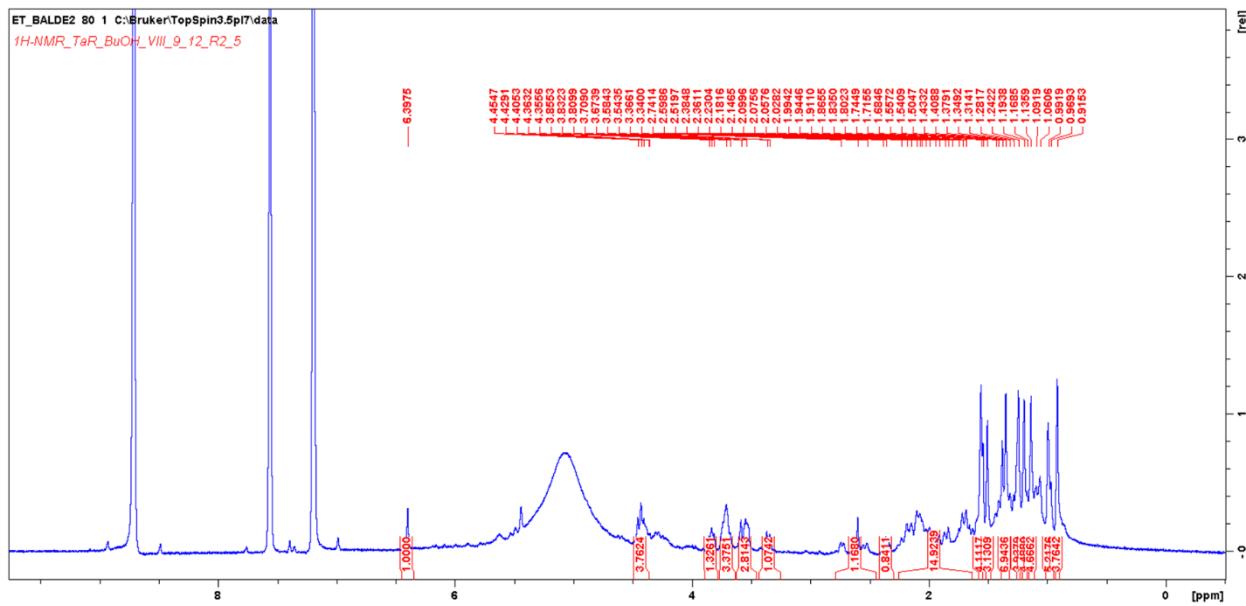


**Figure 5.27.** HMBC spectrum (methanol- $d_4$ ) of compound (5)

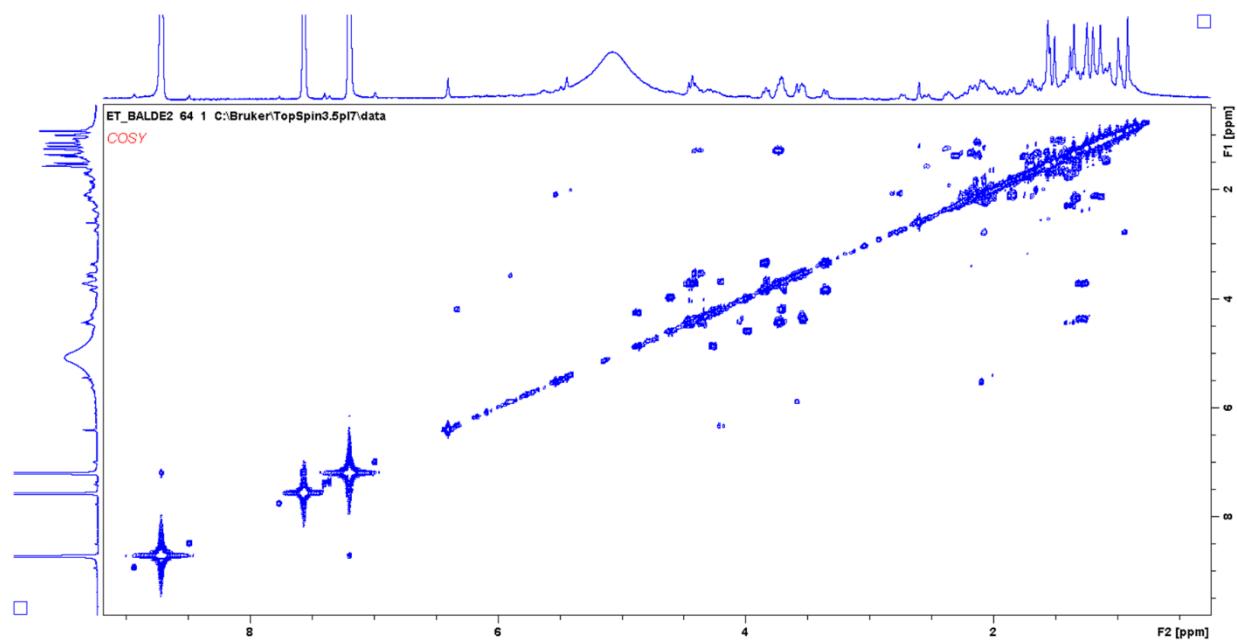


**Figure 5.28.** HR-ESI-MS spectrum of compound (5)

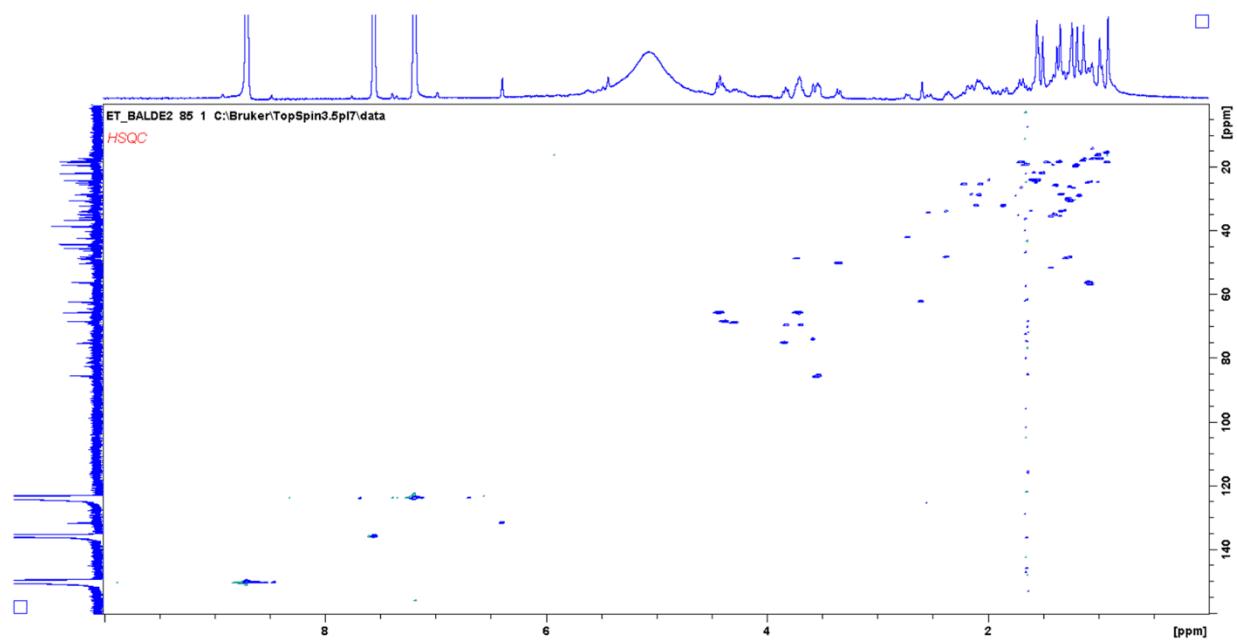
Compound **6** revealed the same monoisotopic mass as compound **4** and the same fragmentation pattern in the HR-ESI-MS spectrum:  $m/z$  517.3163 [M-H]<sup>-</sup> consistent with a molecular formula of C<sub>30</sub>H<sub>46</sub>O<sub>7</sub>, suggesting that compound **6** was an isomer of compound **4**. However, the <sup>1</sup>H- and <sup>13</sup>C-NMR spectra showed some differences in the A-ring, especially due to the orientation of the C-23 methyl group. Indeed, the chemical shift of C-23 ( $\delta_c$  24.1) ( $\alpha$ -orientation) in compound **6** is 9.6 ppm different from the methyl group at  $\delta$  14.5 ( $\beta$ -orientation), assigned to C-24 in compound **4**. Moreover, the chemical shifts of C-3 ( $\delta$  85.4) and C-5 ( $\delta$  56.1) were deshielded compared to compound **4** ( $\delta$  77.8 and  $\delta$  47.7, respectively). The aforementioned spectroscopic analysis suggested that the structure of compound **6** was identical to the aglycone of compound **3**. Therefore, albidic acid (**6**) was identified as 2 $\alpha$ ,3 $\beta$ ,19 $\beta$ ,23 tetrahydroxy-11-oxo-olean-12-en-28-oic acid, isolated for the first time as an aglycone.



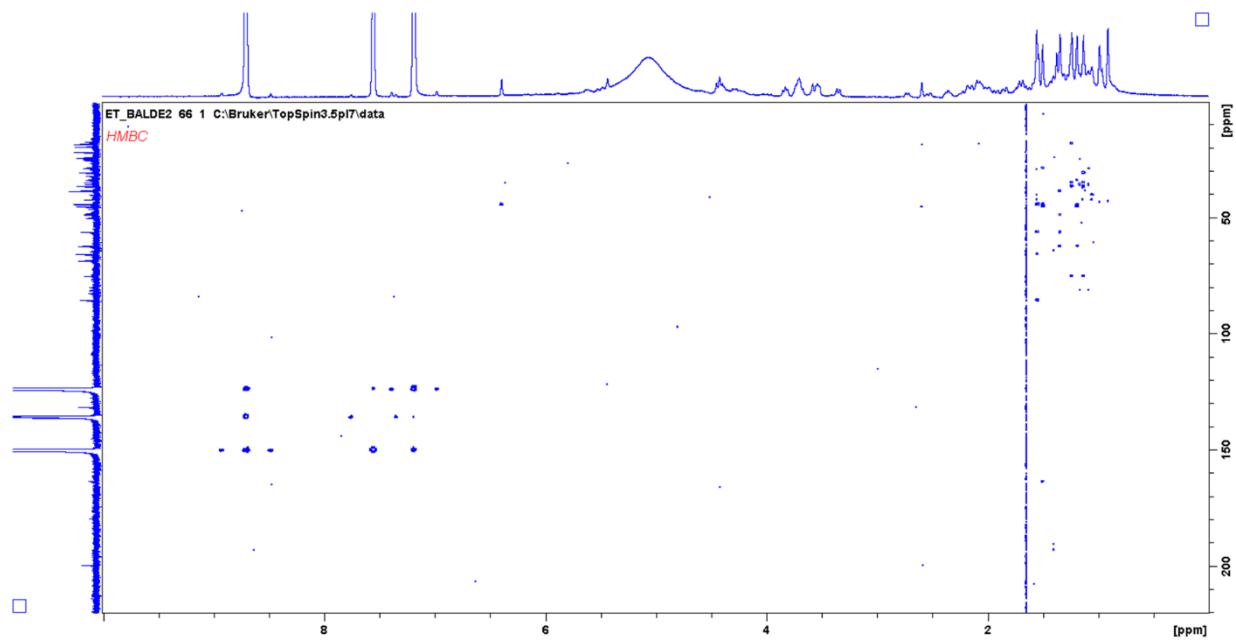
**Figure 5.29.** <sup>1</sup>H NMR spectrum (pyridine-*d*<sub>5</sub>, 400 MHz) of compound **(6)**



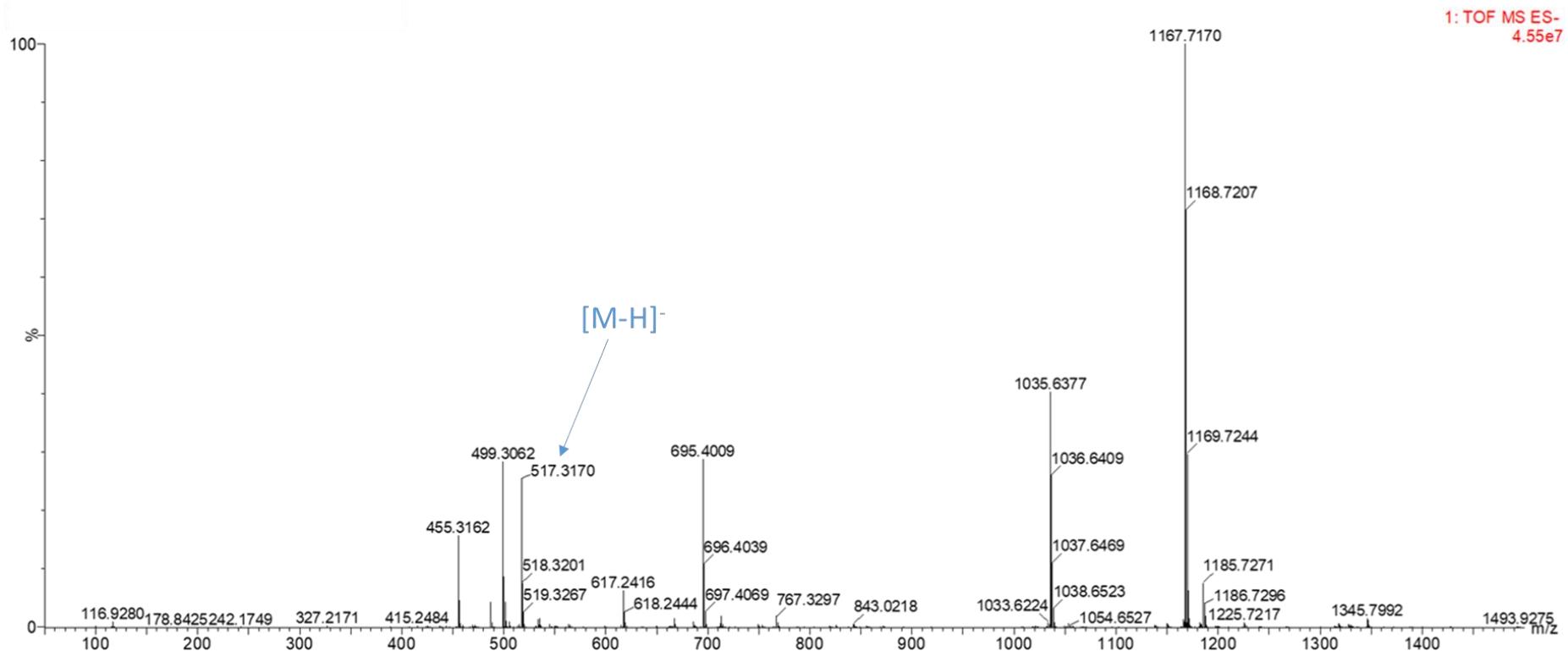
**Figure 5.30.** COSY spectrum (pyridine-*d*<sub>5</sub>) of compound (6)



**Figure 5.31.** HSQC spectrum of (pyridine-*d*<sub>5</sub>) of compound (6)

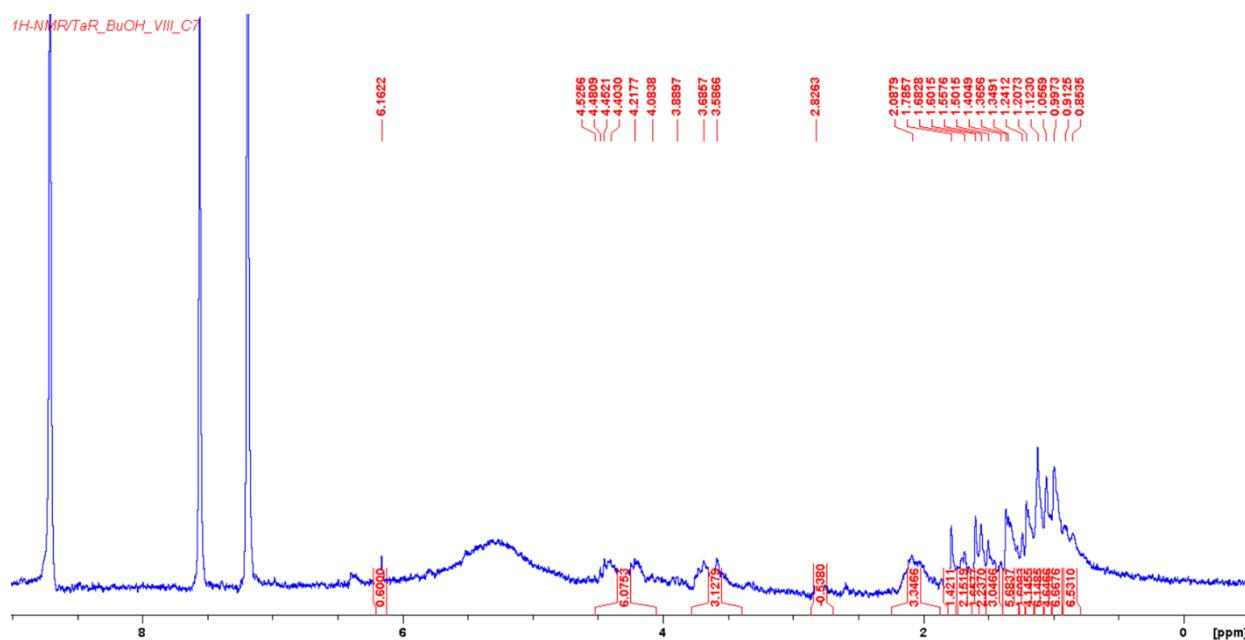


**Figure 5.32.** HMBC spectrum of (pyridine-*d*<sub>5</sub>) of compound (6)

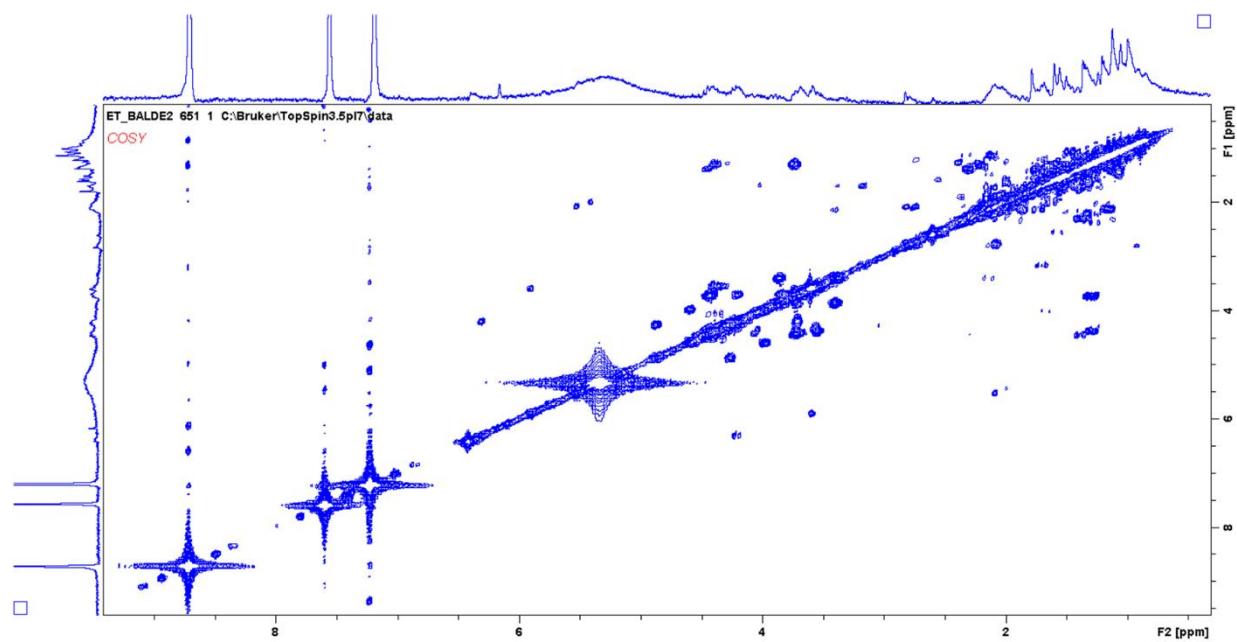


**Figure 5.33.** HR-ESI-MS spectrum of compound (6)

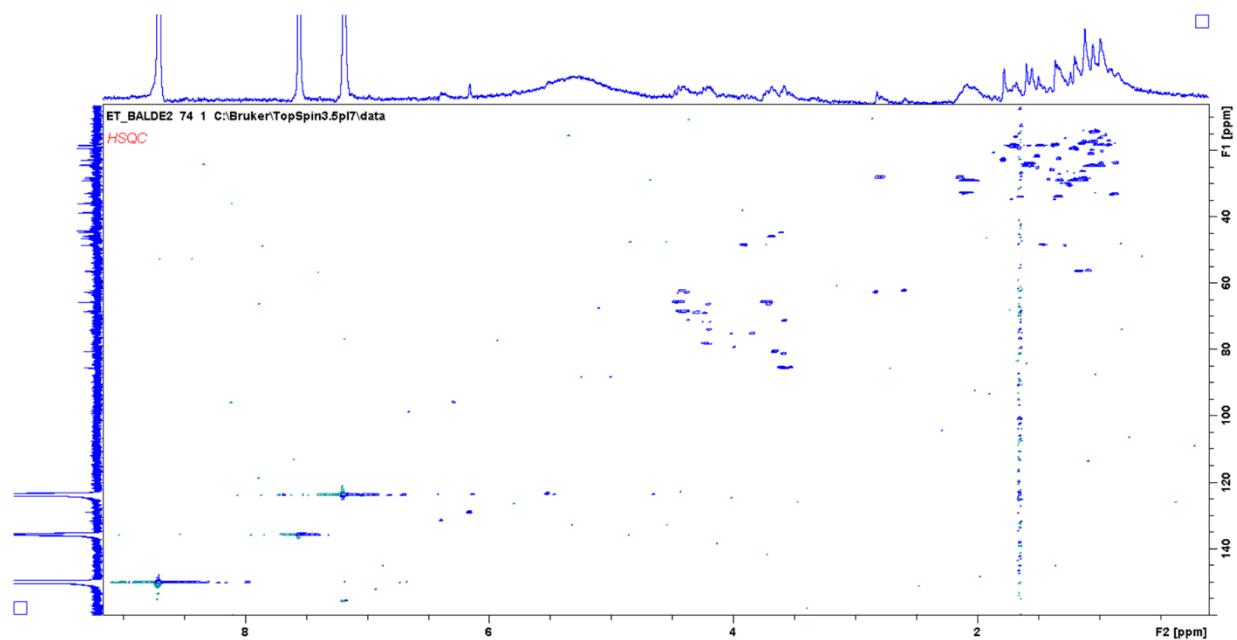
The HR-ESI-MS spectrum of compound **7** showed a deprotonated ion at  $m/z$  517.3154 [M-H]<sup>-</sup>, (molecular formula C<sub>30</sub>H<sub>46</sub>O<sub>7</sub>) suggesting that compound **7** was an isomer of compounds **4** and **6**. Furthermore, its <sup>1</sup>H-and <sup>13</sup>C-NMR data (Table 5.2, and 5.3) were similar to those of compound **6** except for the configuration of C-19 of the E-ring. Indeed, the chemical shift of C-19 ( $\delta_c$  80.5) in compound **7** shows a difference of 5.5 ppm to that of compound **6** ( $\delta_c$  75.0). Furthermore, the chemical shifts of C-12 and C-13 of the C ring and the methyl groups C- 29 and C-30 attached to the E-ring were shifted both in the <sup>1</sup>H- and <sup>13</sup>C NMR spectra in comparison with compound **7**. Comparison of the coupling constants of H-19 of compounds **7** ( $\delta$  3.66 d,  $J$  = 5.1 Hz) and **6** ( $\delta$  3.83 d,  $J$  = 10.2 Hz) confirmed the  $\alpha$ -orientation of the hydroxy group linked to C-19 in compound **7**. The identity of albidic acid C (**7**) was confirmed as 2 $\alpha$ ,3 $\beta$ ,19 $\alpha$ ,24-tetrahydroxy-11-oxo-olean-12-en-28-oic acid, isolated for the first time as an aglycone.



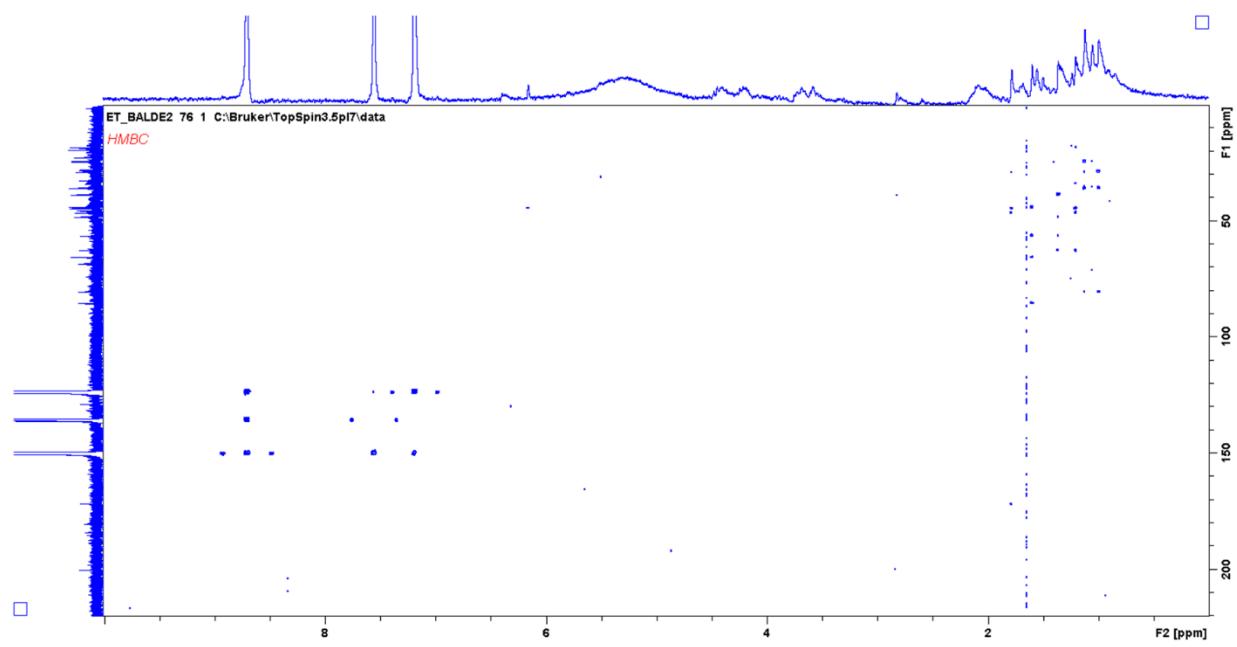
**Figure 5.34.** <sup>1</sup>H NMR spectrum (pyridine-*d*<sub>5</sub>, 400 MHz) of compound (**7**)



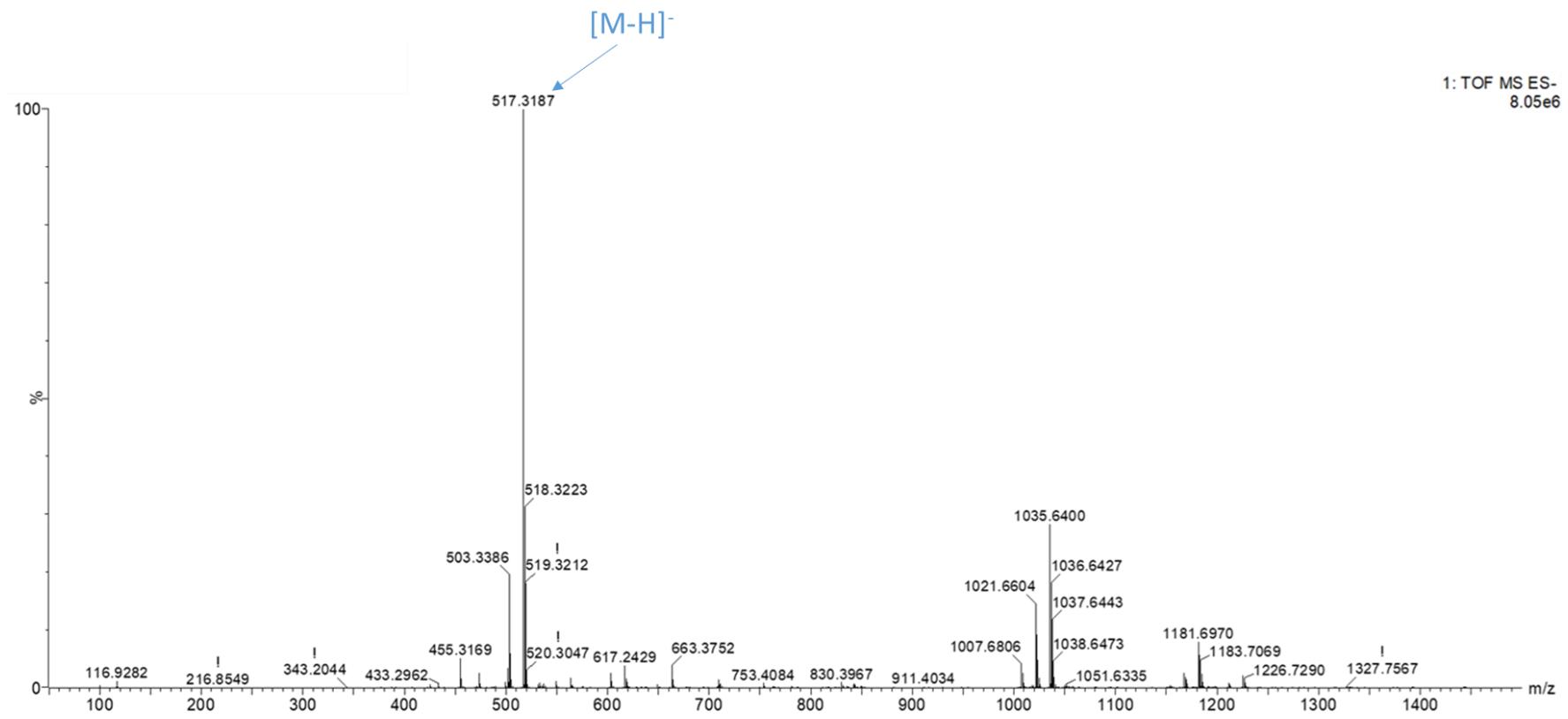
**Figure 5.35.** COSY spectrum of (pyridine-*d*<sub>5</sub>) of compound (7)



**Figure 5.36.** HSQC spectrum of (pyridine-*d*<sub>5</sub>) of compound (7)



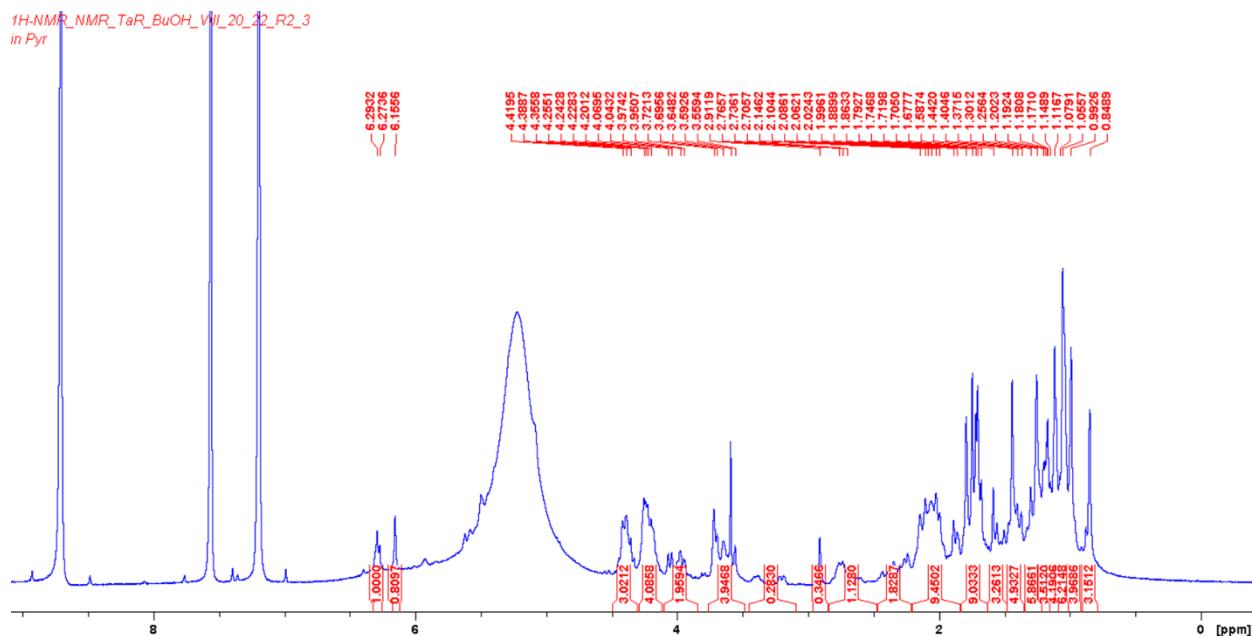
**Figure 5.37.** HMBC spectrum of (pyridine-*d*<sub>5</sub>) of compound (7)



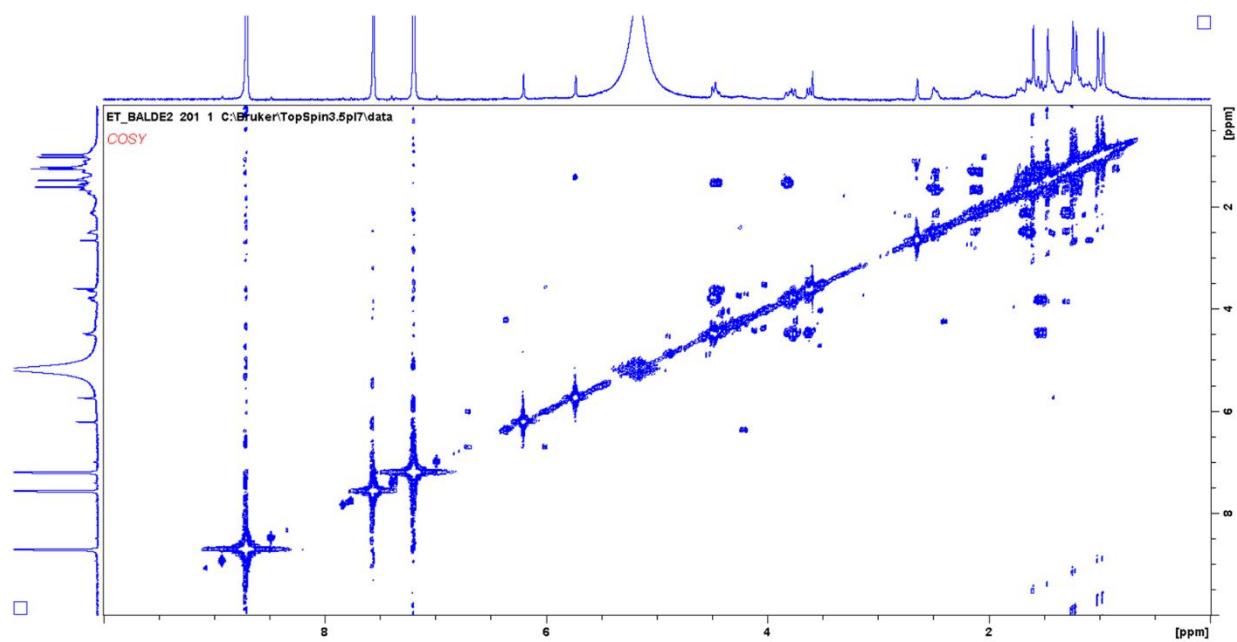
**Figure 5.38.** HR-ESI-MS spectrum of compound (7)

The HR-ESI-MS spectrum of compound **8** exhibited a deprotonated ion at  $m/z$  (499.3065 [M-H] $^-$ , molecular formula C<sub>30</sub>H<sub>46</sub>O<sub>6</sub>) and a fragment at  $m/z$  455.3160 due to the loss of a hydroxycarboxyl moiety. Compared to the other isolated aglycones, compound **8** bears an additional olefinic proton but lacks one hydroxyl group. The <sup>1</sup>H-NMR data (Table 5.2) in pyridine-d<sub>5</sub> displayed six methyl groups ( $\delta_H$  0.96, 1.01, 1.21, 1.24, 1.46 and 1.60), two olefinic protons ( $\delta_H$  5.74 and 6.21), two hydroxymethine protons at  $\delta_H$  3.62 (1H, m), 4.46 (1H, m) and two oxymethylene protons at  $\delta_H$  3.77 (1H, m) and 4.49 (1H, m). The <sup>13</sup>C-NMR data (Table 5.3) showed 30 carbon signals, including six methyl signals, and two olefinic carbons at  $\delta_C$  123.0 (C-12) and  $\delta_C$  138.9 (C-19). Signals observed at  $\delta$  199.7 (C-11), 123.0 (C-12) and 163.8 (C-13) revealed the presence of an  $\alpha,\beta$ -unsaturated carbonyl functionality in the C-ring. The presence of an olefinic proton in position C-19 of the E-ring was supported by the HMBC correlations observed between H-19 ( $\delta_H$  5.74), C-29 ( $\delta$  27.9) and C-30 ( $\delta$  30.0) (figure 1). Accordingly, compound **8** was identified as 2 $\alpha$ ,3 $\beta$ ,23-trihydroxyolean-12-19-dien-28-oic acid, a new compound for which the name albidienic acid was adopted.

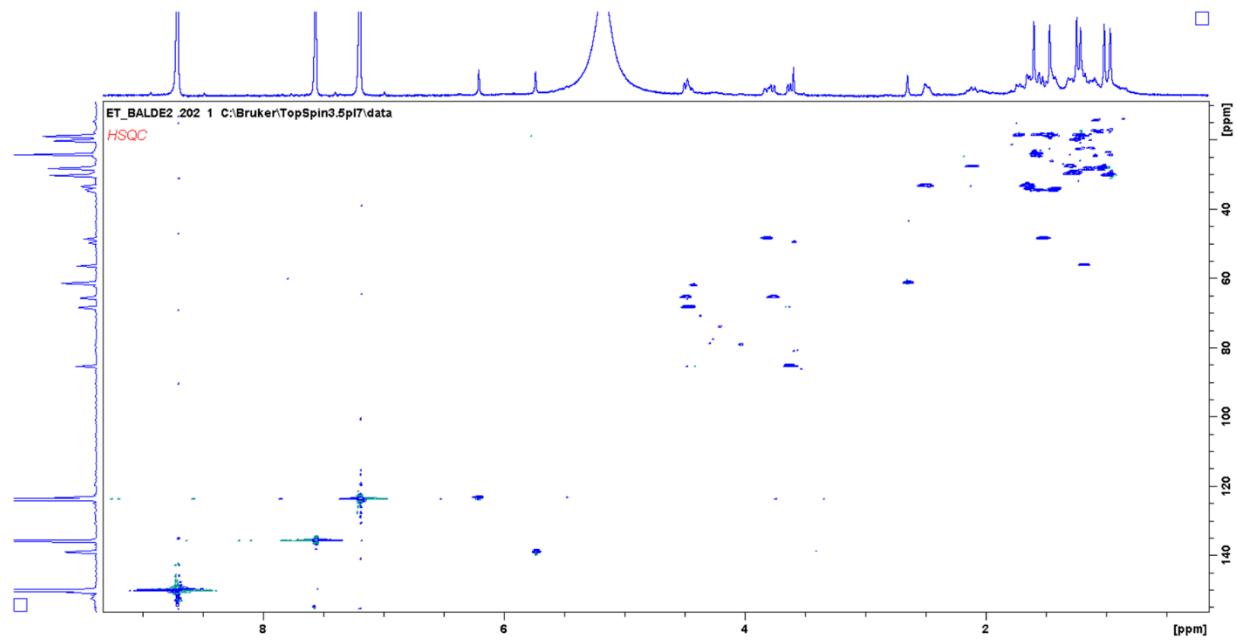
The presence of an olefinic proton at C-19 was supported by the HMBC correlations observed between H-19 ( $\delta_H$  5.74), C-29 ( $\delta$  27.9) and C-30 ( $\delta$  30.0) (Figure 5.3). Accordingly, albidienic acid (**8**) was identified as 2 $\alpha$ ,3 $\beta$ ,23-trihydroxyolean-12-19-dien-28-oic acid.



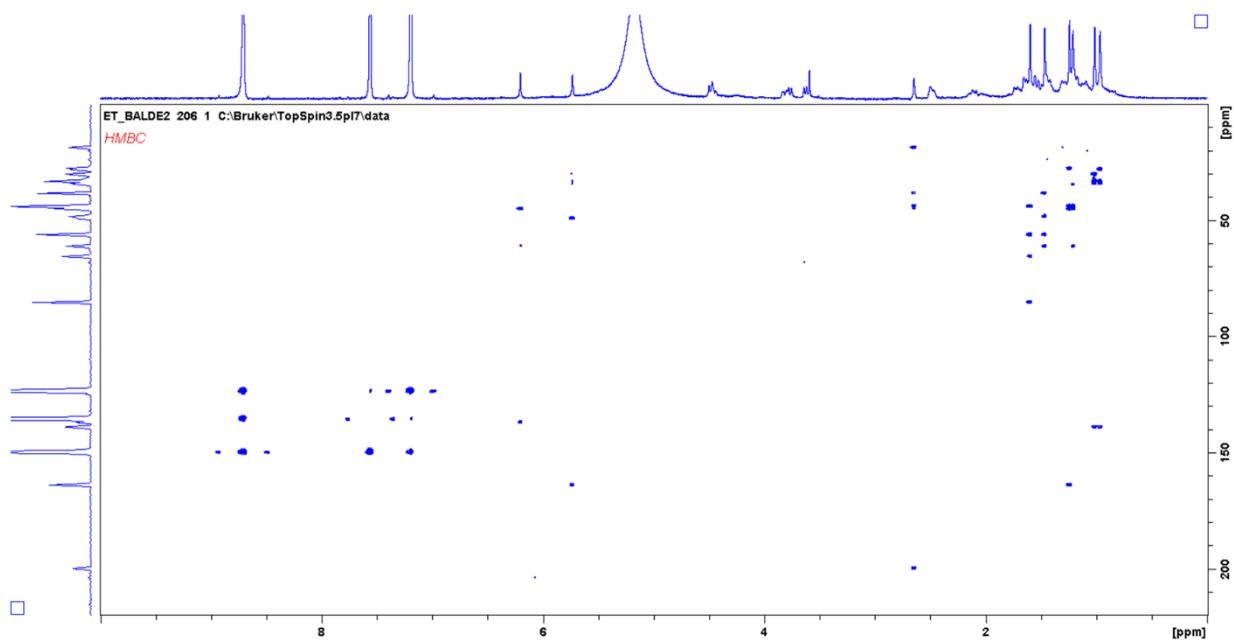
**Figure 5.39.** <sup>1</sup>H NMR spectrum (pyridine-d<sub>5</sub>, 400 MHz) of compound (**8**)



**Figure 5.40.** COSY spectrum (pyridine-*d*<sub>5</sub>) of compound (8)



**Figure 5.41.** HSQC spectrum of (pyridine-*d*<sub>5</sub>) of compound (8)



**Figure 5.42.** HMBC spectrum of (pyridine-*d*<sub>5</sub>) of compound (8)

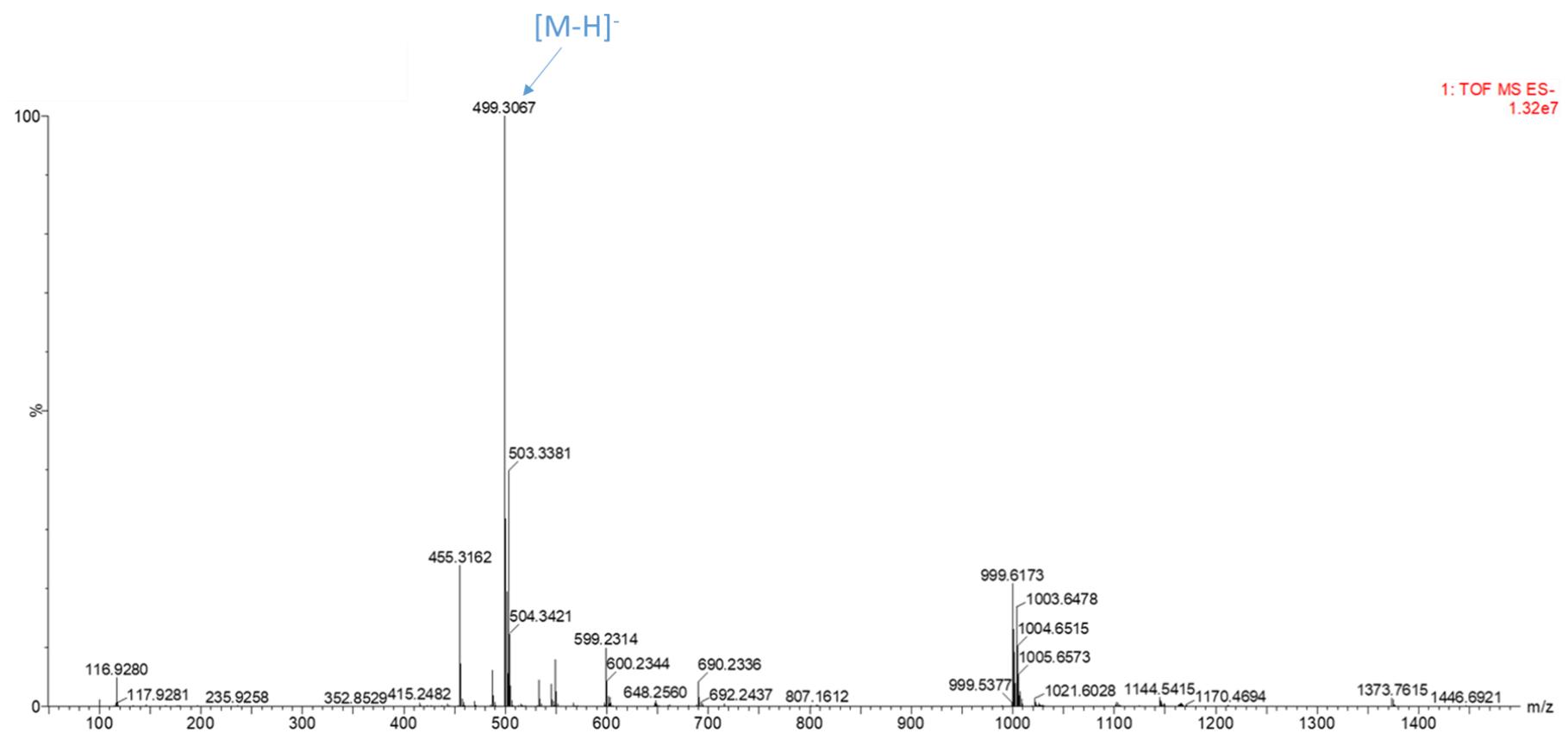
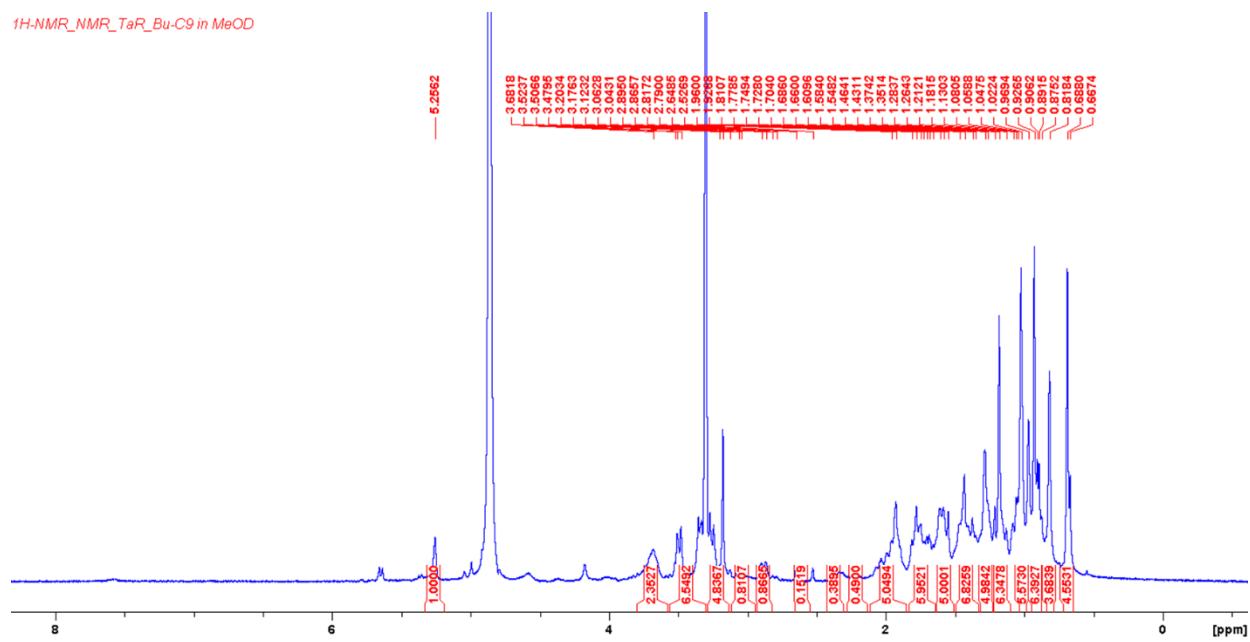
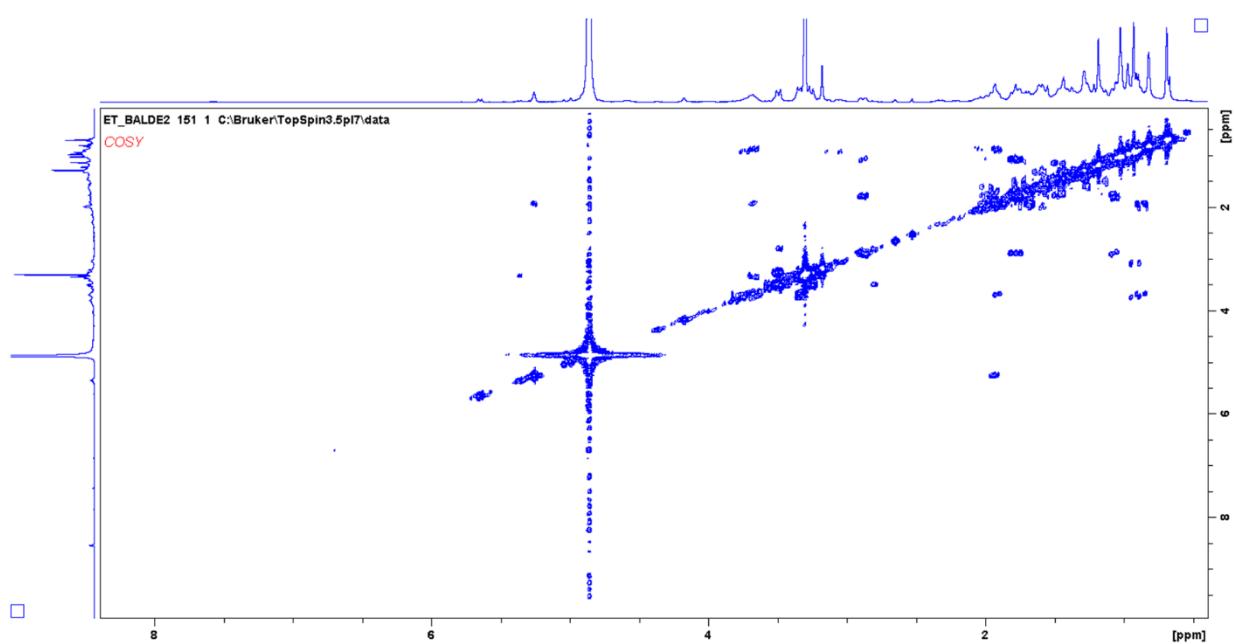


Figure 5.43. HR-ESI-MS spectrum of compound (8)

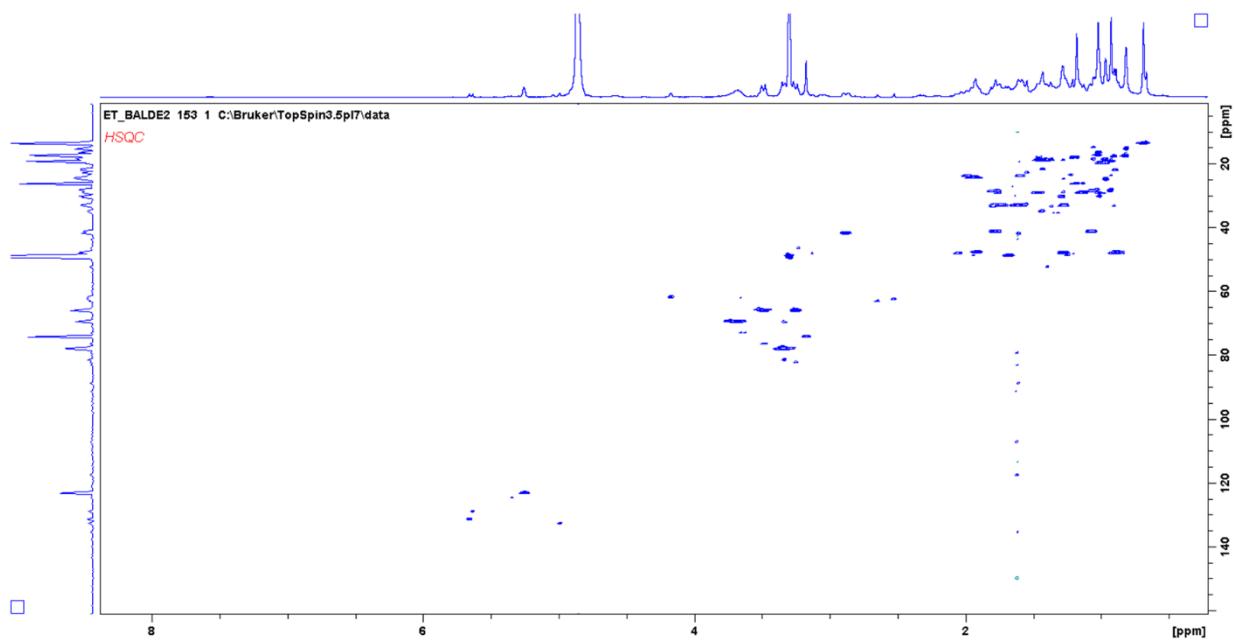
Compound **9** displayed a deprotonated ion at  $m/z$  503.3373 [M-H]<sup>-</sup>, corresponding to a molecular formula of C<sub>30</sub>H<sub>47</sub>O<sub>6</sub> in HR-ESIMS. The molecular ion of compound **9** revealed a difference of 16 mass units in comparison with compound **5** ( $m/z$  519.3330 [M-H]<sup>-</sup>). The <sup>1</sup>H-NMR data (Table 5.2) showed six methyl singlets ( $\delta_H$  0.68, 0.82, 0.92, 0.92, 1.02, 1.18 and 1.43), one olefinic proton at  $\delta_H$  5.25 (1H, br s), three hydroxymethine protons at  $\delta_H$  3.68 (1H, m), 3.34 (1H, m) and 3.18 (1H, s) and two oxymethylene protons at  $\delta_H$  3.48 (1H, d,  $J$  = 11.0 Hz) and 3.25 (1H, d,  $J$  = 11.0 Hz) linked to C-23. The <sup>13</sup>C-NMR data (Table 5.3) displayed 30 carbon signals which were assigned to a triterpenoid skeleton. Comparison of the <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of this compound with previously reported data suggested that compound **9** is a new oleanane triterpenoid. The presence of a hydroxyl group at C-21 was supported by the HMBC correlations of H-21 with C-29 ( $\delta_C$  28.4) and C-30 ( $\delta_C$  19.5). This was also supported by the COSY correlation observed between the protons linked to carbons C-18 ( $\delta$  42.09) and C-19 ( $\delta$  41.5). Therefore, the new albidolic acid (**9**) was identified as 2 $\alpha$ ,3 $\beta$ , 21 $\alpha$ ,23-tetrahydroxyolean-12-en-28-oic acid.



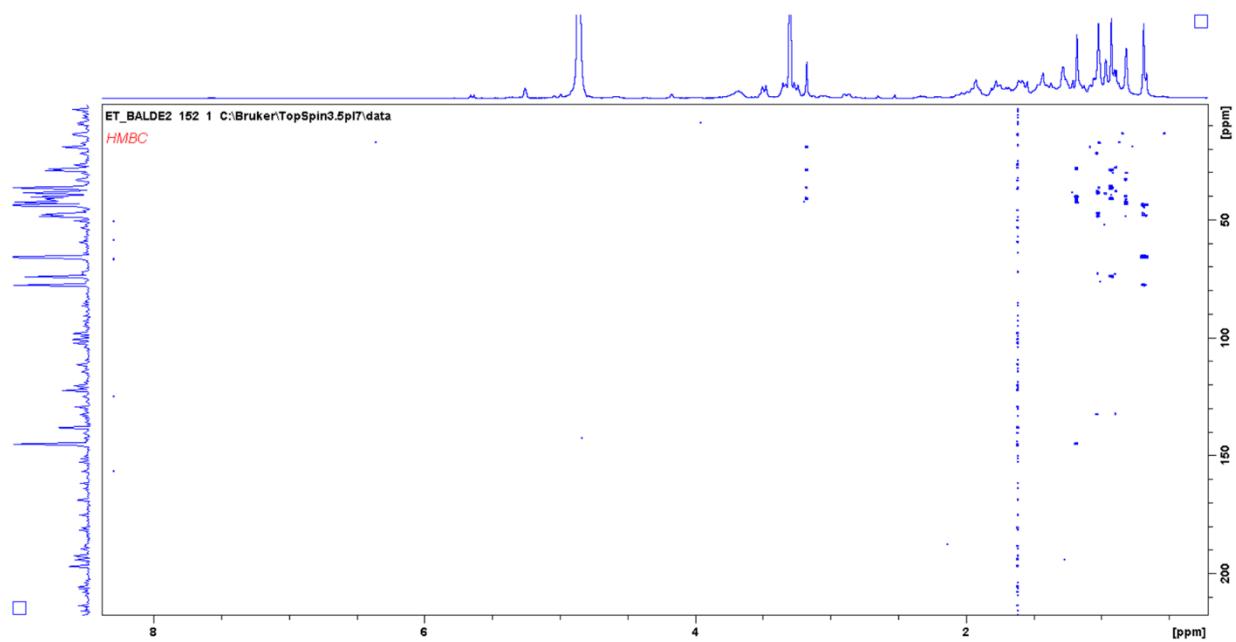
**Figure 5.44.** <sup>1</sup>H NMR spectrum (methanol-*d*<sub>4</sub>, 400 MHz) of compound (**9**)



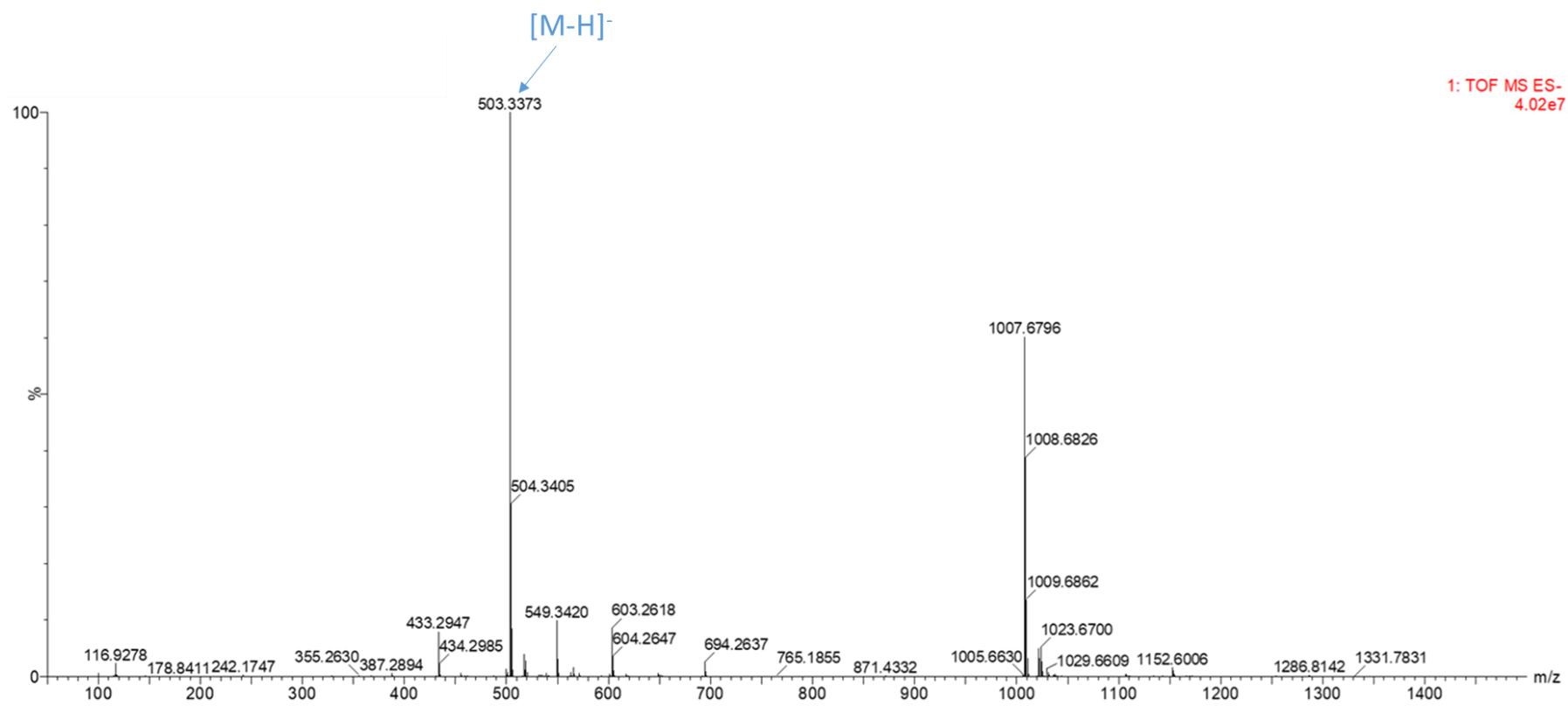
**Figure 5.45.** COSY spectrum (methanol- $d_4$ ) of compound (9)



**Figure 5.46.** HSQC spectrum of (methanol- $d_4$ ) of compound (9)

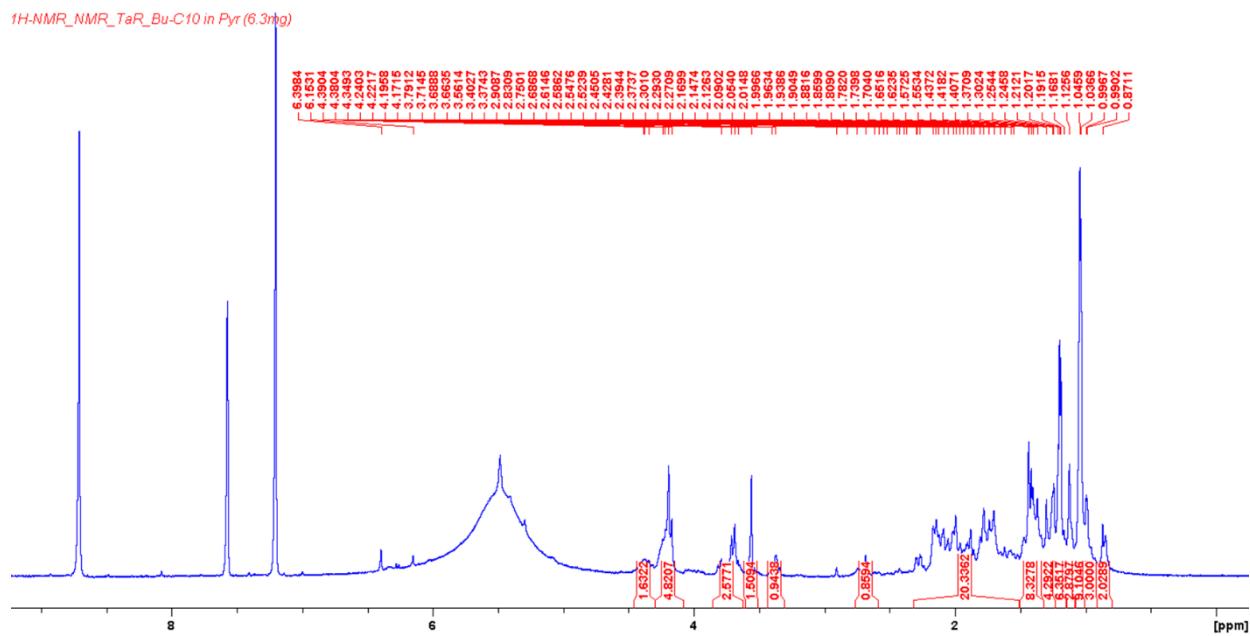


**Figure 5.47.** HMBC spectrum of (methanol-*d*<sub>4</sub>) of compound (9)

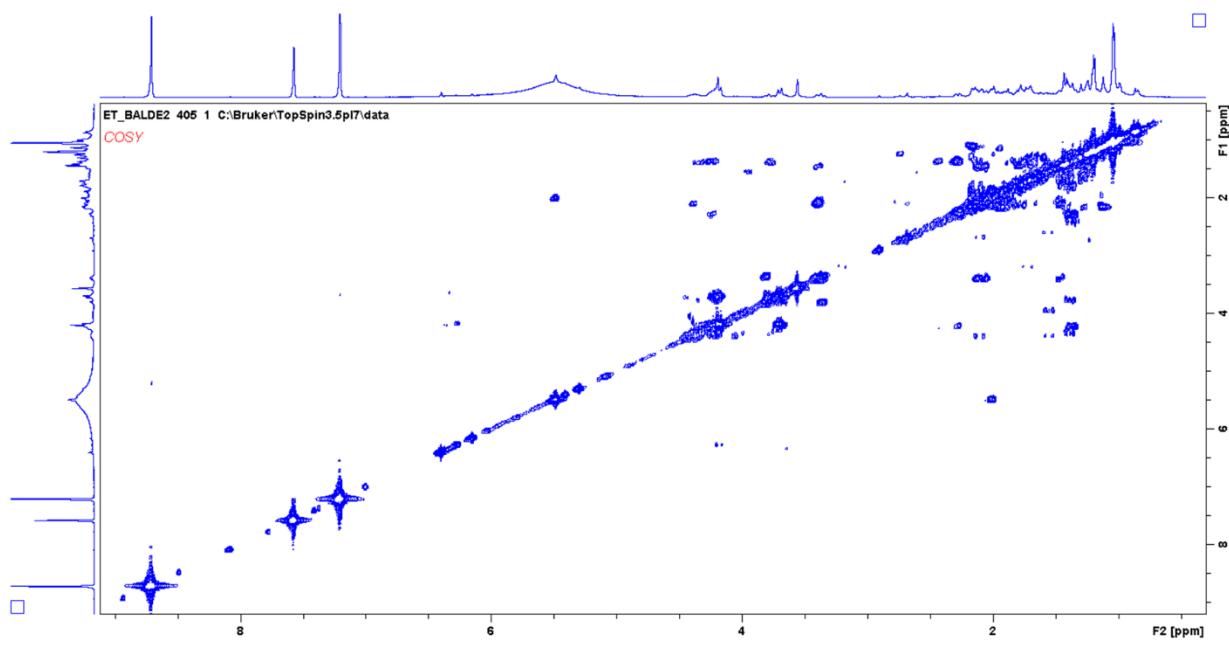


**Figure 5.48.** HR-ESI-MS spectrum of compound (9)

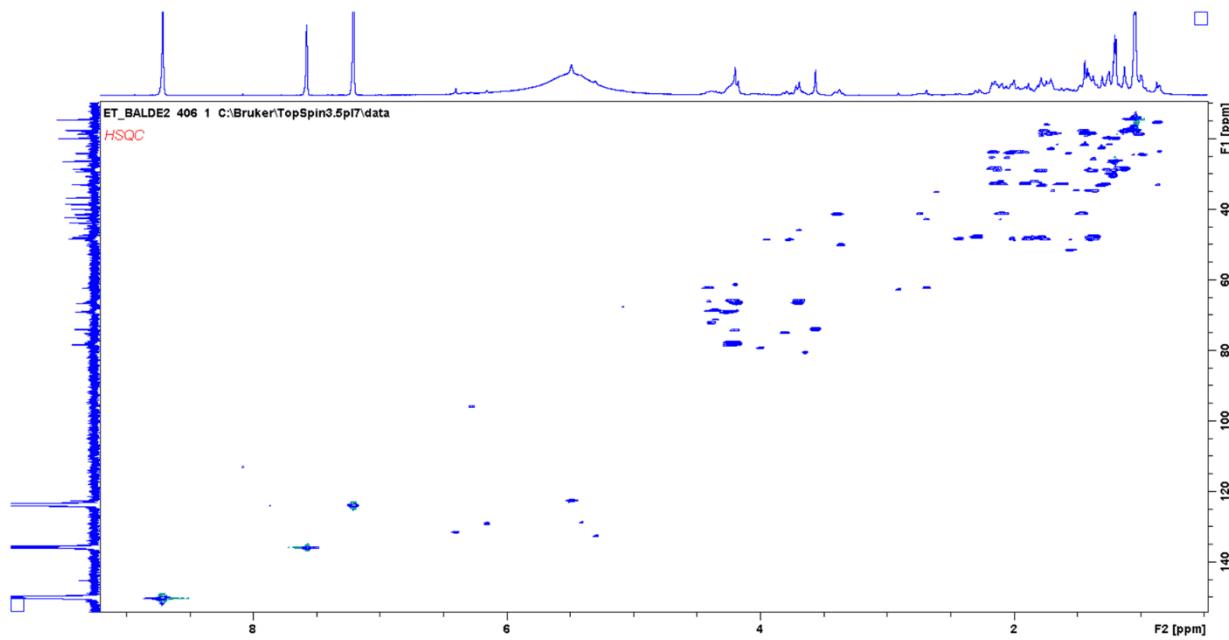
The HR-ESI-MS spectrum of compound **10** exhibited a deprotonated ion at  $m/z$  (503.3370 [M-H] $^-$ , molecular formula C<sub>30</sub>H<sub>47</sub>O<sub>6</sub>). Compared to compound **9**, compound **10** has two additional oxymethylene protons at  $\delta_H$  3.56 (2H, s) but lacks one hydroxyl group. The <sup>13</sup>C-NMR data (Table 5.3) of compound **10** displayed 30 carbons. The presence of an oxymethylene group at position C-30 of the E-ring was supported by HMBC correlations of H-30 with C-20 ( $\delta_C$  36.6) and C-29 ( $\delta_C$  19.8). This was also supported by the COSY correlation between H-18 and H-19 (Figure 5.4). The position of the hydroxymethylene groups was confirmed by HMBC correlations observed between H-3 ( $\delta_H$  4.19, m) and C-2 ( $\delta_C$  68.9), H-23 ( $\delta_H$  3.70, 4.18) and C-3 ( $\delta_C$  78.2) and C-24 ( $\delta_C$  14.4), and H-29 ( $\delta_H$  1.19) and C-30 ( $\delta_C$  73.9). Comparison of the <sup>1</sup>H and <sup>13</sup>C NMR reported data suggested that albidolic acid (**10**) was a new triterpenoid, and was identified as 2 $\alpha$ ,3 $\beta$ ,23,30-tetrahydroxy-11-oxo-olean-12-en-28-oic acid. Two isomers 2 $\alpha$ , 3 $\alpha$ , 23, 30-tetrahydroxy-11-oxo-olean-12-en-28-oic acid and 2 $\beta$ , 3 $\beta$ , 23, 30-tetrahydroxy-11-oxo-olean-12-en-28-oic acid of this compound were previously identified in other plant species<sup>17,18</sup>.



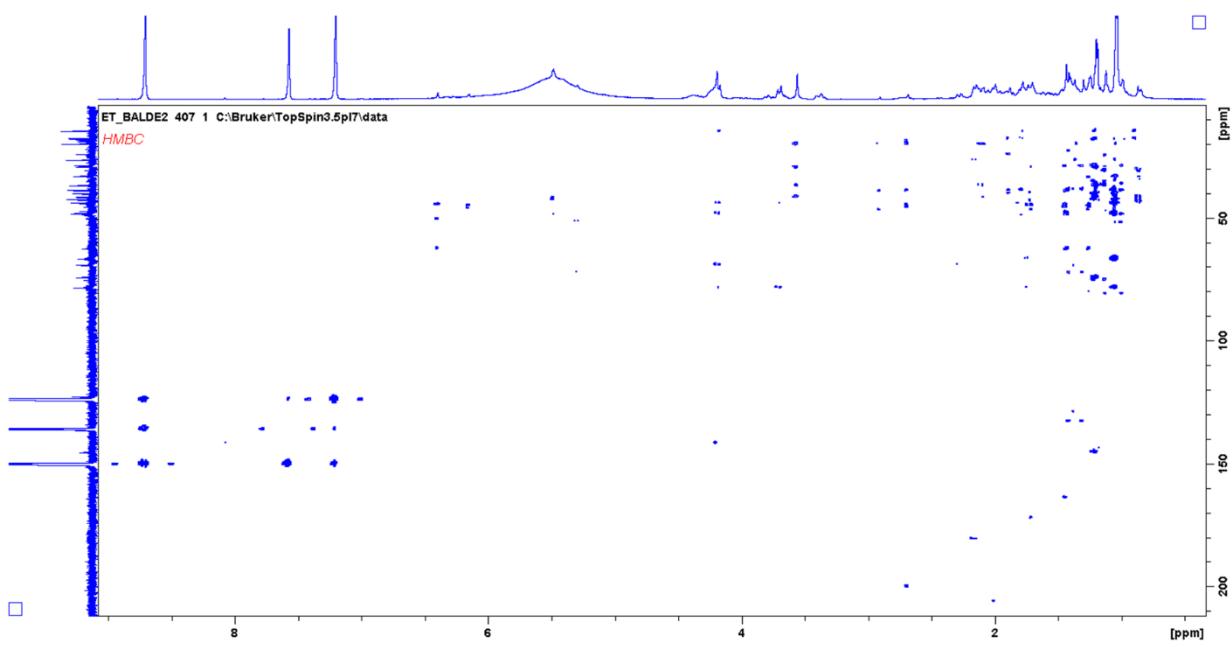
**Figure 5.49.** <sup>1</sup>H NMR spectrum (Pyridine-*d*<sub>5</sub>, 400 MHz) of compound (**10**)



**Figure 5.50.** COSY spectrum (Pyridine-*d*<sub>5</sub>) of compound (**10**)



**Figure 5.51.** HSQC spectrum of (Pyridine-*d*<sub>5</sub>) of compound (**10**)



**Figure 5.52.** HMBC spectrum of (Pyridine-*d*<sub>5</sub>) of compound (**10**)

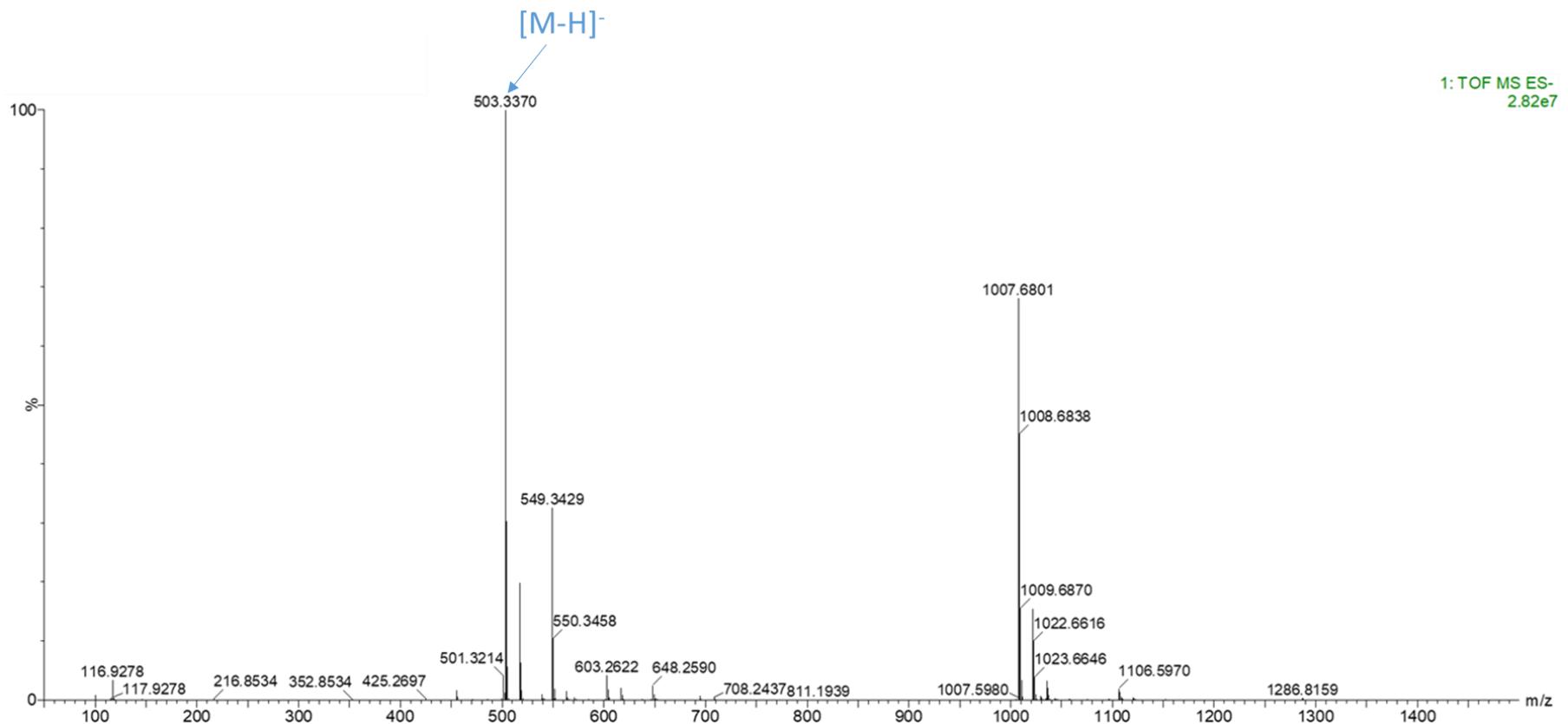


Figure 5.53. HR-ESI-MS spectrum of compound (10)

The compounds isolated in this study have been tested against *P. falciparum*, *Candida albicans* and *Staphylococcus aureus*, and their cytotoxicity was determined against MRC-5 cells (human foetal lung fibroblast cells) (Table 5.4). It emerges from these results that compounds **1 - 4**, **6**, **7** and **8** possess moderate antiplasmodial activity with IC<sub>50</sub> values between 5 and 15 µM, while compound **10** was weakly active (IC<sub>50</sub> 48.9 µM). Notably, albidanoside B (**2**) is the C-28 glucosyl ester of albidic acid A (**4**), and that ivorenoside C (**3**) is the C-28 glucosyl ester of albidic acid B (**6**). None of the active compounds were cytotoxic. Furthermore, all the tested compounds were inactive against *S. aureus* and *C. albicans*.

In conclusion, molecular networking and *in silico* MS/MS dereplication aided in the targeted semi-preparative HPLC-MS-DAD purification of oleanane triterpenoids from the *n*-BuOH fraction of *Terminalia albida*. This resulted in the isolation of six new oleanane triterpenoids named albidanoside A, albidic acid A, albidinol acid, albidienic acid, albidol acid and albidolic acid, together with two triterpenoid aglycones (albidic acid B, albidic acid C) reported here for the first time as aglycones, and two known compounds. The isolated compounds were evaluated for their activity against *P. falciparum*, *C. albicans* and *S. aureus*. Compounds **1-4**, **6**, **7** and **8** demonstrated moderate antiplasmodial activity with IC<sub>50</sub> values between 5 and 15 µM. None of the tested compounds was active against *C. albicans* or *S. aureus*. These findings emphasize the potential of *T. albida* as a source for discovery of new antiplasmodial compounds.

**Table 5.4.** *In vitro* antimicrobial, antiplasmodial and cytotoxic activity of the *n*-BuOH fraction and isolated compounds from *T. albida* root

Fraction and compound names	Antibacterial and antifungal activity ( $IC_{50}$ $\mu M$ )		Antiplasmodial activity ( $(IC_{50} \mu M)$ )	Cytotoxicity ( $CC_{50} \mu M$ )	Selectivity index $MRC-5/PfK1$
	<i>S. aureus</i>	<i>C. albicans</i>			
<i>n</i> -BuOH fraction	>64.0	>64.0	>64.0	>64.0	nd
2 $\alpha$ ,3 $\beta$ ,19 $\alpha$ ,23-tetrahydroxy-11-oxoolean-12-en-28-oic acid 28-O- $\beta$ -D-glucopyranosyl ester ( <b>1</b> )	>64.0	>64.0	13.9	>64.0	>4.6
Albidanoside A ( <b>2</b> )	>64.0	>64.0	<b>11.3</b>	>64.0	>5.7
Ivorenoside C ( <b>3</b> )	>64.0	>64.0	9.1	>64.0	>7.0
Albidic acid A ( <b>4</b> )	>64.0	>64.0	10.1	>64.0	>6.3
Albidic acid B ( <b>6</b> )	>64.0	>64.0	6.1	>64.0	>10.5
Albidic acid C ( <b>7</b> )	>64.0	>64.0	6.1	>64.0	>10.5
Albidienic acid ( <b>8</b> )	>64.0	>64.0	6.9	>64.0	>9.27
Albidiolic acid ( <b>10</b> )	>64.0	>64.0	48.5	>64.0	>1.31
Chloroquine			0.15 ± 0.1 $\mu M$		
Doxycycline		0.28 ± 0.2 $\mu M$			
Flucytosine			0.70 ± 0.01 $\mu M$		
Tamoxifen				10.0 ± 1.5 $\mu M$	

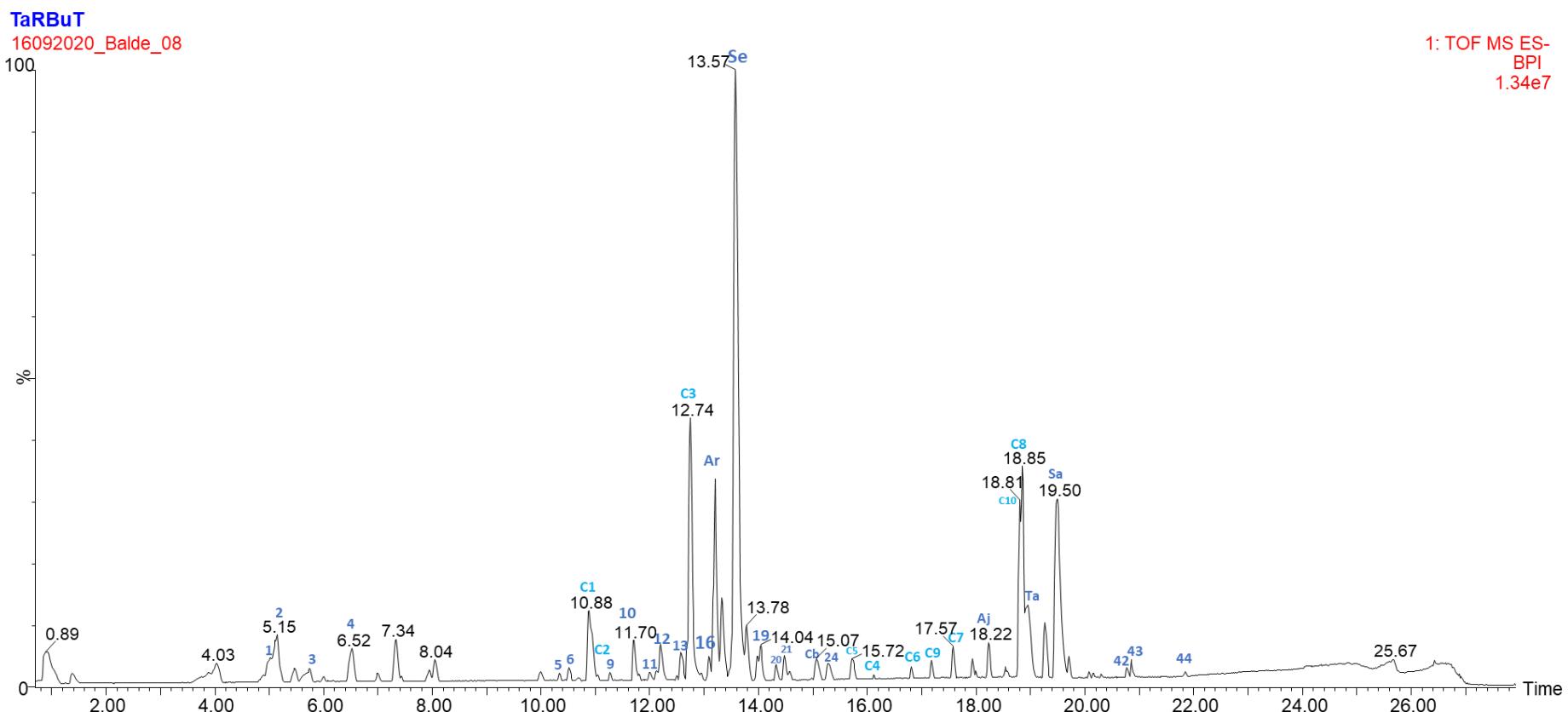
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## Supplementary information



**Figure S 5.1.** base peak intensity chromatogram (BPI) of the *n*-BuOH fraction with peaks labelled with compound number (C1-C10 isolated compounds), Ar (arjunglucoside I), Aj (arjungenin), Cb (Chebuloside II), Se (sericoside), Sa (sericic acid), Ta (Terminolic acid).

**Table S 5.1.**  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR assignments ( $\delta_c$  in ppm) for compounds 1, 3 (100 MHz, pyridine-*d*5)

Position	<b>1<sup>b</sup></b>		<b>3<sup>b</sup></b>	
	$\delta_{\text{H}}$ ( <i>J</i> in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ ( <i>J</i> in Hz)	$\delta_{\text{C}}$
1a; 1b	1.54, t (11.6); 3.94, dd (3.7, 12.8)	48.3, CH <sub>2</sub>	1.26, m; 3.72, dd (3.7, 11.7)	48.4, CH <sub>2</sub>
2	4.38, m	68.5, CH	4.38, m	68.1, CH
3	4.23, m	77.5, CH	3.53, d (9.3)	85.1, CH
4		43.6, C		43.9, C
5	1.87, br d (11.7)	47.4, CH	1.06, d (11.7)	55.9, CH
6a; 6b	1.44, m; 1.73, m	17.5, CH <sub>2</sub>	1.48, m; 1.65, m	18.3, CH <sub>2</sub>
7a; 7b	1.43, m; 1.83, m	32.9, CH <sub>2</sub>	1.59, m; 1.38, m	33.5, CH <sub>2</sub>
8		46.5, C		45.2, C
9	2.90, s	62.5, CH	2.57, s	61.8, CH
10		38.5, C		38.2, C
11		200.2, C		199.4, C
12	6.11, s	128.9, CH	6.36, s	131.4, CH
13		170.6, C		162.2, C
14		44.4, C		44.12, C
15a; 15b	1.28, d (13.7); 1.35 <sup>a</sup>	28.6, CH <sub>2</sub>	1.34 <sup>a</sup> ; 2.44, br t (13.4)	28.1, CH <sub>2</sub>
16a ; 16b	2.12, d (12.5) ; 2.75, t (12.5)	27.4, CH <sub>2</sub>	2.09, br d (13.4) ; 2.20, br t (13.4)	24.8, CH <sub>2</sub>
17		45.7, C		45.3
18	3.57, d (5.2)	45.4, CH	3.23, d (10.6)	49.5, CH
19	3.59, d (5.2)	80.0, CH	3.80, d (10.6)	74.5, CH
20		35.4, C		36.1, C
21a; 21b	0.99, m; 1. 96 <sup>a</sup>	28.4, CH <sub>2</sub>	1.63, m; 1.28, m	34.3, CH <sub>2</sub>
22a; 22b	1.96 <sup>a</sup> ; n.o	31.9, CH <sub>2</sub>	1.75, m; 1.87, dd (14.2, 10.0, 4.3)	31.1, CH <sub>2</sub>
23a; 23b	4.22, m; 3.69, d (10.9)	65.6, CH <sub>2</sub>	1.54, s; n.o	23.8, CH <sub>2</sub>
24a; 24b	1.06, s; n.o	14.6, CH <sub>3</sub>	3.72, d (11.7); 4.45, d (11.7)	65.4, CH <sub>2</sub>
25	1.46, s	18.3, CH <sub>3</sub>	1.37, s	18.6, CH <sub>3</sub>
26	1.37, s	19.4, CH <sub>3</sub>	1.32, s	19.2, CH <sub>3</sub>
27	1.67, s	22.7, CH <sub>3</sub>	1.46, s	21.5, CH <sub>3</sub>

28		176.6, C		175.3, C
29	1.06, s	28.1, CH <sub>3</sub>	1.20, s	30.1, CH <sub>3</sub>
30	0.85, s	24.0, CH <sub>3</sub>	1.01, s	17.6, CH <sub>3</sub>
1'	6.35, d (8.0)	95.8, CH	6.30, d (8.0)	95.8, CH
2'	4.18, t (8.3)	73.9, CH	4.19, t (8.3)	73.8, CH
3'	4.27, t (8.7)	78.6, CH	4.28, t (8.6)	78.5, CH
4'	4.35 <sup>a</sup>	70.8, CH	4.36, t (9.4)	70.8, CH
5'	4.03, m	79.3, CH	4.04, m	79.2, CH
6'	4.40, m, 4.44 m	61.8, CH <sub>2</sub>	4.44, m	61.9, CH <sub>2</sub>

<sup>a</sup>Overlapping signals, <sup>b</sup>Pyridine-*d*<sub>5</sub>



## CHAPTER 6

***Tetracera alnifolia:*** bioassay-guided isolation  
of antiplasmodial and antimicrobial constituents  
from the leaves of *Tetracera alnifolia*

**Manuscript in preparation:** Baldé MA, Tuenter E, Diallo MST, Matheeussen A, Traoré MS, Peeters L, Cos P, Caljon G, Balde AM, Foubert K, Pieters L. Bioassay-guided isolation of antiplasmodial and antimicrobial constituents from the leaves of *Tetracera alnifolia*. Prepared for *Fitoterapia*.



## 6.1. Introduction

The discovery of new bioactive natural products as leads for therapeutic development can be inspired by ethnopharmacological knowledge or achieved by screening a collection of extracts for bioactivity, using several bioassays<sup>1,2</sup>. The traditional workflow for studying complex plant preparations is bioactivity-guided fractionation in which bioactive extracts and subsequent fractions are chromatographically separated and retested for bioactivity until active compounds have been isolated<sup>3,4</sup>. In the last decade, substantial developments have been made in improving extraction and separation efficiency and facilitating the isolation of minor constituents that may contribute to activity<sup>5</sup>. Bioassay-guided fractionation workflow is still in use today by several scientists for the discovery of new therapeutic leads from natural sources including plant species<sup>1</sup>.

The Dilleniaceae family comprises 10-14 genera and ca. 500 species, with a pantropical and subtropical distribution. Around 102 species distributed in six genera viz. Curatella, Davilla, Doliocarpus, Neodillenia, Pinzona, and Tetracera are mainly found in the Neotropics areae. The Dilleniaceae family is divided into four subfamilies and the genus Tetracera belongs to the Delimoideae subfamily<sup>6,7</sup>. The genus Tetracera (family Dilleniaceae) contains about 50 species with a pantropical distribution, of which 20 occur in the Neotropical area. In traditional folk medicine, some species have been used for the management of various diseases and infections<sup>8,9,10</sup>. Species of this genus have been reported to exhibit a broad range of pharmacological activities including antifungal, antiplasmoidal, analgesic, anti-inflammatory, antioxidant, antipyretic, antimycobacterial, anti-HIV, anti-ulcerogenic, hepatoprotective<sup>8,6</sup>. Several *Tetracera* species have been previously investigated for their phytochemical constituents, which resulted in the identification of a broad range of compounds, including flavonoids and terpenoids. Some of the isolated compounds have demonstrated various bioactivities such as antifungal, antibacterial, anti-diabetic, antitumor, anti-HIV-1, antioxidant, and analgesic<sup>11,12</sup>. In the Guinean flora, around 4 *Tetracera* species have been identified and *Tetracera alnifolia* is distributed in all Guinean regions. *T. alnifolia*, a forest liana up to 15 m high, is widely used

in Guinean traditional medicine for the management of several diseases including skin diseases, oral diseases, infectious diseases and malaria<sup>13,14,15</sup>. This plant species is also well known in other African countries for its traditional uses in the management of inflammation, pain, cough, sexually transmitted infections and leprosy<sup>16,9,17</sup>. Promising *in vitro* activities against *Candida albicans*, *Staphylococcus aureus* and *Plasmodium falciparum* were recently obtained in our laboratory for both methanolic and dichloromethane extracts of the leaves of *T. alnifolia*<sup>13</sup>. The focus of this study was consequently to isolate the metabolites possibly contributing to these activities, and which may therefore be promising precursors for the development of new antimicrobial and / or antiplasmoidal agents. A bioguided fractionation of *Teracera alnifolia* leaves extracts has been undertaken in this context.



**Clade :** Angiosperms

**Order :** Dilleniales

**Family :** Dilleniaceae

**Genus :** *Tetracera*

**Species :** *alnifolia*

**Figure 7.1.** *Teracera alnifolia* Willd. Picture: O. Ouédraogo

## 6.2. Materials and methods

### 6.2.1. Solvent and reagents

Dichloromethane, ethyl acetate, *n*-butanol, *n*-hexane, acetone, acetonitrile, acetonitrile far UV and methanol (all HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK). All reagents, such as sulphuric acid and formic acid (eluent additive for HPLC) were

purchased from Acros Organics (Geel, Belgium) or Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and formic acid (both for UPLC-MS) were obtained from Biosolve Chimie (Dieuze, France). Ultrapure water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA). NMR deuterated solvents ( $\text{CDCl}_3$ , acetonitrile-d<sub>3</sub>, methanol-d<sub>4</sub>, DMSO-d<sub>6</sub>, pyridine-d<sub>5</sub>, D<sub>2</sub>O) were obtained from Sigma-Aldrich.

### 6.2.2. General experimental methods

Fractionation of extracts and fractions was carried out using an open column chromatography on Diaion HP-20 resin and a Grace Reveleris X2 flash chromatographic system (Lokeren, Belgium) equipped with an evaporative light scattering detector (ELSD), a diode array detector (DAD), and a fraction collector. The chromatographic profiles of fractions collected during different isolation steps were analysed by an Agilent HPLC system (1200 series) and/or by thin layer chromatography (TLC) on NP F254 plates (20 cm × 20 cm) from Merck (Darmstadt, Germany). The TLC plates were observed under UV light (254 and 366 nm) and under visible light after spraying with a vanillin-sulphuric acid reagent (prepared by mixing 5 g of vanillin with 475 mL of ethanol and 25 mL sulphuric acid).

A semipreparative HPLC system equipped with DAD and ESI-MS detectors was used for the isolation of the pure compounds. The system was composed of a sample manager, injector, and collector (2767), a quaternary gradient module (2545), a System Fluidics Organizer, an HPLC pump (515), a diode array detector (2998), and a Micromass Quattro TQD mass spectrometer , all supplied by Waters (Milford, MA, USA). For data processing MassLynx version 4.1 was used.

Low amount sub-fractions (less than 100 mg) were purified using HPLC-SPE-NMR. All analyses were carried out using an Agilent 1200 series HPLC instrument with degasser, quaternary pump, automatic injection sampler and an a PDA detector (Agilent Technologies). Selected analytes were cumulatively trapped based on their UV thresholds. After the detector, 3 mL/min of water was added to the eluent stream with a make-up pump (Knauer K 120, Berlin, Germany) in order to increase the retention of the analytes on the SPE cartridges using a Bruker / Spark SPE system, equipped with

HySphere Resin General Phase (GP) cartridges (polydivinyl-benzene material with particle size 5–15 µm). Thereafter, each cartridge was dried with pressurized nitrogen gas for 40 min and eluted with 60 µL of deuterated solvent (acetonitrile-d<sub>3</sub> or methanol-d<sub>4</sub>) into 3 mm NMR tubes with a Gilson Liquid Handler 125. Chromatographic separation and analyte trapping on SPE cartridges were controlled using Hystar ver. 3.2 software (Bruker Daltonik, Bremen, Germany), whilst the elution process was controlled by Prep Gilson ST ver. 1.2 software (Bruker Biospin, Karlsruhe, Germany). 1D and 2D Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DRX-400 NMR spectrometer (Rheinstetten, Germany) equipped with either a 3 mm inverse broadband (BBI) probe or a 5 mm dual <sup>1</sup>H/<sup>13</sup>C probe using standard Bruker pulse sequences and operating at 400 MHz for <sup>1</sup>H and at 100 MHz for <sup>13</sup>C NMR spectra. The spectra were processed with Topspin version 4.0.6.

Accurate mass measurements were done using a Xevo-G2XS-QToF mass spectrometer (Waters) coupled with an ACQUITY LC system equipped with MassLynx version 4.1 software. A Waters Acquity UHPLC BEH SHIELD RP18 column (2.1 mm x 100 mm, 1.7 µm) was used with a mobile phase consisting of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), which were pumped at a rate of 0.4 mL/min. The gradient system was set as follows: 2% B (0-1 min), 2-100% B (1-5 min), 100% B (5-7 min), 100-2% B (7-8 min), 2% B (8-10 min). For all analyses, full scan data were recorded in ESI (-) and ESI (+) mode from *m/z* 50 to 1500 in sensitivity mode (approximate resolution: 22000 FWHM) using a spray voltage at either -0.8 kV and +1.0 kV, respectively. Cone gas flow and desolvation gas flow were set at 50.0 L/h and 1000.0 L/h, respectively; and source temperature and desolvation temperature at 120 °C and 550 °C, respectively. Leucine enkephalin was used as lock mass during the analysis.

### 6.2.3. Plant Material

Leaves of *Tetracera alnifolia* were collected in Telimélimé, Republic of Guinea in June 2017. The plant was identified by the botanists from the Research and Valorization Center on Medicinal Plants, Dubréka, Guinea where a voucher specimen (D42HK2) is stored. The collected leaves samples were dried at room temperature and milled.

#### **6.2.4. Extraction bioguided fractionation and isolation**

The dried and powdered leaves (780 g) of *Tetracera alnifolia* were firstly defatted by *n*-hexane (5 x 2.5 L), then extracted with 80% MeOH (5 x 2.5 L), at room temperature with magnetic stirring by renewing the solvent every 24 h during five days for each solvent. Pooled extracts were concentrated under reduced pressure and freeze-dried to obtain the hexane soluble fraction ( $T_E$ ) (12 g) and the crude methanolic fraction ( $T_M$ ) (207.0 g) which was redissolved in water and partitioned with  $\text{CH}_2\text{Cl}_2$  to give a dichloromethane soluble fraction ( $T_D$ ) (10 g). Based on the biological results, the active fractions (hexane, dichloromethane and methanol) were selected and further purified by open column chromatography, flash chromatography semi preparative HPLC-DAD-MS and LC-SPE-NMR.

#### **6.2.5. Purification of the hexane fraction**

The hexane fraction ( $T_E$ ) (9.3 g) was fractionated by flash chromatography using a silicagel column (GraceResolv 120 g), eluted with a gradient of hexane (A), dichloromethane (B) and ethyl acetate (C) at a flow rate of 40 mL/min. The gradient was set as follows: 100% A (0-15 min), followed by an increasing concentration of B (15-65 min) till 50% B, which was maintained for 5 min (65-70 min); then an increasing concentration of B (70-90 min) till 100% B; next the amount of C was increased till 100% (90-105 min); finally this condition was maintained for 10 min (105-115 min). The collected tubes were pooled in 6 fractions ( $T_{E1}$ - $T_{E6}$ ) based on similarity of their TLC profiles (mobile phase: hexane / dichloromethane: 1/9 v/v). The purification of fraction  $T_{E2}$  (2.3 g) by flash chromatography using a silicagel column (GraceResolv 40 g), eluted with a gradient of hexane (A) and dichloromethane (B) at a flow rate of 40 mL/min resulted in the isolation of compounds **1** (16 mg) and **2** (56 mg). The gradient was set as follows: 100% A (0-5 min), followed by an increasing concentration of B (5-55 min) till 20% B; then an increasing concentration of B (55-65 min) till 35% B; next the amount of B was increased till 100% (65-70 min); finally, this condition was maintained for 5 min (70-75 min).

Fraction T<sub>E3</sub> and T<sub>E4</sub> were subjected to TLC preparative (mobile phases: dichloromethane/ hexane: 1/1 v/v and Toluene 100% respectively). Fraction T<sub>E3</sub> afforded compound **3** (7.5 mg) while Fraction T<sub>E4</sub> yielded compounds **4** (3.2 mg) and **5** (8.2 mg).

#### 6.2.6. Purification of the dichloromethane fraction

The dichlorometane fraction (T<sub>D</sub>) (5 g) was subjected to a flash chromatography using a silicagel column (GraceResolv 80 g) eluted with a gradient of dichloromethane (A), ethyl acetate (B) and methanol (C) at a flow rate of 60 mL/min. The gradient was set as follows: 100% A (0-8 min), followed by an increasing concentration of B in A (8-48 min) till 100% B; then an increasing concentration of C in B (48-75 min) till 100% C and an increasing concentration of D in C (75-100 min) till 50% of D. This condition was finally maintained for 10 min (110 min). The collected tubes were pooled in 8 fractions (T<sub>D1</sub>-T<sub>D8</sub>) based on similarity of their TLC profiles (dichloromethane / ethyl acetate: 8/2 v/v). The purification of the fractions T<sub>D1</sub> (0.3 g) by TLC preparative (mobile phase: dichloromethane / hexane: 9/1 v/v) resulting in the isolation of compounds **6** (13.5mg) while fraction T<sub>D2</sub> yielded compound **7** (4.2 mg) and **8** (3.7 mg). In turn the purification of the fraction T<sub>D4</sub> (766,7mg) by flash chromatography using GraceResolv 40 g silica column eluted with a mixture of dichloromethane (A), ethyl acetate (B) and methanol (C) at a flow rate of 40 mL/min led to the isolation of compound **9** (14.6 mg), **10** (15.3 mg) and **11** (9.4 mg). The gradient was set as follows: 100% A (0-10 min), followed by increasing concentration of B till 100% (10-48 min), then the concentration of C was increased till 20% (48-78 min); finally, this condition was maintained for 10 min (78-88 min). The isolation of compound **12** (4.5 mg) from fraction T<sub>D5</sub> (0.3 g) was achieved by semi-preparative HPLC-MS using a C18 Kinetex column (250 mm × 10.0 mm, particle size, 5 µm) from Phenomenex (Utrecht, The Netherlands) and eluted with a gradient of H<sub>2</sub>O + 0.1% formic acid (A) and acetonitrile far UV (B) at a flow rate of 3 mL/min. The DAD spectrum was recorded from 200 to 450 nm, and mass spectra in the ESI (+) and ESI (-) modes, MS scan range: m/z 100 to 1000; capillary voltage 3.00 kV, cone voltage 50 V, extractor voltage 3 V, source temperature 135 °C, desolvation temperature 400 °C, desolvation gas flow 750 L/h, cone gas flow 50 L/h. The sample concentration was 15 mg/mL and 300 µL have been considered as injection volume. The Purification of the fraction T<sub>D7</sub> by LC-SPE-NMR allowed the isolation of compound **13** (1.3 mg). An Agilent Zorbax SB-phenyl column (4.6 x 250 mm, 5 µm) was used with a mobile phase consisting

of water + 0.1% formic acid (A) and acetonitrile (B), which were pumped at a flow rate of 1 mL/min. The following gradient (min/%B): 0.0/20, 5.0/20, 15.0/60, 30.0/96, 35.0/96, 36.0/20, 41.0/20 was used.

#### 6.2.7. Purification of the methanol fraction

The methanolic fraction ( $T_M$ ) (30 g) was dissolved in 3% of methanol and subjected to open column chromatography on Diaion HP-20 resin (450 g) using a stepwise gradient of 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90 and 100% methanol (1L for each step). Collected fractions ( $T_{M1}$ - $T_{M12}$ ) were concentrated under reduced pressure and freeze dried. The antibacterial, antifungal and antiplasmodial activities of all fractions were assessed and the most active fractions were further purified. Indeed, the purification of fraction ( $T_{M3}$ ) (305.0 mg) by reverse phase flash chromatography using a GraceResolv (12 g) Rp18 column eluted with a linear gradient of water (A) and acetonitrile (C) at a flow rate of 30 mL/min yielded three fractions. The gradient was set as follows: (0 min) 4% B, (0-30 min) 4% B, (30-50 min) 50% B, (50-90 min) 100% B and this condition was maintained for 10 min (90-100 min). The purification of fraction  $T_{M32}$  by LC-SPE-NMR yielded compound **14** (2 mg). A mobile phase consisting of water + 0.1% formic acid (A) and acetonitrile (B), which were pumped at a flow rate of 1 mL/min. The following gradient (min/%B): 0.0/20, 5.0/20, 15.0/60, 30.0/96, 35.0/96, 36.0/20, 41.0/20 was used.

In turn the purification of the fraction ( $T_{M9}$ ) by semi preparative HPLC-MS using a C18 Kinetex column (250 cm x 10 mm) and eluted with a mixture of acetonitrile and water yielded compounds **15** (8 mg) and **16** (7.6 mg). The mobile phase was set as follows: (0-5 min) 20% B, (5-40 min) 25% B, (40-50 min) 95% B, (50-55 min) 95% B.

Fractions  $T_{M9}$  and  $T_{M12}$  were further subjected to semi-preparative HPLC-MS analysis. Indeed, the purification of fraction  $T_{M9}$  (0.2 g) with the following gradient (0-5 min) 16% B, (5-40 min) 30% B, (40-50 min) 96% B, (50-55 min) 96% B, afforded compounds **17** (5 mg) and **18** (24 mg). The purification of fraction  $T_{M12}$  (0.25 g) with the gradient (0-5 min) 20% B, (5-60 min) 50% B, (60-70 min) 95% B, (70-75 min) 95% B yielded compounds **19** (3.5 mg). The flow rate was set at 4.75 mL/min for  $T_{M9}$  and  $T_{M10}$  while 3.0 mL/min was used for fraction  $T_{M12}$ . Sample concentration for all fractions was 20 mg/mL and the injection volume for fraction  $T_{M9}$  and  $T_{M12}$  was 300  $\mu$ L while for fraction  $T_{M12}$ , 400  $\mu$ L was injected.

### **6.2.8. LC-ESI-MS**

For the LC-MS analysis the extract was dissolved in 80% MeOH (v/v) at a concentration of 1 mg/mL, which was diluted with water in order to obtain a final concentration of 0.1 mg/mL. The final concentration of fractions was 0.05 mg/mL while that of pure compounds was maintained at 0.01mg/mL.

### **6.2.9. Biological evaluation**

#### **6.2.9.1. Antibacterial and antifungal activity**

The antimicrobial activity of all fractions and pure compounds was evaluated according to Cos et al. (2006) and Baldé et al. (2010). Fractions and pure compounds were tested against the following microorganisms: *Staphylococcus aureus* ATCC 6538 (Gram-positive) and *Candida albicans* ATCC59630 (yeast). The following positive controls were used: flucytosine for *C. albicans* ( $IC_{50}$   $0.70 \pm 0.01 \mu\text{M}$ ) (mean  $\pm$  standard deviation) and doxycycline for *S. aureus* ( $IC_{50}$   $0.28 \pm 0.20 \mu\text{M}$ ). These reference compounds are routinely used in the screening platform and their activities were in the range that is usually observed<sup>18,19</sup>.

#### **6.2.9.2. Antiplasmodial and cytotoxicity assays**

Antiplasmodial activity and cytotoxicity were assessed as previously described by Cos et al. (2006) and Tuenter et al. (2016). Fractions and pure compounds were tested *in vitro* against the chloroquine-resistant strain *Plasmodium falciparum* K1 using the lactate dehydrogenase assay. The most active compounds were tested in triplicate; mean and standard deviation (SD) were calculated. Tamoxifen was used as the positive control for cytotoxicity on MRC-5 cells ( $IC_{50}$   $10.0 \pm 1.5 \mu\text{M}$ ), and chloroquine ( $IC_{50}$   $0.15 \pm 0.10 \mu\text{M}$ ) for *P. falciparum*. These reference compounds are routinely used in the screening platform and their activities were in the range that is usually observed<sup>18,20</sup>.

### 6.2.9.3. Physicochemical and spectral data of isolated compounds

**Squalene (1).** Colorless oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$  400 MHz):  $\delta_{\text{H}}$  1.59 (s, Me-25, Me-26, Me-27, Me-28, Me-29 and Me-30), 1.66 (s, Me-1 and Me-24), 5.06 (m, H-3 and H-20), 5.08 (m, H-7 and H-18), 5.13 (m, H-13 and H-14).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz):  $\delta_{\text{C}}$  25.9 (C-1), 131.4 (C-2), 124.4 (C-3), 26.9 (C-4), 40.0 (C-5), 135.1 (C-6), 124.5 (C-7), 26.9 (C-8), 39.9 (C-9), 135.2 (C-10), 124.6 (C-11), 28.5 (C-12), 28.5 (C-13), 124.6 (C-14), 135.2 (C-15), 39.9 (C-16), 26.9 (C-17), 124.5 (C-18), 135.1 (C-19), 40.0 (C-20), 26.8 (C-21), 124.4 (C-22), 131.4 (C-23), 25.9 (C-24), 17.8 (C-25), 16.2 (C-26), 16.2 (C-27), 16.2 (C-28), 16.2 (C-29), 17.8 (C-30).

**Cycloart-24-en-3 $\beta$ -yl  $\alpha$ -linolenate (2).** Colorless gum;  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ ):  $\delta_{\text{H}}$  4.54 (dd,  $J=4.63$ ; 11.69 Hz, H-3), 0.54 and 0.31 (d,  $J=4.13$  Hz, 2H-19), 1.38 and 1.35 (d,  $J=4.27$  Hz, H-5), 1.50 and 1.47 (d,  $J=4.89$  Hz, 1H-8), 5.07 (m, H-24).  $^{13}\text{C-NMR}$  ( $\text{CDCl}_3$ ): 31.6 (C-1), 26.8 (C-2), 80.2 (C-3), 39.5 (C-4), 47.2 (C-5), 20.9 (C-6), 28.1 (C-7), 47.8 (C-8), 20.1 (C-9), 25.9 (C-10), 25.8 (C-11), 35.6 (C-12), 45.2 (C-13), 48.8 (C-14), 32.8 (C-15), 26.5 (C-16), 52.3 (C-17), 18.0 (C-18), 29.8 (C-19), 35.9 (C-20), 18.3 (C-21), 36.4 (C-22), 24.9 (C-23), 125.3 (C-24), 130.7 (C-25), 25.7 (C-26), 17.6 (C-27), 173.4 (C-1'), 34.8 (C-2'), 25.1 (C-3'), 29.2 (C-4'), 29.1 (C-5'), 29.2 (C-6'), 29.6 (C-7'), 27.2 (C-8'), 130.2 (C-9'), 128.2 (C-10'), 25.6 (C-11') 127.7 (C-12'), 128.2 (C-13'), 25.5 (C-14'), 127.7 (C-15'), 131.9 (C-16'), 20.5 (C-17'), 14.3 (C-18').

**$\alpha$ -Tocopherol (3).** Colorless oil;  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ ):  $\delta_{\text{H}}$  2.15 (3H, s, Me-8a), 2.10 (3H, s, Me-5a), 2.10 (3H, s, Me-7a), 1.22 (3H, s, Me-2a), 0.87 (3H, s, Me-22a), 0.87 (3H, s, Me-22b), 0.85 (3H, s, Me-14a), 0.85 (3H, s, Me-18a), 1.52 (1H, m, H-22), 1.39 (2H, m, H-14 and H-118).  $^{13}\text{C-NMR}$  ( $\text{CDCl}_3$ ):  $\delta_{\text{C}}$  74.5 (s, C-2), 31.5 (d, C-3), 20.7 (d, C-4), 121.0 (s, C-5), 11.3 (q, C-5a), 144.5 (s, C-6), 117.3 (s, C-7), 11.8 (q, C-7a), 118.5 (s, C-8), 12.2 (q, C-8a), 122.6 (s, C-9), 145.5 (s, C-10), 39.8 (t, C-11), 21.0 (t, C-12), 37.5 (t, C-13), 32.8 (d, C-14), 19.6 (q, C-14a), 37.3 (t, C-15), 24.4 (t, C-16), 37.5 (t, C-17), 32.8 (d, C-18), 19.7 (q, C-18a), 37.3 (t, C-19), 24.8 (t, C-20), 39.4 (t, C-21), 27.9 (d, C-22a), 22.6 (q, C-22), 22.7 (q, C-22b).

**Trans-pentamethyl-icosa-tetraene (4).** Colorless oil;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ , 400 MHz):  $\delta_{\text{H}}$  1.57 (s, Me-25, Me-26, Me-27, Me-28, Me-29 and Me-30), 1.65 (s, Me-1 and Me-24), 5.05 (m, H-3 and H-20), 5.07 (m, H-7 and H-18), 5.09 (m, H-13 and H-14).  $^{13}\text{C NMR}$  ( $\text{CDCl}_3$ , 100 MHz):  $\delta_{\text{C}}$  25.9 (C-1), 131.4 (C-2), 124.4 (C-3), 26.9 (C-4), 40.0 (C-5), 135.1 (C-6), 124.5 (C-7), 26.9 (C-8), 39.9 (C-9), 135.2 (C-10), 124.6 (C-11), 28.5 (C-12), 28.5 (C-13), 124.6 (C-14),

135.2 (C-15), 39.9 (C-16), 26.9 (C-17), 124.5 (C-18), 135.1 (C-19), 40.0 (C-20), 26.8 (C-21), 124.4 (C-22), 131.4 (C-23), 25.9 (C-24), 17.8 (C-25), 16.2 (C-26), 16.2 (C-27), 16.2 (C-28), 16.2 (C-29), 17.8 (C-30).

**3- $\beta$ -Hydroxy-olean-12-ene-heptadecanoate (5).** Pale yellow oil;  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta_{\text{H}}$  0.65 (3H, s, Me-26), 0.78 (3H, s, Me-23), 0.78 (3H, s, Me-24), 0.78 (3H, s, Me-28), 0.80 (3H, s, Me-17'), 0.82 (3H, s, Me-30), 0.85 (3H, s, Me-25), 0.85 (3H, s, Me-29), 1.05 (3H, s, Me-27), 5.21 (1H, brs, H-12), 4.43 (1H, m, H-3);  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ , 100 MHz): 38.3 (C-1), 23.3 (C-2), 80.8 (C-3), 37.9 (C-4), 55.5 (C-5), 18.4 (C-6), 32.8 (C-7), 39.4 (C-8), 47.7 (C-9), 37.1 (C-10), 23.6 (C-11), 122.5 (C-12), 144.0 (C-13), 48.1 (C-14), 27.9 (C-15), (C-16), 39.7 (C-17), 41.5 (C-18), 46.0 (C-19), 30.8 (C-20), 35.1 (C-21), 38.3 (C-22), 16.98 (C-23), 28.3 (C-24), 15.6 (C-25), 17.0 (C-26), 26.1 (C-27), 28.3 (C-28), 23.8 (C-29), 33.3 (C-30), 173.9 (C-1'), 35.9 (C-2'), 25.4 (C-3'), 29.7 (C-4'), 29.9 (C-5'), 29.9 (C-6'), 29.9 (C-7'), 29.8 (C-8'), 29.8 (C-9'), 29.7 (C-10'), 29.7 (C-11'), 29.7 (C-12'), 29.6 (C-13'), 29.5 (C-14') 29.4 (C-15'), 25.4 (C-16'), 14.4 (C-17').

**Phytol (6).** Colorless oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta_{\text{H}}$  5.36 (1H, td,  $J$  = 6.9, 1.2 Hz, H-2), 4.11 (2H, d,  $J$  = 6.9 Hz, H-1), 1.95 (2H, m, H-4), 1.63 (3H, bs, H-20);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz):  $\delta_{\text{C}}$  59.3 ( $\text{CH}_2$ , C-1), 123.1 ( $\text{CH}$ , C-2), 140.1 (C, C-3), 16.1 ( $\text{CH}_3$ , C-5), 39.8 ( $\text{CH}_2$ , C-6), 25.1 ( $\text{CH}_2$ , C-7), 36.6 ( $\text{CH}_2$ , C-8), 32.6 ( $\text{CH}$ , C-9), 19.7 ( $\text{CH}_3$ , C-10), 37.4 ( $\text{CH}_2$ , C-11), 24.4 ( $\text{CH}_3$ , C-12), 37.3 ( $\text{CH}_2$ , C-13), 32.7 ( $\text{CH}$ , C-14), 19.7 ( $\text{CH}_3$ , C-15), 37.2 ( $\text{CH}_2$ , C-16), 24.8 ( $\text{CH}_2$ , C-17), 39.3 ( $\text{CH}_2$ , C-18), 27.9 ( $\text{CH}$ , C-19), 22.7 ( $\text{CH}_2$ , C-21), 22.6 ( $\text{CH}_3$ , C-20).

**Isophytol (7).** Colorless oil.  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta_{\text{H}}$  5.90 (1H, dd,  $J$  = 10.7, 17.4 Hz, H-2), 5.15–5.20 (1H, dd,  $J$  = 1.27, 17.4 Hz, H-1a), 5.0–5.03 (1H, dd,  $J$  = 1.27, 10.7 Hz, H-1b), 1.25 (3H, br-s, H-20); The position of the other saturated methylene protons (total 16H); 0.85 (6H, s, H-16,17), 0.83 (3H, s, H-19), 0.83 (3H, s, H-18).  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ , 100 MHz)  $\delta_{\text{H}}$  145.3 (C-2), 111.5 (C-1), 73.3 (C-3), 42.7 (C-4), 39.4 (C-14), 37.4 (C-12), 37.4 (C-10), 37.4 (C-8), 37.3 (C-6), 32.8 (C-7), 29.7 (C-15), 27.9 (C-20), 24.8 (C-13), 24.5 (C-9), 22.7 (C-16), 22.6 (C-17), 21.3 (C-5), 19.7 (C-18), 19.6 (C-19).

**(1, 2) Bis-nor-phytane (8).** Colorless oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta_{\text{H}}$  2.38 (2H, br t,  $J$  = 7.68 Hz, H-2), 2.11 (3H, s, H-18), 1.50 (1H, m, H-13), 0.85 (3H,  $J$  = 6.6 Hz), 0.84 (3H,  $J$  = 6.5 Hz), 0.83 (3H,  $J$  = 6.6 Hz), 0.80 (3H,  $J$  = 6.6 Hz).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz):  $\delta_{\text{C}}$  209.5 (C-1),

44.2 (C-2), 21.4 (C-3), 36.5 (C-4), 32.8 (C-5), 37.3 (C-6), 24.4 (C-7), 37.4 (C-8), 32.7 (C-9), 37.2 (C-10), 24.8 (C-11), 39.4 (C-12), 27.8 (C-13), 22.6 (C-14), 22.7 (C-15), 19.6 (C-16), 19.8 (C-17), 29.9 (C-18).

*Phaeophorbide-a methyl ester (9)* black-green powder;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta_{\text{H}}$  9.29 (1H, s, H-5), 9.47 (1H, s, H-10), 8.61 (1H, s, H-20), 7.90 (1H, dd,  $J = 11.47$ ; 17.77 Hz, H-3<sup>1</sup>), 6.28 (1H, s, H-13<sup>2</sup>), 6.25 (1H, dd,  $J = 17.6$  Hz, H-3<sup>2a</sup>), 6.15 (1H, dd,  $J = 11.64$  Hz, H-3<sup>2b</sup>), 4.49 (1H, q,  $J = 7.5$  Hz, H-18), 4.23 (1H, br d,  $J = 8.5$  Hz H-17), 3.89 (3H, s, C-13<sup>3</sup>, -OMe), 3.59 (3H, s, C-17<sup>3</sup>, -OMe), 3.66 (3H, s, H3-12<sup>1</sup>), 3.59 (2H, q,  $J = 7.5$  Hz, H-8<sup>1</sup>), 3.36 (3H, s, H3-2<sup>1</sup>), 3.12 (3H, s, H3-7<sup>1</sup>), 2.66 (1H, m, H-17<sup>1a</sup>), 2.55 (1H, m, H-17<sup>2a</sup>), 2.32 (1H, m, H-17<sup>1b</sup>), 2.26 (1H, m, H-17<sup>2b</sup>), 1.84 (3H, d,  $J = 7.25$  Hz, H3-18<sup>1</sup>), 1.63 (3H, t,  $J = 7.5$  Hz, H3-8<sup>2</sup>).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz):  $\delta_{\text{C}}$  189.6 (C13<sup>1</sup>), 173.4 (C-17<sup>3</sup>), 172.4 (C-19), 169.6 (C-13<sup>3</sup>), 161.7 (C-16), 154.4 (C-6), 151.0 (s, C-9), 149.9 (C14), 145.0 (C-8), 142.2 (C-1), 137.9 (C-11), 136.3 (C-4), 135.9 (C-7), 136.6 (C-3), 132.1 (C-2), 129.0 (C-12), 128.9 (C-3<sup>1</sup>), 128.9 (C-13), 122.9 (CH<sub>2</sub>, C-3<sup>2</sup>), 105.3 (C-15), 104.4 (CH, C-10), 97.5 (CH, C-5), 93.5 (CH, C-20), 64.8 (d, C-13<sup>2</sup>), 52.9 (CH<sub>3</sub>, C-13<sup>3</sup>, -OMe), 51.8 (CH<sub>3</sub>, C-17<sup>3</sup>, -OMe), 51.2 (CH, C-17), 50.2 (CH, C-18), 31.3 (CH<sub>2</sub>, C17<sup>2</sup>), 29.8 (CH<sub>2</sub>, C-17<sup>1</sup>), 23.2 (CH<sub>3</sub>, C-18<sup>1</sup>), 19.4 (CH<sub>2</sub>, C-8<sup>1</sup>), 17.4 (CH<sub>3</sub>, C-8<sup>2</sup>), 12.1 (CH<sub>3</sub>, C-2<sup>1</sup>), 12.2 (CH<sub>3</sub>, C-12<sup>1</sup>), 11.2 (CH<sub>3</sub>, C-7<sup>1</sup>).

*Phaeophorbide-b methyl ester (10)* brown powder,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 10.92 (1H, s, H-7<sup>1</sup>), 10.13 (1H, s, H-5), 9.38 (1H, s, H-10), 8.53 (1H, s, H-20), 7.89 (1H, dd,  $J = 11.57$ ; 17.8 Hz, H-3<sup>1</sup>), 6.21 (1H, s, H-13<sup>2</sup>), 6.33 (1H, d,  $J = 17.8$  Hz, H-3<sup>2a</sup>), 6.19 (1H, d,  $J = 11.7$  Hz, H-3<sup>2b</sup>), 4.44 (1H, m, H-18), 4.17 (1H, m, H-17), 3.9 (3H, s, C-13<sup>2</sup>, -OMe), 3.59 (3H, s, C-17<sup>3</sup>, -OMe), 3.50 (3H, s, H3-12<sup>1</sup>), 3.78 (2H, q,  $J = 7.2$  Hz, H-8<sup>1</sup>), 3.33 (3H, s, H3-2<sup>1</sup>), 2.56 (1H, m, H-17<sup>1a</sup>), 2.66 (1H, m, H-17<sup>2a</sup>), 2.28 (1H, m, H-17<sup>1b</sup>), 2.33 (1H, m, H-17<sup>2b</sup>), 1.83 (3H, d,  $J = 7.31$  Hz, H3-18<sup>1</sup>), 1.68 (3H, t,  $J = 7.45$  Hz, H3-8<sup>2</sup>).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta_{\text{C}}$  189.6 (C13<sup>1</sup>), 173.4 (C-17<sup>3</sup>), 174.2 (C-19), 169.4 (C-13<sup>3</sup>), 164.4 (C-16), 150.7 (C-6), 146.5 (s, C-9), 150.9 (C14), 159.0 (C-8), 143.8 (C-1), 138.0 (C-11), 137.9 (C-4), 132.5 (C-7), 137.3 (C-3), 132.5 (C-2), 132.8 (C-12), 128.7 (C-3<sup>1</sup>), 129.8 (C-13), 123.8 (CH<sub>2</sub>, C-3<sup>2</sup>), 105.2 (C-15), 106.9 (CH, C-10), 101.5 (CH, C-5), 93.7 (CH, C-20), 64.7 (d, C-13<sup>2</sup>), 53.2 (CH<sub>3</sub>, C-13<sup>2</sup>, -OMe), 51.9 (CH<sub>3</sub>, C-17<sup>3</sup>, -OMe), 51.5 (CH, C-17), 50.3 (CH, C-18), 29.2 (CH<sub>2</sub>, C17<sup>2</sup>), 31.3 (CH<sub>2</sub>, C-17<sup>1</sup>), 23.3 (CH<sub>3</sub>, C-18<sup>1</sup>), 19.5 (CH<sub>2</sub>, C-8<sup>1</sup>), 19.5 (CH<sub>3</sub>, C-8<sup>2</sup>), 12.3 (CH<sub>3</sub>, C-2<sup>1</sup>), 12.4 (CH<sub>3</sub>, C-12<sup>1</sup>), 187.6 (C, C-7<sup>1</sup>).

*Stigma-5-en-3-O-β-glucoside (11)*.  $^1\text{H-NMR}$  ( $\text{DMSO-d}_6$ ): 0.64 (s, 3H-18), 0.95 (s, 3H-19), 0.89 (d,  $J = 6.51$  Hz, 3H-21), 0.79 (d,  $J = 7.05$ , 3H-26), 0.80 (d,  $J = 6.8$ , 3H-27), 0.81 (d,  $J =$

6.83, 3H-29), 3.44 (m, H-3), 5.31 (d,  $J$  = 4.8 Hz, H-6), 1.37 (d,  $J$  = 4.5 Hz, H-8), 0.89 (d,  $J$  = 6.51 Hz, H-9), 0.95 (s, H-14), 1.07 (d,  $J$  = 9.9 Hz, H-17), 1.34 (m, H-20), 0.90 (d,  $J$  = 6.49 Hz H-24), 1.63 (m, H-25), 4.22 (d,  $J$  = 7.80 Hz H-1'), 2.89 (dt,  $J$  = 4.55; 8.15 Hz H-2'), 3.12 (m, H-3'), 3.01 (m, H-4'), 3.06 (dd,  $J$  = 1.97; 5.90 Hz, H-5').  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ ): 36.9 (C-1), 29.3 (C-2), 76.8 (C-3), 38.4 (C-4), 140.5 (C-5), 121.4 (C-6), 31.4 (C-7), 31.5 (C-8), 49.6 (C-9), 36.3 (C-10), 20.6 (C-11), 39.17 overlapping with solvent (C-12), 40.8 (C-13), 56.2 (C-14), 23.9 (C-15), 27.9 (C-16), 55.4 (C-17), 11.7 (C-18), 19.0 (C-19), 35.5 (C-20), 18.7 (C-21), 33.4 (C-22), 25.45 (C-23), 45.2 (C-24), 28.7 (C-25), 18.55 (C-26), 19.83 (C-27), 28.7 (C-27), 22.6 (C-28), 11.7 (C-29), 108 (C-1'), 73.5 (C-2'), 77.0 (C-3'), 70.2 (C-4'), 76.8 (C-5').

*Vanillic acid (12)*. colorless needles,  $^1\text{H}$ -NMR ( $\text{CD}_3\text{OD}$ ):  $\delta$  6.84 (d,  $J$  = 8.79 Hz, H-5), 7.56 (d,  $J$  = 8.79 Hz, H-2), 7.55 (dd,  $J$  = 1.88; 8.79 Hz, H-6), 3.88 (s,  $\text{OCH}_3$ ).  $^{13}\text{C}$ -NMR ( $\text{CD}_3\text{OD}$ )  $\delta$ : 123.4 (C-1), 113.7 (C-2), 148.7 (C-3), 152.7 (C-4), 115.8 (C-5), 125.3 (C-6), 170.0 (C-7), 56.4 (-OMe).

*Vanillic acid (12)*. colorless needles,  $^1\text{H}$ -NMR ( $\text{CD}_3\text{OD}$ ):  $\delta$  6.84 (d,  $J$  = 8.79 Hz, H-5), 7.56 (d,  $J$  = 8.79 Hz, H-2), 7.55 (dd,  $J$  = 1.88; 8.79 Hz, H-6), 3.88 (s,  $\text{OCH}_3$ ).  $^{13}\text{C}$ -NMR ( $\text{CD}_3\text{OD}$ )  $\delta$ : 123.4 (C-1), 113.7 (C-2), 148.7 (C-3), 152.7 (C-4), 115.8 (C-5), 125.3 (C-6), 170.0 (C-7), 56.4 (-OMe).

*Abscisic acid (13)*. White oil,  $^1\text{H}$  NMR ( $\text{CD}_3\text{CN}$ , 400 MHz):  $\delta_{\text{H}}$  5.71 (1H, s, H-2), 7.78 (1H, d,  $J$  = 15.95 Hz, H-4), 6.24 (1H, d,  $J$  = 16.2 Hz, H-5), 5.82 (1H, s, H-3'), 3.26 (2H, brs, H-5'), 1.99 (3H, s, H-6), 1.83 (3H, s, H-7'), 1.01 (3H, s, H-8'), 0.95 (3H, s, H-9').  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{CN}$ , 100 MHz).  $\delta_{\text{C}}$  170.6 (C-1), 119.2 (C-2), 164.2 (C-3), 129.2 (C-4), 138.6 (C-5), 21.9 (C-6), 80.8 (C-1'), 151.5 (C-2'), 128.1 (C-3'), 198.9 (C-4'), 50.9 (C-5'), 42.8 (C-6'), 19.8 (C-7'), 24.1 (C-8'), 25.2 (C-9').

*Gallic acid (14)*. White, needle.  $^1\text{H}$ -NMR ( $\text{CD}_3\text{OD}$ ):  $\delta_{\text{H}}$  7.07 (s, 2 H).  $^{13}\text{C}$ -NMR ( $\text{CD}_3\text{OD}$ ):  $\delta_{\text{C}}$  110.3 (C-2 and C-6), 121.9 (C-1), 139.5 (C-4); 146.3 (C-3 and C-5), 169.8 (C-7).

*Myricetin-3-O-rhamnoside (15)*. Pale yellow powder ; HR-ESI-MS  $m/z$  463.0883 [M-H]<sup>-</sup> (calculated for  $\text{C}_{21}\text{H}_{19}\text{O}_{12}$ , 463.0877,  $\Delta$  -1.29 ppm),  $^1\text{H}$  NMR ( $\text{DMSO-d}_6$ ):  $\delta_{\text{H}}$  0.83 (3H, d,  $J$  = 6.20 Hz), 5.19 (1H, d,  $J$  = 1.17 Hz), 6.19 (1H, d,  $J$  = 1.95 Hz), 6.36 (1H, d,  $J$  = 1.95 Hz), 6.88 (2H, s), 3.97 (1H, dd,  $J$  = 1.58; 3.04 Hz), 3.56 (1H, dd,  $J$  = 3.29; 9.38 Hz), 3.15 (1H, t,  $J$  = 9.37), 3.36 (1H, m).  $^{13}\text{C}$  NMR ( $\text{DMSO-d}_6$ )  $\delta$ : 157.5 (C-2), 134.3 (C-3), 177.8 (C-4), 161.3 (C-5), 98.7 (C-6), 164.4 (C-7), 93.6 (C-8), 156.4 (C-9), 104.0 (C-10), 119.6 (C-1'), 107.9 (C-2'),

145.8 (C-3'), 136.4 (C-4'), 145.8 (C-5'), 107.9 (C-6'); 3-O-Rhamnose: 101.9 (C-1''), 70.0 (C-2''), 70.3 (C-3''), 71.2 (C-4''), 70.6 (C-5''), 17.6 (C-6'').

*Quercetin-3-O-rhamnoside (16).* Pale yellow powder; HR-ESI-MS  $m/z$  447.0930 [M-H]<sup>-</sup> (calculated for C<sub>21</sub>H<sub>19</sub>O<sub>11</sub>, 447.0927,  $\Delta$  -0.67 ppm), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$ : 0.82 (3H, d,  $J$  = 6.02 Hz), 5.22 (1H, d,  $J$  = 1.25 Hz), 6.19 (1H, d,  $J$  = 1.88 Hz), 6.38 (1H, d,  $J$  = 1.88 Hz), 6.87 (1H, d,  $J$  = 8.32 Hz), 7.26 (1H, dd,  $J$  = 2.1; 8.28 Hz), 7.30 (1H, d,  $J$  = 2.1 Hz). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$ : 157.1 (C-2), 134.1 (C-3), 177.7 (C-4), 161.3 (C-5), 98.8 (C-6), 164.8 (C-7), 93.7 (C-8), 156.5 (C-9), 103.8 (C-10), 120.6 (C-1'), 115.4 (C-2'), 145.3 (C-3'), 148.6 (C-4'), 115.6 (C-5'), 121.05 (C-6'); 3-O-Rhamnose: 101.8 (C-1''), 70.3 (C-2''), 70.5 (C-3''), 71.2 (C-4''), 70.0 (C-5''), 11.5 (C-6'').

*Myricetin-3',5'dimethylether-3-O-Galactopyranoside (17).* yellow powder, <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$ <sub>H</sub> 6.16 (1H, s,), 6.34 (1H, s), 7.19 (1H, s), 3.45 (1H, m), 3.28 (1H, m). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$ <sub>C</sub> 156.0 (C-2), 133.8 (C-3), 177.3 (C-4), 161.1 (C-5), 98.4 (C-6), 164.4 (C-7), 94.1 (C-8), 156.0 (C-9), 103.6 (C-10), 119.7 (C-1'), 108.5 (C-2'), 145.3 (C-3'), 136.6 (C-4'), 145.3 (C-5'), 108.5 (C-6'); 3-O-Glucose: 101.7 (C-1''), 73.1 (C-2''), 71.1 (C-3''), 68.7 (C-4''), 75.7 (C-5''), 59.8 (C-6'').

*Quercetin-3-O-galactopyranoside (18).* yellow powder; HR-ESI-MS  $m/z$  463.0882 [M-H]<sup>-</sup> (calculated for C<sub>21</sub>H<sub>19</sub>O<sub>12</sub>, 463.0877,  $\Delta$  1.07 ppm), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$ <sub>H</sub> 6.16 (1H, s,), 6.37 (1H, s), 6.79 (1H, d,  $J$  = 1.95 Hz), 7.51 (1H, d,  $J$  = 2.1 Hz), 7.65 (1H, dd,  $J$  = 2.1, 8.5 Hz), 3.44 (1H, m), 3.29 (1H, m). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$ <sub>C</sub> 156.5 (C-2), 133.8 (C-3), 165.6 (C-4), 156.7 (C-5), 99.3 (C-6), 161.1 (C-7), 94.1 (C-8), 156.8 (C-9), 104.1 (C-10), 121.5 (C-1'), 122.4 (C-2'), 116.0 (C-3'), 145.3 (C-4'), 149.0 (C-5'), 115.6 (C-6'); 3-O-Glucose: 102.3 (C-1''), 71.6 (C-2''), 73.5 (C-3''), 68.4 (C-4''), 76.2 (C-5''), 60.6 (C-6'').

*Epicatechin-3-galloylester (19).* yellow powder; HR-ESI-MS  $m/z$  441.0824 [M-H]<sup>-</sup> (calculated for C<sub>22</sub>H<sub>17</sub>O<sub>10</sub>, 441.0822,  $\Delta$  0.45 ppm) <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$ <sub>H</sub> 6.85 (1H, s,), 6.81 (1H, s), 6.73 (1H, s), 5.93 (1H, s), 5.83 (1H, s), 5.33 (1H, s), 5.24 (1H, s), 5.06 (1H, s), 5.02 (1H, s), 5.24 (1H, s). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$ <sub>C</sub> 77.2 (C-2), 68.2 (C-3), 25.6 (C-4), 156.4 (C-5), 94.4 (C-6), 155.5 (C-7), 95.4 (C-8), 156.4 (C-9), 97.3 (C-10), 129.3 (C-1'), 117.7 (C-2'), 115.0 (C-3'), 144.9 (C-4'), 144.9 (C-5'), 114.2 (C-6'); 3-O-Galloyl ester: 165.3 (C-1''), 108.7 (C-2''), 145.5 (C-3''), 138.5 (C-4''), 145.5 (C-5''), 108.7 (C-6'').

### 6.3. Results and Discussion

Preliminary biological studies have shown that extracts from the leaves of *T. alnifolia* were active against *Plasmodium falciparum* and *Candida albicans*. In this study, a classical activity-guided fractionation approach was used in order to characterize the active metabolites of *T. alnifolia* leaves. The most active fractions (hexane, dichloromethane and methanol) against *P. falciparum* and *Candida albicans* (Table 6.1) were subjected to normal-phase and reverse-phase flash chromatography and semipreparative HPLC with DAD and ESIMS detection to afford 22 compounds. Five compounds were isolated from the hexane fraction, 8 compounds were isolated from the dichloromethane fraction and 9 compounds from the methanol fraction. Chemical structures of the isolated compounds were established by HR-ESI-MS combined with 1D NMR ( $^1\text{H}$ ,  $^{13}\text{C}$ -NMR, DEPT 135, DEPT 90) and 2D NMR (COSY, HSQC, HMBC). The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectroscopic data of these compounds were in agreement with previously published assignments, allowing the identification of squalene (**1**)<sup>21,22</sup>, cycloart-24-en-3 $\beta$ -yl  $\alpha$ -linolenate (**2**)<sup>23</sup>,  $\alpha$ -tocopherol (**3**)<sup>24,25</sup>, *trans*-pentamethyl-icosa-tetraene (**4**)<sup>26</sup>, 3- $\beta$ -hydroxy-olean-12-ene-heptadecanoate (**5**), phytol (**6**)<sup>27</sup>, iso-phytol (**7**)<sup>28</sup>, (1, 2)-bis-nor-phytane (**8**)<sup>29</sup>, pheophorbide-a methyl ester (**9**)<sup>30,31</sup>, pheophorbide-b methyl ester (**10**)<sup>32</sup>, stigma-5-en-3-O- $\beta$ -glucoside (**11**)<sup>33</sup>, vanillic acid (**12**)<sup>34</sup>, abscisic acid (**13**)<sup>35,36</sup>, gallic acid (**14**)<sup>37</sup>, myricetin-3-O-*rhamnoside* (**15**)<sup>38</sup>, quercetin-3-O-*rhamnoside* (**16**)<sup>39</sup>, myricetin-3',5'dimethylether-3-O-galactopyranoside (**17**)<sup>40</sup>, quercetin-3-O- $\beta$ -D-galactopyranoside (**18**)<sup>41</sup> and epicatechin-3-galloylester (**19**)<sup>42</sup>. The antiplasmodial activity of most of these compounds has been evaluated and the highest activity was obtained for pheophorbide-b methyl ester ( $1.0 \pm 0.7 \mu\text{M}$ ), (1,2)-bis-nor-phytane ( $2.0 \mu\text{M}$ ), isophytol ( $4.0 \mu\text{M}$ ), pheophorbide-a methyl ester ( $2.8 \pm 1.2 \mu\text{M}$ ), epicatechin-3-galloylester ( $5.5 \pm 2.1 \mu\text{M}$ ) and phytol ( $6.9 \pm 2.4 \mu\text{M}$ ) (Table 6.2). Apart from myricetin-3-O-rhamnopyranoside, ( $14.3 \mu\text{M}$ ),  $\alpha$ -tocopherol ( $13.5 \mu\text{M}$ ), cycloart-24-en-3 $\beta$ -yl  $\alpha$ -linolenate ( $25.0 \mu\text{M}$ ), which gave weak antiplasmodial activity, all other tested compounds were completely inactive.

The antimalarial activity of pheophorbide-a methyl ester (**9**) ( $2.8 \pm 1.2 \mu\text{M}$ ) found in our study is comparable to that previously reported by Wael et al. (2014) against a chloroquine-resistant *Plasmodium falciparum* strain ( $\text{IC}_{50} 3.9 \mu\text{g/mL}$ ). Moreover, the antiplasmodial activity found for this compound was slightly higher than for pheophorbide a ( $\text{IC}_{50} 6.7 \mu\text{g/mL}$ ) and its derivative hydroxylated at C-13 ( $\text{IC}_{50} 5.1 \mu\text{g/mL}$ ),

previously isolated from *Mezoneuron benthamianum*<sup>44</sup>. We can therefore note that the hydroxylation of C-13 and the esterification of the carboxylic acid function carried at C-17 result in the improvement of the antiplasmoidal activity of pheophorbide a. Pheophorbide derivatives are considered as breakdown products that are structurally related to chlorophyll, which are formed following a loss of the phytol chain and the central magnesium atom of the tetrapyrrole macrocycle of chlorophyll a<sup>44,45</sup>. Even though these compounds (pheophorbide-b methyl ester and pheophorbide-a methyl ester) have been previously isolated from several plant extracts including *Piper penangense*, *Clerodendrum species* and *Garuga pinnata*, this is the first report of these compounds in *T. alnifolia*<sup>46,32</sup>.

Pheophorbide-b methyl ester (**10**), which differs from its counterpart (pheophorbide-a methyl ester) by the presence of an aldehydic function at C-7 was effective against *P. falciparum* ( $1.0 \pm 0.7 \mu\text{M}$ ). The pheophorbide derivatives are used both for malaria vector control and for the elimination of *Plasmodium falciparum* through a photosensitization effect. In another antiplasmoidal study, the photosensitization and red light illumination of synthetic lipophilic pheophorbide derivatives eradicated *Plasmodium falciparum*. Moreover, it has been reported that photosensitization with pheophorbide derivatives could be a promising approach for inactivation of transfusion-transmissible parasites and viruses in blood bank units<sup>47</sup>.

The polyphenolic compound epicatechin-3-galloylester (**19**) ( $\text{IC}_{50} 5.5 \pm 2.1 \mu\text{M}$ ) has previously been isolated from *Camellia cinensis*<sup>48</sup> and *Sclerocarya birrea*<sup>42</sup>. The antiplasmoidal activity of epicatechin-3-galloylester has been previously reported against *P. falciparum* chloroquine sensitive (3D7) ( $10.8 \mu\text{M}$ ) and chloroquine-resistant (FCR-1/FVO) ( $7.2 \mu\text{M}$ ) strains<sup>48</sup>.

Phytol (**6**) and two of its derivatives (isophytol and (1,2)-bis-nor-phytane) were among the most active compounds found in our studies. Being a part of the chlorophyll molecule, phytol is a compound abundantly found in nature where it is produced by almost all photosynthetic organisms<sup>49</sup>. The antiplasmoidal activity of this compound was previously reported against *Plasmodium falciparum* chloroquine sensitive strain (D10) ( $\text{IC}_{50} 5.60 \pm 0.18 \mu\text{g/mL}$ ). Furthermore, previous bioguided fractionation has also identified phytol as one of the most active compounds against both *P. falciparum* chloroquine resistant PoW

(8.5  $\mu\text{M}$ ) and chloroquine sensitive Dd2 (11.5  $\mu\text{M}$ ) strains<sup>50</sup>. Although phytol and its derivatives isophytol (**7**) and (1,2)-bis-nor-phytene (**8**) have been reported in other plant species such as *Calotropis procera*<sup>51</sup>, *Glycine hispida*<sup>52</sup>, *Pellia epiphylla*, *Microglossa pyrifolia*<sup>53</sup>, *Brassica oleracea*<sup>54</sup> and *Strobilanthes crispus*<sup>55</sup>, to the best of our knowledge, this is the first time that these compounds were isolated from *T. alnifolia*, and that antiplasmodial activity was reported for isophytol and (1,2)-bis-nor-phytene. Isophytol is generally considered as an intermediate in the degradation of the chlorophyll phytol side chain and has been reported in the essential oil of several terrestrial plants<sup>56</sup>. It is important to stress that the antiplasmodial activity of phytol (6.9  $\pm$  2.4  $\mu\text{M}$ ) is slightly lower than that found for isophytol (4.0  $\mu\text{M}$ ) which in turn was less active than bis-nor-phytene (2.0  $\mu\text{M}$ ). This shows therefore, that the position of the double bond and its oxidation could have an influence on the antiplasmodial activity of these compounds.

In order to determine the specificity of the antiplasmodial activity of the isolated compounds, their cytotoxicity on MRC-5 cells was evaluated. Apart from pheophorbide-a methyl ester (SI 1.7), pheophorbide-b methyl ester (SI 1.6) and phytol (SI 1.3), all other compounds did not show any cytotoxicity up to the highest concentration tested (>64  $\mu\text{M}$ ) (Table 6.2). Several studies have previously reported the cytotoxicity of pheophorbide derivatives. Indeed a number of the photosensitizers that have been investigated for clinical photodynamic therapy (PDT) of cancer are constituents from plants. Some examples including degradation derivatives of chlorophylls such as pheophorbides, which are present in green leaves, are well-studied photosensitizers in photodynamic cancer therapy<sup>46</sup>. Moreover, most of the photosensitizers clinically approved or in clinical trials are derived from naturally occurring structures based on cyclic tetrapyrroles such as chlorophyll-based compounds from higher plant<sup>57</sup>. This result suggest that the antiplasmodial activity of these three compounds could in all likelihood be linked to their cytotoxicity.

Myricetin-3-*O*-rhamnopyranoside (**18**) has shown moderate antiplasmodial activity (IC<sub>50</sub> 14.3  $\mu\text{M}$ ), while quercetin-3-rhamnoside (**19**) was completely inactive at the highest concentration tested (IC<sub>50</sub> >64.0  $\mu\text{M}$ ). These two compounds are structurally related. However, their difference is particularly located in position C-5' of their aglycone moiety where myricetin has an additional substitution with a hydroxyl group. It seems that addition of the hydroxyl group in position C-5' as in myricetin-3-*O*-rhamnoside could have

a strong influence on the antiplasmodial activity. In contrast to our result, a previous study carried out by Ganesh et al. (2012) revealed that quercetin-3-O-rhamnoside demonstrated weak activity against fresh *P. falciparum* field isolates from Bangladesh ( $IC_{50}$   $20.4 \pm 14.5 \mu M$ ), *P. falciparum* chloroquine-sensitive 3D7 ( $IC_{50}$   $35.4 \pm 6.4$ ) and *P. falciparum* chloroquine-resistant K1 ( $IC_{50}$   $11.7 \pm 27.2$ )<sup>58</sup>. Quercetin and myricetin can be found both in their free form (aglycon) and in their combined form (heteroside) in several plant organs. Previous studies have revealed that the antiplasmodial activity found for myricetin-3-O-rhamnopyranoside and quercetin-3-O-rhamnopyranoside were much lower than their corresponding aglycons myricetin ( $IC_{50}$   $8.9 \mu M$ ) and quercetin ( $IC_{50}$   $12.9 \mu M$ )<sup>59,58</sup>. To date, quercetin and myricetin, were found to exhibit strong inhibitory activity towards three important enzymes (FabG, FabZ, and FabI) involved in the fatty acid biosynthesis of *P. falciparum* and these flavonoids showed *in vitro* activity against chloroquine-sensitive (NF54) and chloroquine-resistant (K1) *P. falciparum* strains. Moreover, myricetin and quercetin inhibited the intraerythrocytic growth of the chloroquine-sensitive (3D7) and chloroquine-resistant (7G8) strains of *P. falciparum*<sup>60</sup>.

The isolated compounds were tested against *Staphylococcus aureus* and *Candida albicans*. Apart from gallic acid which gave a weak activity against *Staphylococcus aureus* all the other compounds did not show any antimicrobial activity. The antibacterial activity of gallic acid against *S. aureus* in suspension ( $MIC$   $2 \text{ mg/mL}$ ) and in biofilms ( $MIC$   $4 \text{ mg/mL}$ ) has previously been reported<sup>61</sup>. Furthermore, a study carried out by Chanwitheesuk et al. (2007) has reported the effectiveness of gallic acid against *S. aureus* ( $MIC$   $1250 \mu g/mL$ )<sup>62</sup>. In contrast to our result, a previous study carried out by Motlhatlego et al. 2020 showed that myricetin-3-O-rhamnoside isolated from *Newtonia buchananii* demonstrated antibacterial activity against *S. aureus* ( $MIC$   $62.5 \mu g/mL$ )<sup>63</sup>. Previous studies suggested that pythol exhibited broad-spectrum antimicrobial effects <sup>49</sup>. Indeed, Inoue et al. 2005 have observed a good antimicrobial activity of this compound against *Staphylococcus aureus* ( $MIC$   $0.15 \mu g/mL$ ).

## 6.4. Conclusion

The results of this study give a possible explanation of the traditional use of *T. alnifolia* against microbial diseases, more in particular malaria. This study reported the bioassay-guided isolation of constituents from the leaves of *T. alnifolia*, which are active against *Plasmodium falciparum* from the hexane, dichloromethane and methanol extracts. Bioassay-guided fractionation of *Tetracera alnifolia* leaves led to the isolation of 19 compounds mostly reported for the first time in this species. The highest antiplasmodial activity was obtained for pheophorbide-b methyl ester, (1,2)-bis-nor-phytene, isophytol, pheophorbide-a methyl ester and phytol. Other compounds, including myricetin-3-O-rhamnopyranoside,  $\alpha$ -tocopherol and cycloart-24-en-3 $\beta$ -yl  $\alpha$ -linolenate were less active. A hight cytotoxicity was found for pheophorbide-b methyl ester, pheophorbide-a methyl ester and phytol. These results may provide some explanations related to the antiplasmodial activity of the leaves extract of *T. alnifolia*.

**Table 6.1.** *In vitro* antimicrobial, antiplasmodial and cytotoxic activity of extracts, fractions from *T. alnifolia* leaves

Fractions	Antimicrobial activity			Antiplasmodial activity (IC <sub>50</sub> , µg/mL)	Cytotoxicity (CC <sub>50</sub> , µg/mL)	Selectivity index
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>Pf-K1</i>	<i>MRC-5</i>	<i>MRC-5/PfK1</i>
T <sub>E</sub>	>64.0	>64.0	>64.0	2.2	32.7	14.8
T <sub>D</sub>	>64.0	>64.0	6.8	1,81	3.6	2.0
T <sub>M</sub>	>64.0	>64.0	18.3	46.4	>64.0	>1.3
T <sub>M1</sub>	>64.0	>64.0	>64.0	34.5	>64.0	>1.8
T <sub>M2</sub>	>64.0	>64.0	>64.0	7.1	>64.0	8.9
T <sub>M3</sub>	>64.0	>64.0	>64.0	1.3	31.0	23.4
T <sub>M4</sub>	>64.0	>64.0	>64.0	8.8	30.4	3.4
T <sub>M5</sub>	>64.0	>64.0	>64.0	19.6	>64.0	>3.3
T <sub>M6</sub>	>64.0	>64.0	>64.0	25.2	30.1	1.2
T <sub>M7</sub>	>64.0	>64.0	3.6	23.8	28.5	1.19
T <sub>M8</sub>	>64.0	>64.0	1.7	16.0	26.3	1.6
T <sub>M9</sub>	>64.0	>64.0	1.6	18.8	29.4	1.5
T <sub>M10</sub>	>64.0	>64.0	5.9 ± 4.7*	4 ± 0.8*	>64.0	>13.0
T <sub>M11</sub>	>64.0	>64.0	25.2	18.7	>64.0	>3.4

$T_{M12}$	>64.0	>64.0	$3.6 \pm 3.0^*$	37.07	>64.0	>1.7
Chloroquine				$0.15 \pm 0.1 \mu M^*$		
Doxycycline	$0.28 \pm 0.2 \mu M^*$	$0.6 \pm 0.3 \mu M^*$				
Flucytosine			$0.70 \pm 0.01 \mu M^*$			
Tamoxifen					$10.0 \pm 1.5 \mu M^*$	

\*n = 3

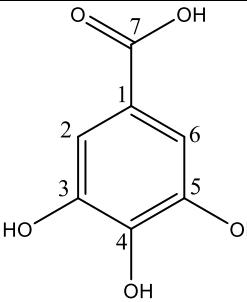
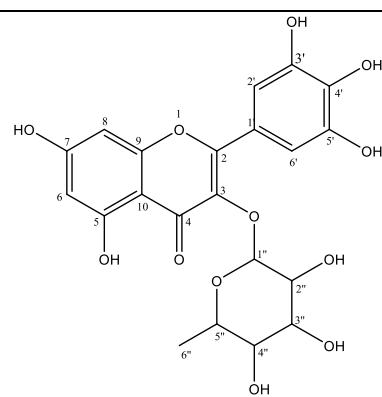
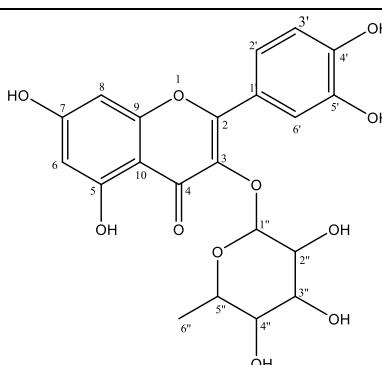
**Table 6.2.** *In vitro* antimicrobial, antiplasmoidal and cytotoxic activity of isolated compounds from *T. alnifolia* leaves

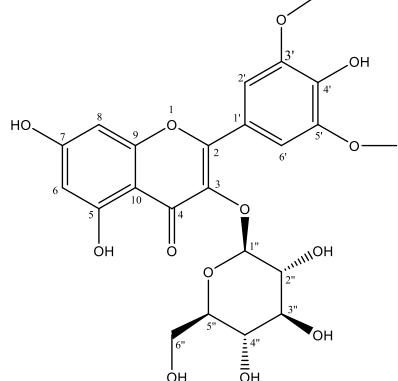
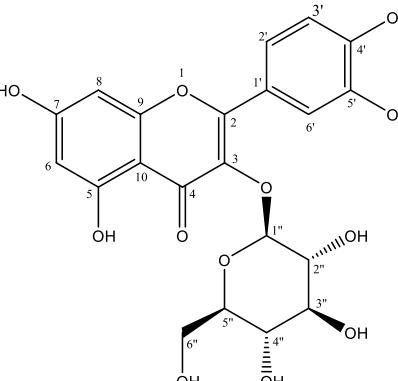
Compound names	Structures	Antimicrobial activity		Antiplasmodal activity (IC <sub>50</sub> μM)	Cytotoxicity (CC <sub>50</sub> , μM)	Selectivity index
		<i>S. aureus</i>	<i>C. albicans</i>	<i>Pf-K1</i>	<i>MRC-5</i>	<i>MRC-5/PfK1</i>
Squalene (1)		>64.0	>64.0	Nd	20.6	Nd

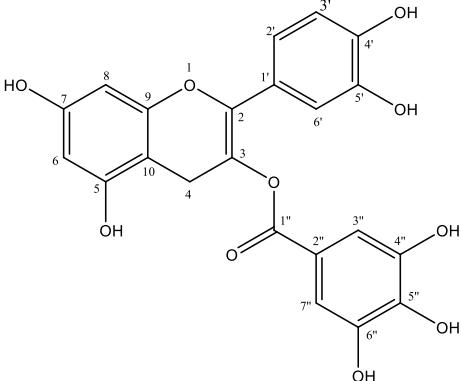
Cycloart-24-en-3 $\beta$ -yl linolenate ( <b>2</b> )	$\alpha$ - 	>64.0	>64.0	25.0	>64.0	>2.4
$\alpha$ -Tocopherol ( <b>3</b> )		>64.0	>64.0	13.5	>64.0	>4.4
<i>Trans</i> -pentamethyl-Icosa-tetraene ( <b>4</b> ).  Chemical structure of <i>Trans</i> -pentamethyl-Icosa-tetraene ( <b>4</b> ), showing a branched hydrocarbon chain with numbered carbons from 1 to 25.		nd	nd	nd	nd	nd
3- $\beta$ -hydroxy-olean-12-ene-heptadecanoate ( <b>5</b> ).  Chemical structure of 3-β-hydroxy-olean-12-ene-heptadecanoate (5), showing a triterpenoid saponin with a hydroxyl group at C3 and a heptadecenoate ester side chain.		>64.0	>64.0	>64.0	>64.0	>64.0
Phytol ( <b>6</b> )  Chemical structure of Phytol (6), showing a branched hydrocarbon chain with numbered carbons from 1 to 16.		>64.0	>64.0	6.9±2.4*	8.0	1.3

Isophytol ( <b>7</b> )		>64.0	>64.0	4.0	>64.0	>64.0
(1,2)-Bis-nor-phytane ( <b>8</b> )		>64.0	>64.0	2.0	>64.0	>64.0
Pheophorbide-A methyl ester ( <b>9</b> )		64.00	>64.0	2.8±1.2*	7.5	1.7
Pheophorbide-B methyl ester ( <b>10</b> )		>64.0	>64.0	1.4±0.4*	0.7	0.5

Stigma-5-en-3-O- $\beta$ -glucoside ( <b>11</b> )		>64.0	>64.0	>64.0	>64.0	Nd
Vannilic acid ( <b>12</b> )		>64.0	>64.0	>64.0	>64.0	Nd
Abscisic acid ( <b>13</b> )		>64.0	>64.0	>64.0	>64.0	>64.0

Gallic acid ( <b>14</b> )		32.46	>64.0	Nd	8.06	Nd
Myricetin-3-O-Rhamnopyranoside ( <b>15</b> )		>64.0	>64.0	14.3	>64.0	Nd
Quercetin-3-O-Rhamnoside ( <b>16</b> )		>64.0	>64.0	>64.0	>64.0	Nd

Myricetin-3',5'dimethylether-3-O-Galactopyranoside ( <b>17</b> )		>64.0	>64.0	>64.0	>64.0	Nd
Quercetin-3-O-galactopyranoside ( <b>18</b> )		>64	>64	>64	>64	Nd

Epicatechin-3-galloylester <b>(19)</b>		>64	>64	5.5±2.1*	>64	>11.6
Chloroquine				0.15 ± 0.1 μM*		
Doxycycline		0.28 ± 0.2 μM*				
Flucytosine			0.70±0.01 μM*			
Tamoxifen					10.0 ± 1.5 μM*	

\*n = 3

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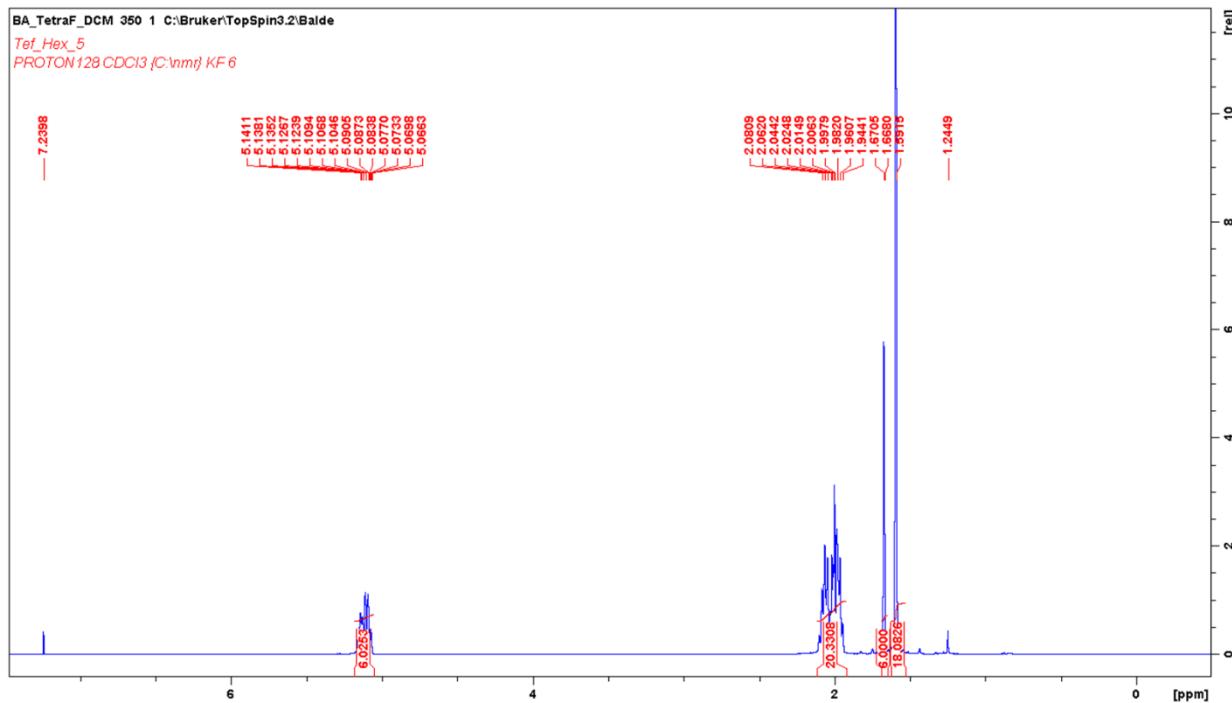
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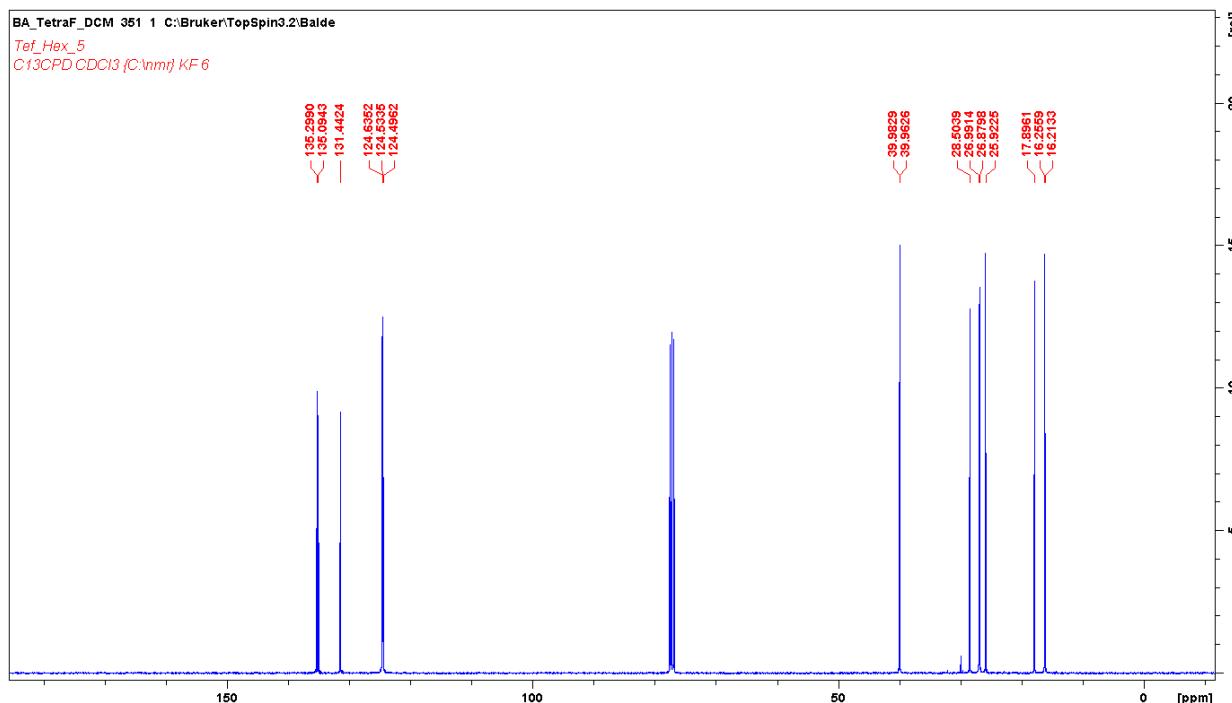
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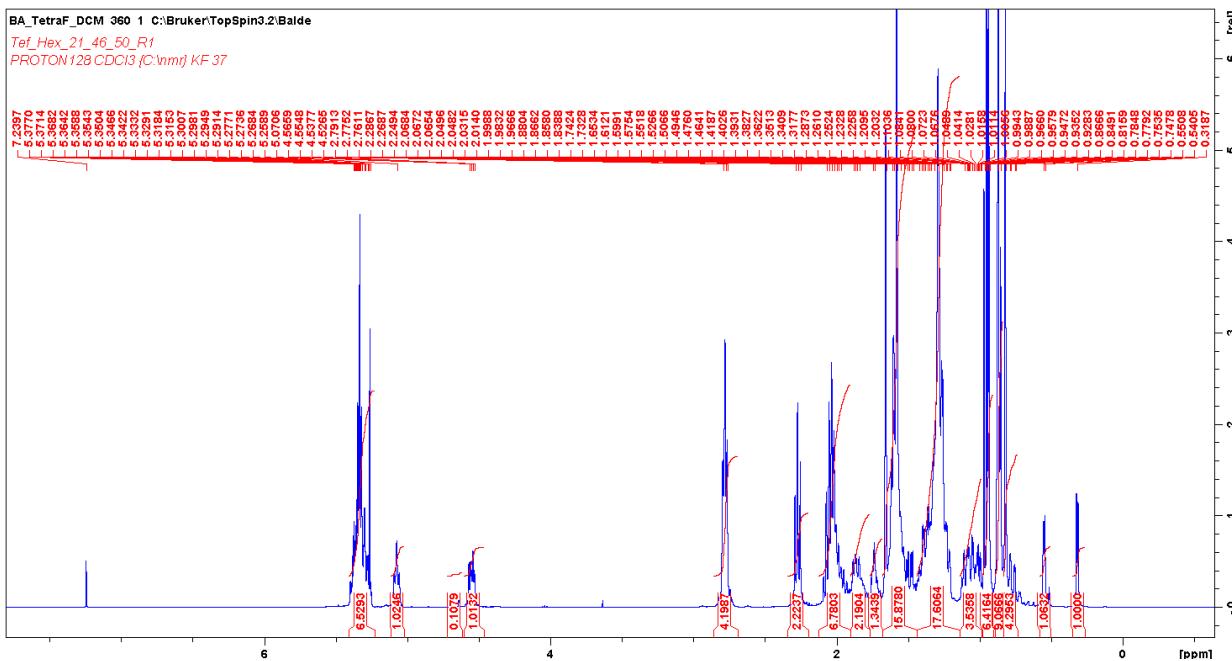
## Supplementary information



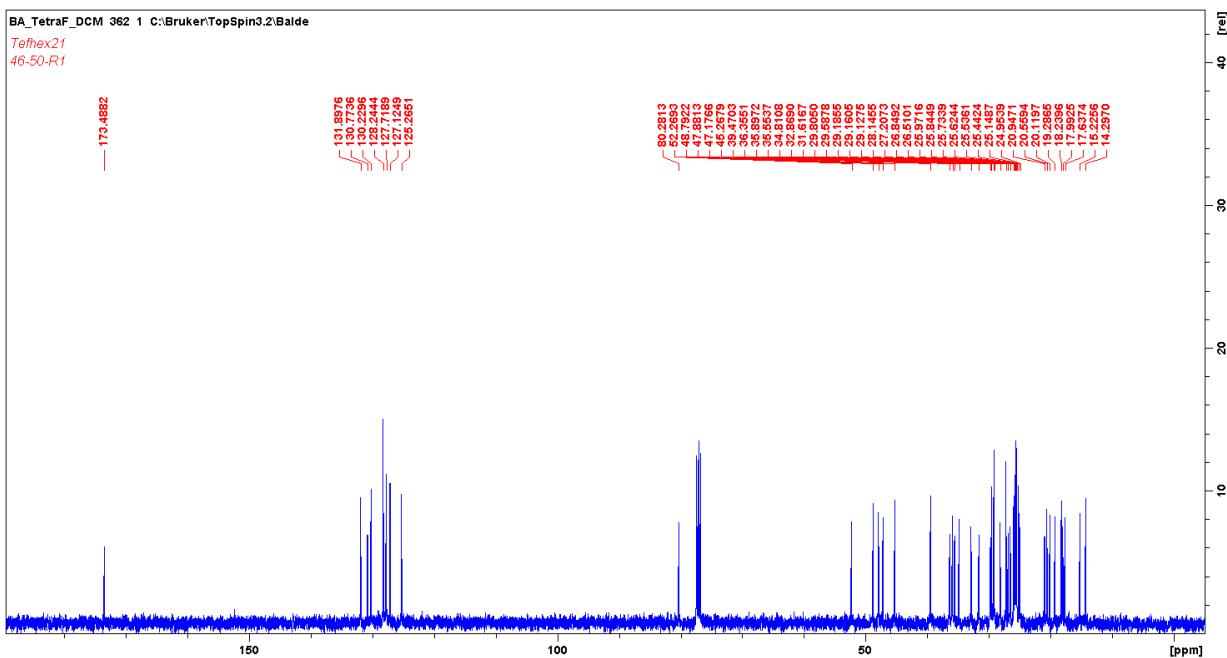
**Figure 6.1.** <sup>1</sup>H NMR spectrum (CDCl<sub>3</sub>, 400 MHz) of squalene (**1**).



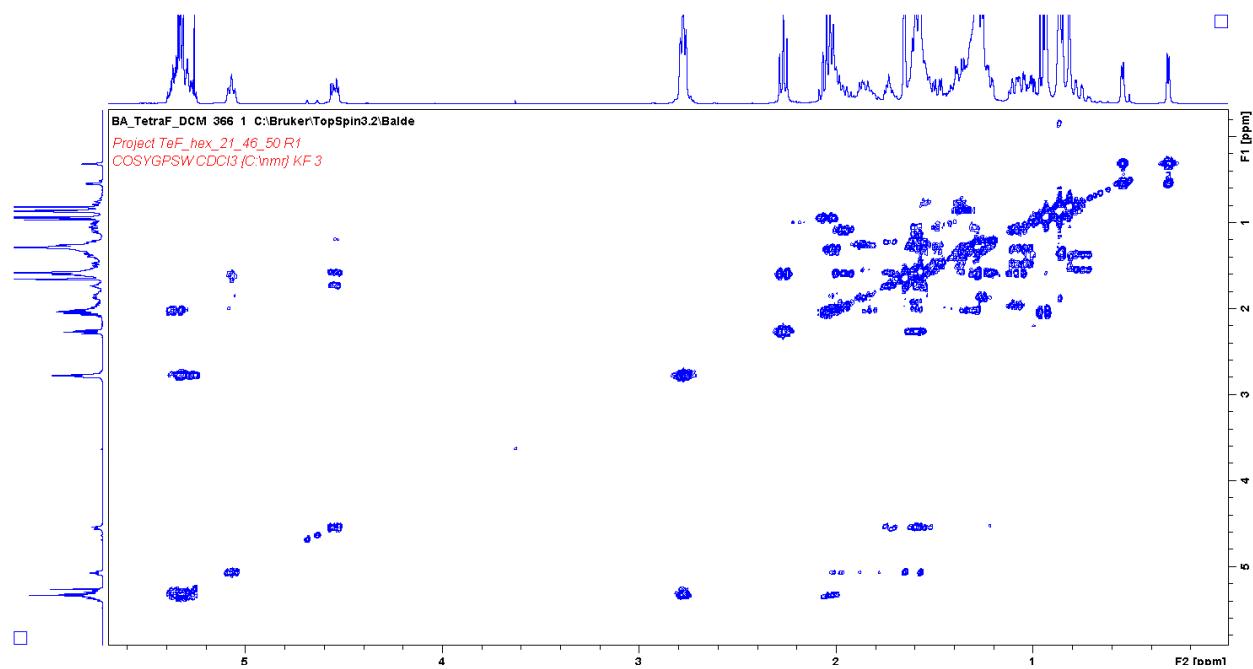
**Figure 6.2.** <sup>13</sup>C NMR spectrum (CDCl<sub>3</sub>, 100 MHz) of squalene (**1**).



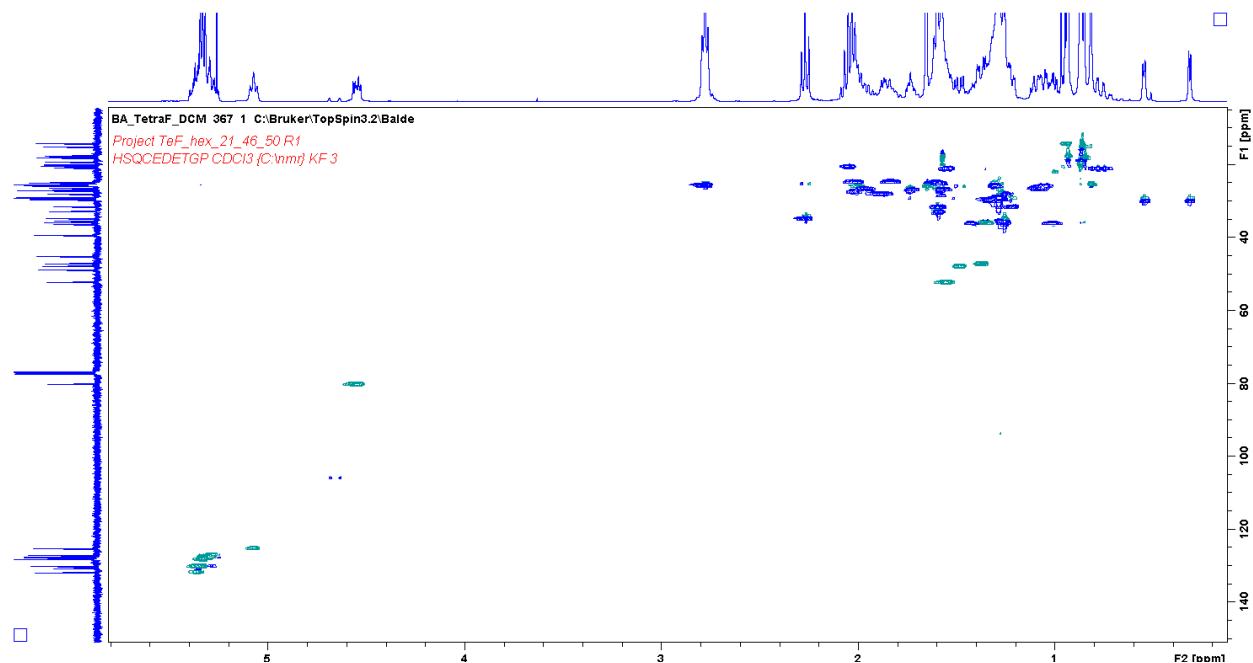
**Figure 6.3.** <sup>1</sup>H NMR spectrum (CDCl<sub>3</sub>, 400 MHz) of cycloart-24-en-3β-yl α-linolenate (**2**)



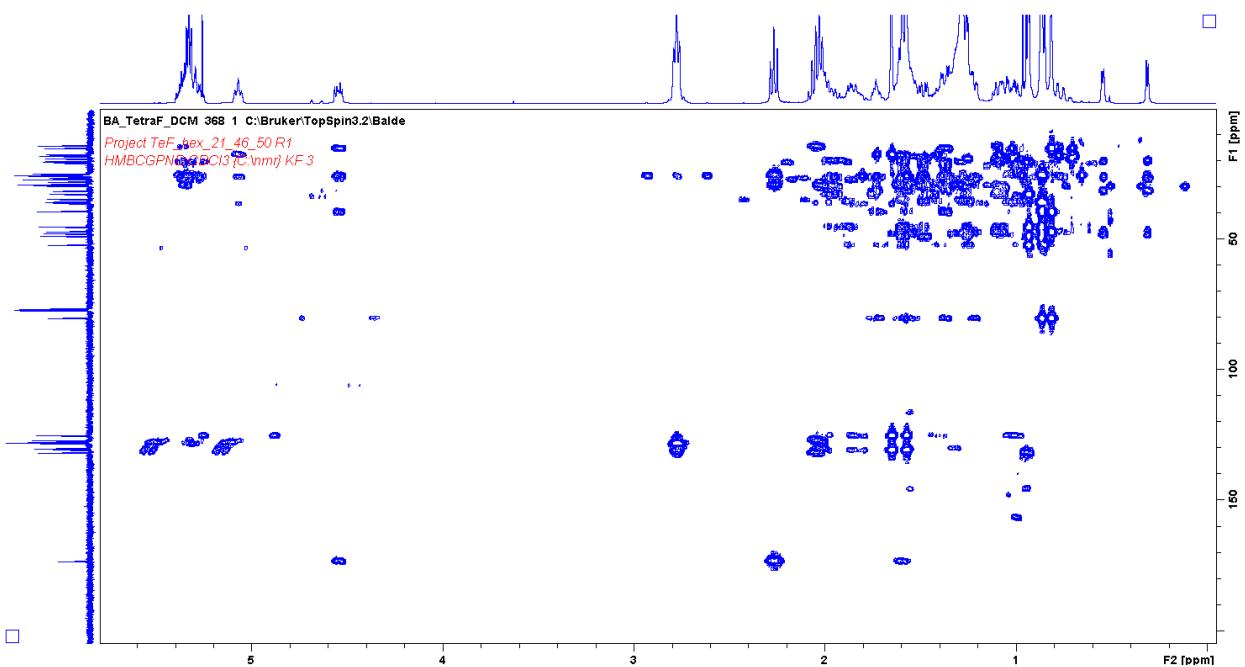
**Figure 6.4.** <sup>13</sup>C NMR spectrum (CDCl<sub>3</sub>, 100 MHz) of cycloart-24-en-3β-yl α-linolenate (**2**)



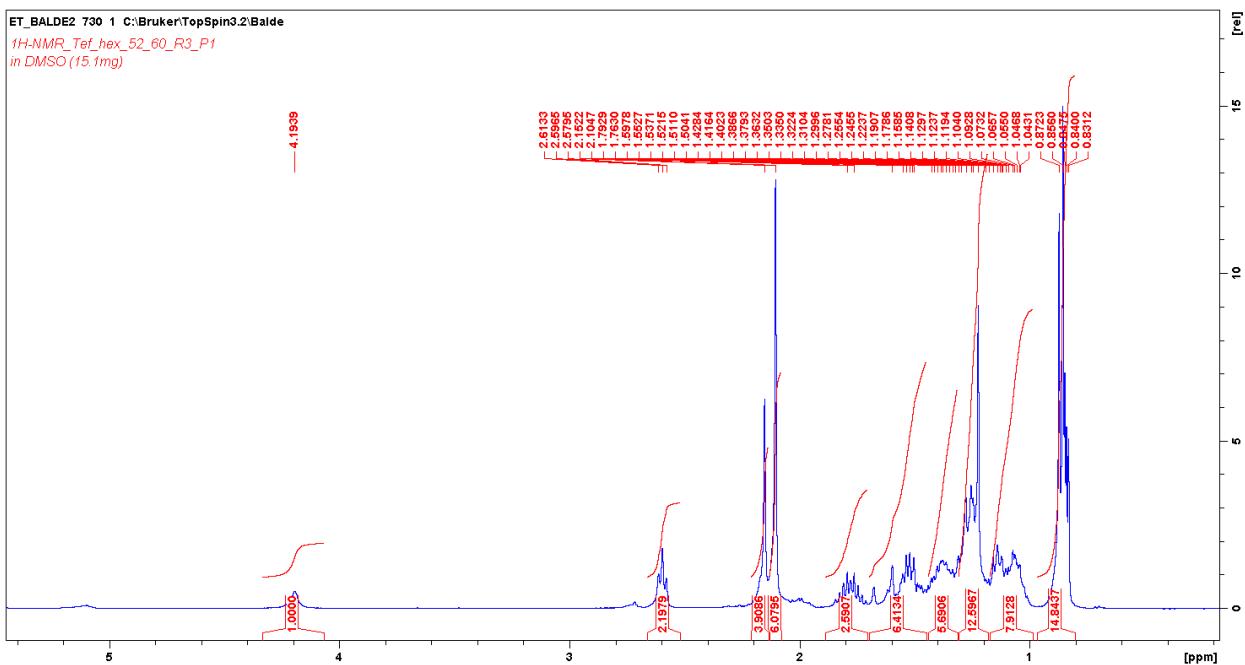
**Figure 6.5.** COSY spectrum ( $\text{CDCl}_3$ ) of Cycloart-24-en-3 $\beta$ -yl  $\alpha$ -linolenate (**2**)



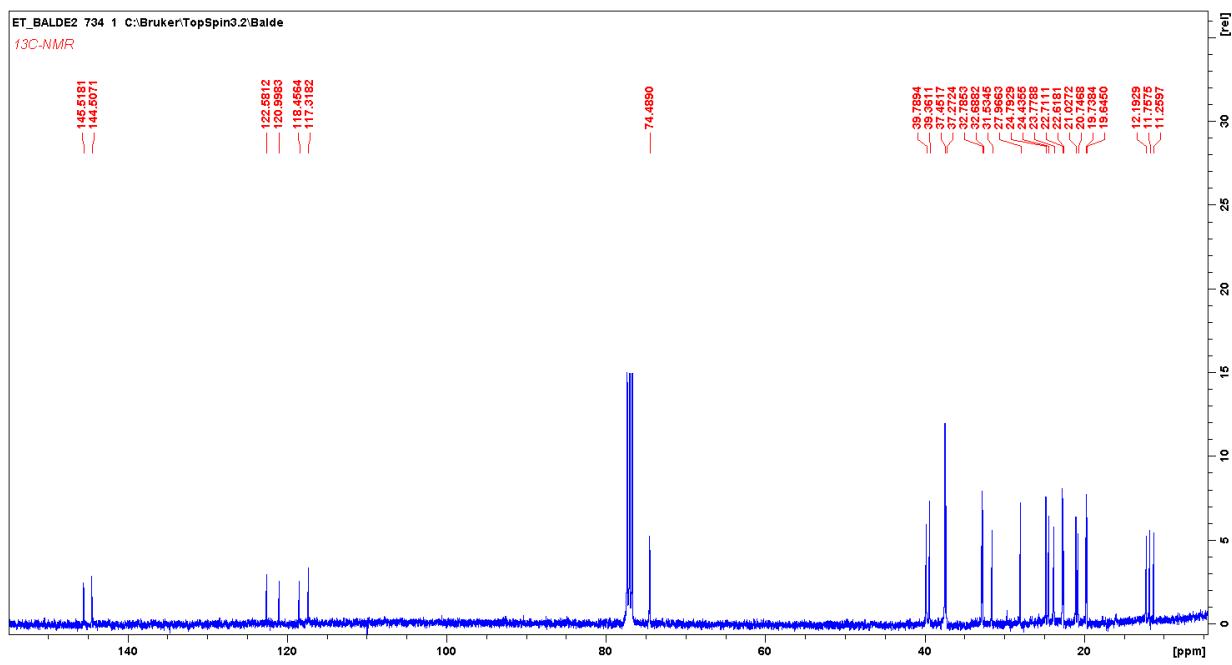
**Figure 6.6.** COSY spectrum ( $\text{CDCl}_3$ ) of Cycloart-24-en-3 $\beta$ -yl  $\alpha$ -linolenate (**2**)



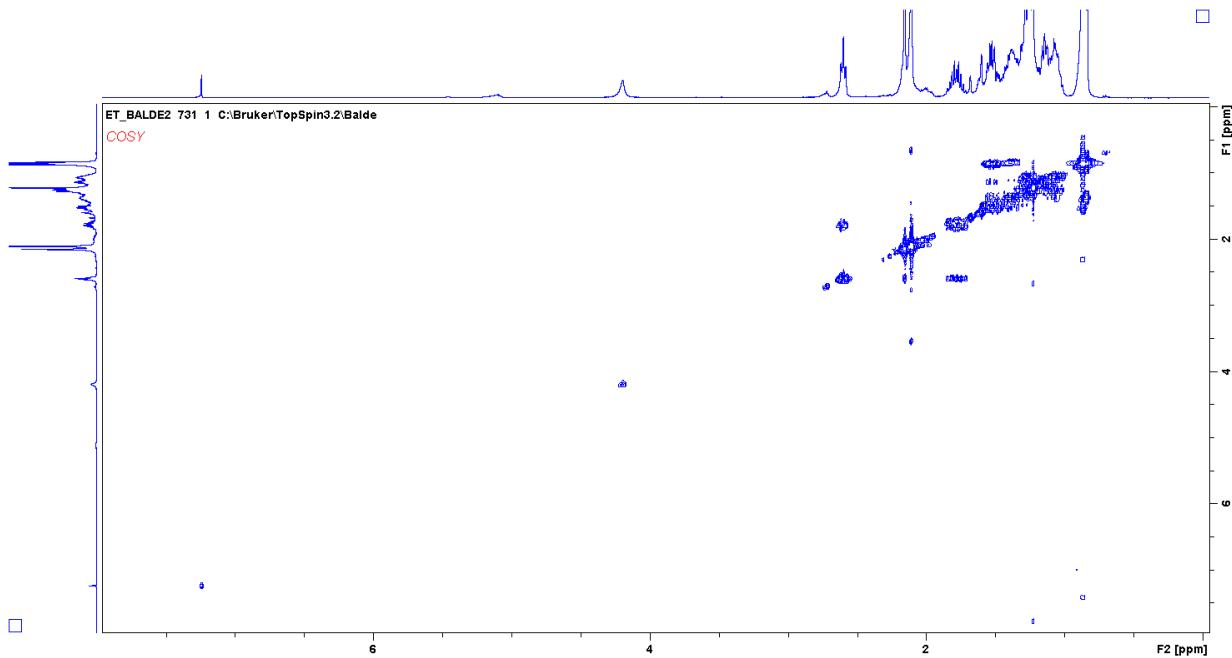
**Figure 6.7.** COSY spectrum ( $\text{CDCl}_3$ ) of Cycloart-24-en-3 $\beta$ -yl  $\alpha$ -linolenate (**2**)



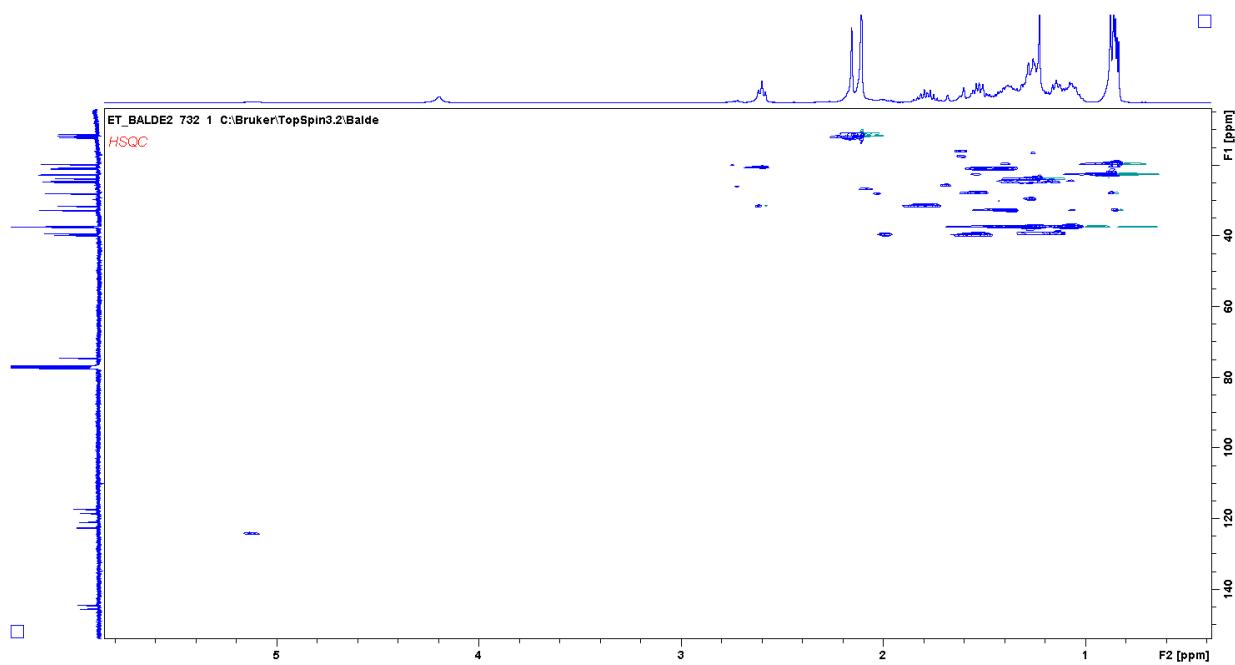
**Figure 6.8.**  $^1\text{H}$  NMR spectrum (DMSO, 400 MHz) of  $\alpha$ -Tocopherol (**3**)



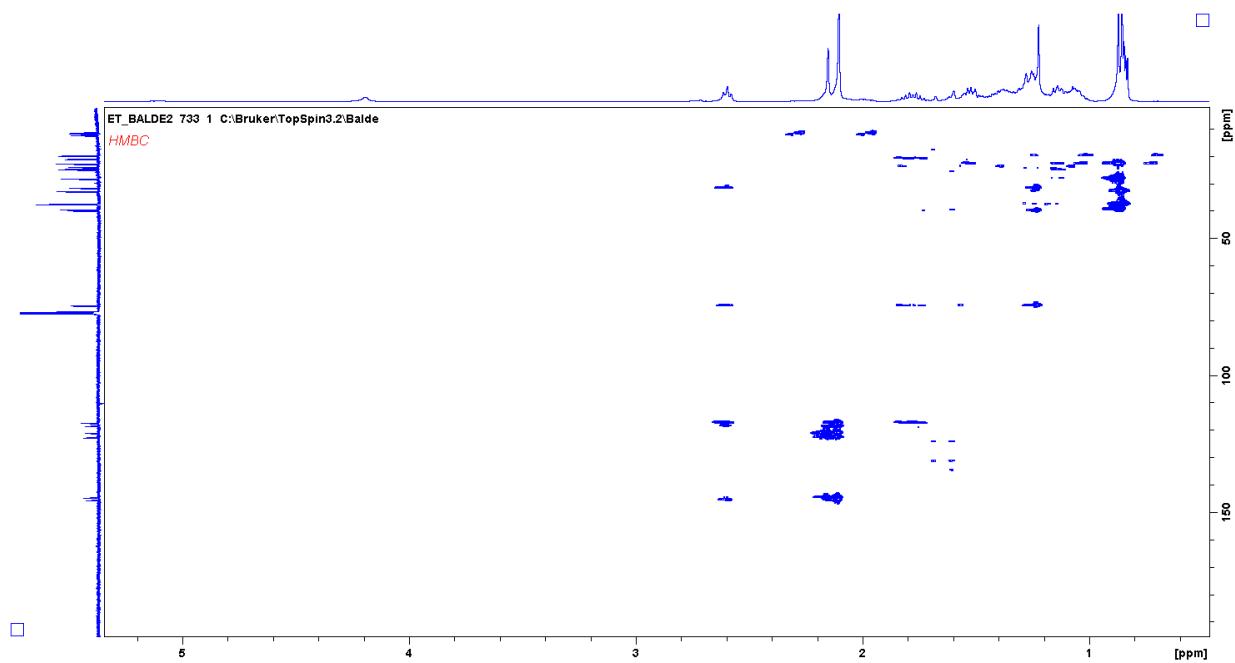
**Figure 6.9.** <sup>13</sup>C NMR spectrum (DMSO, 100 MHz) of  $\alpha$ -Tocopherol (**3**)



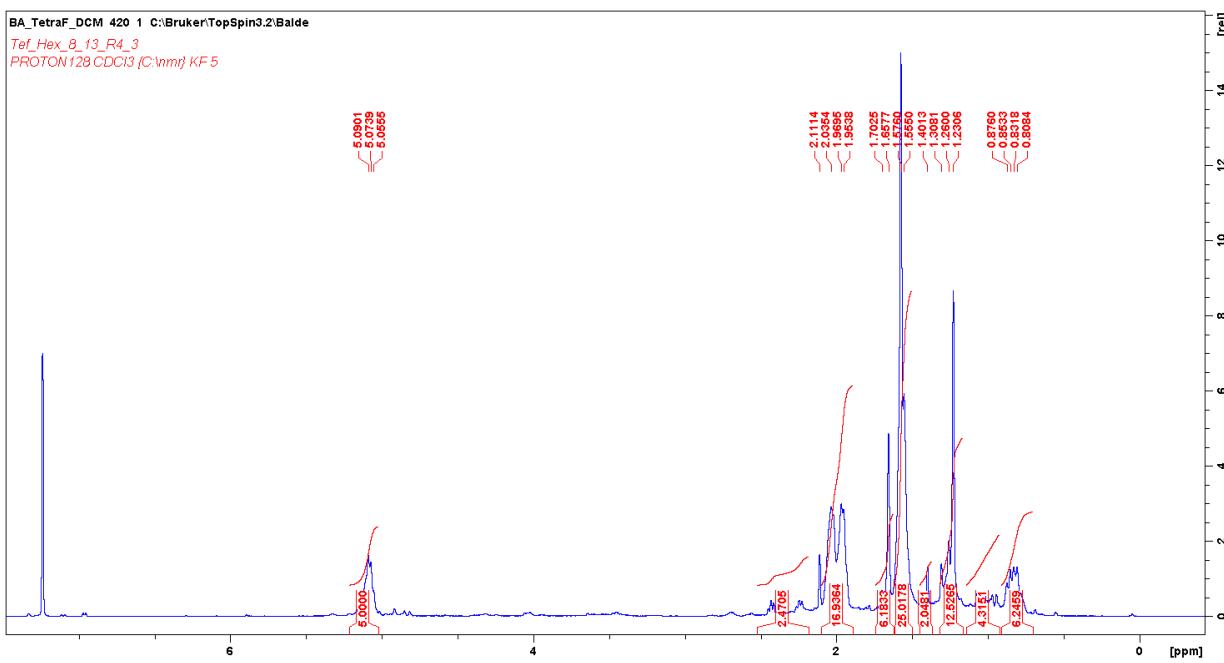
**Figure 6.10.** COSY spectrum (CDCl<sub>3</sub>) of  $\alpha$ -Tocopherol (**3**)



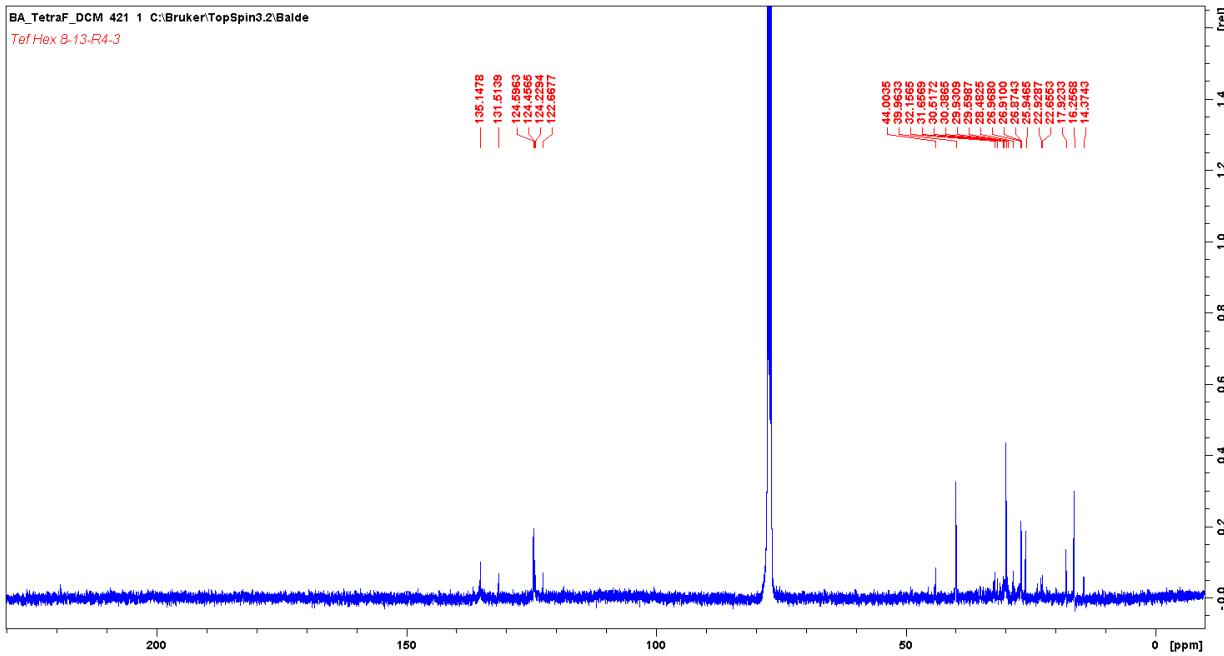
**Figure 6.11.** HSQC spectrum ( $\text{CDCl}_3$ ) of  $\alpha$ -Tocopherol (3).



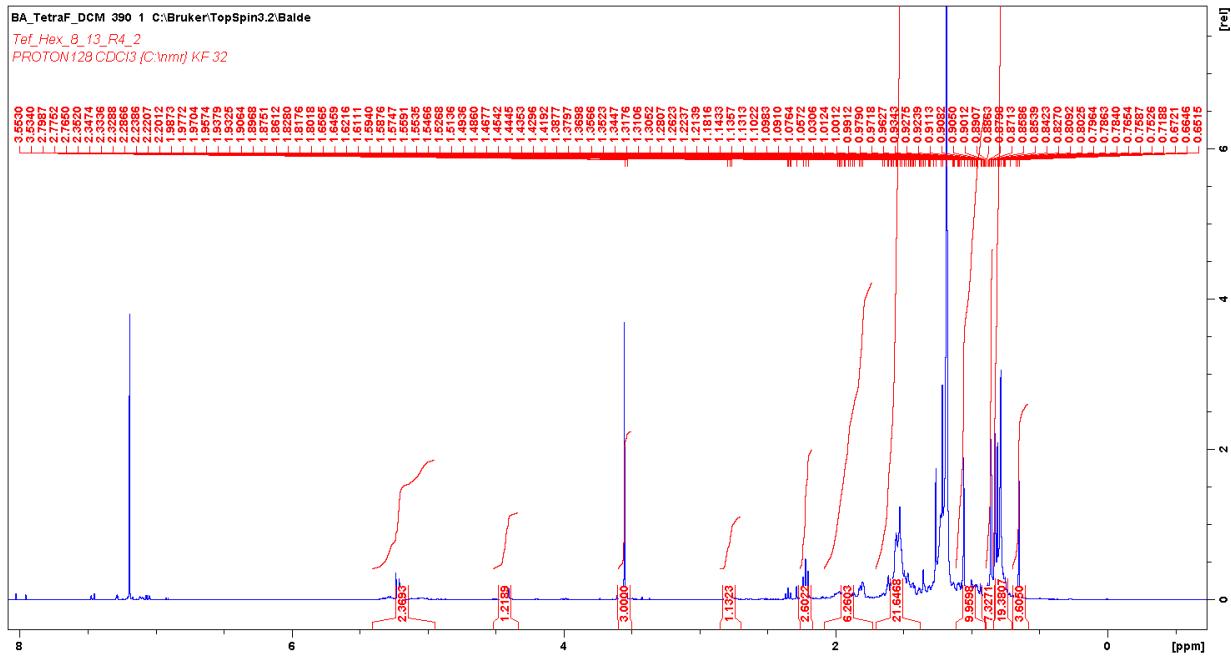
**Figure 6.12.** HMBC spectrum ( $\text{CDCl}_3$ ) of  $\alpha$ -Tocopherol (3).



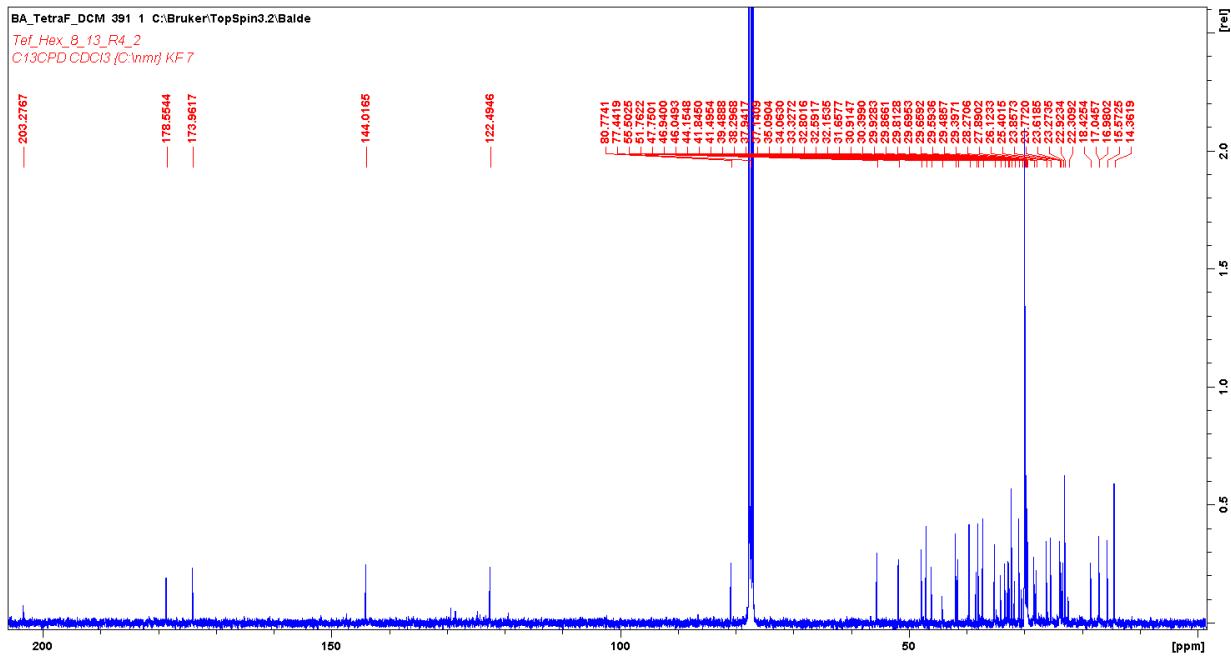
**Figure 6.13.** <sup>1</sup>H NMR spectrum (CDCl<sub>3</sub>, 400 MHz) of Trans-pentamethyl-Icosa-tetraene (**4**).



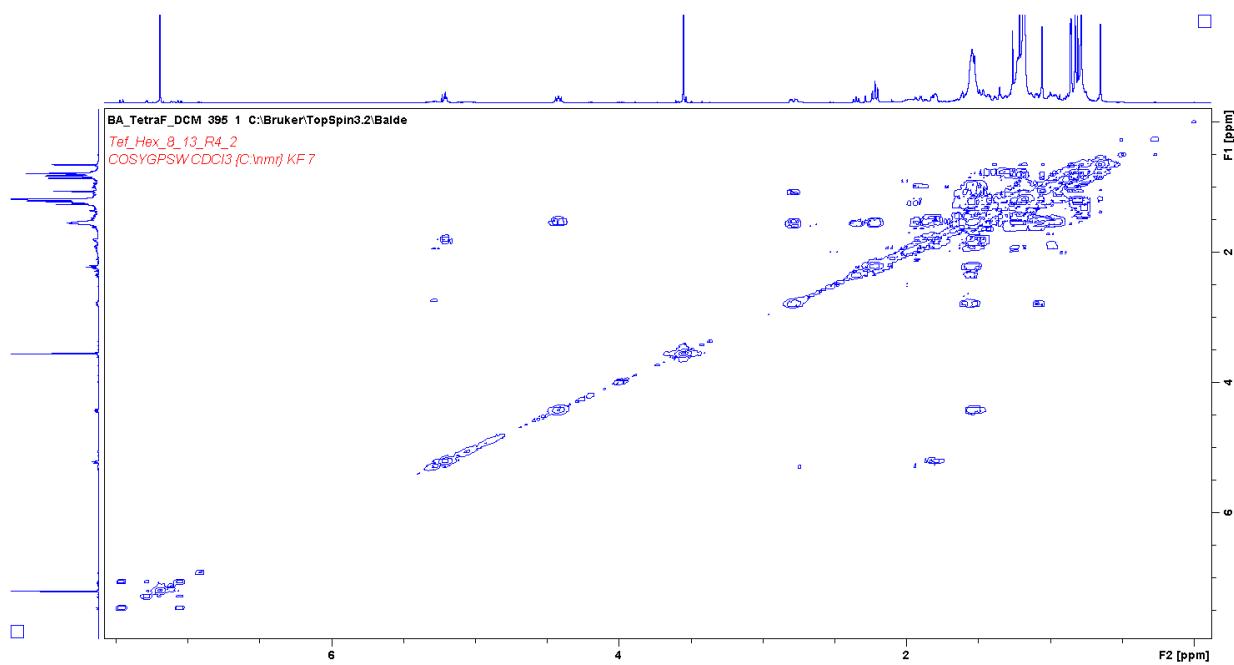
**Figure 6.14.** <sup>13</sup>C NMR spectrum (CDCl<sub>3</sub>, 100 MHz) of Trans-pentamethyl-Icosa-tetraene (**4**).



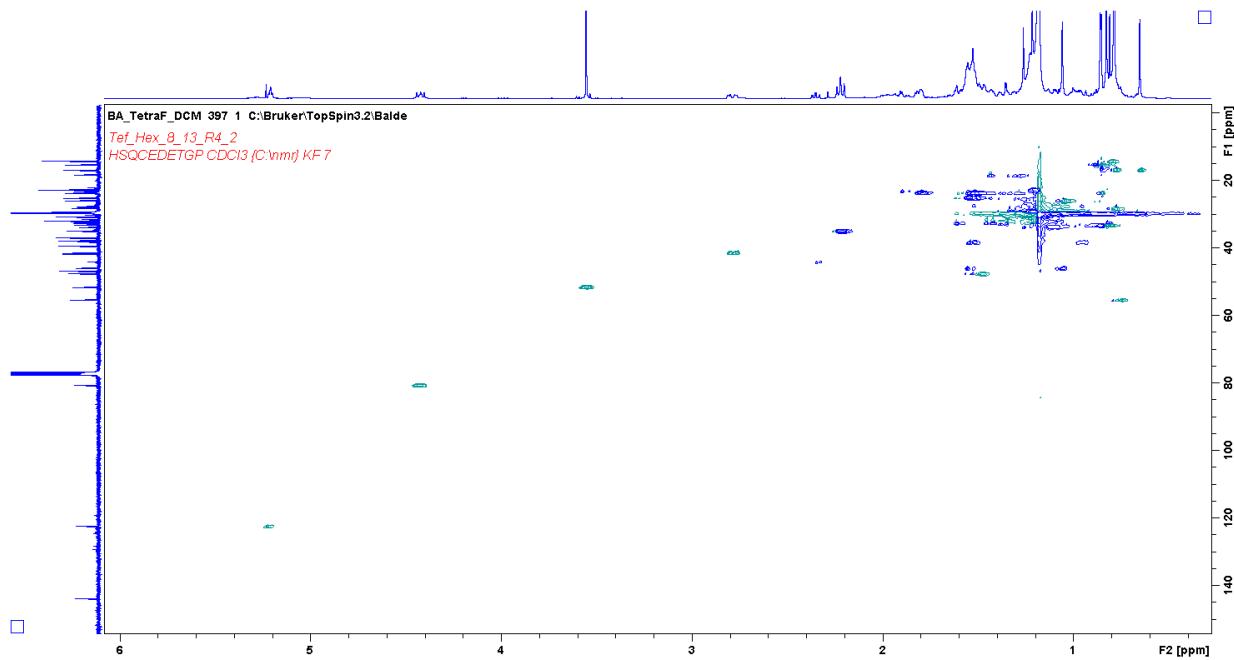
**Figure 6.15.**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of 3- $\beta$ -hydroxy-olean-12-ene-heptadecanoate (5).



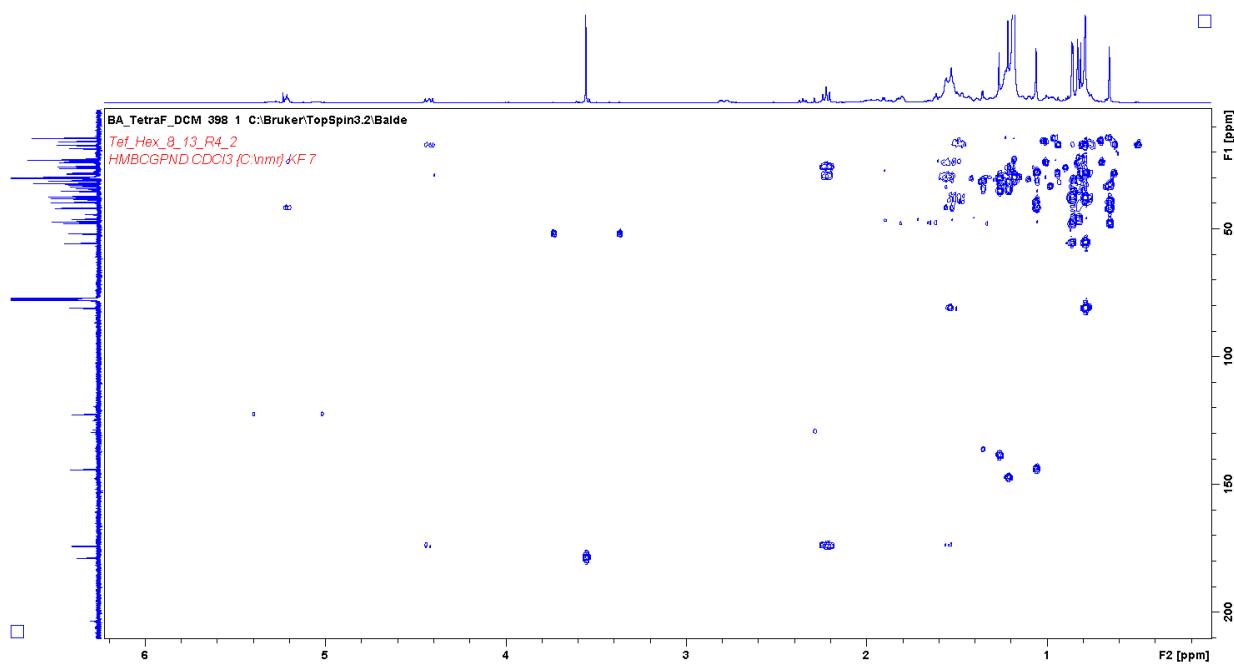
**Figure 6.16.**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of 3- $\beta$ -hydroxy-olean-12-ene-heptadecanoate (5).



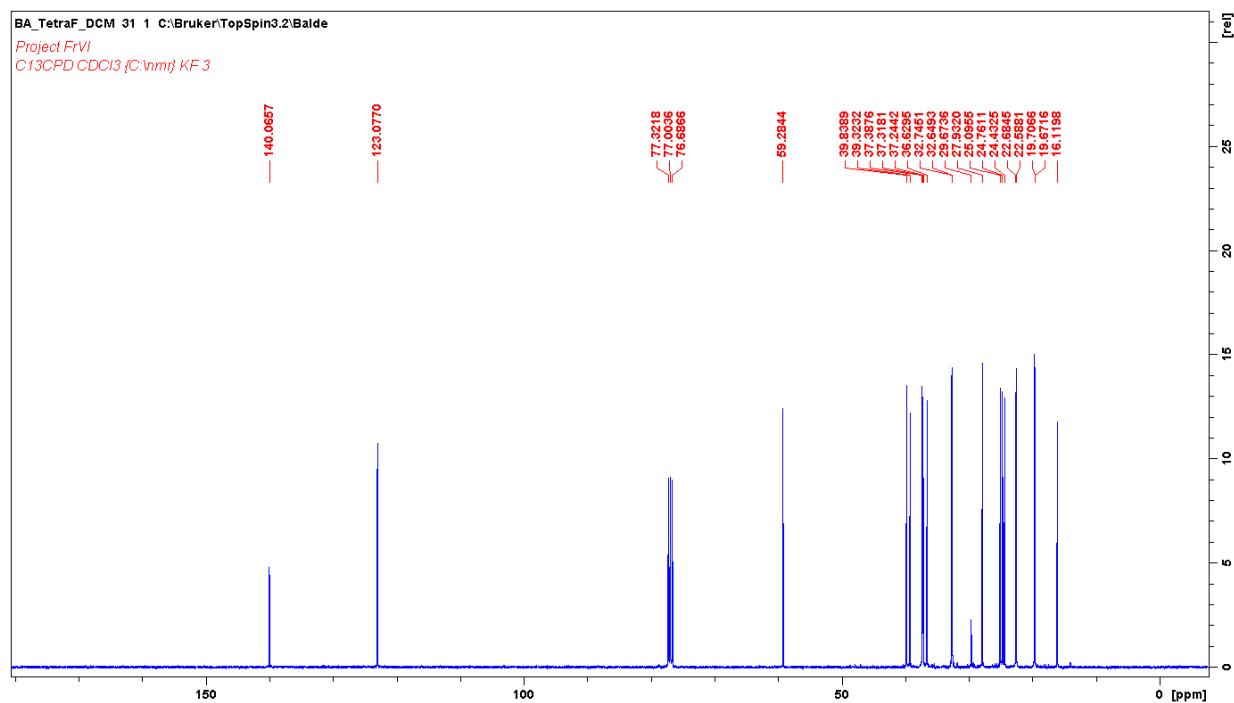
**Figure 6.17.** COSY spectrum ( $\text{CDCl}_3$ ) of 3- $\beta$ -hydroxy-olean-12-ene-heptadecanoate (**5**).



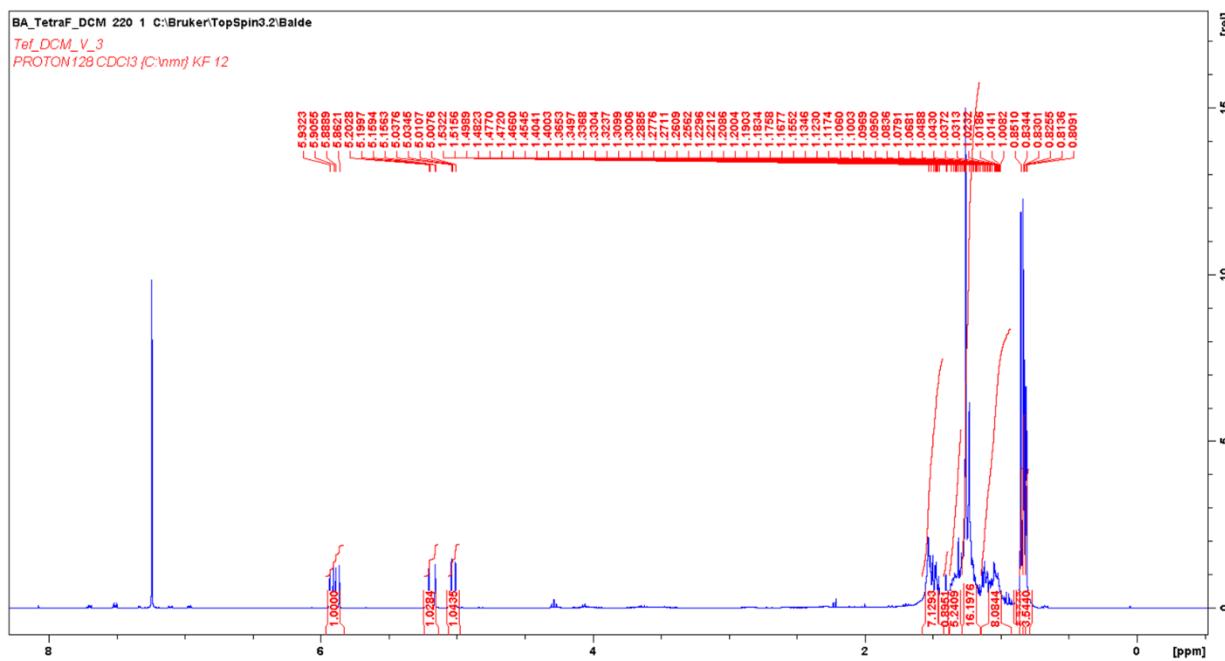
**Figure 6.18.** HSQC spectrum ( $\text{CDCl}_3$ ) of 3- $\beta$ -hydroxy-olean-12-ene-heptadecanoate (**5**).



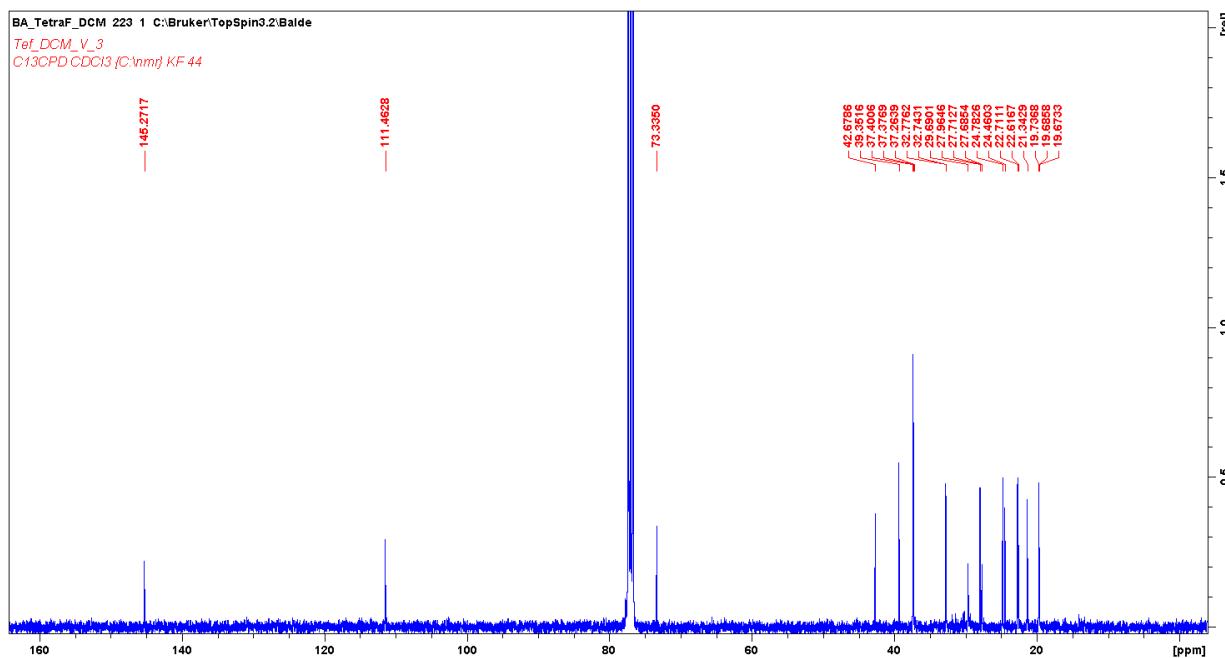
**Figure 6.19.** HMBC spectrum ( $\text{CDCl}_3$ ) of 3- $\beta$ -hydroxy-olean-12-ene-heptadecanoate (**5**)



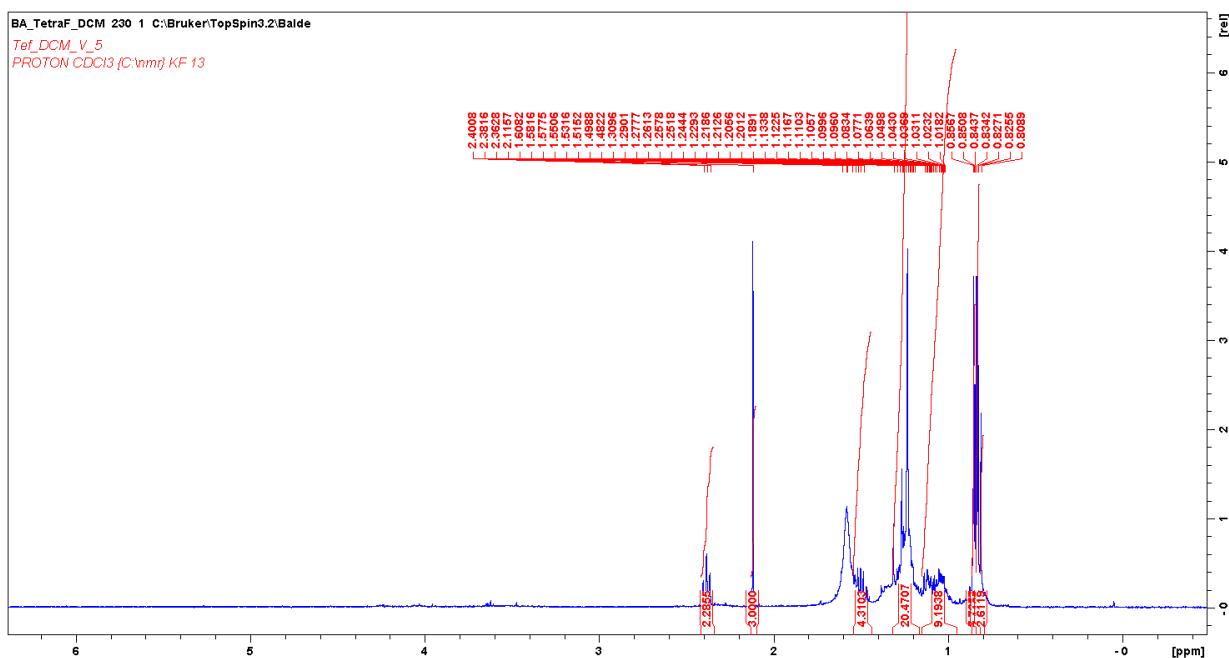
**Figure 6.20.**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of Phytol (**6**)



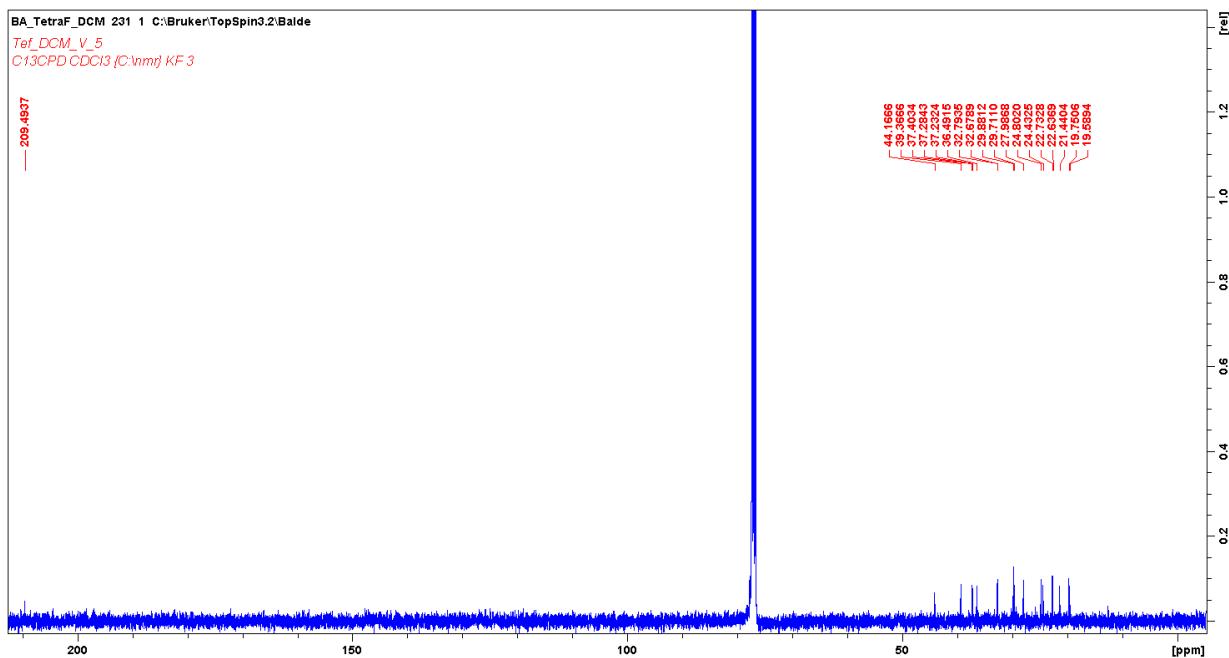
**Figure 6.21.** <sup>1</sup>H NMR spectrum (CDCl<sub>3</sub>, 400 MHz) of Isophytol (**7**)



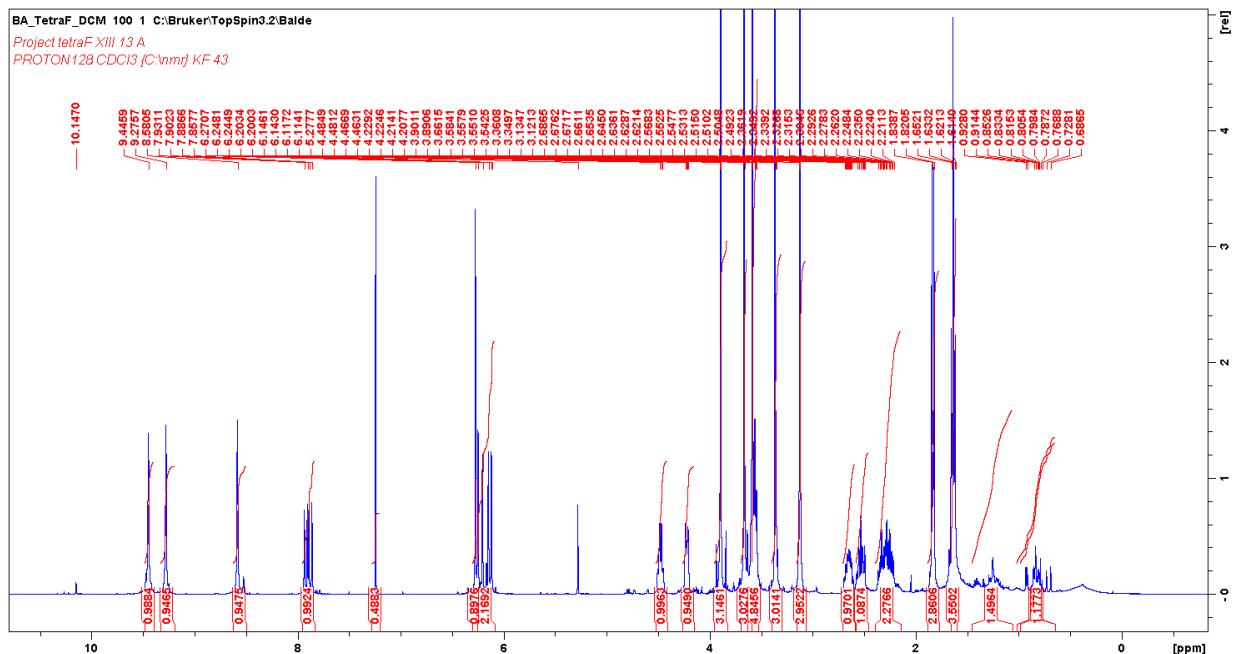
**Figure 6.22.** <sup>13</sup>C NMR spectrum (CDCl<sub>3</sub>, 100 MHz) of Isophytol (**7**).



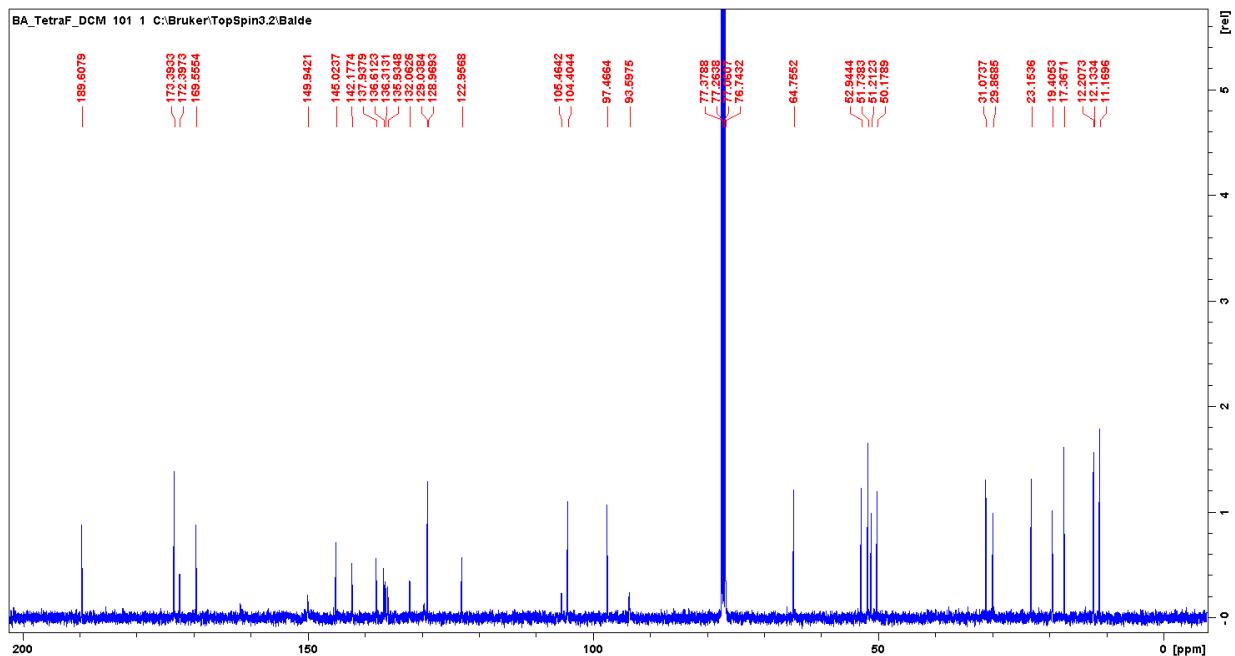
**Figure 6.23.**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of (1,2)-Bis-nor-phytene (8)



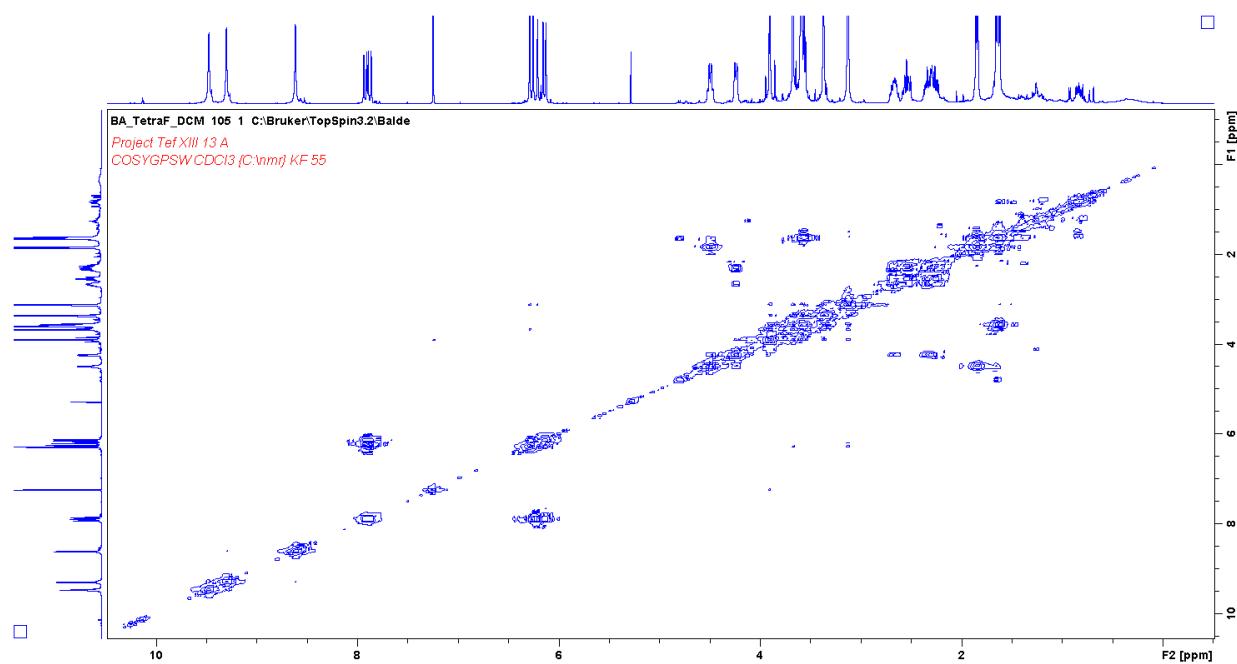
**Figure 6.24.**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of (1,2)-Bis-nor-phytene (8)



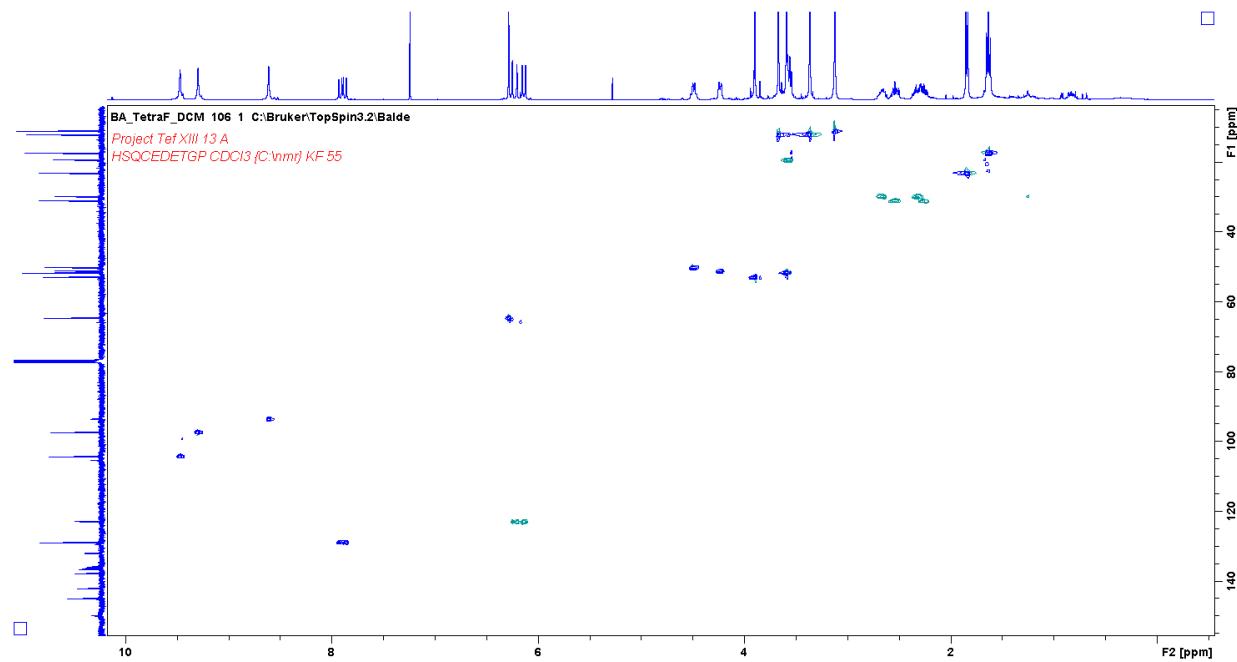
**Figure 6.25.**  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of Pheophorbide-A methyl ester (9)



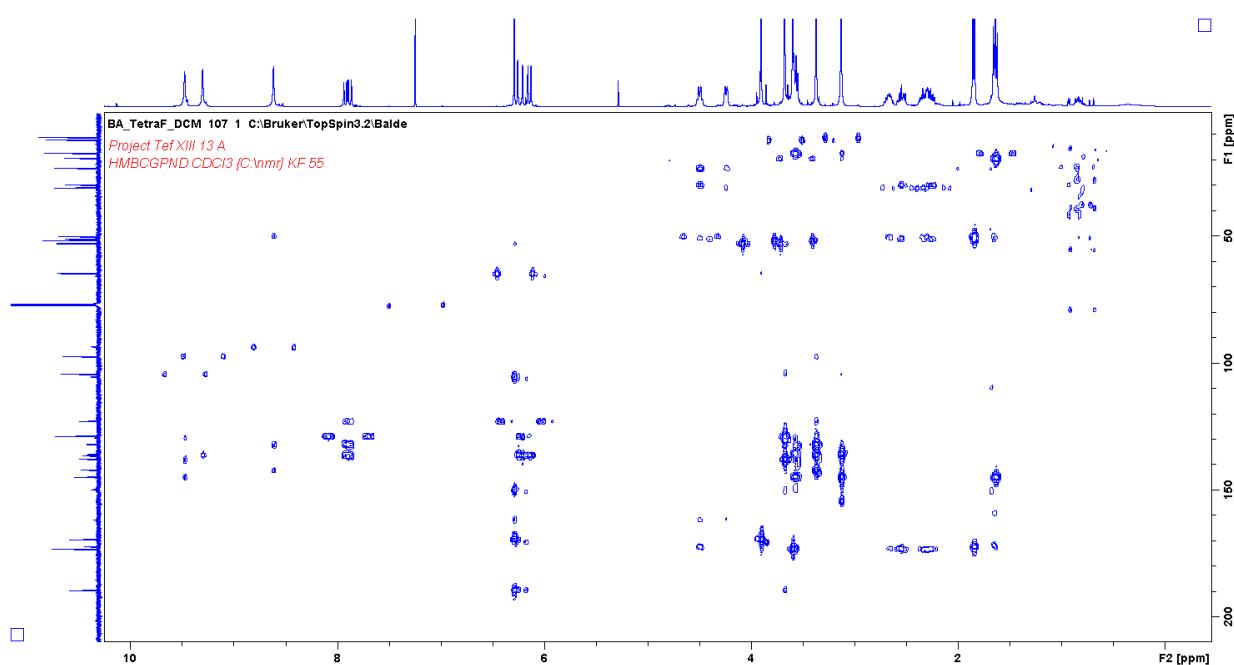
**Figure 6.26.**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of Pheophorbide-A methyl ester (**9**)



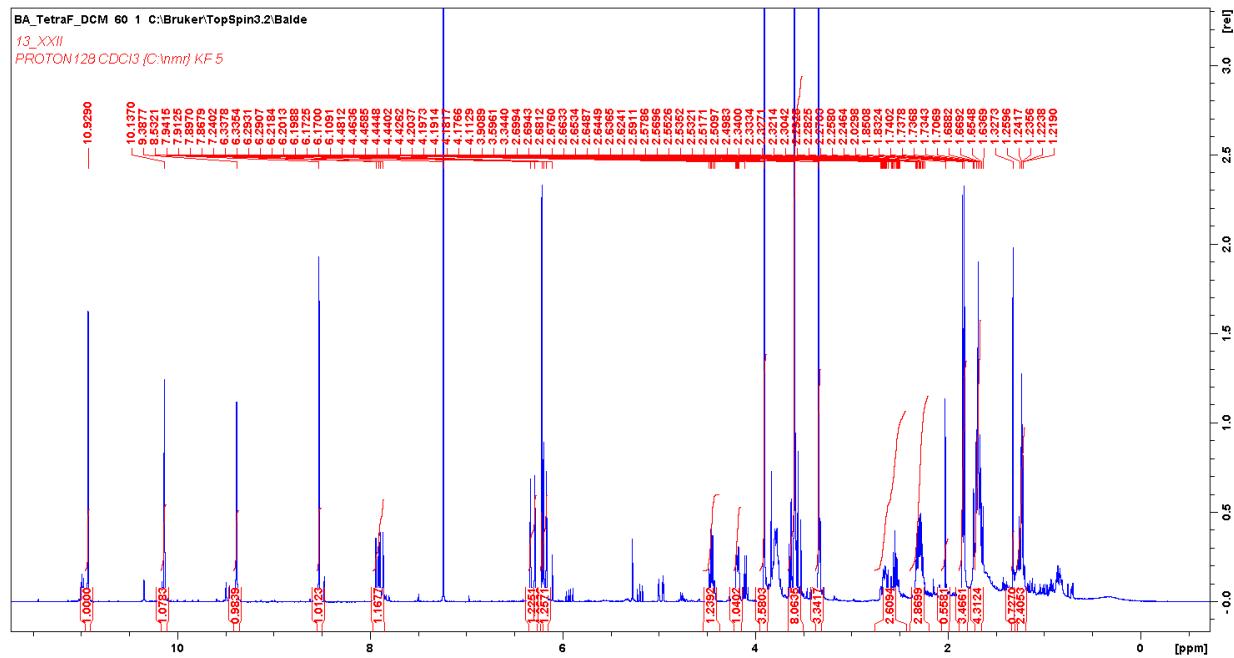
**Figure 6.27.** COSY spectrum ( $\text{CDCl}_3$ ) of Pheophorbide-A methyl ester (**9**)



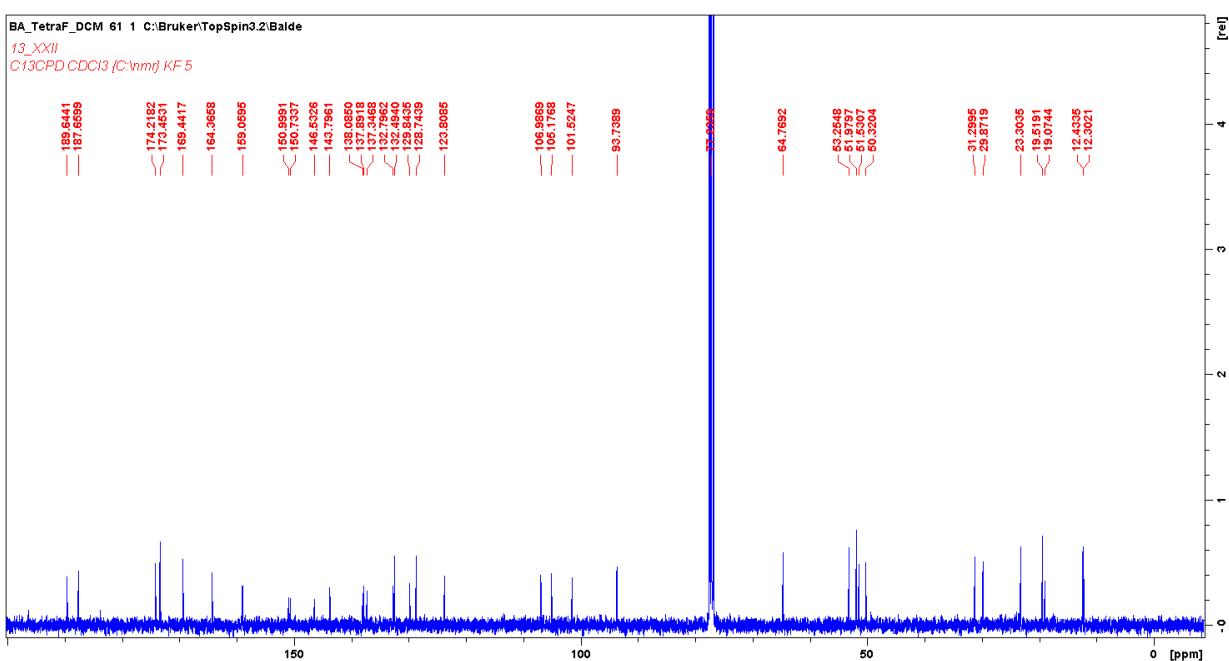
**Figure 6.28.** HSQC spectrum ( $\text{CDCl}_3$ ) of Pheophorbide-A methyl ester (**9**)



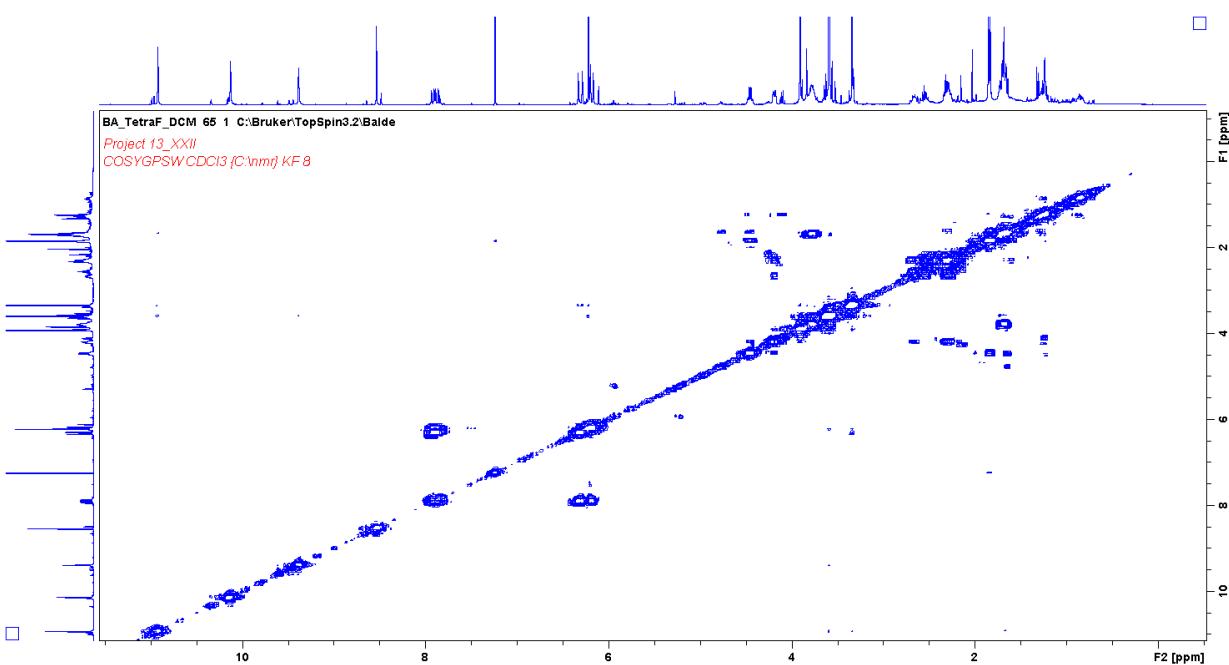
**Figure 6.29.** HMBC spectrum (CDCl<sub>3</sub>) of Pheophorbide-A methyl ester (**9**)



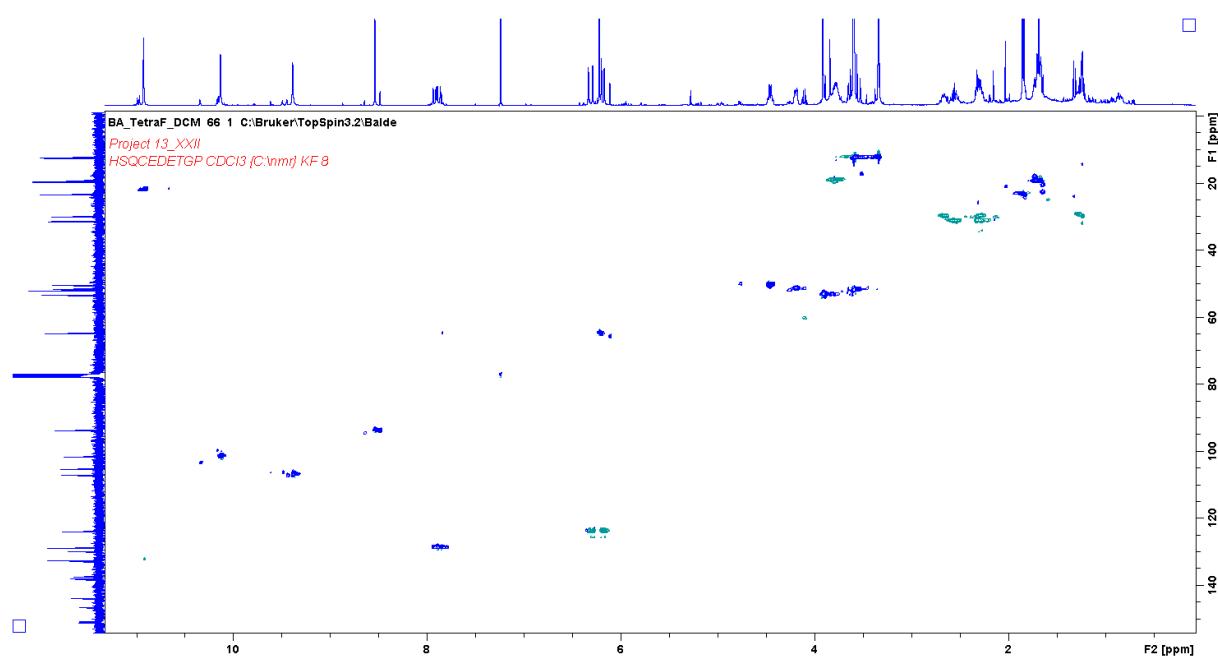
**Figure 6.30.** <sup>1</sup>H NMR spectrum (CDCl<sub>3</sub>, 400 MHz) of Pheophorbide-B methyl ester (**10**)



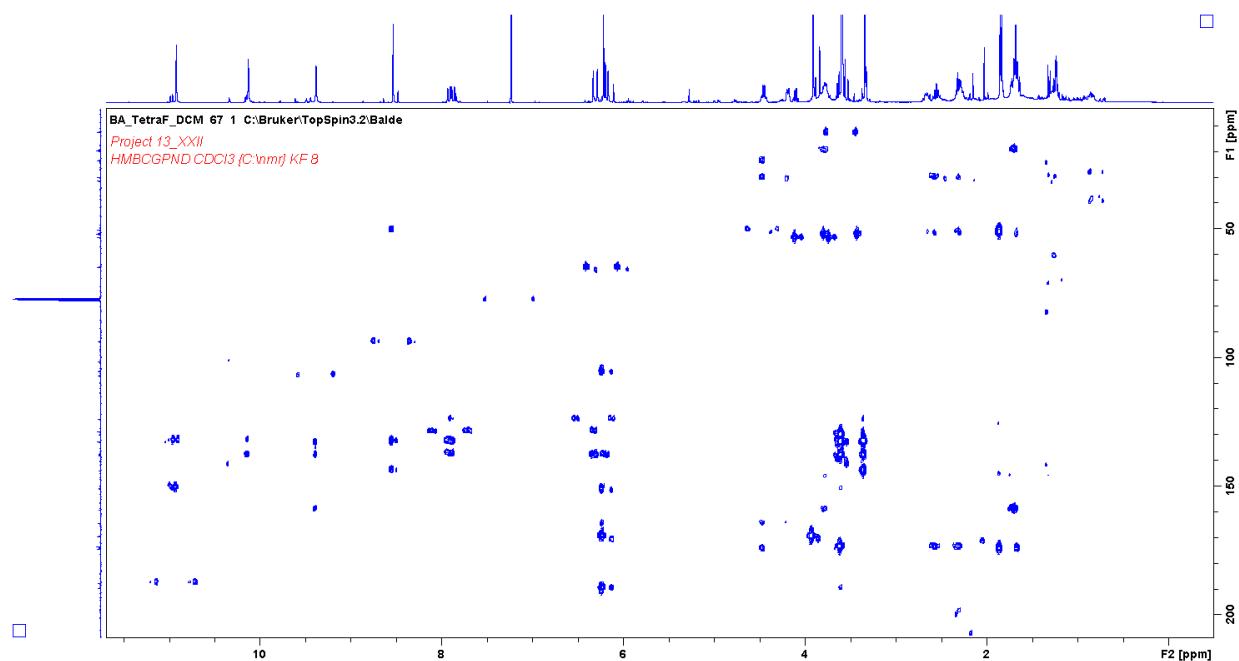
**Figure 6.31.**  $^{13}\text{C}$  NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) of Pheophorbide-B methyl ester (**10**)



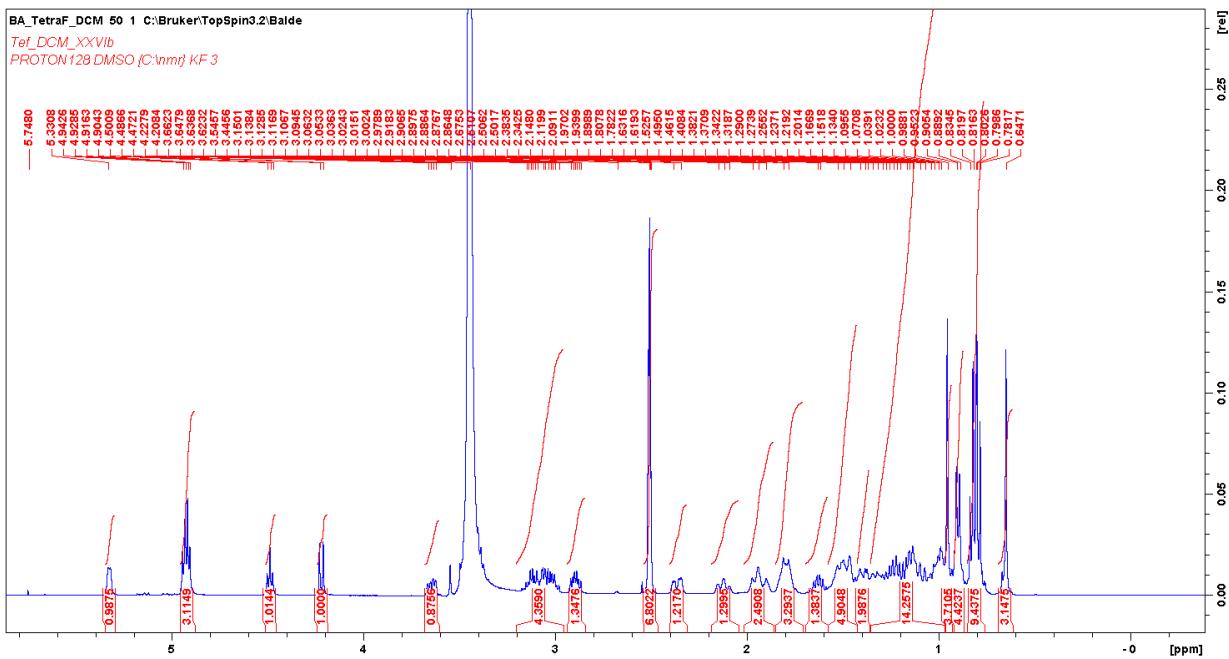
**Figure 6.32.** COSY spectrum ( $\text{CDCl}_3$ ) of Pheophorbide-B methyl ester (**10**)



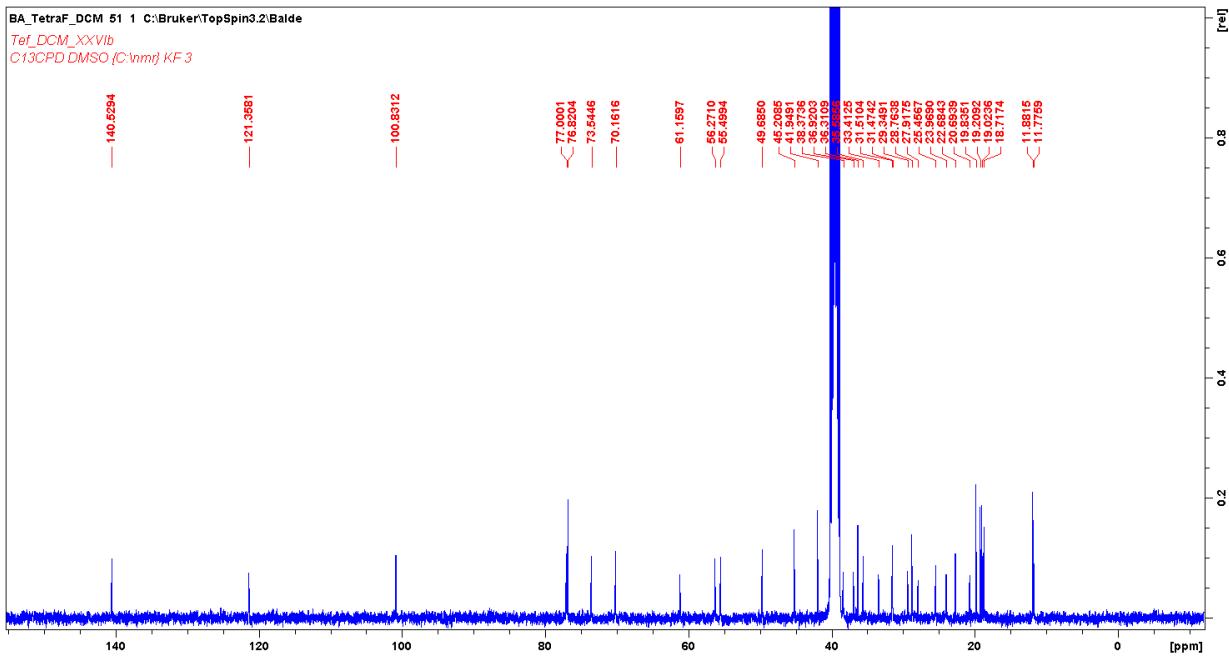
**Figure 6.33.** HSQC spectrum (CDCl<sub>3</sub>) of Pheophorbide-B methyl ester (**10**)



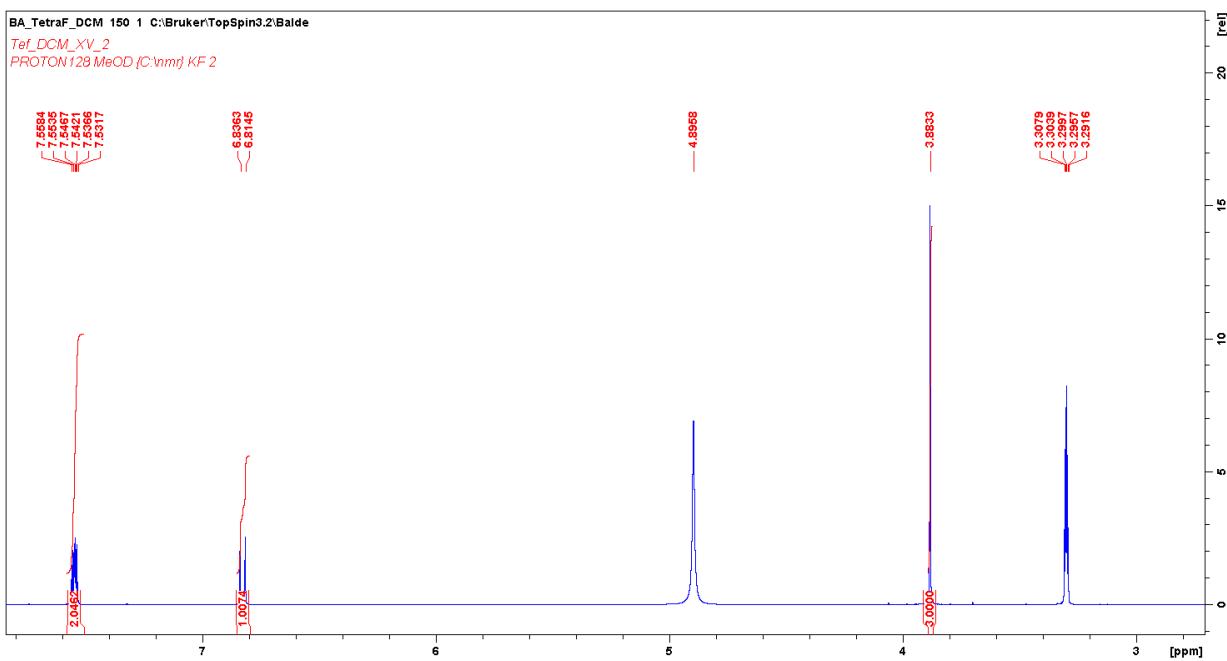
**Figure 6.34.** HMBC spectrum (CDCl<sub>3</sub>) of Pheophorbide-B methyl ester (**10**)



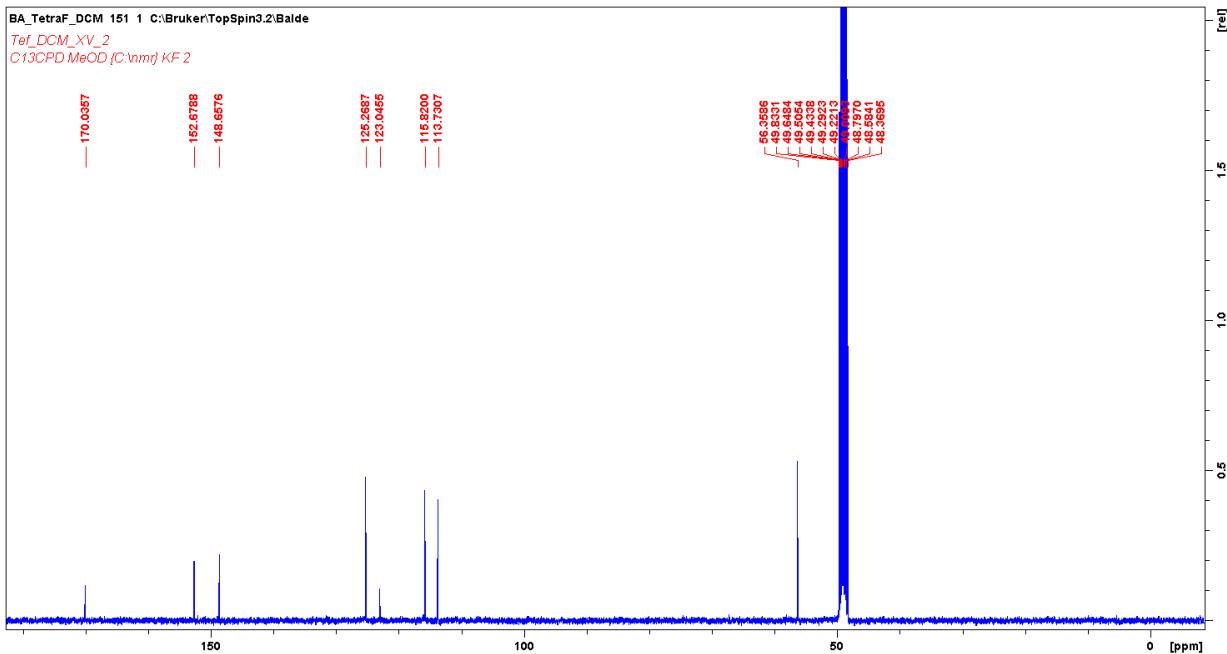
**Figure 6.35.**  $^1\text{H}$  NMR spectrum (DMSO, 400 MHz) of Stigma-5-en-3-O- $\beta$ -glucoside (**11**)



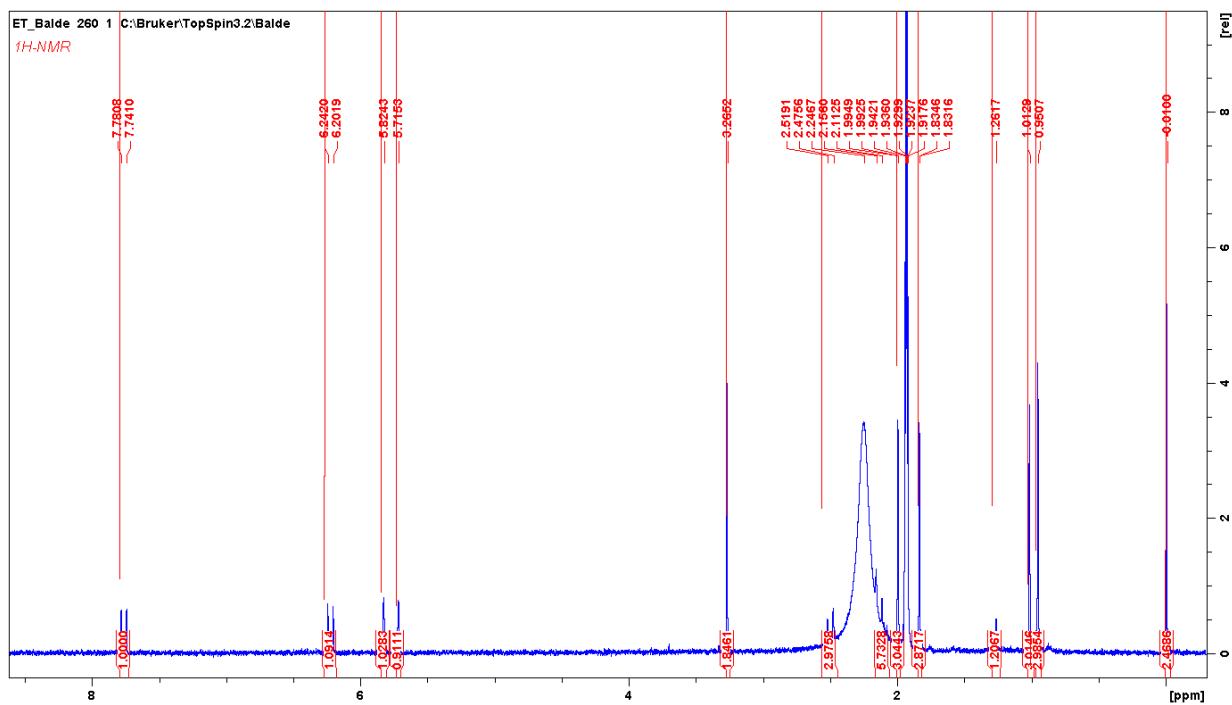
**Figure 6.36.**  $^{13}\text{C}$  NMR spectrum (DMSO, 100 MHz) of Stigma-5-en-3-O- $\beta$ -glucoside (**11**)



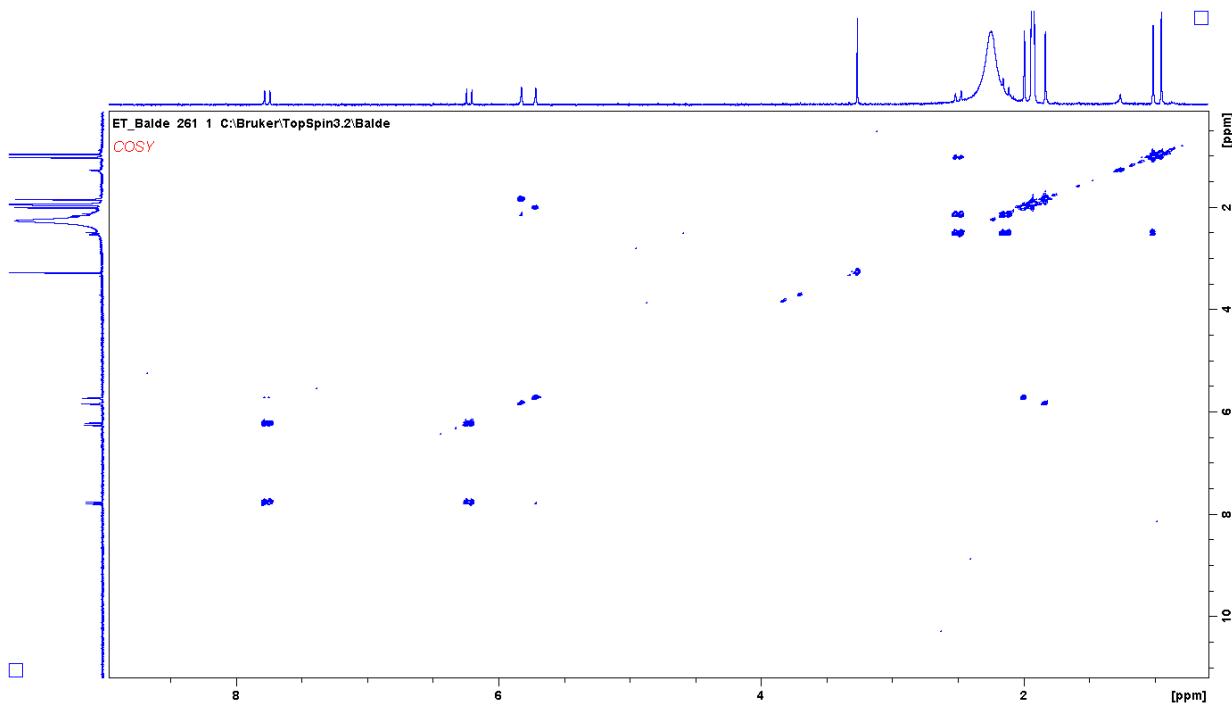
**Figure 6.37.**  $^1\text{H}$  NMR spectrum (DMSO, 400 MHz) of Vannilic acid (**12**)



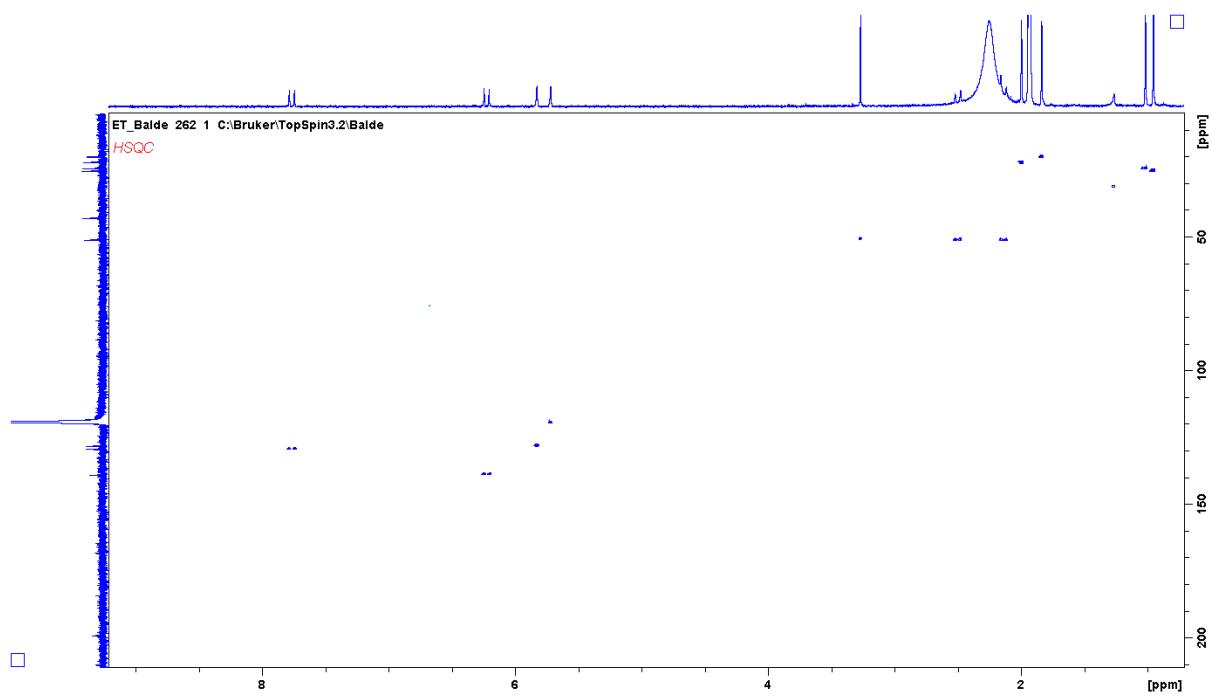
**Figure 6.38.**  $^{13}\text{C}$  NMR spectrum (DMSO, 100 MHz) of Vannilic acid (**12**)



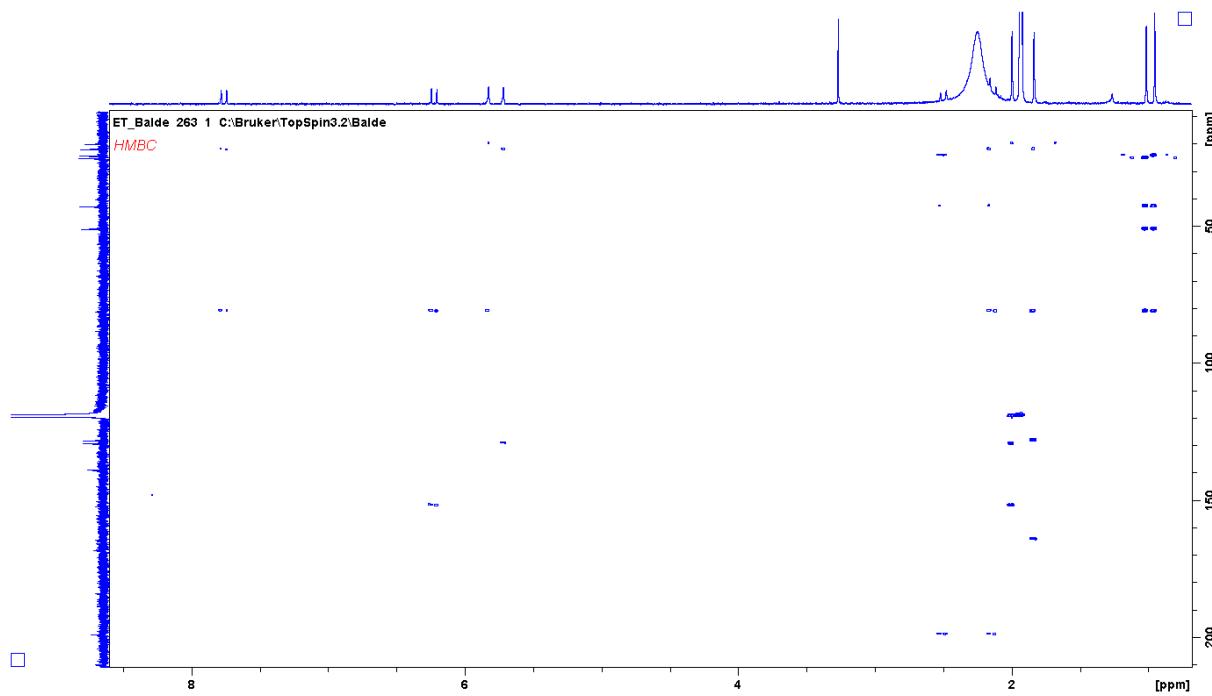
**Figure 6.39.** <sup>1</sup>H NMR spectrum (CD<sub>3</sub>CN, 400 MHz) of Abscisic acid (**13**)



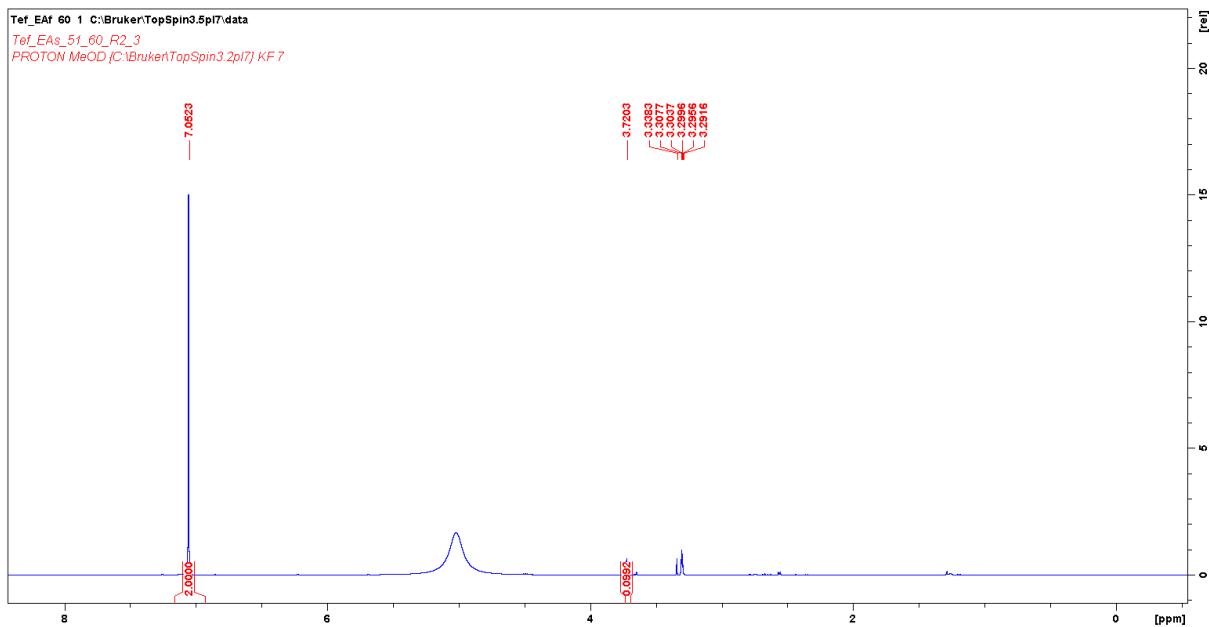
**Figure 6.40.** COSY spectrum (CD<sub>3</sub>CN) of Abscisic acid (**13**)



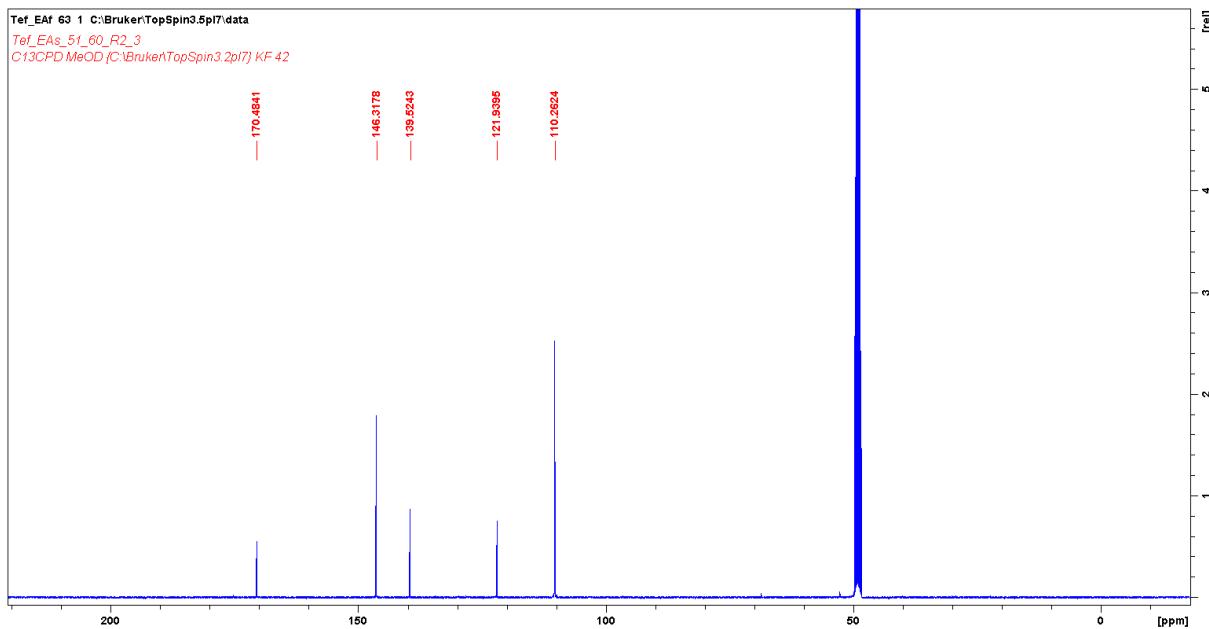
**Figure 6.41.** HSQC spectrum ( $\text{CD}_3\text{CN}$ ) of Abscisic acid (**13**)



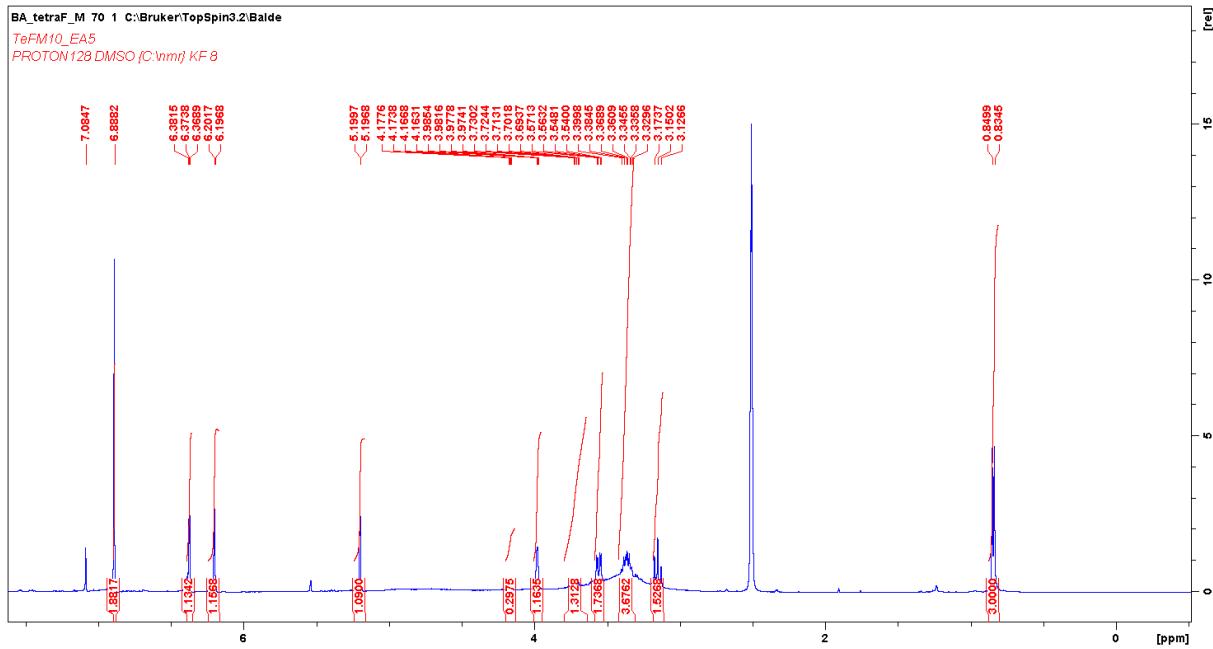
**Figure 6.42.** HMBC spectrum ( $\text{CD}_3\text{CN}$ ) of Abscisic acid (**13**)



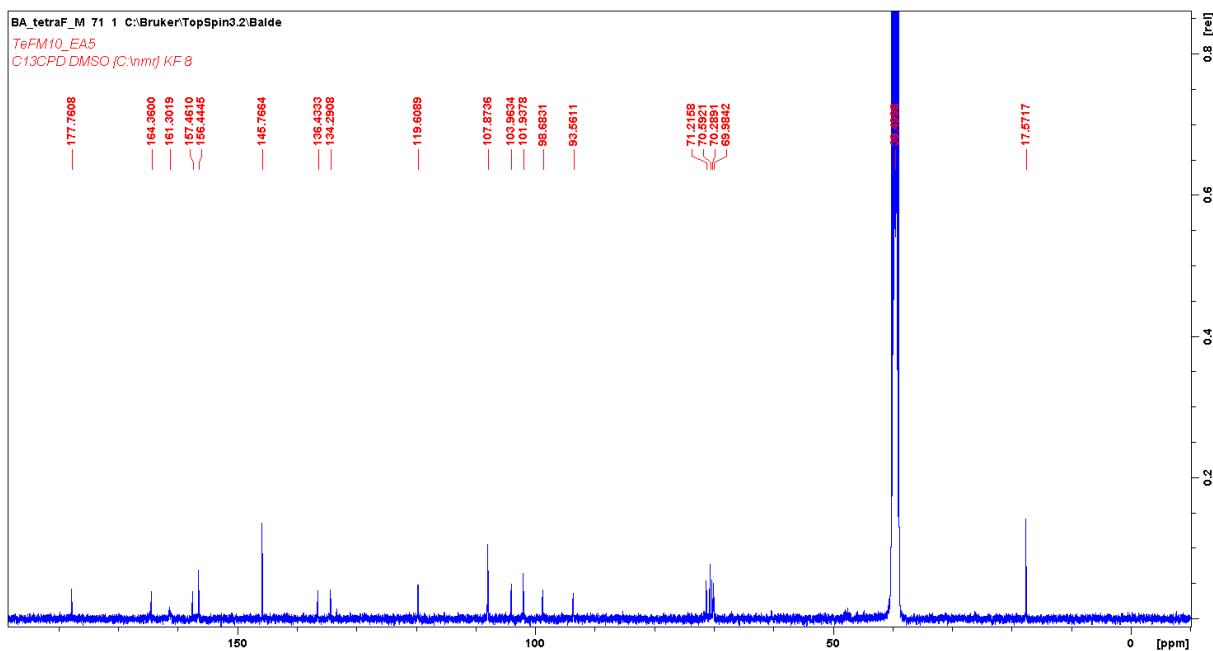
**Figure 6.43.**  $^1\text{H}$  NMR spectrum ( $\text{CD}_3\text{OD}$ , 400 MHz) of Gallic acid (**14**)



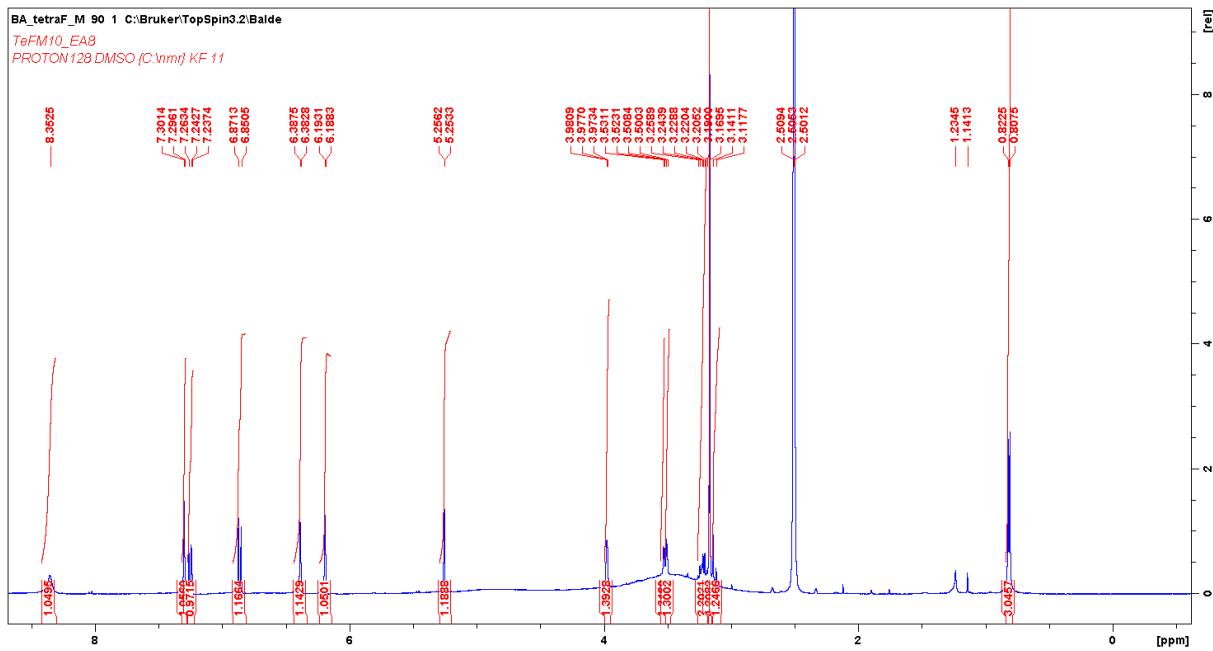
**Figure 6.44.**  $^{13}\text{C}$  NMR spectrum ( $\text{CD}_3\text{OD}$ , 100 MHz) of Gallic acid (**14**)



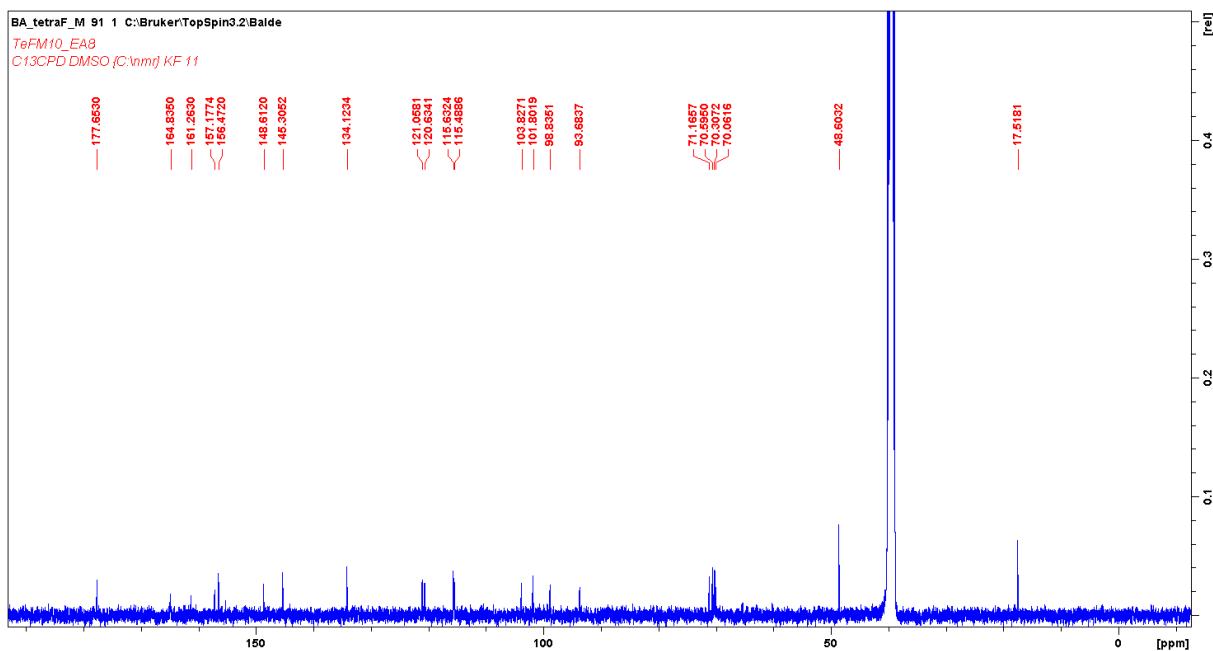
**Figure 6.45.**  $^1\text{H}$  NMR spectrum (DMSO, 400 MHz) of Myricetin-3-O- rhamnoside (**15**)



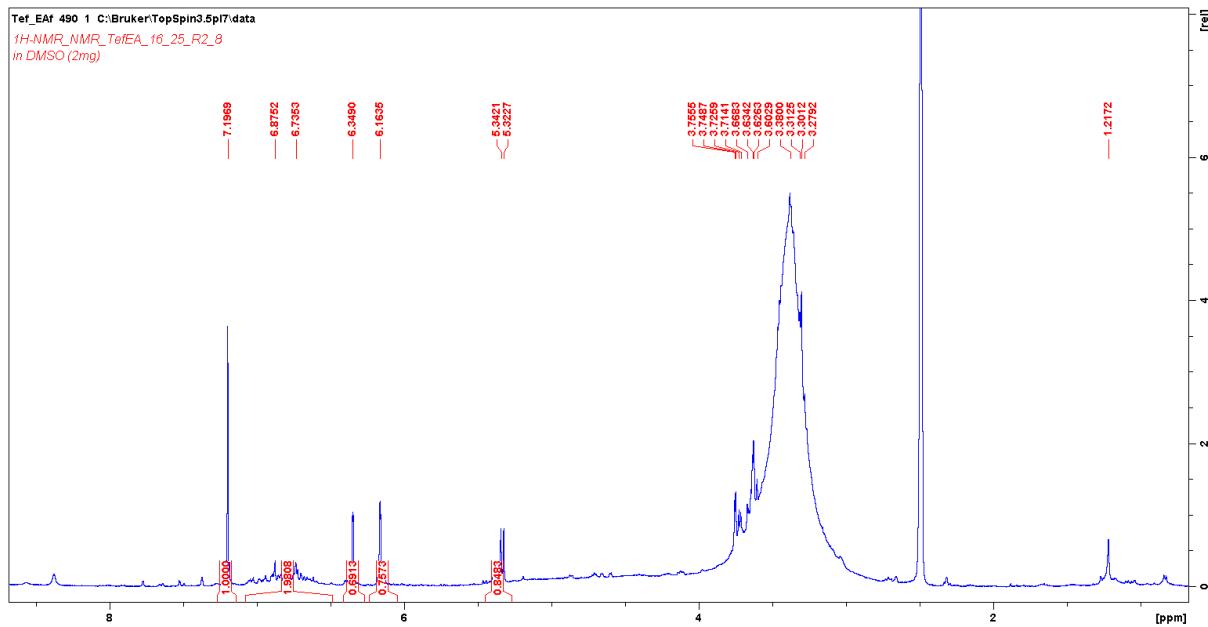
**Figure 6.46.**  $^{13}\text{C}$  NMR spectrum (DMSO, 100 MHz) of Myricetin-3-O- rhamnoside (**15**)



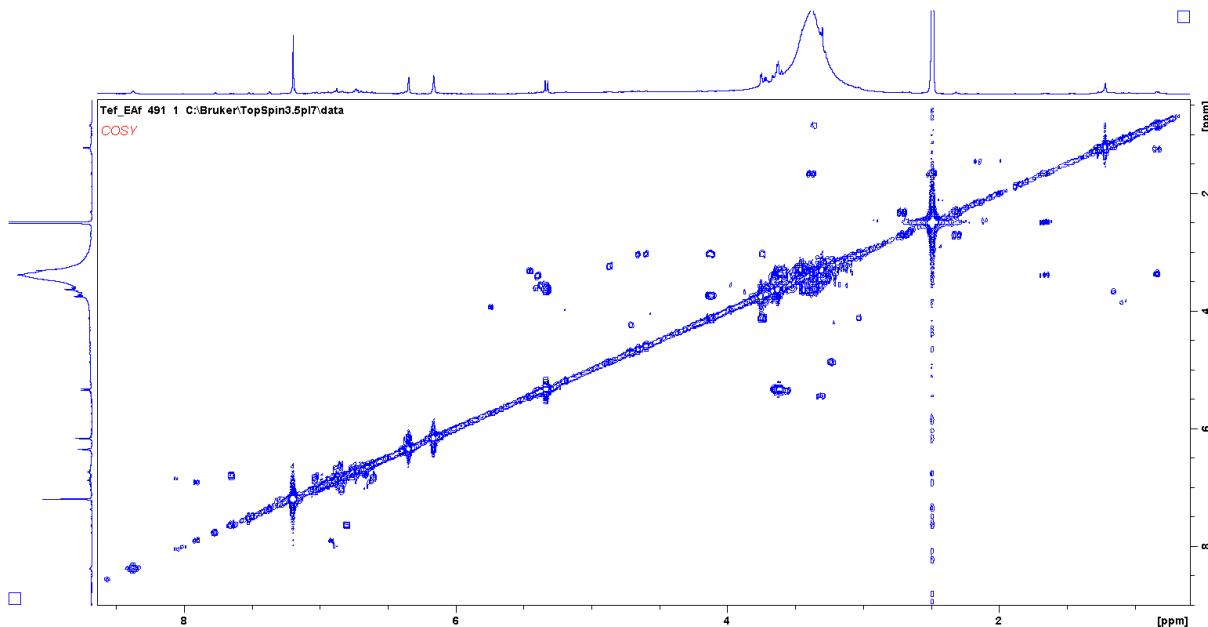
**Figure 6.47.**  $^1\text{H}$  NMR spectrum (DMSO, 400 MHz) of Quercetin-3-O- rhamnoside (**16**)



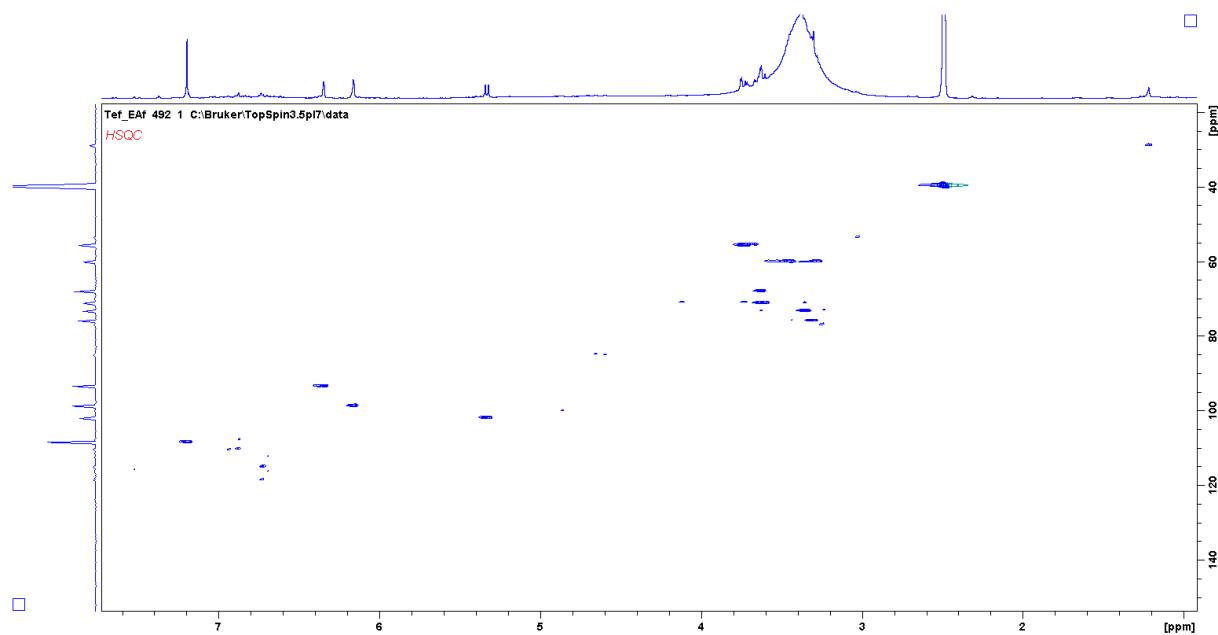
**Figure 6.48.**  $^{13}\text{C}$  NMR spectrum (DMSO, 100 MHz) of Quercetin-3-O- rhamnoside (**16**)



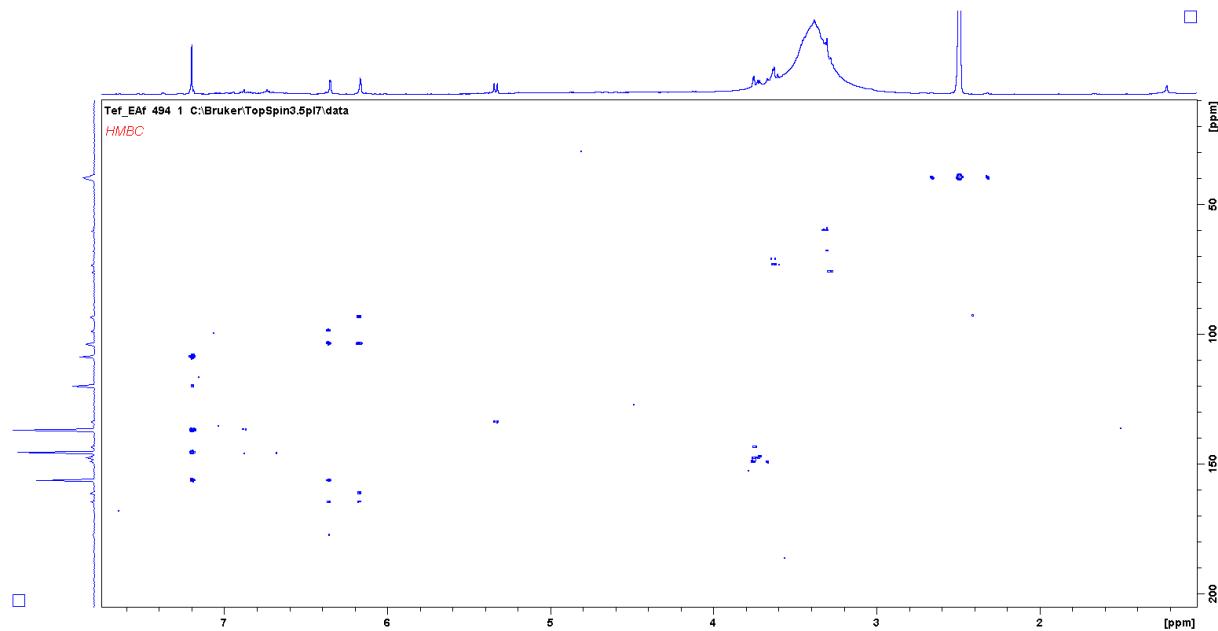
**Figure 6.49.** <sup>1</sup>H NMR spectrum (DMSO, 400 MHz) Myricetin-3',5'dimethylether-3-O-Galactopyranoside (**17**)



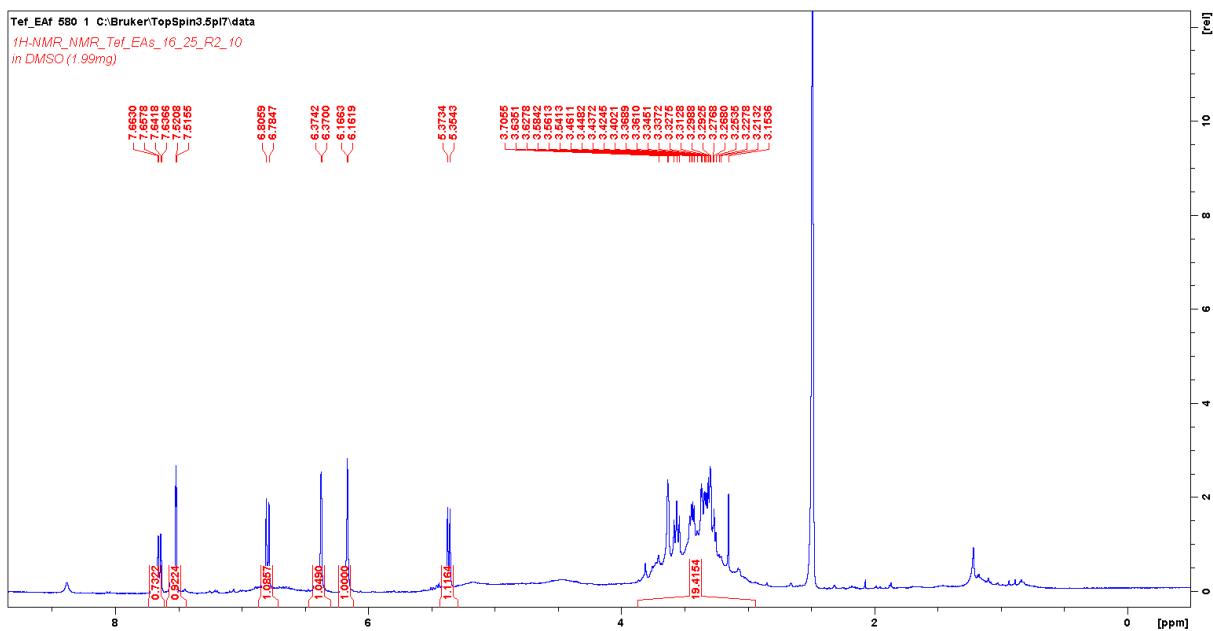
**Figure 6.50.** COSY spectrum (DMSO) Myricetin-3',5'dimethylether-3-O-Galactopyranoside (**17**)



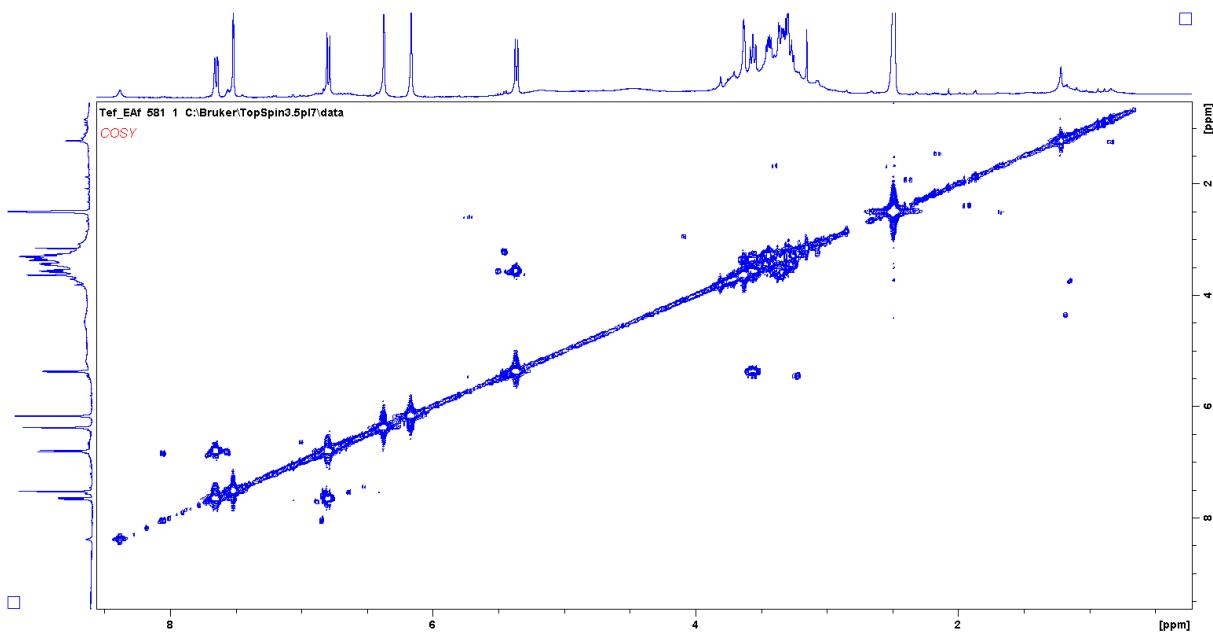
**Figure 6.51.** HSQC spectrum (DMSO) Myricetin-3',5'dimethyl-ether-3-O-Galactopyranoside (**17**)



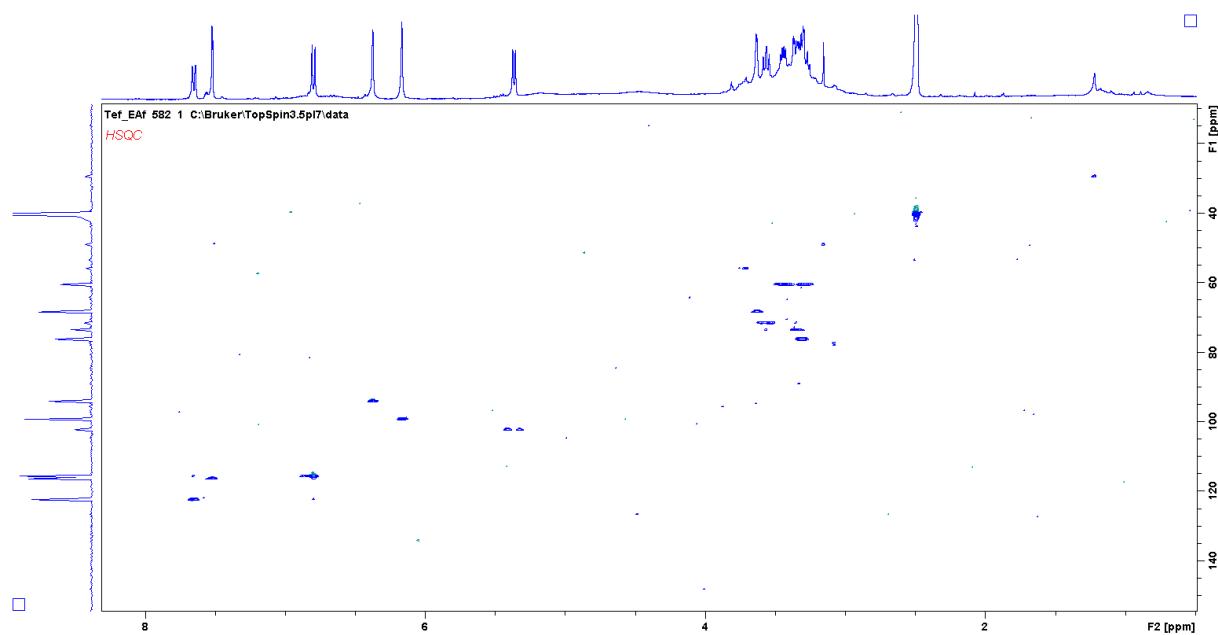
**Figure 6.52.** HMBC spectrum (DMSO) Myricetin-3',5'dimethyl-ether-3-O-Galactopyranoside (**17**)



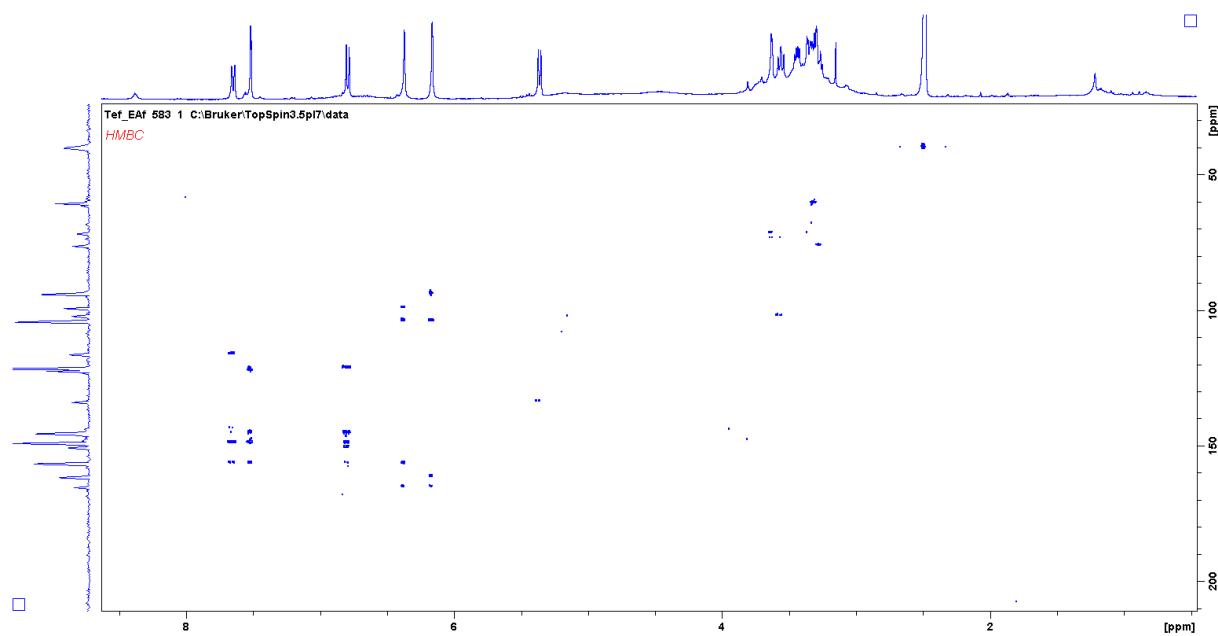
**Figure 6.53.** <sup>1</sup>H NMR spectrum (DMSO, 400 MHz) of Quercetin-3-O-galactopyranoside (**18**)



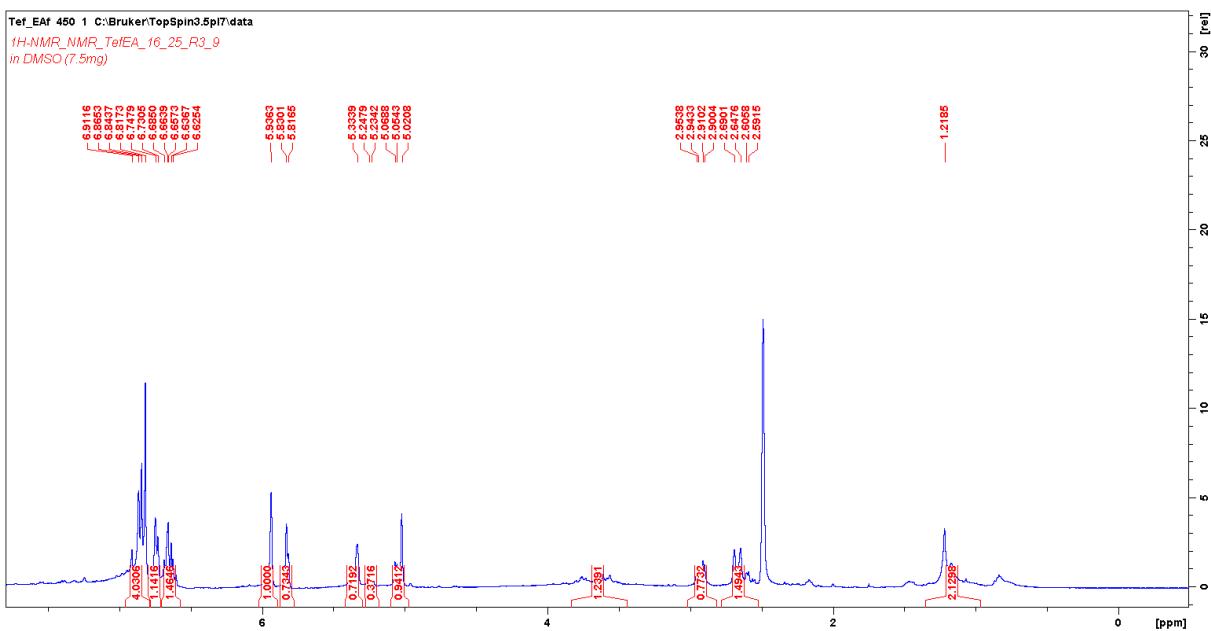
**Figure 6.54.** COSY spectrum (DMSO) of Quercetin-3-O-galactopyranoside (**18**)



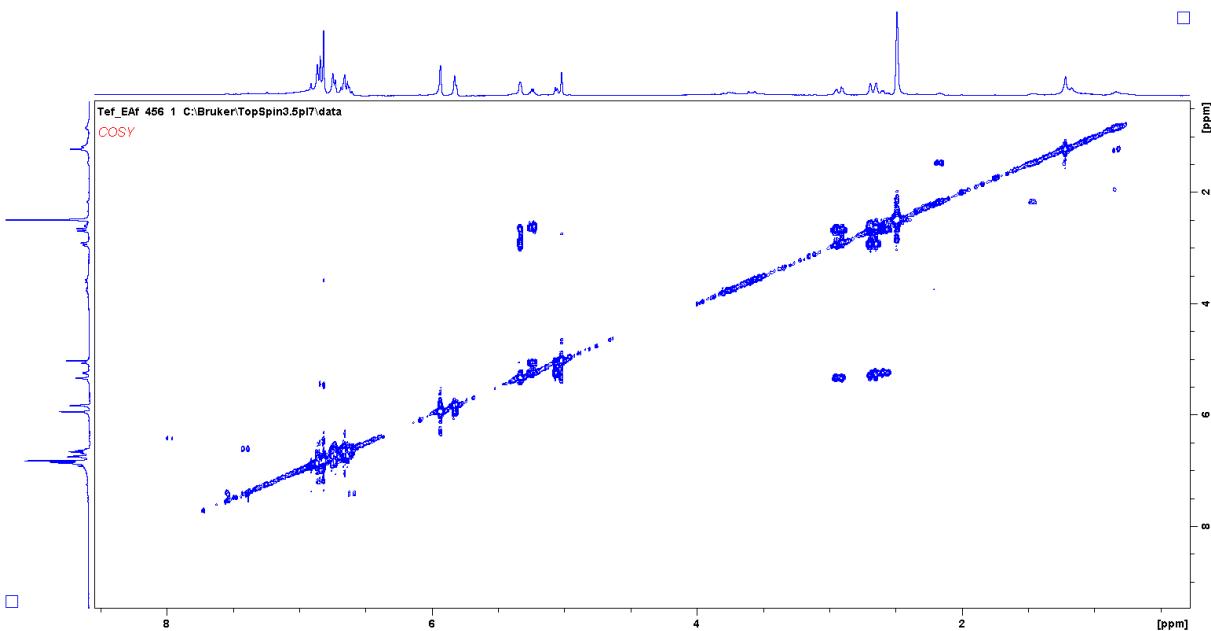
**Figure 6.55.** HSQC spectrum (DMSO) of Quercetin-3-*O*-galactopyranoside (**18**)



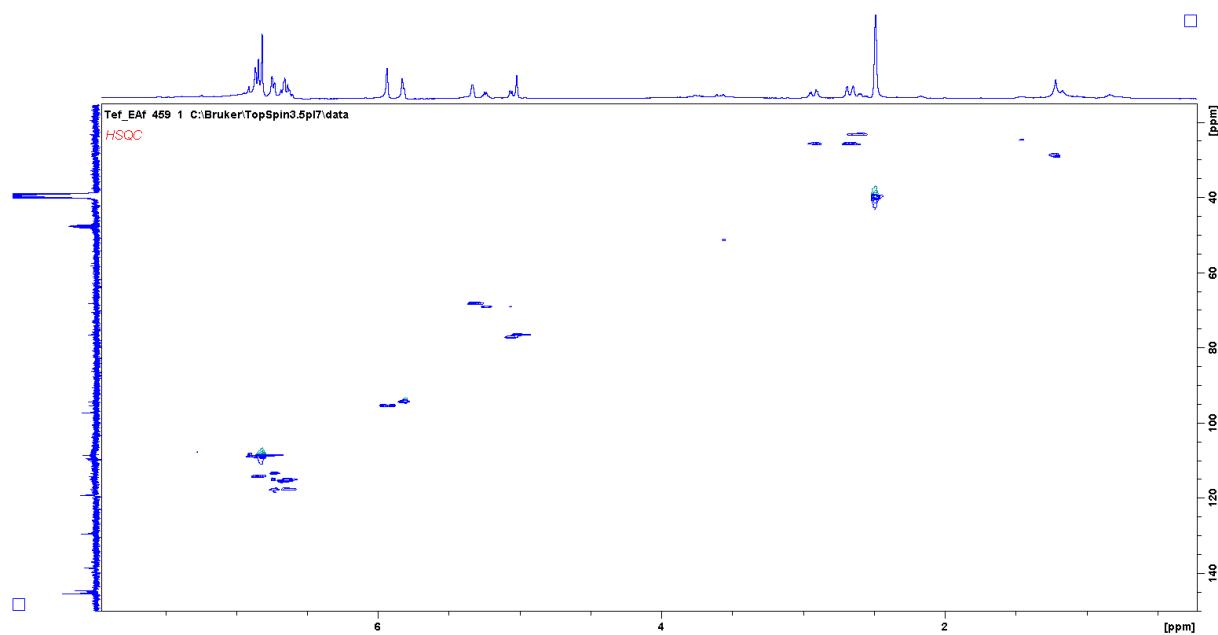
**Figure 6.56.** HMBC spectrum (DMSO) of Quercetin-3-*O*-galactopyranoside (**18**)



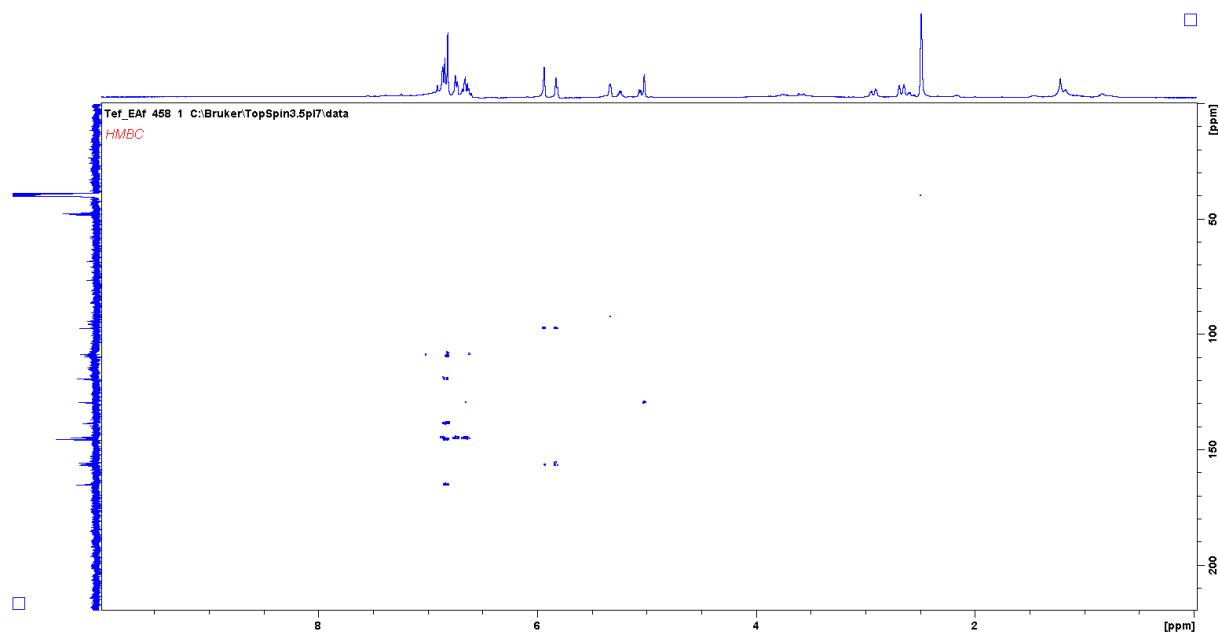
**Figure 6.57.** <sup>1</sup>H NMR spectrum (DMSO, 400 MHz) of Epicatechin-3-galloylester (**19**)



**Figure 6.58.** COSY spectrum (DMSO) of Epicatechin-3-galloylester (**19**)



**Figure 6.59.** HSQC spectrum (DMSO) of Epicatechin-3-galloylester (**19**)



**Figure 6.60.** HMBC spectrum (DMSO) of Epicatechin-3-galloylester (**19**)



## CHAPTER 7

***Combretum paniculatum:*** integration of dereplication and regression analysis for rapid characterization of antiplasmodial compounds from the leaves of *Combretum paniculatum*

**Manuscript in preparation:** Integration of dereplication and regression analysis for rapid characterization of antiplasmodial compounds from the leaves of *Combretum paniculatum*



## 7.1. Introduction

The Combretaceae is a large family of herbs, shrubs and trees, comprising about 20 genera and 600 species with tropical and subtropical distribution around the world<sup>1</sup>. The genus *Combretum* is the most represented, with about 370 species<sup>2</sup>. Some *Combretum* species are extensively used in African traditional medicine against inflammation, infections, malaria, diabetes, sore throats, dental caries, bleeding, diarrhea, jaundice, leprosy, oedema, pneumonia, skin diseases, HIV/AIDS infections, bilharzia, cancer, digestive disorders and yellow fever among others<sup>1,3</sup>. Several *Combretum* species have been previously explored for their phytochemical constituents, which resulted in the identification of many classes of compounds, including triterpenes, flavonoids, lignans and non-protein amino acids, among others. Moreover, since the 1970s, several unusual compounds including dihydrophenanthrenes and a substituted bibenzyl have also been isolated from *Combretum* species<sup>1</sup>.

Among the 24 *Combretum* species inventoried in the Guinean flora, *Combretum paniculatum* is one of the most widespread. *C. paniculatum*, a vigorous evergreen or deciduous liana up to 15 m long or scrambling shrub with deep root system and vivid scarlet flowers, is well known in Guinean traditional medicine for the treatment of several ailments including malaria, skin and oral diseases<sup>4,5,6</sup>. Previous studies have reported activity of *C. paniculatum* against ringworms, and significant cytotoxicity against breast cancer cells<sup>7,8</sup>. In our research program for new potential antiplasmodial and antimicrobial agents from Guinean medicinal plants, promising antiplasmodial activity and a moderate cytotoxicity has been obtained for both the dichloromethane and methanolic extracts of *C. paniculatum*<sup>4</sup>. Following these preliminary results, in-depth studies combining advanced research techniques are needed to isolate and determine the potentially active compounds.

Despite centuries of use, the activity of medicinal plants is only partially understood, and for many herbal preparations on the market, there is a lack of knowledge as to which constituents are responsible for the purported biological activity. Scientific investigation of botanical products is challenging because of their immense complexity and variability<sup>9</sup>.

This process often requires time consuming conventional techniques which involve extraction, bio-guided fractionation, isolation, and characterisation steps<sup>10</sup>. Despite the historical effectiveness of these techniques, loss of activity during the fractionation process is very common. Additionally, because structural information is usually not used to guide separations, this approach may result in the repeated isolation of previously described molecules. To overcome these limitations, preliminary structural assessment steps called “dereplication” can be applied in order to identify and discard samples containing known active constituents<sup>9,11</sup>. Indeed, dereplication strategies rely on analytical techniques and database searching to determine the identity of an active compound at the earliest possible stage in the discovery process<sup>12</sup>. The dereplication approach is often achieved by comparing the spectral patterns of mixtures through the application of chromatographic analysis coupled to spectroscopic or spectrometric approaches (IR, UV, MS, or NMR) and searching for known compounds by matching spectral fingerprints in a dereplication database<sup>13,9,14</sup>.

Nowadays, the rapid development of modern hyphenated techniques and various novel bioinformatics approaches such as molecular networking (MN) and *in silico* tools to predict fragmentation during mass spectrometric analysis provide new perspectives for early metabolite identification in natural products (NPs) research<sup>15,16</sup>. Since compounds with similar structures tend to have similar MS/MS fragmentation patterns, information from MS/MS data with chemical similarity is used for molecular networking, which is considered as an effective dereplication strategy. Indeed, the Global Natural Products Social Molecular Networking (GNPS) website is an open-access web-based mass spectrometry, facilitating high-throughput online dereplication and molecular networking analysis<sup>17</sup>. This method provides an ability to quickly and efficiently categorize hundreds or thousands of samples collected from various origins based on their chemical structures. In this manner, an unknown compound spectrally matched to a structurally annotated compound is resolved as well as elucidated in a more logical way<sup>18</sup>. To date bioassay guided fractionation can be coupled to a dereplication approach and multivariate data analysis in order to rank and identify compounds in mixtures responsible for the biological effects of plant extracts, and provide a better understanding of the mechanisms of action of medicinal plants. Recently, several approaches have been developed for correlating metabolite profiles with biological data sets<sup>19,10</sup>. In this regard

multivariate data analysis (MVA) is commonly used. Orthogonal partial least square (OPLS) regression models enable the prediction of features involved in a given activity for each sample<sup>20,21</sup>. By using this combination of methods, the bioassay-guided isolation route is getting shorter and dereplication of known activities is rapidly delivered<sup>22</sup>. In our research program for new potential antiplasmoidal compounds, this innovative approach combining dereplication, multivariate data analysis and bioassay guided fractionation was applied in order to efficiently identify and isolate metabolites most probably involved in antiplasmoidal activity from *Combretum paniculatum* leaves extract.



**Clade :** Angiosperms

**Order :** Myrtales

**Family :** Combretaceae

**Genus :** *Combretum*

**Species :** *paniculatum*

**Figure 7.1.** *Combretum paniculatum* Vent. Picture: Rainer vent

## 7.2. Materials and methods

### 7.2.1. Solvent and reagents (For more details see chapter 2 and 3)

### 7.2.2. General experimental procedures.

Purification of extracts and fractions was carried out using a MCI open column and a Grace Reveleris X2 flash chromatographic system (Lokeren, Belgium) equipped with an evaporative light scattering detector (ELSD), a UV detector and a fraction collector. The ELSD carrier solvent was isopropyl alcohol and the column used was a pre-packed Flash Pure BUCHI C18 (40 g, particle size of 40 µm). A semi-preparative HPLC system equipped with DAD and ESI-MS detectors was used for the isolation of compounds. The system was

composed of a sample manager, injector, and collector (2767), a quaternary gradient module (2545), a System Fluidics Organizer, a HPLC pump (515), a diode array detector (2998), and a Micromass Quattro TQD mass spectrometer, all supplied by Waters (Milford, MA, USA). For data processing MassLynx version 4.1 was used. Optical rotations were measured on a JASCO P-2000 spectropolarimeter (Easton, MD, USA). 1D and 2D Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DRX-400 NMR spectrometer (Rheinstetten, Germany) equipped with either a 3 mm inverse broadband (BBI) probe or a 5 mm dual  $^1\text{H}/^{13}\text{C}$  probe using standard Bruker pulse sequences and operating at 400 MHz for  $^1\text{H}$  and at 100 MHz for  $^{13}\text{C}$  NMR spectra. The spectra were processed with Topspin version 4.0.6. Chemical shifts are expressed in  $\delta$  (ppm) and referenced to the residual solvent signals.

### 7.2.3. Plant Material

Leaves of *Combretum paniculatum* Vent. (Combretaceae) were collected in Labé, Republic of Guinea in June 2016. The plant was identified by the botanists from the Research and Valorization Center on Medicinal Plants, Dubréka, where a voucher specimen (D36HK10) is kept. The collected samples were dried at room temperature and milled. A "Material Transfer Agreement" has been established between the University of Antwerp and the Department of Pharmacy, University Gamal Abdel Nasser of Conakry / Research and Valorization Center on Medicinal Plants.

### 7.2.4. Extraction and fractionation

The dried leaves of *Combretum paniculatum* (624.6 g) were ground and extracted 6 times with MeOH 80% (each for 24 h) at room temperature. After removal of the solvent by evaporation, the crude extract (180 g) was redissolved in water (1 L) and successively partitioned with  $\text{CH}_2\text{Cl}_2$  (12 x 1 L), EtOAc (10 x 1 L) and *n*-BuOH (10 x 2 L) to give a  $\text{CH}_2\text{Cl}_2$  (16.3 g), an EtOAc soluble fraction (19.6 g), and a *n*-BuOH soluble fraction (26.4 g). The dichloromethane fraction (10.1 g) was fractionated by flash chromatography using a silicagel column (GraceResolv 80 g), eluted with a gradient of dichloromethane (A), ethyl acetate (B) and methanol (C) at a flow rate of 80 mL/min. The gradient was set as follows: 100% A (0–10 min), followed by an increasing concentration of B (10–50 min) till 100% B; this concentration of B was maintained for 10 min; then an increasing concentration of C (60–75 min) till 100% C; finally, this condition was maintained for 10 min (85–100 min).

The collected tubes were pooled in 8 fractions (S<sub>1</sub>-S<sub>8</sub>) based on similarity of their chromatogram on TLC (mobile phase: dichloromethane/ethyl acetate: 1/1 v/v) and HPLC profiles. A Kinetex C18 (100 mm × 2.10 mm, 2.6 µm) (Phenomenex, Torrance, CA, USA) column was used with H<sub>2</sub>O + 0.1% formic acid (A) and acetonitrile (B) as the mobile phase. For all fractions a flow rate of 1 mL/min of the following gradient (min/%B) was used: 0.0/5, 5.0/5, 50.0/95, 55.0/95, 57.0/5, 62.0/5.

The EtOAc fraction (15 g) was subjected to MCI column chromatography and eluted with 3%, 5%, 10%, 15%, 20%, 30%, 40% 50%, 60%, 70%, 80% and 100% MeOH-H<sub>2</sub>O to give eight pooled fractions (fractions S<sub>9</sub> to S<sub>16</sub>) according to their HPLC profiles obtained according to the aforementioned methods.

The *n*-BuOH fraction (17.1 g) was in turn subjected to MCI column chromatography and eluted with 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 100% MeOH-H<sub>2</sub>O to give seven pooled fractions (fractions S<sub>17</sub> to S<sub>23</sub>) according to their HPLC profile. The LC-MS profile of all these fractions (S<sub>1</sub> to S<sub>23</sub>) was recorded for further analysis.

### **7.2.5. Sample preparation and LC-ESI-MS analysis**

Fractions were prepared by dissolving 1 mg of sample in 1 mL of 80% MeOH (v/v) or acetone/methanol (1/9:V/V), which was diluted in order to obtain a final concentration of 0.1 mg/mL. Analysis were carried out on a Xevo-G2XS-QTof mass spectrometer (Waters) coupled with an Acquity LC system equipped with MassLynx version 4.1 software. A Waters Acquity UHPLC BEH Shield RP18 column (2.1 mm × 100 mm, 1.7 µm) was used with a mobile phase consisting of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), which were pumped at a rate of 0.4 mL/min. The gradient system was set as follows: 2% B (0–1 min), 2–100% B (1–15 min), 100% B (15–16 min), 100–2% B (16–17 min), 2% B (17–20 min). For all analyses, full scan data were recorded in ESI (-) and ESI (+) mode from *m/z* 50 to 1500 in sensitivity mode (approximate resolution: 22,000 FWHM) using a spray voltage at either - 0.8 kV and +1 kV, respectively. Cone gas flow and desolvation gas flow were set at 50.0 L/h and 1000.0 L/h, respectively; and source temperature and desolvation temperature at 120 °C and 550 °C, respectively. Leucine enkephalin was used as lock mass during the analysis.

### **7.2.6. Data preprocessing in MZmine 2 software**

Firstly, data were converted to the open-source mzData format to allow further processing<sup>23</sup>. Then, the raw data files were converted to mzXML format using the Msconvert software (ProteoWizard Software Foundation, Los Angeles, CA, USA). After conversion, the spectra were processed in MZmine 2.41.2. software using the following modules: mass detection (RT 2.5–20 min, centroid); chromatogram builder (MS level 1; minimum height  $3 \times 10^3$ ; minimum time span 0.01 min; *m/z* tolerance 5 ppm); deconvolution of the spectra (Algorithm Savitzky-Golay); isotopic peaks grouper (*m/z* tolerance 5 ppm; RT tolerance 0.1 min); duplicate peak filtering; smoothing; data alignment (Join aligner; *m/z* tolerance 5 ppm; RT tolerance 0.2 min); gap-filling (intensity tolerance 10%; *m/z* tolerance 5 ppm, RT tolerance 0.2 min); and peak filtering range (0.00–0.5 min). Then, the chromatograms were aligned, considering MS1 data only and exported as comma-separated values (.csv). These data were finally exported and uploaded to R and metaboanalyst for further analysis.

### **7.2.7. Molecular networking and *in-silico* MS/MS derePLICATION of fractions**

#### **7.2.7.1. In-silico MS/MS derePLICATION**

The UHPLC–HR-MS raw data were firstly converted to Abf files (Reifycs Abf Converter) and processed with MS-DIAL (version 4.16)<sup>24</sup> for mass signal extraction between 50 and 1500 Da from 0 till 20 min. Respective MS1 and MS2 tolerances were set to 0.01 and 0.05 Da in centroid mode. The optimized detection threshold was set to  $1 \times 10^4$  for MS1. The peaks were finally aligned on a quality control (QC) reference file with a retention time tolerance of 0.1 min and a mass tolerance of 0.025 Da. MS-FINDER software ([http://prime.psc.riken.jp/Metabolomics\\_Software/](http://prime.psc.riken.jp/Metabolomics_Software/))<sup>25</sup> was used for the *in silico* fragmentation predictions.

#### **7.2.7.2. GNPS molecular networking**

After MS-DIAL processing, two files (the feature quantification table “TXT file” and the MS/MS spectral summary “MGF file”) were exported and uploaded to the GNPS website (<http://gnps.ucsd.edu>). The molecular network was created using the GNPS web platform<sup>26</sup>. All MS/MS signals within 17 Da of the precursor *m/z* were removed, and only

the top six fragment peaks were compared for analysis. The data were then clustered with MS-Cluster with a precursor ion mass tolerance of 0.05 Da and a MS/MS fragment ion tolerance of 0.5 Da. Next, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.65 and more than one matched peak. Edges between two nodes were kept in the network if, and only if, each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. The data were then imported into Cytoscape v3.7.2 (The Cytoscape Consortium, New York, NY, USA) for visualisation. All matches kept between network spectra and library spectra were required to have a score above 0.6 and at least 1 matched peak. Analogue search was enabled against the library with a maximum mass shift of 100.0 Da.

## 7.2.8. Biological evaluation

### 7.2.8.1. Antibacterial and antifungal activity

The antimicrobial activity of all fractions and pure compounds was evaluated according to Cos et al. (2006) and Baldé et al. (2010). Fractions and pure compounds were tested against *Staphylococcus aureus* ATCC 6538 (Gram-positive) and *Candida albicans* ATCC59630 (yeast). For more details see chapter 2 and 3.

### 7.2.8.2. Antiplasmodial and cytotoxicity assays

Antiplasmodial activity and cytotoxicity were assessed as previously described by Cos et al. (2006) . Fractions and pure compounds were tested *in vitro* against the chloroquine-resistant strain *Plasmodium falciparum* K1. For more details see chapter 2 and 3.

### 7.2.8.3. Isolation of targeted compounds

In this first step of our purifications, fractions S7 rich in metabolites of interest strongly correlated with antiplasmodial activity was selected and purified. Indeed, the purification of fraction **S7** (0.3 g) by semi-preparative HPLC-MS resulted in the isolation of compounds **10** (8,6 mg), **12** (24,8 mg), **18** (5,4 mg) and **20** (8,3 mg), **26** (5.3 mg) and **30** (6.8 mg). The instrument operated with a C18 Kinetex column and eluted with H<sub>2</sub>O + 0.1% formic acid (A) and acetonitrile (B). The flow rate was set at 3 mL/min and the gradient was

programmed as follows: (0-5 min) 15% B, (5-30 min) 30% B, (30–40 min) 50% B, (40–50 min) 95% B, (50–52 min) 15% B and (52–57 min) 15% B. Mass-based fraction collection was used to enable highly specific peak triggering with the fragment masses of *m/z* 302 (compounds **10**, **20**, **26** and **30**) and *m/z* 120 (compound **18**) set as the target masses in negative ion mode.

Chemical structures of the isolated compounds were established by HR-ESI-MS combined with 1D NMR (<sup>1</sup>H-, <sup>13</sup>C-NMR, DEPT 135, DEPT 90) and 2D NMR (COSY, HSQC, HMBC). The <sup>1</sup>H and <sup>13</sup>C -NMR spectroscopic as well as mass spectrometric data of these compounds were in agreement with previously published data, allowing the identification of rutin (quercetin 3-*O*-(6"-*O*- $\alpha$ -rhamnopyranosyl)- $\beta$ -D-glucopyranoside) (**10**)<sup>34</sup>, catechin (**12**)<sup>30,31</sup>, foliasalacioside F (**18**)<sup>32,33</sup>, quercetin-3-*O*-(2"-*O*- $\beta$ -D-glucopyranosyl)- $\alpha$ -L-rhamnopyranoside (**20**)<sup>36,37</sup>, quercetin-3-*O*- $\beta$ -D-glucuronide (**24**)<sup>35</sup>, and quercetin 3-*O*- $\alpha$ -L-xylopyranosyl-(1->2)- $\alpha$ -L-rhamnopyranoside (**29**)<sup>38</sup> (Figure 7.2).

### 7.2.9. Physicochemical and spectral data of isolated compounds

*Quercetin-3-*O*-(6"-*O*- $\alpha$  rhamnopyranosyl)- $\beta$ -D-glucopyranoside (10)*. Yellow powder; <sup>1</sup>H NMR (Pyridine-*d*<sub>5</sub>, 400 MHz)  $\delta_H$ : 1.51 (3H, d, *J* = 5.67 Hz, H''), 5.34 (1H, s, H-1''), 5.99 (1H, d, *J* = 7.37 Hz, H-6), 6.62 (1H, d, *J* = 2.04 Hz, H-8), 6.66 (1H, d, *J* = 2.04 Hz, H-8), 6.94 (1H, d, *J* = 8.30 Hz, H-5'), 7.35 (1H, d, *J* = 8.44 Hz, H-6'), 8.11 (1H, dd, *J* = 2.18; 8.4 Hz, H-2'), 8.35 (1H, d, *J* = 2.17 Hz, H-) 4.09-4.33 (6H, m, sugar protons), 3.63 (4H, m H-3''), 3.86 (1H, dd, *J* = 3.52 ; 9.87 Hz, H-3''), 4.26 (1H, m, H-2''), 4.37 (1H, d, *J* = 7.77 Hz, H-1''). <sup>13</sup>C NMR (CD<sub>3</sub>OD, 100 MHz)  $\delta_C$ : 158.1 (C-2), n.o (C-3), 178.6 (C-4), 162.7 (C-5), 100.2 (C-6), 166.4 (C-7), 95.1 (C-8), 158.2 (C-9), 104.9 (C-10), 122.8 (C-1'), 122.8 (C-2'), 116.8 (C-3'), 151.2 (C-4'), 147.2 (C-5'), 118.4 (C-6'); rhamnose: 102.6 (C-1''), 71.7 (C-2''), 72.6 (C-3''), 74.5 (C-4''), 70.0 (C-5''), 18.6 (C-6''); glucose: 105.2 (C-1''), 76.5 (C-2''), 78.7 (C-3''), 70.0 (C-4''), 77.5 (C-5''), 68.9 (C-6'').

*Catechin (12)*. Colourless powder; <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz);  $\delta_H$ : 3.76 (d, *J*= 9.69 Hz, H-3) 3.54 (m, H-4), 5.32 (d, *J* = 7.6 Hz, H-2) 6.18 (d, *J* = 2.4 Hz, H-8), 6.35 (d, *J* = 2.4 Hz, H-6) 7.63 (d, *J* = 2.1 Hz, H-2') 6.85 (d, 8.4 Hz, H-5') 7.61 (dd, *J* = 2.1, 8.4 Hz, H-6'). <sup>13</sup>C-NMR (CD<sub>3</sub>OD, 100 MHz)  $\delta_C$ : 82.9 (C-2), 68.8 (C-3), 28.5 (C-4), 156.9 (C-5), 96.3 (C-6), 175.6 (C-

7), 95.5 (C-8), 100.8 (C-9), 157.8 (C-10), 132.2 (C-1'), 120.1 (C-2'), 116.1 (C-3'), 146.2 (C-4'), 146.3 (C-5'), 115.3 (C-6').

*Foliasalacioside F (18)*. Amorphous powder;  $^1\text{H}$  NMR ( $\text{Pyridine}-d_5$ , 400 MHz)  $\delta_{\text{H}}$ : 1.05 (3H, s, H3-11), 1.08 (3H, s, H3-12), 1.35 (3H, d,  $J$  = 6.10 Hz, H3-10), 1.61 (3H, s, H3-13), 1.67 (2H, m, H2-8), 1.71 (1H, m, H-2<sub>a</sub>), 2.05 (1H, m, H-7a), 2.21 (1H, m, H-2eq), 2.23 (1H, dd,  $J$  = 16, 9.5 Hz, H-4ax), 2.46 (2H, dd,  $J$  = 5; 14.2 Hz, H-7b, H-4ex), 3.99 (1H, t,  $J$  = 7.6 Hz, H-9), 4.29 (1H, dd,  $J$  = 11, 1 Hz, H6'<sub>a</sub>), 4.38 (1H, m, H-3), 4.82 (1H, dd,  $J$  = 11, 1 Hz, H-6'<sub>b</sub>), 4.93 (1H, d,  $J$  = 7.66 Hz, H-1''), 4.99 (1H, d,  $J$  = 6.67 Hz, H-1').  $^{13}\text{C}$ -NMR ( $\text{Pyridine}-d_5$ , 100 MHz)  $\delta_{\text{C}}$ : 38.4 (C-1), 47.4 (C-2), 72.1 (C-3), 39.9 (C-4), 125.1 (C-5), 138.4 (C-6), 25.6 (C-7), 41.2 (C-8), 68.1 (C-9), 24.6 (C-10), 28.9 (C-11), 30.4 (C-12), 20.3 (C-13), 102.8 (C-1'), 75.6 (C-2') 78.9 (C-3'), 72.3 (C-4'), 69.6 (C-5'), , 70.0 (C-6'), 105.8 (C-1''), 72.8 (C-2''), 77.5 (C-3''), 74.8 (C-4''), 67.0 (C-5'').

*Quercetin-3-O-(2''-O- $\beta$ -D-glucopyranosyl)- $\alpha$ -L-rhamnopyranoside (20)*. yellow powder;  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 400 MHz)  $\delta_{\text{H}}$ : 0.97 (3H, d,  $J$  = 6.2 Hz, H''), 5.62 (1H, d,  $J$  = 1.23 Hz, H-64 H-1''), 6.2 (1H, d,  $J$  = 2.06 Hz, H-6), 6.39 (1H, d,  $J$ =2.06 Hz, H-8), 6.94 (1H, d,  $J$  = 8.30Hz, H-5'), 7.32 (1H, dd,  $J$  = 2.14; 8.34 Hz H-6'), 7.35 (1H, d,  $J$  = 2.1 Hz, H-2'), 3.13-3.36 (6H,m, sugar protons), 3.63 (4H, m H-3''), 3.86 (1H, dd,  $J$  = 3.52 ; 9.87 Hz, H-3'''), 4.26 (1H, m, H-2'''), 4.37 (1H, d,  $J$  = 7.77 Hz, H-1'').  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ , 100 MHz)  $\delta_{\text{C}}$ : 159.3 (C-2), 136.5 (C-3), 179.6 (C-4), 163.0 (C-5), 100.0 (C-6), 166.0 (C-7), 94.9 (C-8), 158.6 (C-9), 105.9 (C-10), 122.8 (C-1'), 122.9 (C-2'), 116.6 (C-3'), 149.9 (C-4'), 146.5 (C-5'), 117.0 (C-6'); 3-O-glucopyranosyl: 107.2 (C-1''), 75.3 (C-2''), 77.8 (C-3''), 70.7 (C-4''), 77.8 (C-5''), 62.1 (C-6'') ; rhamnopyranoside: 102.6 (C-1'''), 82.8 (C-2'''), 71.7 (C-3'''), 73.5 (C-4'''), 72.0 (C-5'''), 17.7 (C-6''').

*Quercetin-3-O- $\beta$ -D-glucoronide. (26)*. yellow powder;  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta_{\text{H}}$ : 3.46-3.61 (3H, m, H2'', H3'', H4''), 3.76 (1H, d,  $J$  = 9.67 Hz, H-5''), 5.32 (1H, d,  $J$  = 7.60 Hz, H-1''), 6.18 (1H, d,  $J$  = 2.0 Hz, H-8), 6.37 (1H, d,  $J$  = 2.0 H-6), 6.85 (1H, d,  $J$  = 8.43 Hz, H-5'), 7.61 (1H, dd,  $J$  = 2.20 ; 8.4 H-6'), 7.63 (1H, d,  $J$  = 2.11 Hz, H-2'),  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta_{\text{C}}$ : 159.1 (C-2), 135.4 (C-3), 179.2 (C-4), 162.9 (C-5), 99.9 (C-6), 165.9 (C-7), 94.9 (C-8), 158.4 (C-9), 105.6 (C-10), 122.8 (C-1'), 123.5 (C-2') 117.3 (C-3'), 149.9 (C-4'), 145.9 (C-5'), , 116.1 (C-6'), , 3-O-glucoronide: 104.2 (C-1''), 75.4 (C-2''), 77.6 (C-3''), 72.8 (C-4''), 77.1 (C-5''), 172.2 (C-6'').

*Quercetin-3-O- $\alpha$ -L-xylopyranosyl-(1->2)- $\alpha$ -L-rhamnopyranoside (30).* yellow powder;  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 400 MHz)  $\delta_{\text{H}}$ : 1.02 (3H, d,  $J$  = 6.2 Hz, H'''), 5.32 (1H, d,  $J$  = 1.23 Hz, H-64 H-1'''), 6.2 (1H, d,  $J$  = 2.05 Hz, H-6), 6.38 (1H, d,  $J$  = 2.04 Hz, H-8), 6.94 (1H, d,  $J$  = 8.34 Hz, H-5'), 7.31 (1H, dd,  $J$  = 2.06; 8.32 Hz H-6'), 7.32 (1H, d,  $J$  = 2.06 Hz, H-2'), 4.18 (1H, dd,  $J$  = 1.47; 3.36 Hz H-2'''), 3.62 (1H, dd,  $J$  = 5.2; 11.5 Hz, H-3'''), 3.40 (1H, m, H-4'''), 3.86 (1H, m, H-5'''), 4.25 (1H, d,  $J$  = 7.62 Hz H-1''), 3.19 (1H, dd,  $J$  = 7.7; 8.8 Hz H-2''), 3.40 (1H, m, H-3''), 3.50 (1H, m, H-4''), 3.86 (1H, m, H-5'') 3.06 (1H, t,  $J$  = 11.34 Hz H-5'').  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ , 400 MHz)  $\delta_{\text{C}}$ : 158.5 (C-2), 136.8 (C-3), 179.8 (C-4), 159.3 (C-5), 100.1 (C-6), 165.9 (C-7), 94.9 (C-8), 163.2 (C-9), 107.8 (C-10), 122.8 (C-1'), 122.7 (C-2'), 116.6 (C-3'), 149.9 (C-4'), 146.5 (C-5'), 116.9 (C-6'); rhamnose: 103.3 (C-1'''), 82.7 (C-2'''), 70.9 (C-3'''), 73.6 (C-4'''), 71.9 (C-5'''), 17.7 (C-6'''); xylose: 107.8 (C-1''), 75.2 (C-2''), 77.7 (C-3''), 71.8 (C-4''), 62.0 (C-5'').

### 7.3. Results and Discussion

The present study reports the characterization of metabolites with potential antiplasmoidal activity from the leaves of *C. paniculatum*. After successive extraction of the plant material, antiplamodal screening of the 23 fractions obtained from respectively the dichloromethane, ethylacetate or n-butanolic fraction from the leaves of *C. paniculatum* revealed that 10 (8 from the dichloromethane fraction, 1 from the ethyl acetate fraction and 1 from the *n*-butanolic fraction) of them were active against *P. falciparum* ( $\text{IC}_{50} > 20 \mu\text{g/mL}$ ) (Table 7.2). The dichloromethane fractions (S<sub>8</sub>, S<sub>2</sub>, S<sub>5</sub>, and S<sub>1</sub>) were the most active with IC<sub>50</sub> values of 1.77, 2.65, 3.73, and 5.17  $\mu\text{g/mL}$ , respectively. The dichlomethane fractions S<sub>3</sub>, S<sub>6</sub>, S<sub>7</sub>, the ethyl acetate fraction S<sub>16</sub> and the *n*-butanolic fraction S<sub>23</sub> showed moderate activity (IC<sub>50</sub> ranging between 5 to 20  $\mu\text{g/mL}$ ). The LC-MS/MS profile of all fractions (active and inactive) was recorded and the resulting spectra were processed for further analysis.

### **7.3.1. UHPLC-HRMS and regression analysis**

A total of 1769 metabolite ions were extracted from all 23 fractions of *C. paniculatum*. In order to detect differences between active and inactive samples, the R software and the MetaboAnalyst platform were used. This exploratory statistical analysis platform considers the retention time and molecular weight of compounds (LC-MS data). The input file is a table with feature (*m/z*), sample name, group (active/inactive) and the area of each peak. An orthogonal partial least-squares discrimination analysis (OPLS-DA) regression model was constructed to examine the metabolites difference between fraction groups. Using the OPLS-DA model, a clear discrimination was achieved between an active and inactive group (Figure 7.3 and Figure S 7.1). This approach allowed to highlight putative features involved in the antiplasmodial activity according to their correlations scorers values and position on an OPLS S-loading plot (Figure 7.4). The resulting values of R<sub>2Y</sub> and Q<sub>2Y</sub> of 0.988 and 0.669, respectively, give an indication of an acceptable fitness and predictability of the OPLS regression model. Moreover, the pQ<sub>2</sub> = 0.05, indicated a significant association between PC1 and the outcome variable (IC<sub>50</sub> values of *P falciparum*) (Figure 7.3B). By using the OPLS scores plot, it was possible to generate a contribution plot which takes the loading plots of both the inactive and active fractions of *C. paniculatum* into consideration. Thus, the detailed analysis of the S-loadings plot (Figure 7.4A) led to the identification of some compounds strongly correlating with the antiplasmodial activity.

### **7.3.2. Identification of compounds based on molecular networking and *in silico* MS/MS dereplication.**

In order to have a broad vision on the metabolites present in the fractions from the leaves of *C. paniculatum*, an extensive dereplication combinatory molecular networking and *in silico* MS/MS dereplication was carried out. For the *in silico* MS/MS dereplication, compounds were tentatively identified according to their similarity score, which was based on comparison between experimental MS/MS fragments and *in silico* spectra. After processing the raw MS spectra by MS-DIAL, data were then transferred in MSFINDER for *in silico* fragmentation. The natural product databases integrated in MSFINDER (PlantCyc,

UNPD, KNAPSAcK and NANPDB) have been used for compound identification. To find the potential candidates, compounds consisting essentially of C, H and O have been considered. For some, the identification has been completed by manual searches by querying the generated molecular formula in different natural product databases such as Dictionary of Natural Products, ChemSpider and Scifinder in order to obtain the molecular structures of the compounds.

Molecular networking has been used as a complementary tools for the dereplication approach used in this study. All acquired MS/MS raw data were converted to Abf files (Reifycs Abf Converter) and processed with MS-DIAL. subsequently, the generated data were uploaded to create molecular networking on the GNPS website (<http://gnps.ucsd.edu>). The resultant network of the analysed metabolites is comprised of clusters of nodes with compounds of higher similarity interconnected and often showing relatively high cosine scores<sup>39</sup>.

This step resulted in the characterization of 61 compounds belonging to different chemical families such as alkaloids, tannins, flavonoids, terpenes, lignans ... (Table 7.1). Likewise these compounds were ranked according to their p-value (probability of being responsible for the activity).

Despite this significant number of compounds tentatively identified, it is important to emphasize that several metabolites showing high correlation scores to the antiplasmodial activity have so far not been identified (Figure S7.1, supplementary information). Thus, the presence of these compounds within the fractions of *C. paniculatum* indicated potential novel compounds. On the other hand, few compounds which showed high, medium and low correlation scores have been identified (Figure 7.4B) and some of them were isolated (Figure 7.2). For instance, the ion peak at *m/z* 635.4156 [M -H]<sup>-</sup> eluted at 11.18 min was tentatively identified as liguveitoside B. The ion peak at peak at *m/z* 306.0755 [M -H]<sup>-</sup> eluted at 0.69 min showed high correlation scores. The predicted molecular formula of this compound C<sub>18</sub>H<sub>13</sub>NO<sub>4</sub> could in all likelihood match to an acridon-type alkaloid (hallacridone). In addition, the ion peak at *m/z* 441.2023 [M-H]<sup>-</sup> (T<sub>R</sub> 14.33 min) with a predicted molecular formula (C<sub>22</sub>H<sub>17</sub>O<sub>10</sub>) has been identified as being catechin gallate. The ion peak at *m/z* 447.0933[M-H]<sup>-</sup> (T<sub>R</sub> 5.58 min) with a predicted

molecular formula C<sub>36</sub>H<sub>56</sub>O<sub>12</sub> has been in turn matched to a quercetin-3-O-rhamnoside. The chemical identity of these compounds was especially supported by their strong correlation that they formed within the network. Indeed an obvious connection of catechin gallate with procyanidin B and catechin was observed in the 1st cluster (Figure 7.5). A similar observation was noted for quercetin-3-O-rhamnoside. In fact, the in-depth analysis of the cluster 2 (Figure 7.6), mainly formed by flavonoid-type compounds, revealed a strong connection between quercetin-3-O-rhamnoside and other flavonoid derivatives such as kaempferol-3-O-(6 "-O-acetyl) glycoside with *m/z* 489.1032 [M-H]<sup>-</sup> (T<sub>R</sub> 4.47 min), rutin *m/z* 609.1449 [M-H]<sup>-</sup> (T<sub>R</sub> 4.93 min) and quercetin-3-O-rhamnosyl-pentoside *m/z* 579.1345 [M-H]<sup>-</sup> (TR 5.54 min).

Although these compounds were not on top of the list of compounds showing high OPLS scores, their antiplasmodial activity was evaluated in order to ensure the effectiveness of our approach. As a result, rutin (**10**) and foliasalacioside F (**18**) showed moderate antiplasmodial activity with an IC<sub>50</sub> value of 6.7 μM and 10.6 μM respectively. Catechin (**12**), quercetin-3-O-β-D-glucoronide (**26**), quercetin 3-O-(2"-O-β-D-glucopyranosyl)-α-L-rhamnopyranoside (**20**), quercetin 3-O-α-L-xylopyranosyl-(1->2)-α-L-rhamnopyranoside (**30**) were weakly active or even inactive (Table 7.3).

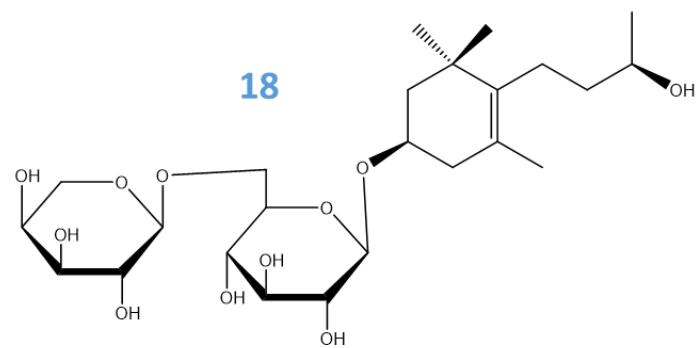
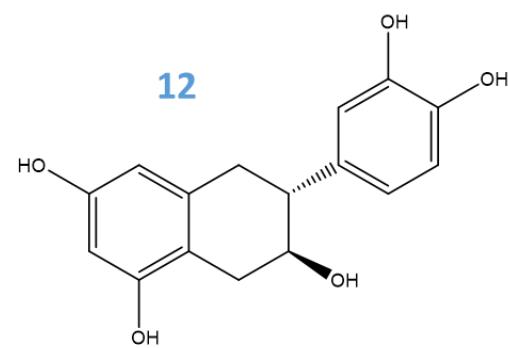
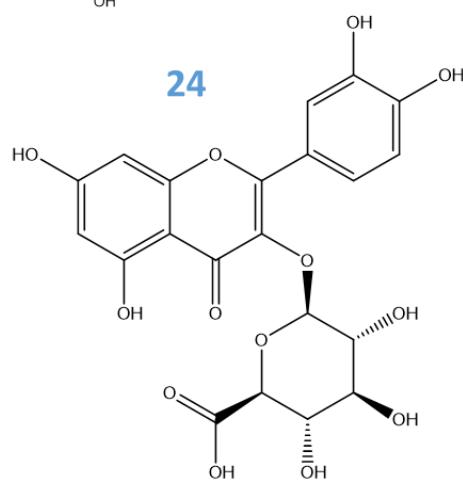
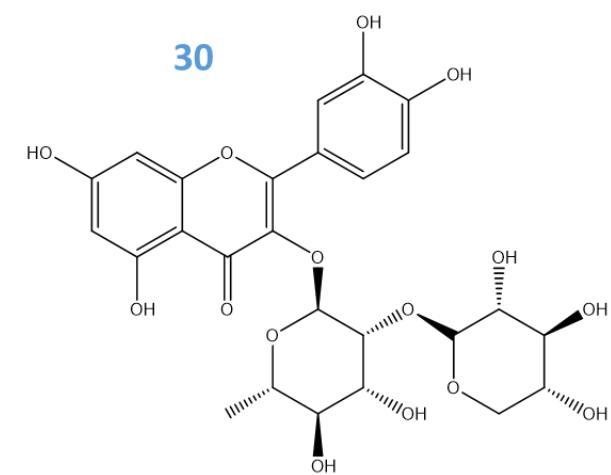
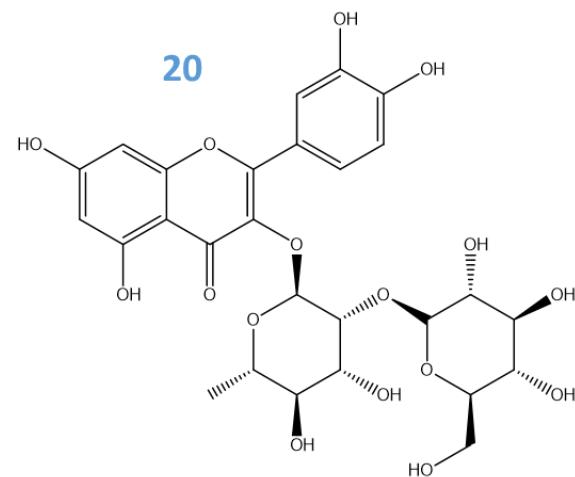
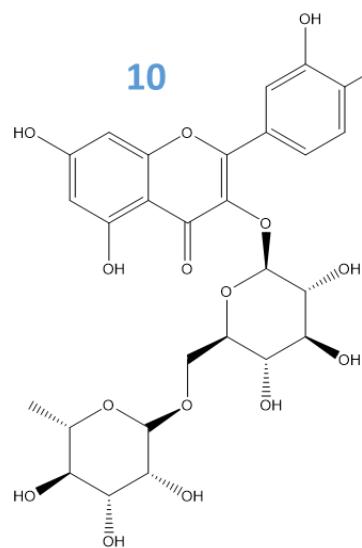
The antiplasmodial activity of rutin have been previously reported. however, to best of our knowledge, this is the first time that the antiplasmodial activity of, foliasalaciosides F (**18**), quercetin-3-O-β-D-glucoronide (**26**), quercetin 3-O-(2"-O-β-D-glucopyranosyl)-α-L-rhamnopyranoside (**20**), and quercetin 3-O-α-L-xylopyranosyl-(1->2)-α-L-rhamnopyranoside (**30**) was reported.

The antiplasmodial activity of rutin has been previously studied. In fact, Ganhes et al. (2012) have previously reported the effectiveness of rutin (**10**) against *P. falciparum* clones 3D7 and K1 clone with IC<sub>50</sub> values (3.53 ± 13.34 μM and 10.38± 15.08 μM), respectively<sup>40</sup>. In addition, an *in vivo* study showed that a combination of rutin and swertiamarin possessed potential antimalarial activity which is similar to that found for chloroquine phosphate<sup>41</sup>. Although these compounds have not yet been isolated and tested against *P. falciparum* in our study, it is important to stress that the antiplasmodial activity of some of them has already been reported. For instance, two catechin gallate

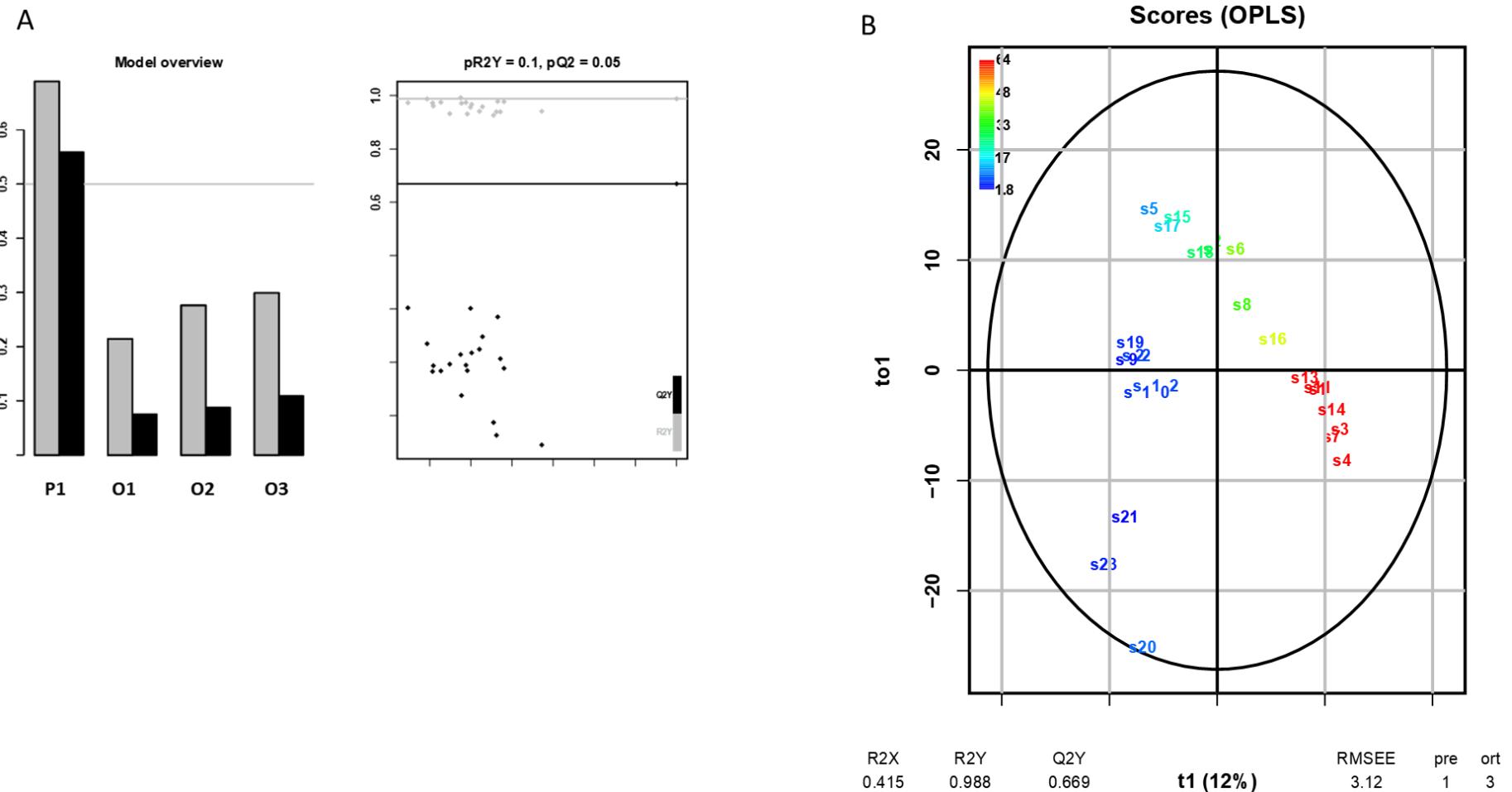
isomers (+)-catechin 3-gallate and (+)-catechin 5-gallate were found to be active against *P. falciparum* chloroquine-resistant strain FcB1 with IC<sub>50</sub> values of 1.2 µM and 1.0 µM, respectively<sup>42</sup>.

The megastigmane glycoside, foliasalacioside F (**18**) was among the metabolites mostly correlated with the antiplasmodial activity with an OPLS coefficient scores slightly higher than to that of rutin (**10**) (Figure 7. 4B).

Previous studies have demonstrated the effectiveness of quercetin-3-O-rhamnoside against *P. falciparum*. Indeed, Zofou et al (2013) have shown that quercetin-O-rhamnoside isolated from *Dacryodes edulis* (Burseraceae) was effective against both 3D7 (chloroquine-sensitive) (IC<sub>50</sub> 5.96 ± 0.51 µg/mL) and Dd2 (multidrug-resistant) (IC<sub>50</sub> 2.26 ± 0.28 µg/mL) strains of *Plasmodium falciparum*<sup>43,44</sup>. Although reported here for the first time in *C. paniculatum*, quercetin-O-rhamnoside has been previously isolated from other *Combretum* species such as *C. leprosum* and *C. erythrophyllum*<sup>45,46</sup>.



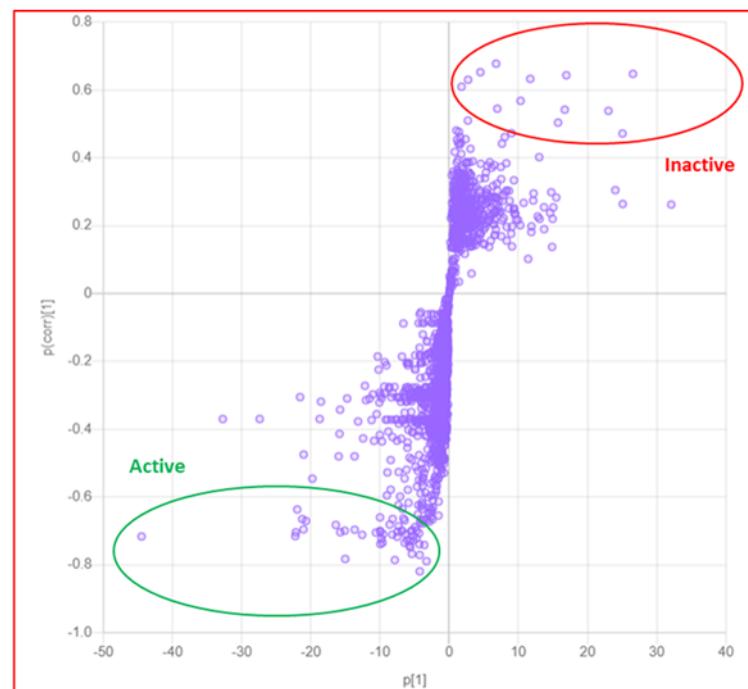
**Figure 7.2.** Compounds isolated from *Combretum paniculatum*



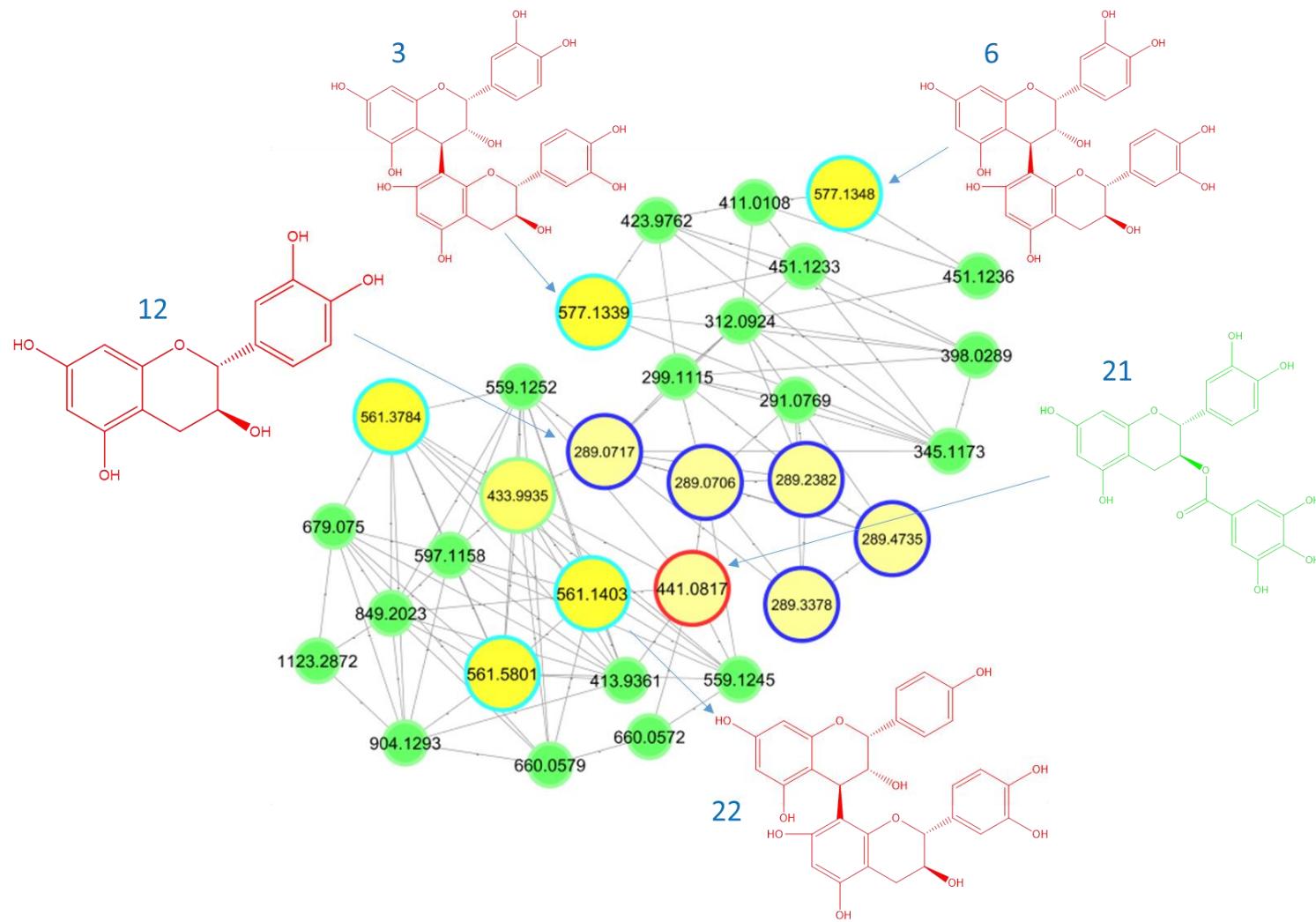
**Figure 7.3.** (A) OPLS-DA model overview and (B) OPLS-DA scores plot of *C. paniculatum* fractions ( $R2Y = 0.988$ ,  $Q2Y = 0.669$ )

**B**

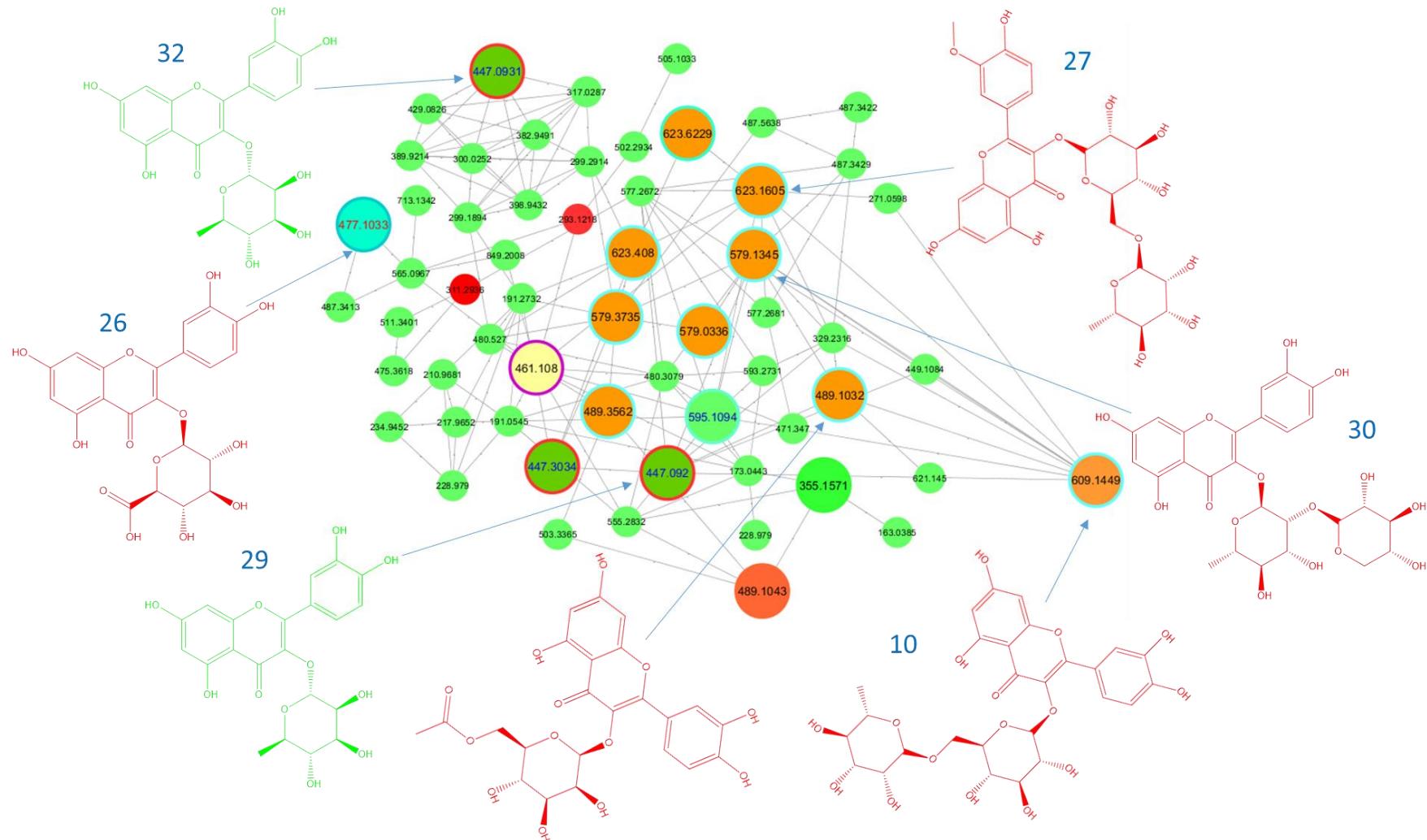
RT (min)	Tentatively identified names	MF	[M-H] <sup>-</sup>	OPLS coefficients
11.18	Liguveitoside B	C <sub>36</sub> H <sub>59</sub> O <sub>9</sub>	635.4156	-0.72828
0.69	Hallacridone	C <sub>18</sub> H <sub>13</sub> NO <sub>4</sub>	306.0755	-0.71663
5.00	Catechin gallate	C <sub>22</sub> H <sub>17</sub> O <sub>10</sub>	441.0817	-0.69677
11.24	acylated triterpenoid	C <sub>39</sub> H <sub>53</sub> O <sub>7</sub>	633.3792	-0.37046
4.64	Foliasalaciosides F	C <sub>39</sub> H <sub>53</sub> O <sub>7</sub>	505.2650	-0.30487
12.82	Coumaroyl maslinic acid	C <sub>39</sub> H <sub>53</sub> O <sub>6</sub>	617.3839	-0.29304
4.09	Epiafzelechin-(4 β→8)-epicatechin (isomer)	C <sub>30</sub> H <sub>25</sub> O <sub>11</sub>	561.1396	0.26384
5.22	Quercetin-3-O-rutinoside	C <sub>27</sub> H <sub>29</sub> O <sub>6</sub>	609.1456	0.25398
5.58	Quercetin-3-O-rhamnoside	C <sub>36</sub> H <sub>56</sub> O <sub>12</sub>	447.0931	-0.20019
3.69	Procyanidin B	C <sub>30</sub> H <sub>25</sub> O <sub>12</sub>	577.1343	0.1811
5.62	Quercetin-3-O-glucoside	C <sub>21</sub> H <sub>19</sub> O <sub>12</sub>	463.0875	-0.15096
4.02	catechin	C <sub>15</sub> H <sub>19</sub> O <sub>12</sub>	289.0917	-0.17633
5.68	Cyanidin-3-O-rhamnoside	C <sub>21</sub> H <sub>21</sub> O <sub>10</sub>	433.1133	-0.061389
5.90	Apigenin 6-C-pentosyl-8-C-hexoside	C <sub>26</sub> H <sub>27</sub> O <sub>14</sub>	563.1411	-0.05521

**A**

**Figure 7.4.** (A) S-loading plot of active versus inactive fractions showing some metabolites highly correlated to the antiplasmodial activity; (B) some identified compounds with high and low correlation scores of *C. paniculatum* fractions.



**Figure 7.5.** Molecular network of the molecular family of tannins extracted from the molecular network of the leaves extract of *Combretum paniculatum*



**Figure 7.6.** Molecular network of the molecular family of flavonoids extracted from the molecular network of the leaves extract of *Combretum paniculatum*.

**Table 7.1.** Summary of dereplicated compounds from *C. paniculatum* fractions

N°	RT (min)	Compound	Molecular Formula	ESI negative full		ESI negative mode MS/MS	Δ ppm
1	0.71	Procyanidin B1	C <sub>30</sub> H <sub>25</sub> O <sub>12</sub>	577.1348	[M-H] <sup>-</sup>	289.0702	0.34
2	0.69	Hallacridone	C <sub>18</sub> H <sub>13</sub> NO <sub>4</sub>	306.0755	[M-H] <sup>-</sup>	288.0649, 272.0878	
3	3.02	Procyanidin B1	C <sub>30</sub> H <sub>25</sub> O <sub>12</sub>	577.1339	[M-H] <sup>-</sup>	289.0698	-1.21
4	3.32	Cynaroside A	C <sub>21</sub> H <sub>31</sub> O <sub>10</sub>	443.1912	[M-H] <sup>-</sup>	281.1203	-0.45
5	3.61	3-p-Coumaroylquinic acid	C <sub>16</sub> H <sub>17</sub> O <sub>8</sub>	451.2182	[M-H] <sup>-</sup>	337.0917	1.77
6	3.66	Procyanidin B2	C <sub>30</sub> H <sub>25</sub> O <sub>12</sub>	577.1348	[M-H] <sup>-</sup>	289.0704	0.34
7	3.73	Procyanidin B2 (isomer)	C <sub>30</sub> H <sub>25</sub> O <sub>12</sub>	577.1343	[M-H] <sup>-</sup>	289.0701	-0.51
8	3.87	Dactylorhin C	C <sub>14</sub> H <sub>23</sub> O <sub>10</sub>	351.1281	[M-H] <sup>-</sup>		-2.84
9	3.99	Epiafzelechin-(4 β→8)-epicatechin	C <sub>30</sub> H <sub>25</sub> O <sub>11</sub>	561.1404	[M-H] <sup>-</sup>	289.0706	1.24
10	3.99	Quercetin 3-O-(6"-O-α rhamnopyranosyl)-β-D-glucopyranoside	C <sub>28</sub> H <sub>31</sub> O <sub>18</sub>	655.1500	[M+HCOO] <sup>-</sup>	609.1454	-1.52

11	4.09	Epiafzelechin-(4 (isomer)	$\beta\rightarrow8$ )-epicatechin	C <sub>30</sub> H <sub>25</sub> O <sub>11</sub>	561.1396	[M-H] <sup>-</sup>	289.0703	-0.17
12	4.02	Catechin		C <sub>15</sub> H <sub>13</sub> O <sub>6</sub>	289.0717	[M-H] <sup>-</sup>	271.0611	1.72
13	4.21	Diphyllin-hexose-pentose	(isomer)	C <sub>32</sub> H <sub>35</sub> O <sub>16</sub>	675.1921	[M-H] <sup>-</sup>	511.2372	-0.59
14	4.23	Rossoliside		C <sub>17</sub> H <sub>19</sub> O <sub>8</sub>	351.1066	[M-H] <sup>-</sup>		-3.98
15	4.41	Diphyllin-hexose-pentose		C <sub>32</sub> H <sub>35</sub> O <sub>16</sub>	675.1918	[M-H] <sup>-</sup>	511.2372	-1.03
16	4.46	(+)-Catechin 3,5-di-O-gallate		C <sub>29</sub> H <sub>22</sub> O <sub>14</sub>	595.1082	[M-H] <sup>-</sup>	577.1330, 533.1082	-1.0
17	4.47	Kaempferol 3-O-(6"-O-acetyl) glycoside		C <sub>23</sub> H <sub>21</sub> O <sub>12</sub>	489.1032	[M-H] <sup>-</sup>		-0.20
18	4.64	Foliasalaciosides F		C <sub>39</sub> H <sub>53</sub> O <sub>7</sub>	551.2448	[M-H] <sup>-</sup>	505.2650	-2.17
19	4.83	Euodionoside E		C <sub>20</sub> H <sub>35</sub> O <sub>9</sub>	447.0562	[M+HCOO] <sup>-</sup>	373.2177	-1.19
20	4.93	Quercetin 3-O-(2"-O- $\beta$ -D- glucopyranosyl)- $\alpha$ -L-rhamnopyranoside		C <sub>27</sub> H <sub>29</sub> O <sub>16</sub>	609.1449	[M-H] <sup>-</sup>	593.1506, 549.2534	0.00
21	5.00	Catechin gallate		C <sub>22</sub> H <sub>17</sub> O <sub>10</sub>	441.0817	[M-H] <sup>-</sup>	272.0661	-1.13

22	5.08	Epiafzelechin-(4 $\beta$ -8)-epicatechin (isomer)	C <sub>30</sub> H <sub>25</sub> O <sub>11</sub>	561.1403	[M-H] <sup>-</sup>	289.0705	1.24
23	5.13	Luteolin 3'-methyl ether 4'-glucoside	C <sub>22</sub> H <sub>21</sub> O <sub>11</sub>	461.1080	[M-H] <sup>-</sup>		-0.86
24	5.15	Isoastilbin	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub>	449.1084	[M-H] <sup>-</sup>	283.0389	0.00
25	5.20	Astilbin or Taxifolin 3- <i>O</i> -rhamnoside (isomer)	C <sub>21</sub> H <sub>21</sub> O <sub>9</sub>	449.1087	[M-H] <sup>-</sup>	285.0393	0.00
26	5.23	Quercetin 3- <i>O</i> -glucuronide	C <sub>21</sub> H <sub>17</sub> O <sub>13</sub>	477.0667	[M-H] <sup>-</sup>	299.0185	0.41
27	5.29	Isorhmnetin 3- <i>O</i> -rutinoside	C <sub>28</sub> H <sub>31</sub> O <sub>16</sub>	623.1605	[M-H] <sup>-</sup>	477.0923	-1.12
28	5.32	Quercetin 3- <i>O</i> -glucuronide (isomer)	C <sub>21</sub> H <sub>17</sub> O <sub>13</sub>	477.0670	[M-H] <sup>-</sup>		0.20
29	5.53	Quercetin 3- <i>O</i> -rhamnoside (isomer)	C <sub>21</sub> H <sub>19</sub> O <sub>11</sub>	447.0920	[M-H] <sup>-</sup>		-3.13
30	5.54	Quercetin 3- <i>O</i> - $\alpha$ -L-xylopyranosyl-[1->2]- $\alpha$ -L-rhamnopyranoside	C <sub>26</sub> H <sub>27</sub> O <sub>15</sub>	579.1345	[M-H] <sup>-</sup>		-0.86
31		Quercetin-3- <i>O</i> -rhamnosylpentoside (isomer)	C <sub>26</sub> H <sub>27</sub> O <sub>15</sub>	579.0336	[M-H] <sup>-</sup>		1.55
32	5.58	Quercetin-3- <i>O</i> -rhamnoside	C <sub>36</sub> H <sub>56</sub> O <sub>12</sub>	447.0931	[M-H] <sup>-</sup>	331.0314	1.34
33	5.60	5,8-dihydroxy-2-(4-hydroxyphenyl)-7-methoxy-3-[(2S,3R,4R,5R,6S)-3,4,5-	C <sub>22</sub> H <sub>21</sub> O <sub>11</sub>	461.0715	[M-H] <sup>-</sup>		

		trihydroxy-6-methyloxan-2-yl]oxychromen-4-one					
34	5.62	Quercetin-3-O-glucoside	C <sub>21</sub> H <sub>19</sub> O <sub>12</sub>	463.0875	[M-H] <sup>-</sup>		-0.43
35	5.68	Cyanidin-3-O-rhamnoside	C <sub>21</sub> H <sub>21</sub> O <sub>10</sub>	433.1133	[M-H+FA] <sup>-</sup>	266.0444	-0.46
36	5.73	Triterpenoid saponin-O-hexoside	C <sub>37</sub> H <sub>59</sub> O <sub>13</sub>	711.3962	[M-H+FA] <sup>-</sup>	665.3879, 503.3386,	0.84
37	5.90	Kaempferol-3-O-beta-(cetyl)-galactopyranoside-7-O-alpha arabinoside	C <sub>36</sub> H <sub>58</sub> O <sub>12</sub>	621.1458	[M-H] <sup>-</sup>		0.32
38	5.90	Apigenin 6-C-pentosyl-8-C-hexoside	C <sub>26</sub> H <sub>27</sub> O <sub>14</sub>	563.1411	[M-H] <sup>-</sup>		1.77
39	6.00	Flavonol glycosides	C <sub>28</sub> H <sub>29</sub> O <sub>16</sub>	621.1454	[M-H] <sup>-</sup>		-0.31
40	6.02	Afzelein	C <sub>21</sub> H <sub>19</sub> O <sub>10</sub>	431.0982	[M-H] <sup>-</sup>		0.92
41	6.16	Flavonol glycosides (isomer)	C <sub>28</sub> H <sub>29</sub> O <sub>16</sub>	621.1445	[M-H] <sup>-</sup>	300.0258	-1.77
42	6.51	Triterpenoid saponin-O-hexoside	C <sub>37</sub> H <sub>60</sub> O <sub>13</sub>	711.3951	[M-H+FA] <sup>-</sup>	665.3841, 549.3423, 503.3373	-0.70
43	6.59	Triterpenoid saponin-O-hexoside	C <sub>37</sub> H <sub>60</sub> O <sub>13</sub>	711.3935	[M-H+FA] <sup>-</sup>	665.3937, 549.3420, 503.3356	-2.95
44	6.70	Triterpenoid saponin-O-hexoside	C <sub>37</sub> H <sub>59</sub> O <sub>12</sub>	695.4000	[M-H+FA] <sup>-</sup>	533.3462, 487.3426	-1.00

45	6.84	Triterpenoid saponin- <i>O</i> -hexoside	C <sub>37</sub> H <sub>59</sub> O <sub>12</sub>	695.3992	[M-H+FA] <sup>-</sup>	533.3467, 487.3422	-2.15
46	6.88	Triterpenoid saponin- <i>O</i> -hexoside	C <sub>37</sub> H <sub>59</sub> O <sub>12</sub>	695.3992	[M-H+FA] <sup>-</sup>	649.3702, 533.3488, 487.3404	-2.15
47	7.20	Platycodigenin- <i>O</i> -hexoside	C <sub>36</sub> H <sub>57</sub> O <sub>12</sub>	681.3841	[M-H] <sup>-</sup>	519.3225	
48	7.61	Triterpenoid saponin- <i>O</i> -hexoside	C <sub>37</sub> H <sub>59</sub> O <sub>12</sub>	695.4002	[M-H+FA] <sup>-</sup>	649.3934, 533.3483, 487.3426	-0.71
49	7.75	Triterpenoid saponin- <i>O</i> -hexoside	C <sub>37</sub> H <sub>59</sub> O <sub>12</sub>	695.3997	[M-H+FA] <sup>-</sup>	649.3851, 533.3351, 487.3318	-1.43
50	8.00	Triterpenoid	C <sub>30</sub> H <sub>47</sub> O <sub>6</sub>	503.3366	[M-H] <sup>-</sup>	485.3258	-1.39
51	8.08	Triterpenoid (isomer)	C <sub>30</sub> H <sub>47</sub> O <sub>6</sub>	503.3373	[M-H] <sup>-</sup>	485.3175	0.00
52	8.50	(epi)allocatechin (isomer)	C <sub>15</sub> H <sub>13</sub> O <sub>7</sub>	305.1751	[M-H] <sup>-</sup>	287.1646	
53	9.18	Chryseriol	C <sub>16</sub> H <sub>11</sub> O <sub>6</sub>	299.0183	[M-H] <sup>-</sup>		-1.00
54	9.71	Medicagenic acid 3a,21b,29-Trihydroxy-16-oxoserrat-14-en-24-oic Acid	C <sub>30</sub> H <sub>45</sub> O <sub>6</sub>	501.3213	[M-H] <sup>-</sup>	483.3130, 465.1602	-0.59
55	11.14	Triterpenoid saponin- <i>O</i> -hexoside	C <sub>36</sub> H <sub>59</sub> O <sub>9</sub>	635.4141	[M-H] <sup>-</sup>	471.3465	-2.8
56	11.18	Liguveitoside B	C <sub>36</sub> H <sub>59</sub> O <sub>9</sub>	635.4156	[M-H] <sup>-</sup>		-0.47

57	11.21	Triterpenoid saponin- <i>O</i> -hexoside	C <sub>36</sub> H <sub>59</sub> O <sub>9</sub>	635.4138	[M-H] <sup>-</sup>	471.3498	-3.3
58	11.24	acylated triterpenoid	C <sub>39</sub> H <sub>53</sub> O <sub>7</sub>	633.3792	[M-H] <sup>-</sup>		0.15
59	12.82	Coumaroyl maslinic acid	C <sub>39</sub> H <sub>53</sub> O <sub>6</sub>	617.3839	[M-H] <sup>-</sup>		0.48
60	14.24	Tamanolide D	C <sub>23</sub> H <sub>27</sub> O <sub>5</sub>	383.1874	[M-H] <sup>-</sup>		4.15
61	14.99	Cholesteryl-alpha-D-glucoside	C <sub>32</sub> H <sub>53</sub> O <sub>6</sub>	533.3847	[M-H] <sup>-</sup>		0.93

**Table 7.2.** *In vitro* antimicrobial, antiplasmodial and cytotoxic activity of extracts, fractions from *C. paniculatum* leaves

Fractions	Antimicrobial activity		Antiplasmodial activity (IC <sub>50</sub> , µg/mL)	Cytotoxicity (CC <sub>50</sub> , µg/mL)	Selectivity index
	<i>S. aureus</i>	<i>C. albicans</i>			
Cpf_DCM	>64.0	>64.0	1.4	2.8	2.0
Cpf_EA	>64.0	>64.0	5.1	50.0	9.7
Cpf_BuOH	>64.0	>64.0	6.5	64.0	9.7
S1	>64.0	>64.0	5.1	>64.0	>12.3
S2	>64.0	>64.0	2.6	21.9	8.2
S3	>64.0	>64.0	6.3	43.6	6.8
S4	>64.0	>64.0	4.4	2.6	0.6
S5	>64.0	>64.0	3.7	43.6	11.7
S6	>64.0	>64.0	5.6	40.0	7.0
S7	>64.0	20.7	7.9	47.7	5.9
S8	>64.0	>64.0	1.7	>64	>36.1
S9	>64.0	>64.0	>64.0	>64.0	Nd
S10	>64.0	>64.0	>64.0	>64.0	Nd
S11	>64.0	>64.0	36.0	>64.0	>1.77

S12	>64.0	>64.0	>64.0	>64.0	Nd
S13	>64.0	18.3	46.4	>64.0	>1.3
S14	>64.0	>64.0	21.9	45.0	2.0
S15	>64.0	>64.0	27.2	64.0	>2.3
S16	>64.0	>64.0	14.6	38.3	2.6
S17	>64.0	>64.0	>64.0	>64.0	Nd
S18	>64.0	>64.0	>64.0	>64.0	Nd
S19	>64.0	>64.0	>64.0	>64.0	Nd
S20	>64.0	>64.0	>64.0	>64.0	Nd
S21	>64.0	>64.0	40.7	>64.0	>1.5
S22	>64.0	>64.0	31.2	>64.0	>2.0
S23	>64.0	>64.0	10.3	>64.0	>6.2

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**Table 7.3.** *In vitro* antimicrobial, antiplasmodial and cytotoxic activity of pure compounds from *C. paniculatum* leaves

Fractions	Antimicrobial activity		Antiplasmodial activity (IC <sub>50</sub> , µg/mL)	Cytotoxicity (CC <sub>50</sub> , µg/mL)	Selectivity index
	<i>S. aureus</i>	<i>C. albicans</i>			
Quercetin 3-O-(6"-O-α rhamnopyranosyl)-β-D-glucopyranoside ( <b>10</b> )	>64.0	>64.0	6.7	25.4	3.7
Catechin ( <b>12</b> )	>64.0	>64.0	>64.0	>64.0	>64.0
Foliasalaciosides F ( <b>18</b> )	64.0	>64.0	10.6	>64.0	>6.0
Quercetin 3-O-(2"-O-β-D-glucopyranosyl)-α-L-rhamnopyranoside ( <b>20</b> )	>64.0	>64.0	31.5	>64.0	>2.0
Quercetin-3-O-β-D-glucoronide ( <b>24</b> )	>64.0	>64.0	>64.0	>64.0	>64.0
Quercetin 3-O-α-L-xylopyranosyl-(1->2)-α-L-rhamnopyranoside ( <b>30</b> )	64.0	>64.0	51.8	>64.0	>1.2

## 7.4. Conclusion

The integration of dereplication analysis, chemometrics and bioassay-guided fractionation provided access to a shorter route to explore compounds responsible for the antiplasmodial activity from *C. paniculatum*. In this regard dereplication strategy combining UPLC-MS/MS-based molecular networking and *in silico* analysis were employed and several compounds strongly correlated with antiplasmodial activity have been tentatively identified. A total of 61 compounds belonging to large chemical families such as alkaloids, tannins, terpenoids, flavonoids and lignans have been tentatively identified. Six compounds including rutin, foliasalacioside F, catechin, quercetin-3-O- $\beta$ -D-glucoronide, quercetin 3-O-(2''-O- $\beta$ -D-glucopyranosyl)- $\alpha$ -L-rhamnopyranoside and quercetin 3-O- $\alpha$ -L-xylopyranosyl-(1->2)- $\alpha$ -L-rhamnopyranoside have been isolated and their antiplasmodial activity evaluated. The OPLS predicted scores values of all these compounds were in agreement with their antiplasmodial results found *in vitro*. These preliminary results provide a better understanding on the effectiveness of using these innovative methods for the rapid identification of active metabolites in plant extracts.

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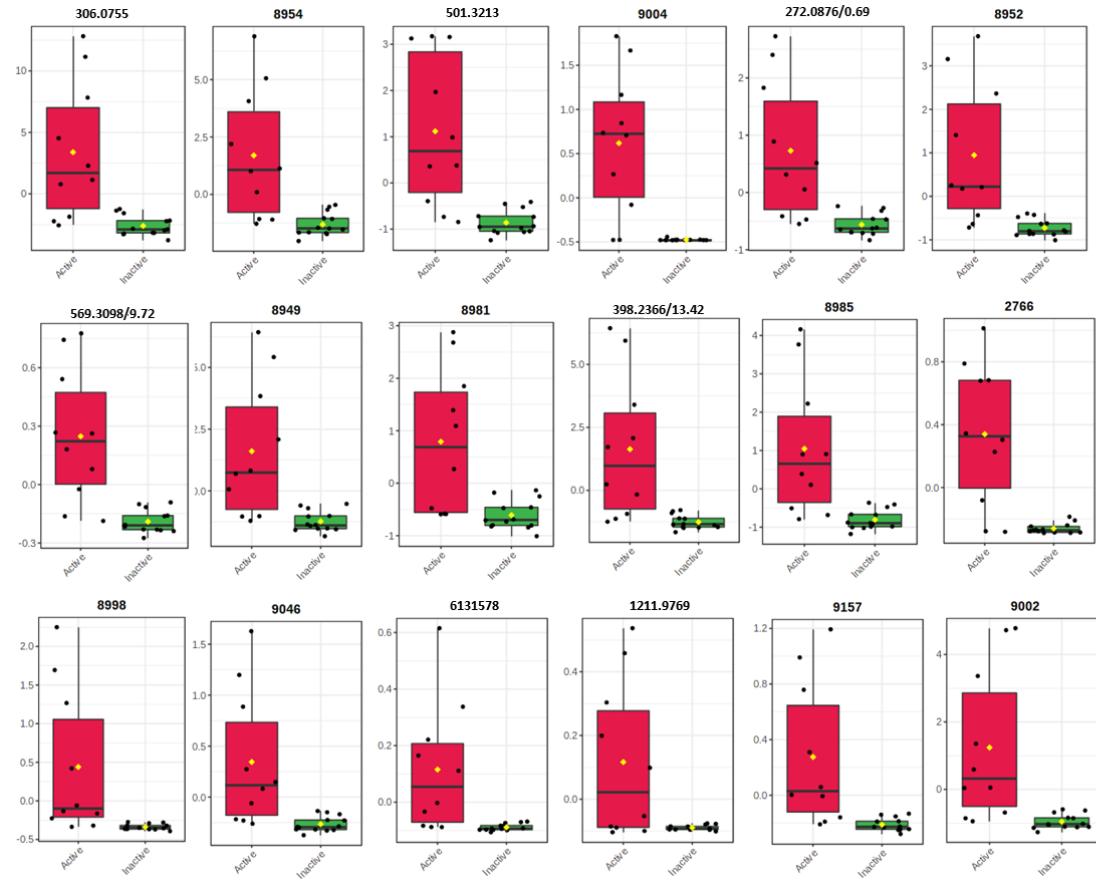
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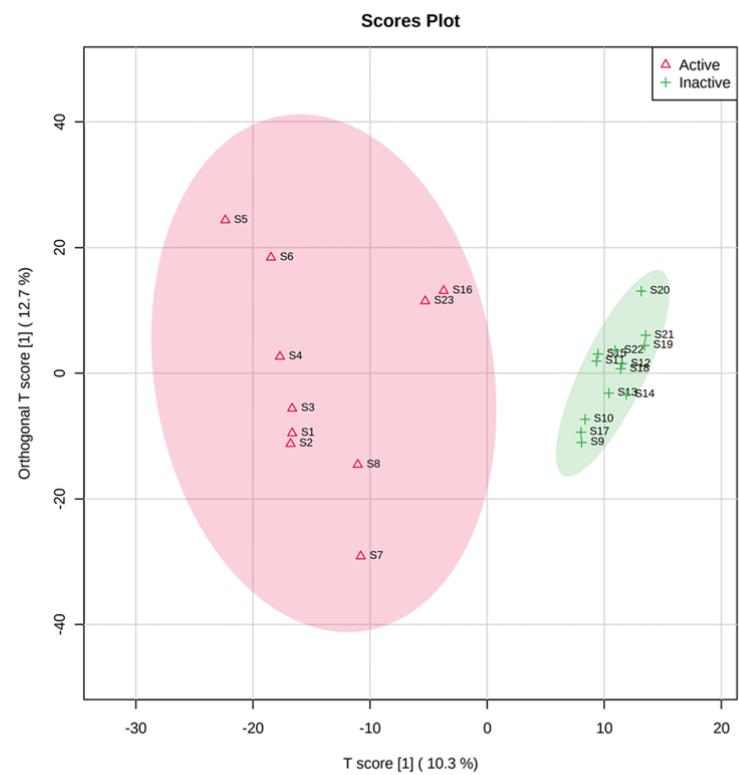
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## Supplementary information

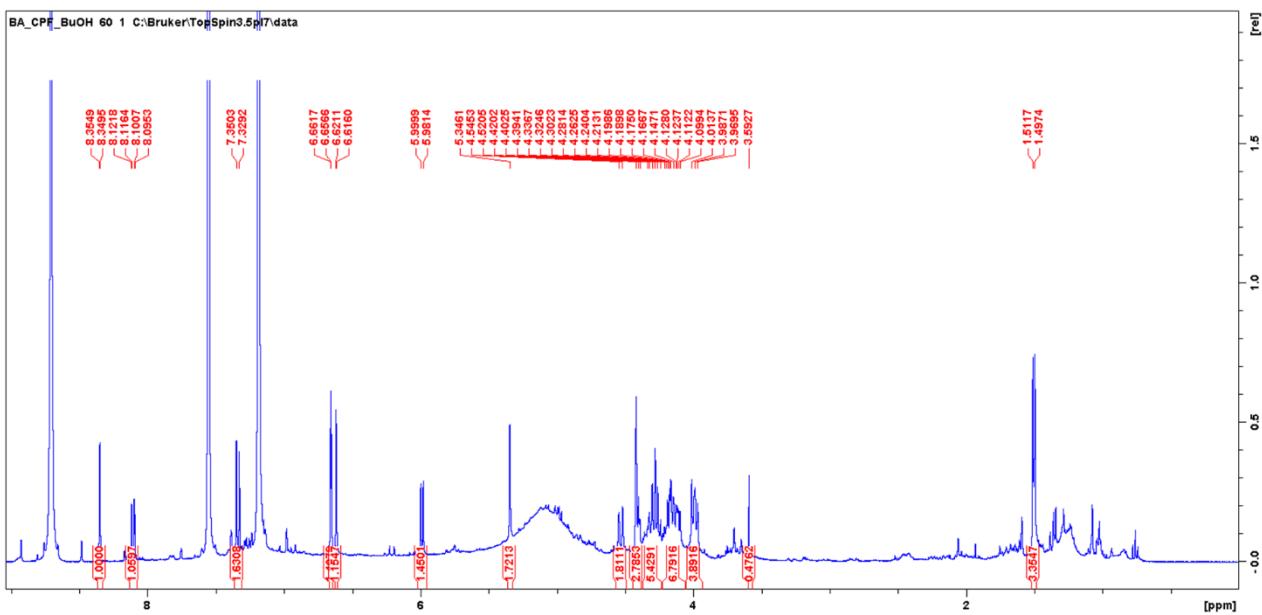
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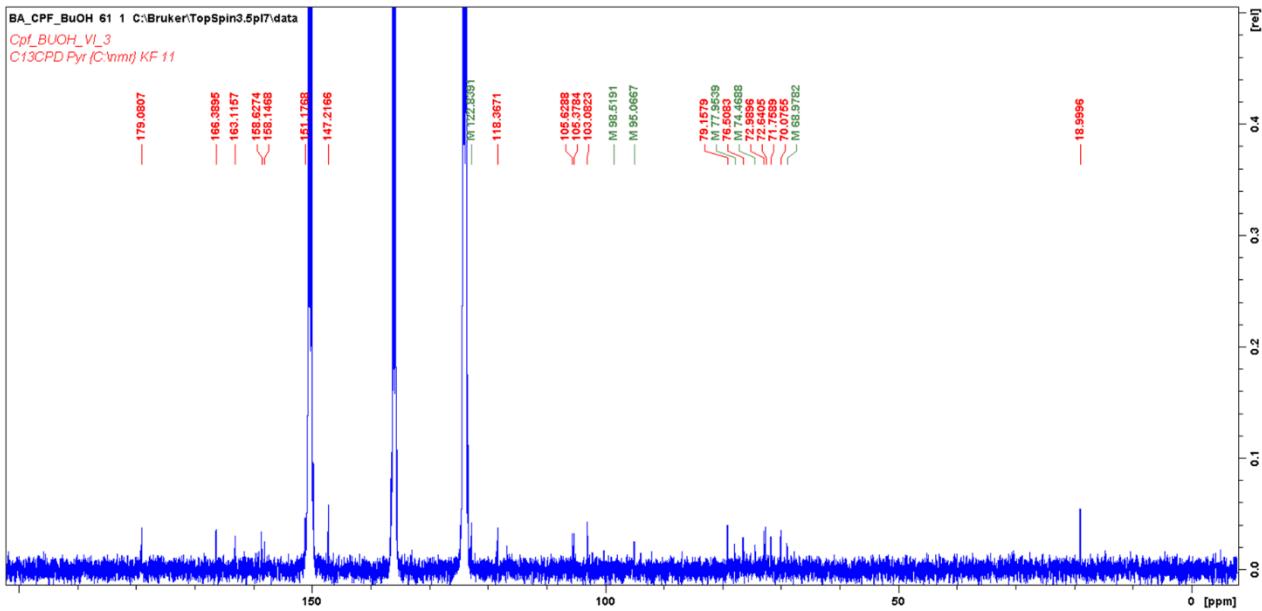
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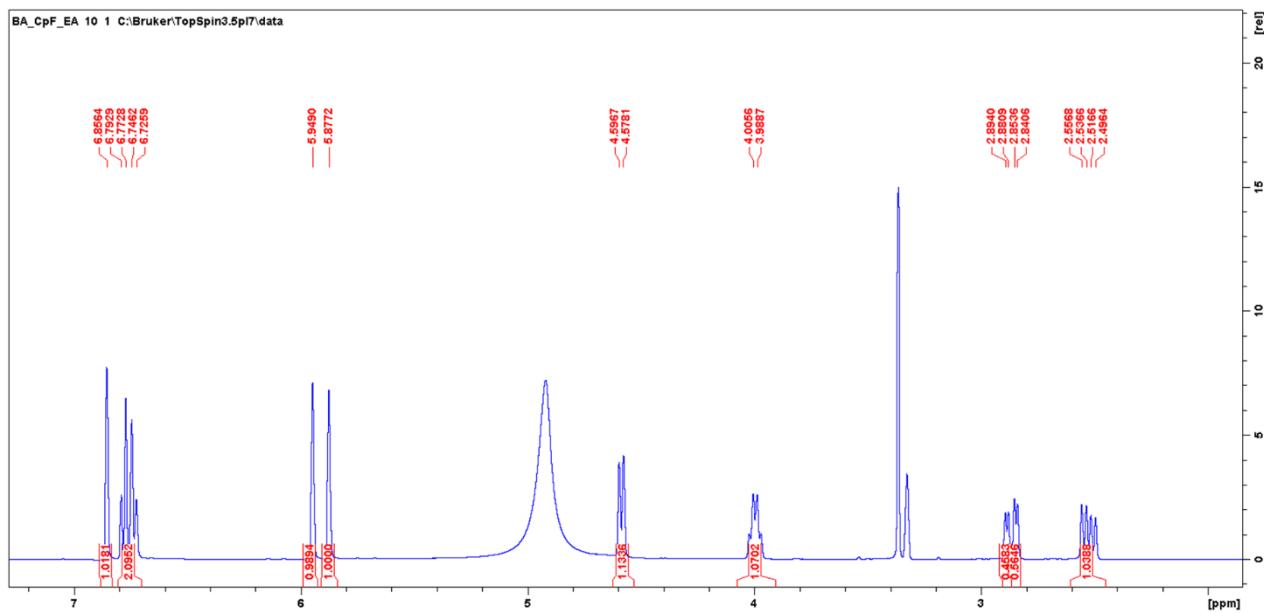
**Figure S 7.1.** (A) some unidentified compound with high correlation scores; (B) OPLS scores plots of active and inactive fractions from *C. Paniculatum*.



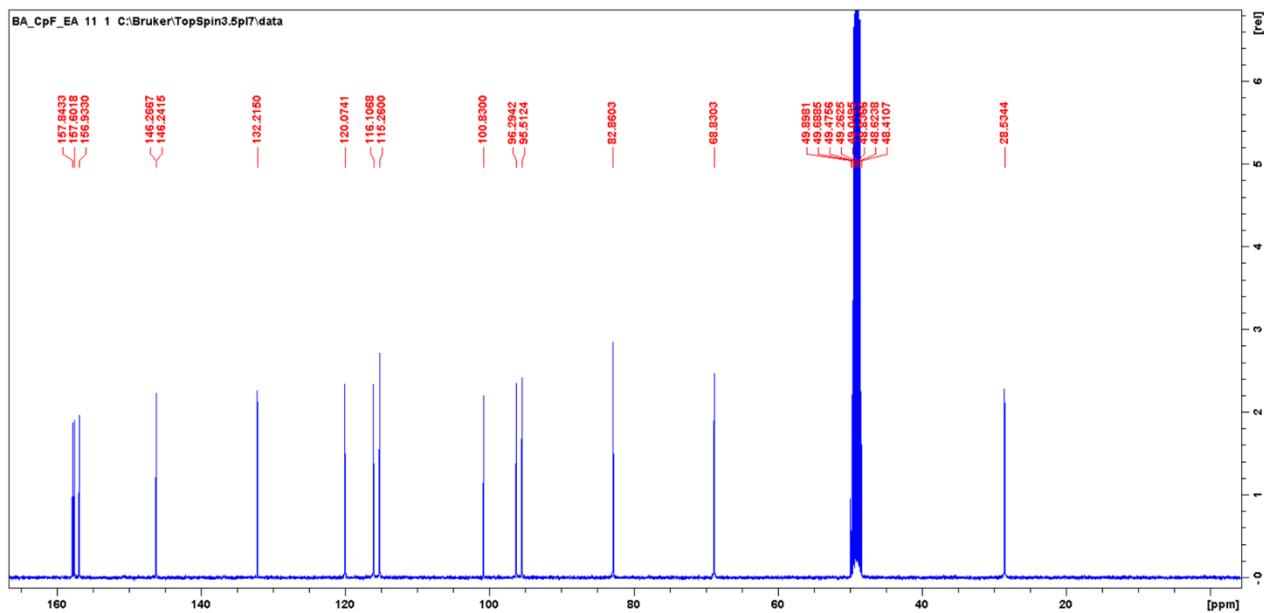
**Figure S 7.2.**  $^1\text{H}$  NMR spectrum ( $\text{CD}_3\text{OD}$ , 400 MHz) of Rutin (**10**).



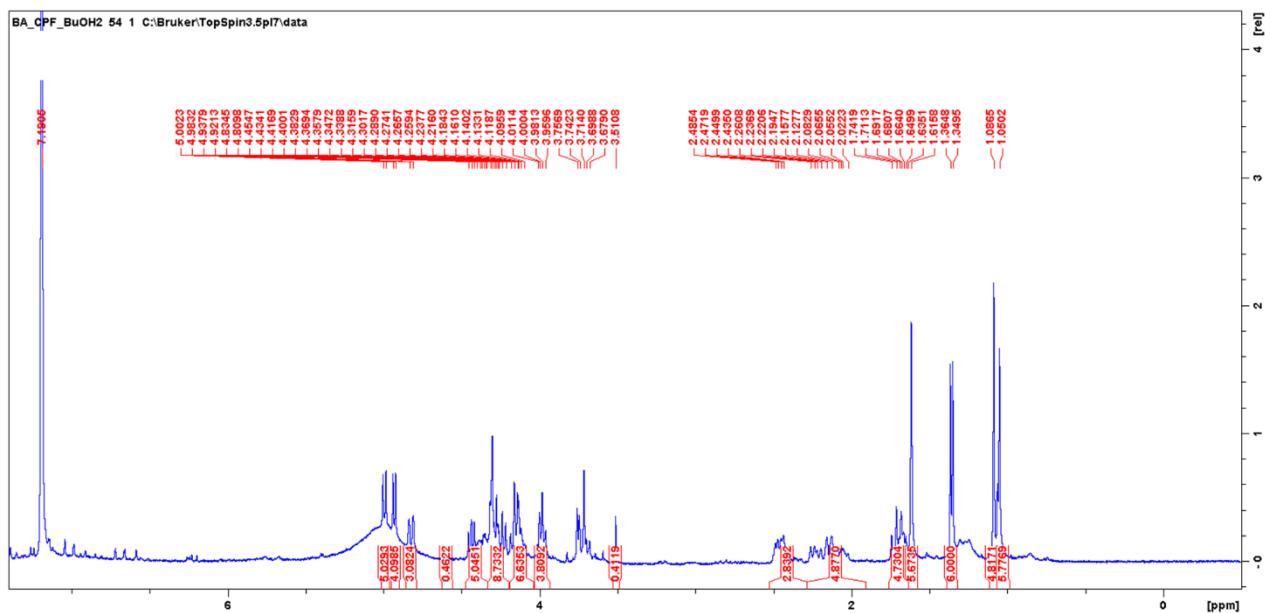
**Figure S 7.3.**  $^{13}\text{C}$  NMR spectrum ( $\text{CD}_3\text{OD}$ , 100 MHz) of Rutin (**10**).



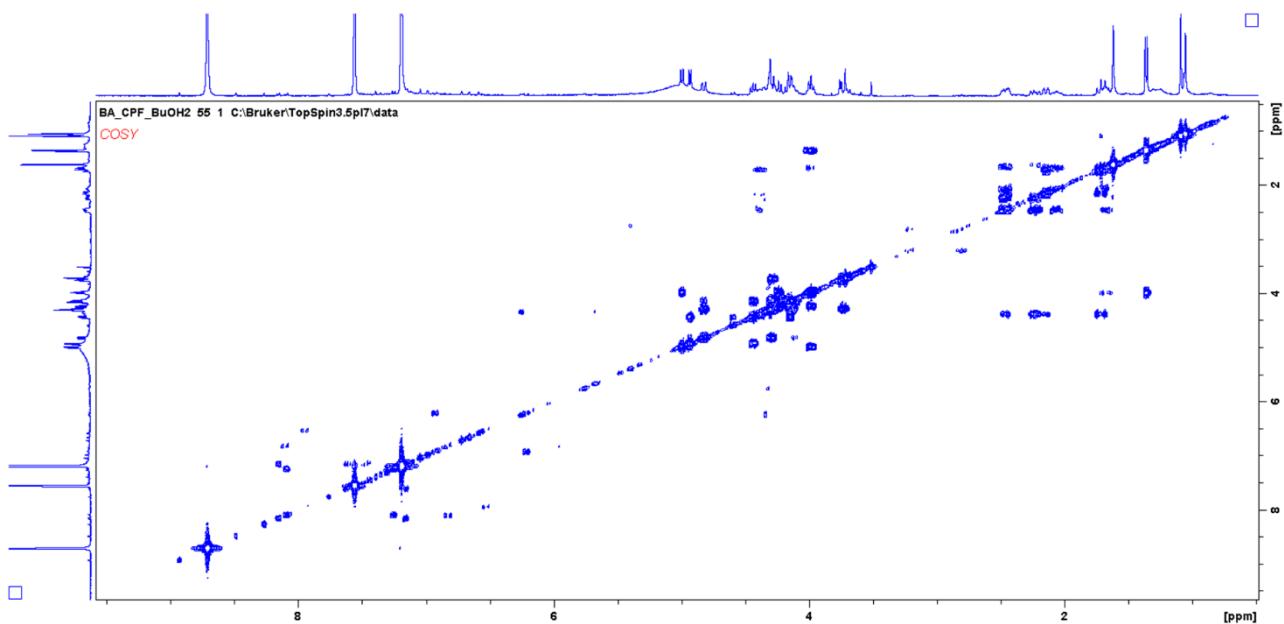
**Figure S 7.4.**  $^1\text{H}$  NMR spectrum ( $\text{CD}_3\text{OD}$ , 400 MHz) of Catechin (12).



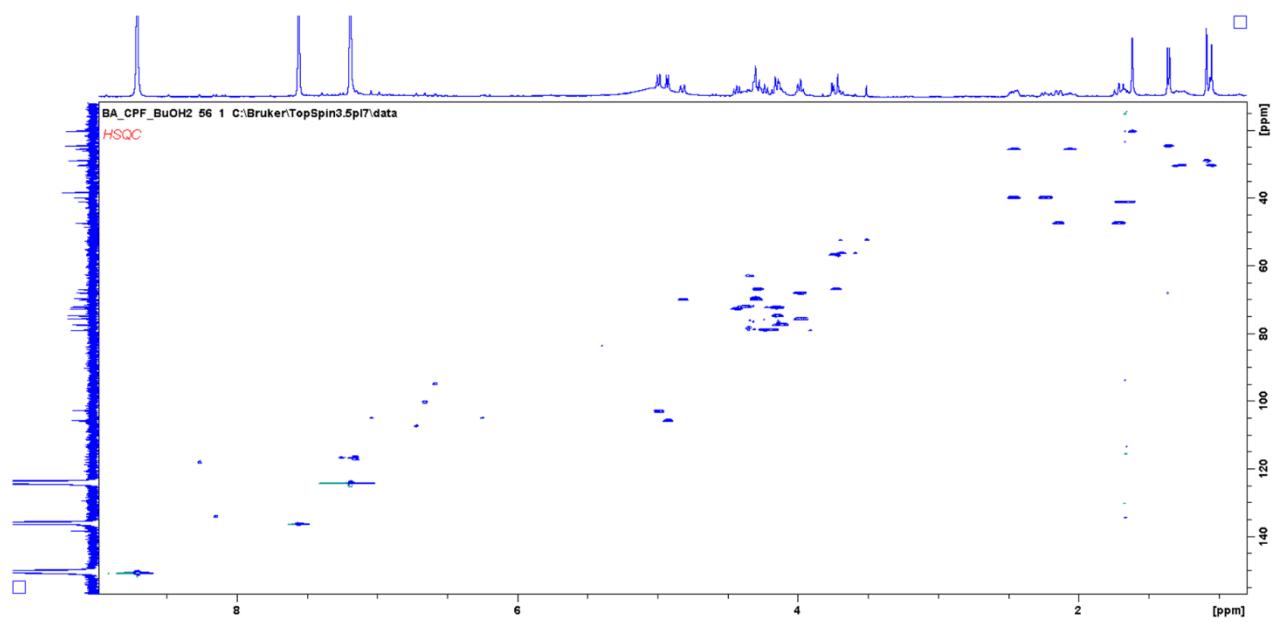
**Figure S 7.5.**  $^{13}\text{C}$  NMR spectrum ( $\text{CD}_3\text{OD}$ , 100 MHz) of Catechin (12).



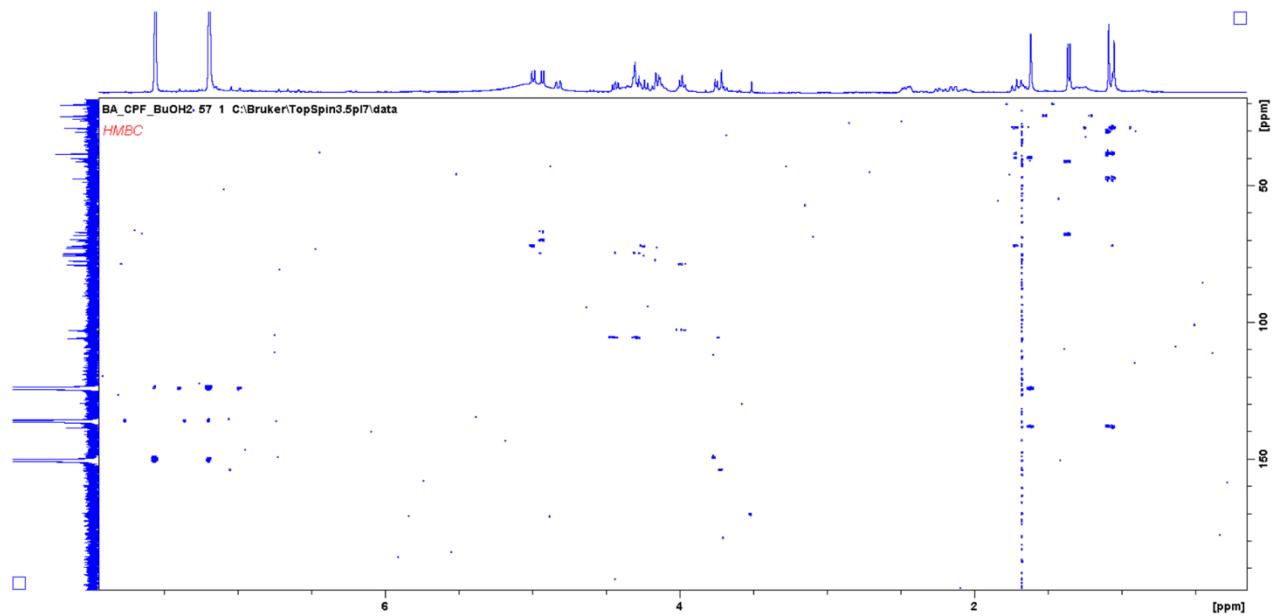
**Figure S 7.6.** <sup>1</sup>H NMR spectrum (Pyridine-*d*<sub>5</sub>, 400 MHz) of foliasalaciosides F (**18**).



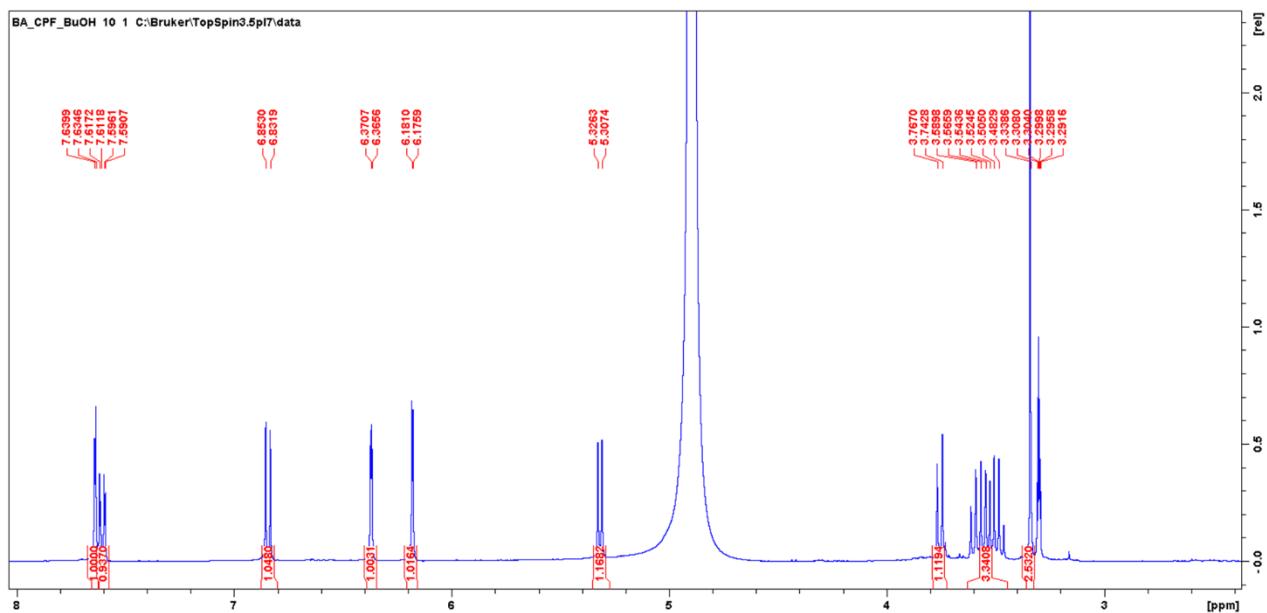
**Figure 7.7.** COSY spectrum (Pyridine-*d*<sub>5</sub>) of foliasalaciosides F (**18**).



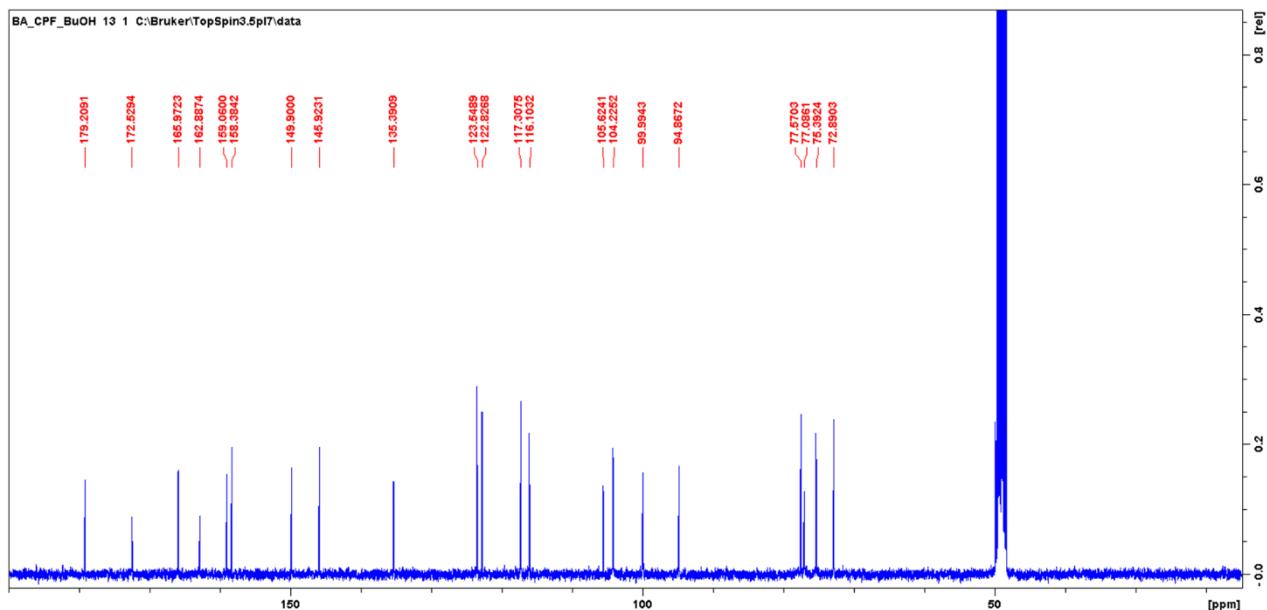
**Figure S 7.8.** HSQC spectrum (Pyridine-*d*<sub>5</sub>) of foliasalaciosides F (**18**).



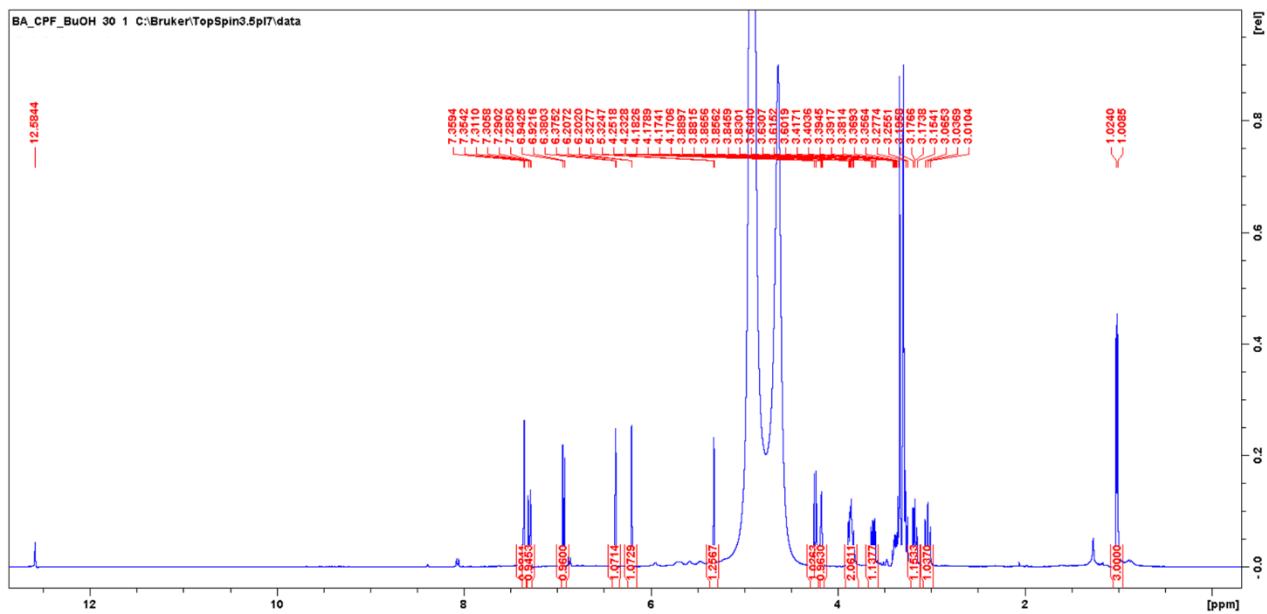
**Figure S9.** HMBC spectrum (Pyridine-*d*<sub>5</sub>) of foliasalaciosides F (**18**).



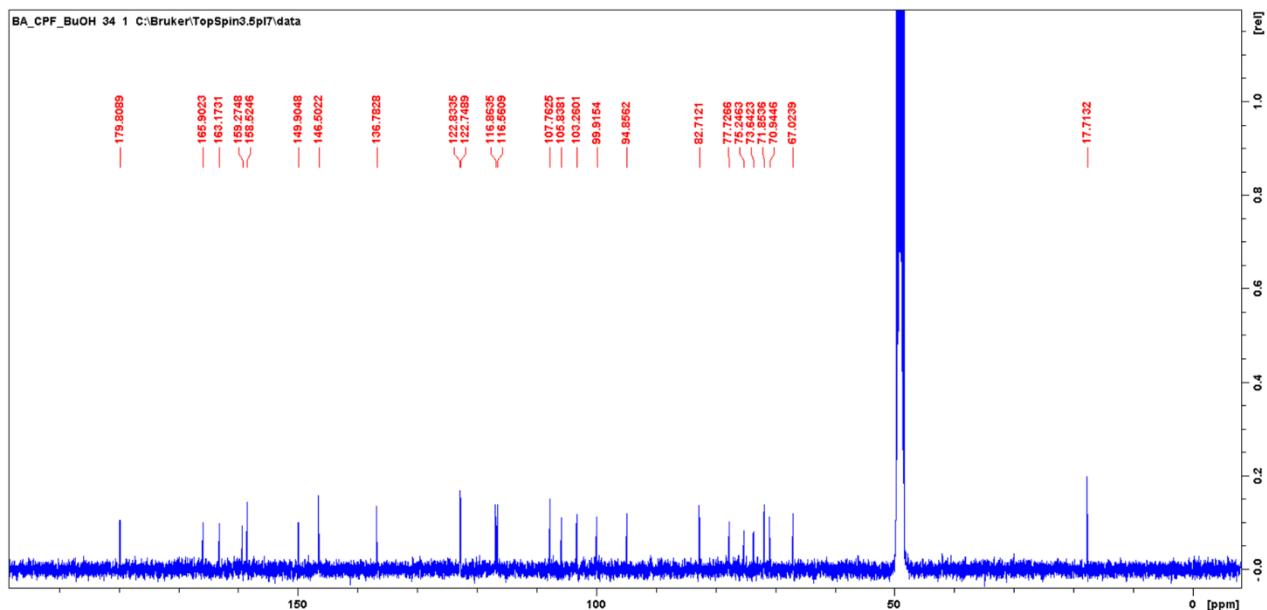
**Figure S10.**  $^1\text{H}$  NMR spectrum (CD<sub>3</sub>OD, 400 MHz) of Quercetin-3- $O$ - $\beta$ -D-glucoronide (**24**).



**Figure S11.**  $^{13}\text{C}$  NMR spectrum (CD<sub>3</sub>OD, 100 MHz) of Quercetin-3- $O$ - $\beta$ -D-glucoronide (**24**).



**Figure S12.**  $^1\text{H}$  NMR spectrum ( $\text{CD}_3\text{OD}$ , 400 MHz) of Quercetin 3- $O$ - $\alpha$ -L-xylopyranosyl-(1->2)- $\alpha$ -L-rhamnopyranoside (**29**)



**Figure S13.**  $^{13}\text{C}$  NMR spectrum ( $\text{CD}_3\text{OD}$ , 100 MHz) of Quercetin 3- $O$ - $\alpha$ -L-xylopyranosyl-(1->2)- $\alpha$ -L-rhamnopyranoside (**29**)

# **CHAPTER 8**

## **General conclusions and future perspectives**



Microbial infections including malaria appear among the main causes of morbidity and mortality of people living in many countries including Guinea. Although the use of antimicrobial agents has significantly increased human lifespan over the past century, it is important to emphasize that the emergence of pathogen strains that are resistant to antimicrobials threatens to reverse these gains. To face these pathologies an important fringe of the affected people use medicinal plants. However, despite the widespread use of these plants, relatively few studies, compared to synthetic medicines, have been undertaken in order to identify the active compounds and therefore justify their traditional use. The aim of this research was to evaluate the potential antimicrobial and antiplasmodial properties of some selected Guinean plant extracts and the corresponding active constituents. Foremost, a comprehensive overview of plant species employed in traditional medicine for the management of various microbial diseases, including malaria, has been carried out. Three plants species (*Terminalia albida*, *Tetracera alnifolia* and *Combretum paniculatum*) were subjected to an extensive phytochemical and biological studies. The general approach used in this present research combined different phytochemical and biological methods.

The bioassay-guided isolation of constituents from *T. albida* root extracts resulted in the isolation of 14 compounds which had never been reported before from this plant species. Moreover, compounds from different phytochemical classes, including pantolactone, 3,4,3'-tri-O-methyl-ellagic-acid, calophyemembraside-B, and a range of hydroxylated oleanane-type triterpenes, were found to contribute to the antiplasmodial activity. Based on the activity of arjungenin, an attempt was made to establish some structure-activity relationships (SAR) of isolated oleanane-type triterpenes. It emerges from this study that the configuration of the hydroxymethyl group linked to C-4 and the position of the hydroxyls groups could have a strong influence on antiplasmodial activity. These results deserve to be confirmed by performing both qualitative and quantitative structure-activity relationship (SAR) studies, based on a large number of triterpenoids and their corresponding antiplasmodial activiy. Therefore, more in-depth phytochemical studies must be performed to isolate a large number of oleanane triterpenoids present within the

root of *T. albida* and other *Terminalia* species. Although less active than the positive control chloroquine, some of these compounds could constitute a good antiplasmodial

scaffold for the discovery of new potential antimalarial drugs. Although the methanolic extract of the stem bark of *T. albida* has previously been shown to significantly increase the survival rate in mice infected with *Plasmodium berghei*, similar *in vivo* studies of pure compounds and characterized extracts from the root would be advisable.

On the other hand we were able to demonstrate through this research that the n-BuOH fraction of *T. albida* contains some heterosides, which could generate more active aglycones in the gastrointestinal tract, more in particular in the colon after oral administration. Indeed, an in-depth phytochemical study integrating molecular networking, *in silico* MS/MS dereplication and semi-preparative HPLC-MS-DAD purification of this extract resulted in the isolation of some active oleanane triterpenoids. These findings support our initial hypothesis that the n-BuOH fraction of *T. albida* possesses prodrugs which upon metabolization yield more active metabolites. Although these compounds have shown *in vitro* activity, further *in vivo* studies would provide a better appreciation of the effectiveness of these glycosides.

For *T. alnifolia* promising *in vitro* activities against *Candida albicans*, *Staphylococcus aureus* and *Plasmodium falciparum* were obtained in our preliminary biological screening for both methanolic and dichloromethane leaves extracts. The bioassay-guided fractionation of the active extracts resulted in the isolation of a series of pheophorbides, phytol derivative, and flavonoids, among other compounds. The highest antiplasmodial activity was obtained for pheophorbide-b methyl ester, (1,2)-bis-nor-phytene, isophytol, pheophorbide-a methyl ester and phytol. Nonetheless, pheophorbide-b methyl ester, pheophorbide-a methyl ester and phytol were less specific due to their hight cytotoxicity against MRC-5 cells. Moreover, through this research, we were able to demonstrate that the position of the double bond and the presence of a carbonyl function could have an influence on the antiplasmodial activity of phytol and its derivative (1,2-bis-nor-phytene, and isophytol).

It could be concluded that these results may provide a possible explanation related to the traditional use of this plant species against microbial diseases, more in particular malaria. To have a better understanding of the active metabolites of this plant, the *in vitro* results

found in our study deserve to be extended to in vivo studies of both extracts and isolated compounds.

Although the methanolic extracts of both *T. albida* and *T. alnifolia* were active against *S. aureus* and *C. albicans*, none of its fractions and isolated compounds showed antibacterial or antifungal activity. The main reasons of the loss of activity and failure in isolation of active compounds may be the degradation of compounds during the purification process, a low concentration of bioactive compounds making their isolation difficult, and/or potential synergistic effects. This issue may be avoided in “synergy-directed fractionation” which combines chromatographic separation and synergy testing in combination with a known active constituent in the original extract.

In the last decade, substantial developments have been made in improving extraction and separation efficiency and facilitating the isolation of minor constituents that may contribute to activity. The integration of dereplication analysis, chemometrics and bioassay-guided fractionation provided access to a shorter route to explore compounds responsible for the antiplasmodial activity from *C. paniculatum*. By implementing this innovative strategy, we were able to streamline the traditional and laborious process of isolating natural products by targeting unknown active compounds present in the leaves of *C. paniculatum* before the purification process. Therefore a large number of compounds belonging to various chemical families such as alkaloids, tannins, terpenoids, flavonoids and lignans have been tentatively identified and six compounds have been isolated and their antiplasmodial activity was evaluated. Although the OPLS predicted score values of the isolated compounds are in agreement with their in vitro antiplasmodial activity, the isolation followed by the biological evaluation of a larger number of compounds could provide more conclusive results.

Our future investigation will mainly focus on the development and validation of an analytical method for the most active and major constituents, in order to produce standardised extracts, and opening the road to quality control, which could lift traditional medicine to a higher level in terms of efficiency and safety.

As it is well known that *in vitro* assays cannot completely mimic *in vivo* model, it is important to specify that the results of this research project deserve to be supplemented by *in vivo* studies in order to confirm and compare the effectiveness of both extracts and pure compounds. Moreover, in-depth studies based on the assessment of the toxicity of the most active extracts and pure compounds as well as a clinical trial to evaluate the efficacy and tolerability of standardized plant extracts and /or compounds formulations, would provide a better understanding on the traditional use of these plant species in the management of microbial infections including malaria.

# **SUMMARY**



## Summary

Medicinal plants have historically proven their value as sources of molecules with therapeutic potential, and nowadays still represent an interesting pool for the discovery of novel drug leads. Current research in drug discovery from medicinal plants involves a multifaceted approach combining several methods and techniques. Despite the considerable progress in terms of research and development of new treatment and prevention procedures over the last decades, infectious diseases still remain the leading cause of death in many developing countries. In Guinea, medicinal plants play an important role in the management of infectious diseases including malaria, urinary disorders, skin diseases and oral diseases. As part of a valorization program of these plant species, ethnopharmacological investigations have been carried out and plants species employed for the treatment of malaria, skin diseases, oral diseases and urinary disorders were inventoried. An extensive bibliographic review, followed by a preliminary biological screening resulted in the selection of some promising plant species including *Terminalia albida*, *Tetracera alnifolia* *Combretum paniculatum* and *Pavetta crassipes*. In the present research, we propose to deepen the biological and phytochemical investigations on some promising plants extracts, through the evaluation of their potential antimicrobial and antiplasmoidal properties, and the corresponding active constituents.

The bioassay-guided fractionation of *Terminalia albida* root resulted in the isolation of 14 compounds (**1–14**), and their antimicrobial properties were evaluated against *Plasmodium falciparum*, *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli*. Pantolactone ( $IC_{50} 0.60 \pm 0.03 \mu M$ ) demonstrated significant activity against *P. falciparum*. Other compounds, including 3,4,3'-tri-O-methyl-ellagic acid, the triterpenes arjunolic acid, arjungenin, arjunic acid and arjunglucoside II, and the phenol glycoside calophymembranside-B, were less active and showed  $IC_{50}$  values in the range 5 – 15  $\mu M$ . None of the tested compound showed antibacterial or antifungal activity.

Although the *n*-butanolic fraction was not active, the possibility cannot be excluded that this polar fraction contains inactive glycosides, which may release active aglycones after removal of the glycosidic moieties in the gastrointestinal tract, more in particular in the colon. Therefore, the *n*-butanolic fraction of the total root extract of *Terminalia albida* has

been subjected to extensive dereplication studies followed by the isolation of the target compounds. As a result, 10 oleanane triterpenoids (**1-10**), among which six new compounds, i.e. albidanoside A, albidic acid A, albidinolic acid, albidienic acid, albidolic acid, albiololic acid; two triterpene aglycones, i.e. albidic acid B and albidic acid C isolated here for the first time from a natural source; and two known compounds. Isolated compounds were evaluated for their antiplasmodial and antimicrobial activity against the chloroquine-resistant strain *Plasmodium falciparum* K1, *Candida albicans* and *Staphylococcus aureus*. Compounds **1 - 4**, **6**, **7** and **8** demonstrated moderate antiplasmodial activity with IC<sub>50</sub> values between 5 and 15 μM. None of the tested compounds was active against *C. albicans* or *S. aureus*. These findings emphasize the potential of *T. albida* as a source for discovery of new antiplasmodial compounds.

The bioassay-guided fractionation of *Tetracera alnifolia* leaves extracts led to the isolation of 19 compounds (**1-19**). Purification of fractions was performed by flash chromatography, followed by semi-preparative HPLC-DAD-MS and LC-SPE-NMR, while the structural elucidation of the isolated compounds was carried out by 1D and 2D NMR and HR-ESI-MS. Isolated compounds were screened against *Plasmodium falciparum*, *Candida albicans* and their cytotoxicity against MRC-5 cells was determined. The highest antiplasmodial activity was obtained for pheophorbide-b methyl ester (1.0 ± 0.7 μM), (1,2)-bis-nor-phytene (2.0 μM), isophytol (4.0 μM), pheophorbide-a methyl ester (2.8 ± 1.2 μM), epicatechin-3-galloylester (5.5 ± 2.1), and phytol (6.9 ± 2.4 μM). Other compounds, including myricetin-3-O-rhamnopyranoside, α-tocopherol and cycloart-24-en-3β-yl α-linolenate were less active and showed IC<sub>50</sub> values in the range 13.5– 25 μM. None of the tested compounds was active against *Candida albicans*. A hight cytotoxicity was found for pheophorbide-b methyl ester, pheophorbide-a methyl ester and phytol. Nowadays, the rapid development of modern analytical techniques and various chemometric approaches provide new perspectives for early metabolite identification in natural products research. These techniques represent a potential strategy to streamline the traditional and laborious process of isolating natural products through targeting of unknown active compounds before purification. These innovative techniques have been applied on extracts of the leaves of *C. paniculatum*, which have demonstrated promising antiplasmodial activity during our preliminary studies, leading to a quick and effective identification of compounds correlated to this activity. The fractionation of crude extracts

was carried out, followed by multivariate data analysis of liquid chromatography–high resolution mass spectrometry (LC–HRMS) profiles of the fractions obtained. In parallel, all fractions were screened against *Plasmodium falciparum*, and their cytotoxicity against MRC-5 cells was determined. Dereplication studies combining UPLC-MS/MS-based molecular networking, *in silico* analysis and NMR methods were employed to identify the important metabolites. Several compounds strongly correlated with antiplasmodial activity have been highlighted. Six compounds including rutin ( $IC_{50}$  6.7  $\mu$ M) and foliasalacioside F ( $IC_{50}$  10.6  $\mu$ M) have been isolated and their OPLS predicted score values were in agreement with antiplasmodial results found *in vitro*. These preliminary results provided clear evidence on the effectiveness of using these innovative methods (chemometrics and dereplication analysis) for the rapid identification of active metabolites in plant extracts.

Further research aiming for the isolating of additional promising compounds which have shown a strong correlation with antiplasmodial activity is ongoing.



# **SAMENVATTING**



## **Samenvatting**

Medicinale planten hebben in de loop van de geschiedenis reeds hun waarde bewezen als bron van moleculen met therapeutische eigenschappen, en ook de dag van vandaag vormen ze nog steeds een interessant startpunt voor de ontdekking van nieuwe “leads” voor geneesmiddelen. Hedendaags geneesmiddelenonderzoek omvat een brede waaier aan benaderingen, waarbij verscheidene methoden en technieken gecombineerd worden. Ondanks de aanzienlijke vooruitgang op gebied van onderzoek en ontwikkeling van nieuwe behandelings- en preventieve-methoden gedurende de laatste decennia, blijven infectieziekten de voornaamste doodsoorzaak in tal van ontwikkelingslanden. In Guinea spelen medicinale planten een belangrijke rol bij de beheersing van infectieziekten zoals malaria, urineweginfecties, huid- en mondaandoeningen. Als onderdeel van een valorisatie-programma van deze planten, werden ethnofarmacologische studies uitgevoerd, en de soorten gebruikt voor de behandeling hiervan werden geïnventariseerd. Uitgebreid literatuuronderzoek, gevolgd door een eerste biologische screening, leidde tot de selectie van enkele veelbelovende plantensoorten, meer bepaald *Terminalia albida*, *Tetracera alnifolia* en *Combretum paniculatum*. In dit onderzoek stellen we voor om de biologische een fytochemische kennis over deze soorten uit te diepen, door de evaluatie van hun antimicrobiële en antiplasmodiale eigenschappen, en de overeenkomstige actieve inhoudsstoffen.

Biologisch gestuurde fractionering van *Terminalia albida* wortel extracten, resulteerde in de isolatie van 14 producten, waarvan de antimicrobiële en antiplasmodiale eigenschappen geëvalueerd werden tegen *Plasmodium falciparum*, *Candida albicans*, *Staphylococcus aureus* en *Escherichia coli*. Pantolacton ( $IC_{50} 0.60 \pm 0.03 \mu M$ ) vertoonde een belangrijke activiteit tegen *P. falciparum*. Andere producten, zoals 3,4,3'-tri-*O*-methyl-ellagzuur, de triterpenen arjunolic zuur, arjungenin, arjunic zuur en arjunglucoside II, en het fenol glycoside calophymembranside-B, waren minder actief en vertoonden  $IC_{50}$  waarden in het gebied 5 – 15  $\mu M$ . Geen van de geteste producten vertoonde antibacteriële of antifungale activiteit.

Alhoewel de *n*-butanol fractie niet actief was, kan de mogelijkheid niet uitgesloten worden dat deze polaire fractie inactieve glycosiden bevat, die mogelijk actieve aglyconen vrijstellen na afsplitsing van de glycosiden in het spijsverteringsstelsel, meer bepaald in

het colon. Daarom werd de *n*-butanol fractie onderworpen aan een dereplicatie-studie, gevolgd door isolatie van de doelproducten. Op deze manier werden 10 oleaan triterpenoiden verkregen, waaronder zes nieuwe producten, meer bepaald albidanoside A, albidic zuur A, albidinolic zuur, albidienic zuur, albidolic zuur en albidiolic zuur; twee triterpeen aglyconen, meer bepaald albidic zuur B en albidic zuur C, nu voor de eerste maal geïsoleerd uit een natuurlijke bron; en twee gekende producten. Deze werden alle geëvalueerd voor hun antiplasmodiale en antimicrobiële werking tegen *Plasmodium falciparum*, *Candida albicans* en *Staphylococcus aureus*. Alhoewel bekomen uit een inactieve fractie, vertoonden enkele producten een matige antiplasmodiale activiteit met IC<sub>50</sub> waarden tussen 5 en 15 µM. Geen van de geteste producten was actief tegen *C. albicans* of *S. aureus*. Deze resultaten benadrukken het potentieel van *T. albida* als bron van nieuwe antiplasmodiale verbindingen.

Biologisch gestuurde fractionering van *Tetracera alnifolia* bladextracten leidde tot de isolatie van 19 producten. Deze werden getest tegen *Plasmodium falciparum* en *Candida albicans*, en de cytotoxiciteit bepaald tegen MRC-5 cellen. De hoogste antiplasmodiale activiteit werd bekomen voor pheophorbide-b methyl ester ( $1.0 \pm 0.7 \mu\text{M}$ ), (1,2)-bis-norphyton ( $2.0 \mu\text{M}$ ), isofytol ( $4.0 \mu\text{M}$ ), pheophorbide-a methyl ester ( $2.8 \pm 1.2 \mu\text{M}$ ), epicatechin-3-galloylester ( $5.5 \pm 2.1$ ), en fytol ( $6.9 \pm 2.4 \mu\text{M}$ ). Andere producten, zoals myricetin-3-*O*-rhamnopyranoside, α-tocoferol en cycloart-24-en-3β-yl α-linolenaat waren minder actief, en vertoonden IC<sub>50</sub> waarden in het gebied 13.5–25 µM. Geen van de geteste producten was actief tegen *Candida albicans*. Een hoge cytotoxiciteit werd vastgesteld voor pheophorbide-b methyl ester, pheophorbide-a methyl ester en fytol.

De dag van vandaag bieden de snelle ontwikkelingen op gebied van analytische technieken en chemometrische benaderingen nieuwe perspectieven voor snelle identificatie van natuurstoffen. Op deze manier kan het gebruikelijke en tijdrovende proces van isolatie van natuurproducten vermeden worden, door zich te richten op onbekende actieve componenten. Deze strategie werd gevolgd voor bladextracten van *Combretum paniculatum*, die antiplasmodiale activiteit vertoonden, en leidde tot de snelle isolatie van de inhoudsstoffen, die met deze activiteit correleerden. De fractionering van de extracten werd uitgevoerd, gevolgd door multivariate data analyse van de LC-HRMS profielen. In parallel werden alle fracties getest tegen *Plasmodium falciparum*.

Dereplicatie studies, waarbij UPLC-MS/MS-gebaseerde moleculaire netwerking, *in silico* analyse en NMR methoden gebruikt werden, leidde tot de identificatie van de belangrijke inhoudsstoffen. Zes producten, waaronder rutine ( $IC_{50}$  6.7  $\mu M$ ) en foliasalacioside F ( $IC_{50}$  10.6  $\mu M$ ) werden verkregen, en hun OPLS-voorspelde scores waren in overeenstemming met de *in vitro* antiplasmodiale resultaten. Verder onderzoek naar de isolatie van andere veelbelovende producten die sterk correleerden met antiplasmodiale werking is lopende.



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# **SCIENTIFIC CURRICULUM VITAE**



# Scientific curriculum vitae

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## Education

**2004-2010** Bachelor in Pharmacy, great distinction.

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**2013-2014** Master in Natural products chemistry “Polymères et Principes Actifs d’Origine Naturelle” (M2P2AON) ; UFR of Pharmaceutical Sciences and Health Engineering

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## Scientific Publications

**2021**

**Baldé M.A.**; Tuenter E.; Traoré M.S.; Peeters L.; Matheeussen A.; Cos P.; Caljon G.; Vermeyen T.; Herrebout W.; Balde A.M.; Foubert K.; Pieters L. Antiplasmoidaloleanane triterpenoids from Terminalia albida root bark. *Journal of NaturalProducts*, 2021 Mar 26; 84 (3): 666-675.

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**2015**

Traoré, M.S.; **Baldé M.A.**; Camara, A.; Baldé E.S, E.; Diané, S.; Diallo, M.S.; Keita, A.; Cos, P.; Maes, L.; Pieters, L.; Mamadou Baldé, A. The Malaria Co-Infection Challenge: An Investigation into the Antimicrobial Activity of Selected Guinean Medicinal Plants. *Journal of Ethnopharmacology*, 2015, 174, 576–581.

**Oral presentation**

**2019**

**Balde, M.A.**; M.A. ; Tuenter, E. ; MS Traore, M.S. ; Cos,P. ; Balde A.M. ; Pieters, L.; Foubert, K. Phytochemical and biological investigations on potentially antimicrobial and anticancer Guinean plant species. 20<sup>th</sup> forum of pharmaceutical sciences (Belgian Society of Pharmaceutical Sciences), palace of the academies, Brussels. May 20<sup>th</sup> 2019.

**Poster presentation**

**2019**

**Balde, M.A.**; M.A. ; Tuenter, E. ; MS Traore, M.S. ; Cos,P. ; Balde A.M. ; Pieters, L.; Foubert, K. Phytochemical and biological investigations on potentially antimicrobial and anticancer Guinean plant species. 67<sup>th</sup> International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Innsbruck, Austria. *Planta Medica*. 2019; 85(18): 1499; DOI: 10.1055/s-0039-3399924



# **ANNEX**



Fiche d'enquête ethnopharmacologique N°.....

Centre de Recherche et de Valorisation des Plantes Médicinales

(CNRVPM-DUBREKA)

Enquêteur :.....

Lieu :.....

Date de l'enquête :.....

**Renseignements sur le guérisseur :**

Nom :..... Prénoms :.....

Sexe :..... Age :..... Région :.....

Préfecture :..... Sous-préfecture :.....

Recensé comme guérisseur :  OUI  NON

Numéro de la carte professionnelle :.....

Spécialité :.....

**Statut :** 1.Chasseur  2.Marabout  3.Féticheur

4.Forgeron  5.Herboriste  6.Charlatan

7.Planteur  8. Vendeur  9.Cultivateur

10.Autres

**Mode d'acquisition de la recette :**

1. Familiale : Grand-père  ; Grand-mère  ; Père  ; Mère

2. Communautaire  ; 3. Apprentissage  ; 4. Ancien Malade

5. Expérience personnelle  ; 6.Rêve

Renseignements sur la maladie

Nom de la maladie dans la langue du lieu d'enquête :.....

Nom de la maladie dans la langue du guérisseur :.....

Manifestation de la maladie selon le guérisseur :.....

Traitemennt de la maladie par le guérisseur par des :

- Produits végétaux
- Produits animaux
- Produits minéraux

Cas des produits végétaux

Nom local de la plante :.....

Langue nationale correspondante : 1.Maninka  ;2.Soussou

3.Poular  ;4.Guerzé ;5.Toma  ;6.Kissi  7.Autres

Nom scientifique de la plante :.....

Synonyme :.....Nom vulgaire :.....

Habitat :.....Altitude.....Latitude.....Longitude.....

Humidité :....Type de sol :.....Type de végétation.....

Abondante ; Très commune; Commune ; Rare ; En danger

- Parties de la plante utilisées

Feuilles ; Ecorces de tige; Ecorces de racine; Racines

Graines; Fruits ; Fleurs; Plantes entières

- Stade de développement de la plante :

Floraison ; Fructification; Végétatif  ; Présence de latex : OUI

Saisonnier ; Pérenne

- Mode de préparation du médicament

Macération ; Décoction ; Infusion  ; Carbonisation; Autres

- Mode d'administration du médicament

Orale; Massage ; Inhalation ; Bain  ; Toilette intime

Badigeonnage ; Application locale ; fumigation ; instillation

Avec rite ou incantation

Posologie :.....

Durée du traitement :.....