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Discovery of Secondary Metabolites from Rwandese Medicinal Plants

Isolation, Characterization and Biological Activity

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ACTA UNIVERSITATIS UPSALIENSIS UPPSALA 2022

ISSN 1651-6214
ISBN 978-91-513-1522-5
URN urn:nbn:se:uu:diva-472783

Dissertation presented at Uppsala University to be publicly examined in A1:111a, BMC, Husargatan 3, Uppsala, Friday, 10 June 2022 at 13:15 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Yngve Stenstrøm (Norwegian University of Life Sciences).

Abstract

Umereweneza, D. 2022. Discovery of Secondary Metabolites from Rwandese Medicinal Plants. Isolation, Characterization and Biological Activity. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 2158. 66 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1522-5.

Plants have served as the principal source of medicines in different parts of the world through the ages. Herb-derived medicines have been used as decoctions, infusions, tinctures or single substance drugs. Due to their impressive possibility of diversification, plants have also provided an immense universe of creativity for synthetic chemists, who constantly make useful new molecules inspired by the natural molecular architecture.

The goal of this thesis work was to investigate secondary metabolites isolated from selected Rwandese medicinal plants. It focused specifically on the isolation, the characterization and the determination of the biological activity of natural products. The investigated plants belong to the families of Myrtaceae (*Eucalyptus melliodora* and *Eucalyptus anceps*), Fabaceae (*Eriosema montanum*), Lamiaceae (*Clerodendrum myricoides*) and Asteraceae (*Senecio mannii*). These were selected from the Rwandese flora based on information collected from traditional healers, and from the literature. The study made use of chromatographic, spectroscopic and spectrometric methods for separation, purification and structure elucidation of the plant constituents.

In paper I, the chemical composition and antifungal activity of essential oils of *E. melliodora and E*. anceps were discussed. The essential oils were composed of mono- and diterpenes, and their alcohol derivatives. The essential oil mixtures exhibited antifungal activity against food spoilage fungi.

In paper II, *E. montanum* was investigated and a total of 20 compounds were isolated including two new prenylated dihydrochalcones and eighteen known secondary metabolites. Their antibacterial activities and cytotoxicity were determined.

In paper III, the isolation of three new and two known iridoid glycosides from *C. myricoides* was reported along with the antiviral activities of the crude extract and of the isolates.

In paper IV, the phytochemical investigation of *S. mannii* was reported. It afforded one new silphiperfolanol angelate ester, two new macrocyclic pyrrolizidine alkaloids, and five known secondary metabolites. Two new synthetic derivatives were obtained by structural modification of 2-angeloyloxy-5,8-dihydroxypresilphiperfolane. The relative stereochemistry of senaetnine was investigated by NAMFIS and confirmed to be 7R, 12R, and 13R.

The new compounds isolated in this study have shown biological activities, and may provide lead compounds for drug discovery and technological applications.

Keywords: Natural products, Nuclear Magnetic Resonance (NMR), Dihydrochalcones, Iridoids, Silphiperfolanols, Pyrrolizidine alkaloids (PAs), Rwandese Medicinal Plants

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ISSN 1651-6214
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URN urn:nbn:se:uu:diva-472783 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-472783)

"In all things of nature there is something of the marvellous." Aristotle

To Danick, Daniella and Valentine

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Umereweneza, D.; Muhizi, T.; Kamizikunze, T.; Nkurunziza, J. P., Chemical composition and antifungal activity of essential oils extracted from leaves of *Eucalyptus melliodora* and *Eucalyptus anceps* grown in Rwanda. *J. Essent. Oil-Bear.* **2019**, 22, 151-158.

II. Umereweneza, D.; Atilaw, Y.; Rudenko A.; Bourgard C.; Orthaber, A.; Muhizi, T.; Sunnerhagen, P.; Erdélyi; M., Gogoll, A., Antibacterial and cytotoxic prenylated dihydrochalcones from *Eriosema Montanum*. *Fitoterapia* **2021**, 149, 104809.

III. Umereweneza, D.; Molel, J.; Said, J.; Atilaw, Y.; Muhizi, T.; Trybala, E., Bergström, T.; Gogoll, A.; Erdélyi, M., Antiviral iridoid glycosides from *Clerodendrum myricoides. Fitoterapia* **2021**, 155, 105055.

IV. Umereweneza, D.; Atilaw, Y.; Peintner, S; Rudenko, A.; Bourgard, C.; Xiong, R.; Muhizi, T.; Gogoll, A.; Erdélyi, M., Macrocyclic pyrrolizidine alkaloids and silphiperfolanol angelate esters from *Senecio mannii*. *Submitted.*

Contribution

The author wishes to clarify his contribution to the research work presented in this thesis.

Papers I-IV: The author took part in study design, sample collection, and processing; he performed extraction, isolation, and purification of the isolates. He was actively involved in structure elucidation by conducting characterization experiments and proposing structures, which could be modified after discussions with co-authors. He also wrote the first draft of the manuscripts that were further improved by co-authors. Except indicated otherwise, the author took the pictures presented in this thesis.

Other documents this work is based on

The content of this thesis builds partially upon the author's half-time report, presented on November 6, 2020. Chapter 1 and 2 have been modified, chapter 3 is presented for the first time, new data have been included in chapter 4 and hence it was updated.

Contents

Abbreviations

AMBER	Assisted Model Building with Energy Refinement
ANS	Anthocyanidin Synthase
AURS	Aurone Synthase
CC	Column Chromatography
CHI	Chalcone Isomerase
CHR	Chalcone Reductase
CHS	Chalcone Synthase
4-CL	4-Coumaroyl CoA Ligase
COSY	Correlation Spectroscopy
DAST	Diethylaminosulfur Trifluoride
DFR	Dihydroflavonol Reductase
DMAPP	Dimethylallyl Pyrophosphate
DOXP	Deoxyxylulose Phosphate
EC_{50}	Half Maximal Effective Concentration
ECD	Electronic Circular Dichroism
FLS	Flavonol Synthase
FNS	Flavanone Synthase
FPP	Farnesyl Diphosphate
FUAP	Forskarutbildningsansvarig Professor
GAP	D-glyceraldehyde-3-Phosphate
GBSA	Generalized Born model augmented with the hydrophobic Solvent Accessible Surface Area
GC	Gas Chromatography
GGPP	Geranylgeranyl Pyrophosphate

GPP	Geranyl Pyrophosphate
HEp-2	Human laryngeal Epidermoid carcinoma
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation Spectroscopy
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution Electrospray Ionisation Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence Spectroscopy
IC_{50}	Half Maximal Inhibitory Concentration
IFR	Isoflavone Reductase
IFS	Isoflavone Synthase
IPP	Isopentenyl Pyrophosphate/Isopentenyl Diphosphate (IDP)
IR	Infrared Spectroscopy
LCR	Leucoanthocyanidin Reductase
MCF-7	Michigan Cancer Foundation-7
MCMM	Monte-Carlo Multiple Minimum
MEP	Methyl-Erythrose-4-Phosphate
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MVA	Acetate/Mevalonate
NAMFIS	NMR Analysis of Molecular Flexibility in Solution
NIRDA	National Industrial Research and Development Agency
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
OPLS	Optimized Potentials for Liquid Simulations
ORD	Optical Rotation Dispersion
PA	Pyrrolizidine Alkaloid
PAL	Phenylalanine Ammonia Lyase
PKS	Polyketide Synthase

PP	Pyrophosphate
PRCG	Polak-Ribière-type Conjugate Gradient
RCE	Redundant Conformer Elimination
RMSD	Root-Mean-Square Deviation
RSV	Respiratory Syncytial Virus
SEC	Size-Exclusion Chromatography
TLC	Thin Layer Chromatography
TOCSY	Total Correlation Spectroscopy
UV-Vis	Ultraviolet–Visible Spectroscopy
WHO	World Health Organization
VLC	Vacuum Liquid Chromatography

1 Introduction

1.1 Biologically active natural products from plants

In a broader sense, ''natural products'' refer to any molecules of biological origin. However, this term has usually been used to indicate secondary metabolites produced by plants, fungi, microorganisms, invertebrates and vertebrates. Plant secondary metabolites refer to those compounds that are not directly involved in the photosynthetic or respiratory processes but are necessary for plant's survival in the environment.[1, 2] By contrast, compounds of primary metabolism or primary metabolites such as proteins, nucleic acids and sugars play basic, well-defined, and essential roles in growth and reproduction processes of the organisms.[3] It is important to note the overlap between these terms. The difference is not distinct and hence primary and secondary metabolisms are interrelated.[4] For example, compounds such as cinnamic acid, squalene, gibberellic acid, auxin, and shikimic acid sit at the interface between both metabolisms.[5]

Plants synthesize a variety of secondary metabolites with highly diverse structures. Some of these molecules are used as defence compounds against herbivores and pathogens, others such as coloured flavonoids attract pollinators, whereas volatile terpenoids repel invaders, and some secondary metabolites act as fungicides or antibiotics.[4, 6] They help the plants to be more competitive in harsh environmental conditions.[7]

These secondary metabolites are not only important to the plants producing them, but also humankind has been exploiting them as source of medicines. The use of medicinal plants in folk medicine to treat diseases has been a common practice since thousands of years.[8] According to some estimates, even today 80% of the world's population relies on traditional medicine as the primary source of healthcare.[9] Herbal preparations have been made using different methods such as maceration, decoction, infusion, tincture and sauna.[10-13]

Plant-based natural products have also been used as source of drugs. The World Health Organization (WHO) estimates that 11% of the 252 basic and essential drugs have been isolated from plants, and a significant number of synthetic drugs have been developed from natural precursors.[14] Figure 1 presents a few selected examples of important drugs isolated from plants, such as morphine (**1**) and codeine (**2**) from *Papaver somniferum*,[15] quinine (**3**) and quinidine (**4**) from *Cinchona spp.*,[16, 17] atropine (**5**) from *Atropa belladona*,[18] vinblastine (**6**) and vincristine (**7**) from *Catharanthus roseus*,[19] digoxin (**8**) from *Digitalis lanata*,[20] and paclitaxel (**9**) from *Taxus brevifolia*.[21]

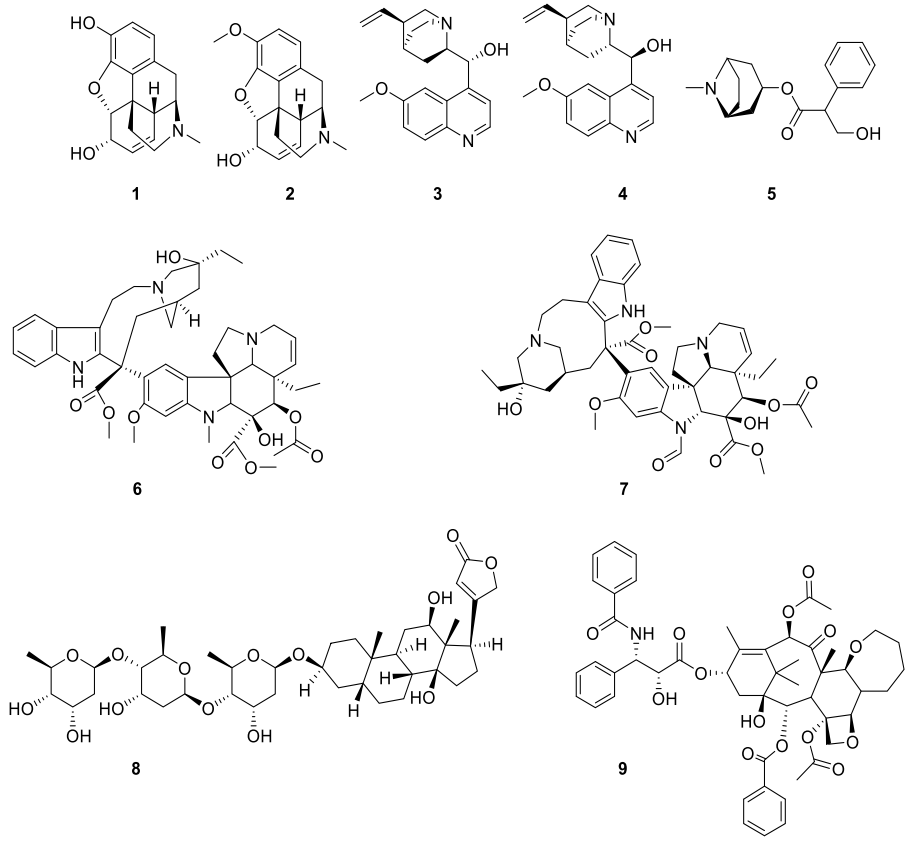


Figure 1. Examples of drugs isolated from medicinal plants.

Although the use of plants as a very important source of medicines dates back to several thousand years, the search for pure active ingredients might have started first in 1806 when Serturner isolated the alkaloid morphine (**1**) from *Papaver somniferum*.[8] The first documented structural modification was achieved in 1839 by Rafaele Piria, who synthesized salicylic acid from salicin, an analgesic and antipyretic constituent of *Salix alba.*[22] It is estimated that only 5–15% of approximately 422 000 species of higher plants have been investigated for bioactive compounds.[23] Most of these botanical species are found in the tropical region of the planet.[24] So far, more than 200 000 natural products have been described, and approximately 4000 new ones are reported every year.[23, 25] Hence, medicinal plants have been proven to be valuable sources of biologically active and pharmacologically important compounds, whereas their exploration has so far not been exhaustive.[26] This thesis aims at investigating the bioactive chemical constituents of selected so far unexplored Rwandese medicinal plants.

1.2 The aims of this thesis

Rwanda (Figure 2) possesses a rich biodiversity of both fauna and flora due to its geographical location characterized by temperate tropical highland climate.

Figure 2. Map of Rwanda. (https://elearning.reb.rw/course/view.php?id=267&section=6)

Rwandans have been using natural resources as traditional medicines to treat human ailments, and cattle as well as plant diseases.[27-32] The goal of the present thesis is to investigate selected Rwandese medicinal plants for biologically active natural products. This thesis specifically deals with isolation, characterization, structure modification and determination of biological activity of different phytoconstituents of Rwandese medicinal plants. The species investigated herein are found in the botanical families Myrtaceae (*E. melliodora* and *E. anceps*), Fabaceae (*E. montanum*), Lamiaceae (*C. myricoides*) and Asteraceae (*S. mannii*).

2 Structural classes of plant secondary metabolites

Plants have the ability to synthesize an enormous variety of structurally diverse natural products. These constituents have important functions in the interaction between plants and their environment, such as structural support, defence mechanisms, molecular signalling, and pollinator attraction.[33] Based on the biosynthetic pathways, these compounds are categorized into four main classes, viz. phenylpropanoids, polyketides, terpenoids and alkaloids.[4, 33, 34]

2.1 Phenylpropanoids

Phenylpropanoids produced by plant secondary metabolism were regarded as either metabolic wastes or substances without important role in fundamental life processes.[35] However, in recent years, they have been proposed to increase the survival chances of plants by coping with worsening environmental conditions and regulating some metabolic processes.[35, 36] For example, phenylpropanoids such as stilbenes, pterocarpans and coumarins are biosynthesized in response to pathogen attack and their level increases to toxic concentration near the site of infection. Whereas deficiency in nitrogen leads to a faster deamination of phenylalanine which results in the accumulation of phenylpropanoids in plant tissue.[32] As a class of natural products, phenylpropanoids are characterized by a C_6-C_3 basic skeleton and comprise cinnamic acid and its derivatives, lignin, lignans, phenylpropenes, and coumarins (Figure 3). It is important to note that, in some naturally occurring cinnamic derivatives, the skeleton is shortened to C_6-C_1 which are referred to as benzenoids. Phenylpropanoids are ubiquitous in plants and serve as precursors of other classes of plant phenolic compounds.[34] In this study, phenylpropanoids were isolated from *E. montanum* (Section 4.2) and *S. mannii* (Section 4.4).

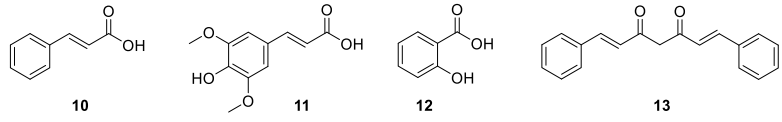
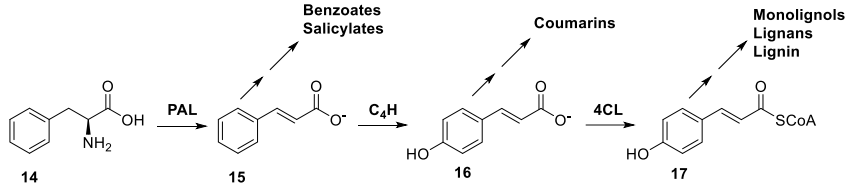


*Figure 3. Cinnamic acid (**10**), sinapic acid (**11**), salicylic acid (**12**), and curcumin (**13**).*

Phenylpropanoids are biosynthesized from phenylalanine (**14**) or tyrosine through the shikimate pathway, as summarized in Scheme 1.[37] The biosynthesis involves a series of enzymes including lyases, transferases, ligases, oxygenases, and reductases.[35]

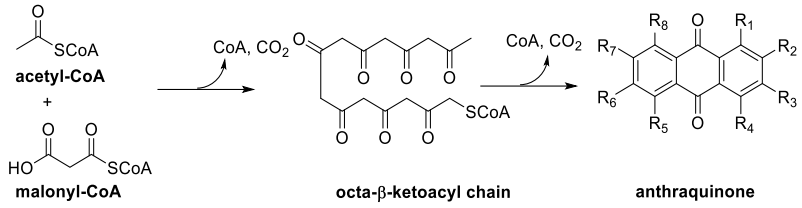


Scheme 1. General phenylpropanoid pathway.[38]

The process starts with phenylalanine derived from shikimic acid, but some monocot species use tyrosine (**14**) instead.[35] The first enzymatic step is the transformation of phenylalanine into cinnamic acid (**15**) by phenylalanine ammonia lyase (PAL). Next, cinnamic acid is hydroxylated by cinnamate 4-hydroxylase (C4H) to produce *p*-coumaric acid (**16**). The latter is converted into *p*-coumaroyl-CoA (**17**) by 4-coumaroyl CoA ligase (4CL), an important precursor to numerous phenylpropanoid compounds through specialized branch pathways.[39, 40] These pathways utilize approximately 30-40% of all organic carbon in vascular plants, and produce a large family of phenolic natural products, including flavonoids and isoflavonoids, stilbenes, coumarins, benzoic acid and benzaldehyde derivatives, phenylpropenes, diarylheptanoids and their glycosides, and other conjugates.[41, 42]

2.2 Polyketides

Polyketides are found in bacteria, fungi and plants. Some of the polyketides, such as daunorubicin, erythromycin, tetracycline, and rapamycin are clinically potent drugs.[43] They are biosynthesized from two-carbon units derived from acetyl-CoA and malonyl-CoA through the acetate/malonate or polyketide pathway, Scheme 2. Polyketide biosynthesis is governed by polyketide synthases (PKS). These enzymes are classified as PKS I, II and III according to the number, organization and function of their active sites. The PKS III is the only type that has been identified in plants and plays key roles in the biosynthesis of plant natural products.[44] The PKSs achieve the synthesis of a huge variety of polyketides through a series of controlled reactions such as chain elongation, reduction and cyclization.[45, 46]



Scheme 2. Anthraquinone biosynthesis via the acetate-malonate pathway.[47]

Natural plant polyketides are not always synthesized from a single acetate pathway, but often are of mixed biosynthetic origins. The acetate moiety is usually combined with a phenylpropanoid and/or a terpenoid part, and parts of the carbon backbone may be synthesized from amino or fatty acids.[33] This combination results in a large array of structurally diverse secondary metabolites.

2.2.1 Polyketides from acetate units

Naphthoquinones, anthraquinones and anthrones (Figure 4) belong to this class of polyketides that are biosynthesized from acetate units. Naphthoquinones (**18**) are built from six acetate units which combine, after decarboxylation, resulting in a skeleton of ten carbon atoms assembled in two six-membered rings. Anthraquinones (**19**) are, in contrast, synthesized from eight acetate units arranged in three six-membered rings affording, following a decarboxylation, a fourteen carbon skeleton. The naturally occurring reduced form of anthraquinones are referred to as anthrones (**20**). It is important to note that naphthoquinones and anthraquinones can also be made by some plants through shikimate pathway.[47, 48]

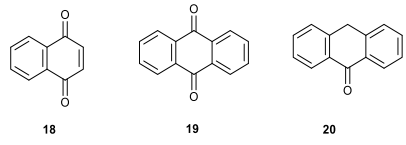
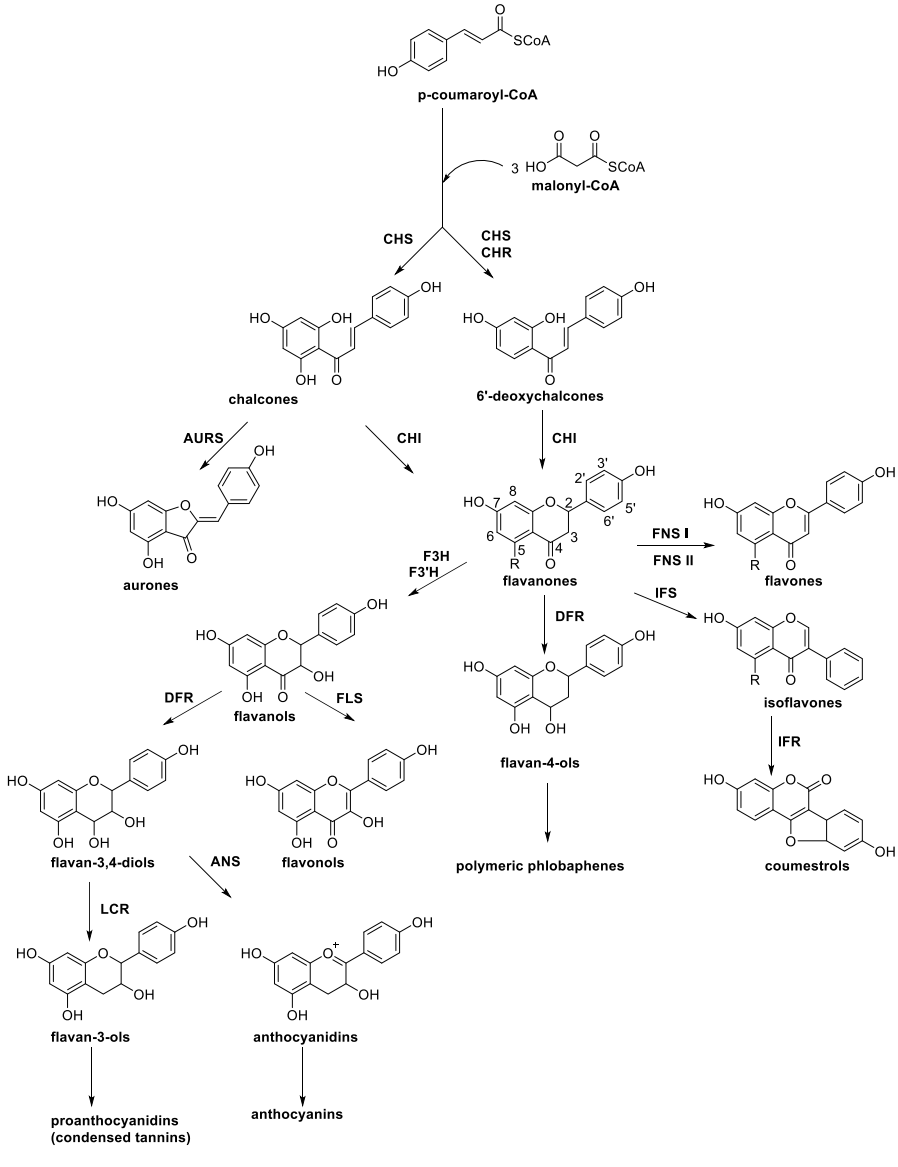


Figure 4. Polyketides from acetate units.

2.2.2 Polyketides with phenylpropanoid moieties

Some natural products are synthesized by the combination of compounds from the shikimate pathway with others having three two-carbon units originating from malonyl-CoA, to afford polyketides of various structures, such as flavonoids, stilbenes, styrylpyrones, and curcuminoids. Flavonoids, characterized by a fifteen-carbon phenylpropanoid core arranged in a C_6-C_3-C_6 carbon framework is the largest group of polyketides. This backbone is usually modified by hydroxylation, methylation, acylation, glycosylation and prenylation

at different positions. They are classified in seven subclasses, namely chalcones, flavones, flavonols, flavandiols, anthocyanins and proanthocyanidins, and aurones.[49, 50] The biosynthesis of flavonoids is one of the specialized pathways stemming from the general phenylpropanoid pathway, Scheme 3.



Scheme 3. Flavonoid biosynthetic pathway.[38] ***ANS****: anthocyanidin synthase;* ***AURS****: aurone synthase;* ***CHI****: chalcone isomerase;* ***CHR****: chalcone reductase;* ***CHS****: chalcone synthase;* ***DFR****: dihydroflavonol reductase;* ***F3/3'H****: flavanone 3/3'-hydroxylase;* ***FLS****: flavonol synthase;* ***FNS I, II****: flavanone synthase I, II;* ***IFS****: isoflavone synthase;* ***IFR****: isoflavone reductase;* ***LCR****: leucoanthocyanidin reductase.*

2.3 Terpenoids

Terpenoids or isoprenoids form a subclass of prenyllipids that are also known as prenol lipids. This group of natural products include terpenoids, prenylquinones, and steroids. These represent the oldest, most widespread, and structurally most diverse group of small molecules synthesized by plants, animals and microbial organisms.[51, 52]

Many plants including cinnamon, ginger, lavender, eucalyptus, peppermint, citrus, and lemongrass exhibit pleasant smell, spicy taste, and intriguing biological activities that are all attributed to the presence of terpenoids.[53] These are often volatile components that are commonly referred to as essential oils. Figure 5 shows isoprene (**21**), tiglic acid (**22**), angelic acid (**23**), myrcene (**24**), prenol (**25**), citral (**26**), geraniol (**27**), phytol (**28**), farnesol (**29**), and geranylgeraniol (**30**) that are some examples of terpenoids.

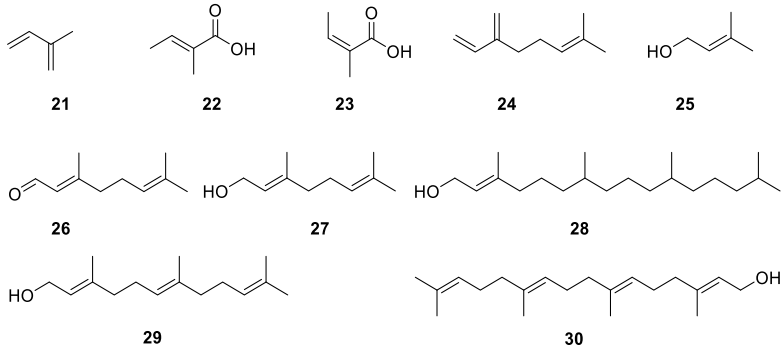


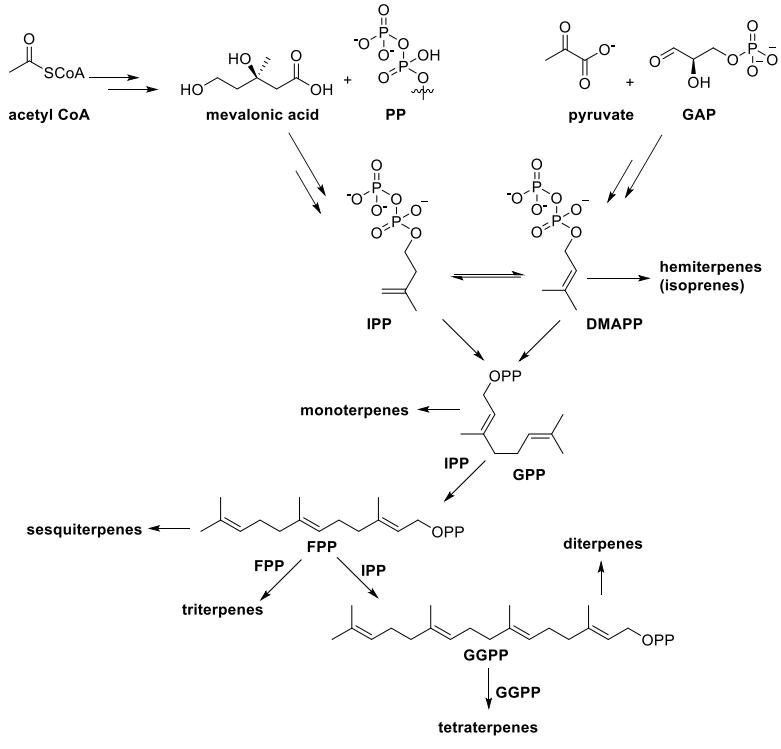
Figure 5. Structures of some terpenoids.

The physiological roles of terpenoids in the producing plants vary from pollinator attraction, photoprotection and communication to defensive mechanisms against herbivore animals, insects and fungi. For example, iridoids, which are oxygenated monoterpenoids with an intense bitter taste, are produced by different plant families including Rubiaceae, Oleaceae, Lamiaceae and Plantaginaceae as feeding deterrents.[33]

Terpenoids are built from isoprene or 2-methyl-1,3-butadiene (**21**), and are classified according to the number of isoprene units assembled: hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}), and polyterpenes.[54]

Terpenoids are biosynthetically derived from two isomeric 5-carbon units known as isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are generated by two independent pathways namely the acetate/mevalonate (MVA) and non-mevalonate or methylerythritol phosphate (MEP) pathway, shown in Scheme 4. In higher plants, the MVA

pathway takes place in the cytosol and mitochondria, while the MEP occurs in plastid. [57, 58]



Scheme 4. Terpenoid biosynthetic pathway.[55, 56] ***DMAPP****: dimethylallyl diphosphate;* ***FPP****: farnesyl diphosphate;* ***GAP****: D-glyceraldehyde-3-phosphate;* ***GGPP****: geranyl-geranyl diphosphate;* ***GPP****: geranyl diphosphate;* ***IPP****: isopentenyl diphosphate;* ***PP****: pyrophosphate*

In the first pathway, the mevalonic acid is produced from the condensation and reduction of three units of acetyl CoA. The mevalonic acid is transformed into IPP by enzymatic phosphorylation followed by decarboxylation. Subsequently, IPP undergoes enzymatic isomerization to afford its highly electrophilic isomer DMAPP.[57, 58]

In the MEP pathway, IPP and DMAPP are obtained from pyruvate and D-glyceraldehyde through a series of enzymatic reactions including the condensation of glyceraldehyde phosphate and pyruvate into 1-deoxyxylulose-5-phosphate (DOXP). In the first step, DOXP is converted into MEP that subsequently undergoes cytidylation, phosphorylation and cyclization to afford 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate which, upon rearrangement and reduction, generates IPP and DMAPP.[56, 59]

The MVA pathway is responsible for the biosynthesis of sterols, sesquiterpenes, triterpenes and ubiquinones. The MEP pathway yields hemi-, mono-,

sesqui- and diterpenes, plastoquinones, carotenoids, and the phytol tail of chlorophyll.[60] The high structural diversity within isoprenoids is generated by numerous reactions including oxidation, reduction, isomerization, hydration, conjugation and other structural modifications involving the diphosphate esters of linear polyunsaturated allylic alcohols, such as prenol (**25**), geraniol (**27**), farnesol (**29**), and geranylgeraniol (**30**).[61, 62] In this study, terpenoids were identified in all plant species investigated (Sections 4.1- 4.4).

2.4 Alkaloids

Alkaloids are naturally occurring, nitrogen-containing organic compounds. With some exceptions, the basic nitrogen is part of a heterocyclic ring, and is not involved in an amide of a peptide bond. Higher plants were the first to be recognized as natural source of alkaloids, but later on alkaloids have also been reported from fungi, bacteria, insects, and other animals.[63, 64] In higher plants, alkaloids are commonly found in the families Chenopodiaceae, Lauraceae, Magnoliaceae, Berberidaceae, Menispermaceae, Ranunculaceae, Papaveraceae, Fumariaceae, Leguminosae, Rutaceae, Apocynaceae, Loganiaceae, Rubiaceae, Boraginaceae, Convolvulaceae, Solanaceae, Campanulaceae, and Compositae.[65]

The exact function of alkaloids in plants is still not fully understood. They are believed to participate in ecological interactions between the plant and its environment. They are involved in defence, feeding attraction and deterrence.[64, 66, 67] Most alkaloids exhibit important biological activities and have therefore been exploited as pharmaceuticals, stimulants, narcotics, poisons or as part of new drug formulations.[68, 69] Morphine (**1**), quinine (**3**), caffeine (**31**) and strychnine (**32**) are examples of alkaloids (Figure 6) that have been utilized in pharmaceutics for human health benefits.[70]

Alkaloids have different biosynthetic pathways as compared to other classes of secondary plant metabolites.

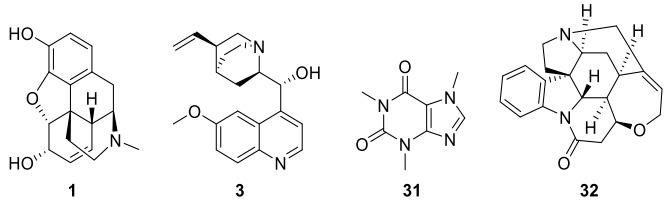


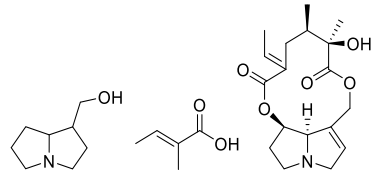
Figure 6. Structures of pharmacologically and historically important alkaloids.

Alkaloids are generally classified based on the starting material from which they are constructed into three main types: true alkaloids, protoalkaloids, and

pseudoalkaloids. True alkaloids are derived from amino acids and have nitrogen as heteroatom in a cyclic subunit. Protoalkaloids are also synthesized from amino acids, but their nitrogen is not part of a cyclic subunit. Pseudoalkaloids are derivatives of terpenes and steroids, and are the results of amination and transamination reactions.[63, 64, 67]

Alkaloid classes are biosynthesized through a wide variety of biosynthetic pathways.[71] Discussing all known pathways that lead to the biosynthesis of alkaloids would be beyond the scope of this thesis. Hence, the discussion herein has been limited to pyrrolizidine alkaloids, PAs, the subclass of alkaloids investigated in course of this project.

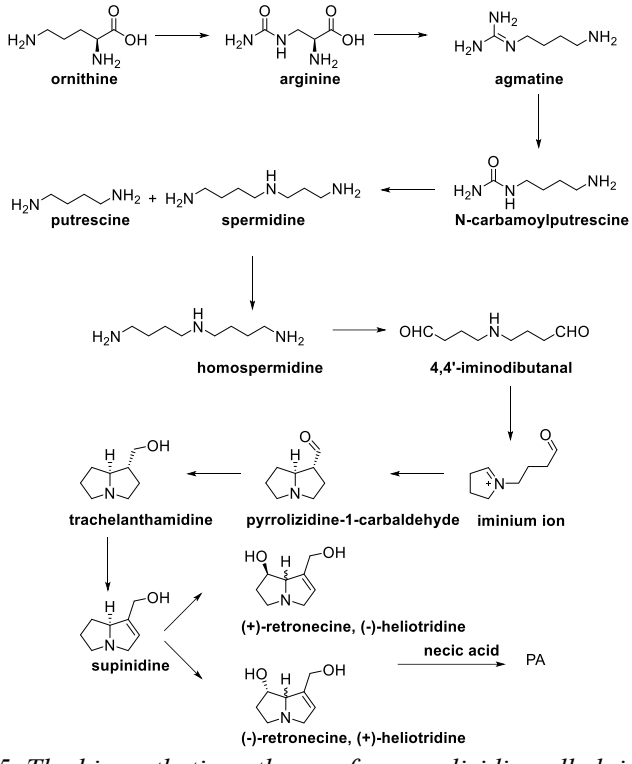
Pyrrolizidine alkaloids are naturally occurring heterocyclic organic compounds composed of hydroxymethylpyrrolizidine (**33**) (necine base) esterified with aliphatic mono- or dicarboxylic acid (necic acid, such as tiglic acid (**34**)), Figure 7.[65, 72] They occur in about 5% of all flowering plants belonging mainly to Asteraceae, Boraginaceae, Fabaceae, and Orchidaceae plant families.[73] Approximately five hundred pyrrolizidine alkaloids have so far been investigated.[51, 72]



33 **34** **35**

*Figure 7. Examples of necine base (**33**), necic acid (**34**), and PA senecionine (**35**).*

Pyrrolizidine alkaloids are biosynthesized from ornithine via putrescine and homospermidine as symmetrical intermediates, Scheme 5. Ornithine generates arginine which is decaboxylated to afford agmatine that is transformed into N-carbamoylputrescine, the precursor of putrescine. Putrescine is converted into spermidine.



Scheme 5. The biosynthetic pathway of a pyrrolizidine alkaloid.[72, 74]

Then, homospermidine synthase catalyses the exchange of 1,3-diaminopropane with putrescine in spermidine to produce homospermidine. The oxidation of homospermidine by copper-dependent diamine oxidase yields 4,4'-iminodibutanal, which cyclizes into pyrrolizidine-1-carbaldehyde. The reduction of the latter by alcohol dehydrogenase affords a 1-hydroxymethylpyrrolizidine, such as trachelanthamidine. Desaturation and hydroxylation result in the formation of retronecine and heliotridine via supinidine. Retronecine and/or heliotridine are subsequently acylated by acyltransferase to finally produce the pyrrolizidine alkaloid.[72, 74] In this study, macrocyclic pyrrolizidine alkaloids were isolated from *S. mannii* (Section 4.4).

3 Overview of methods

3.1 Investigated plant species

Plants are selected for phytochemical studies following five common approaches: (i) random screening, (ii) selection of specific taxonomic groups, (iii) chemotaxonomics focusing on specific class of secondary metabolites, (iv) information database driven, and (v) selection based on ethnomedical uses guided by traditional healers.[75] In this project, *E. anceps*, *E. melliodora*, *E. montanum*, *S. mannii* and *C. myricoides* were selected based on combined information database driven and the ethnomedicinal use based approaches.

The *Eucalyptus* genus belongs to the Myrtaceae family and is diverse.[76, 77] This genus includes about 900 species. *Eucalyptus* (Figure 8) is an evergreen tall tree, native to Australia but also extensively cultivated in many other countries worldwide.

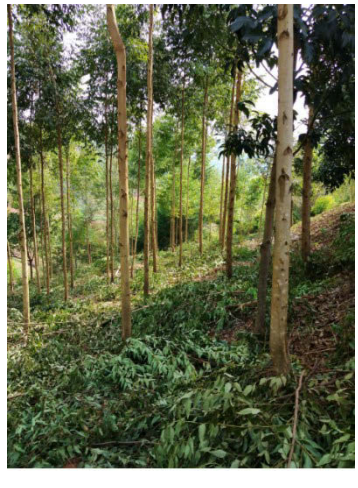


Figure 8. Eucalyptus, Ruhande arboretum, Rwanda.

A wide variety of secondary metabolites are found in the leaves, stem, bark and roots of *Eucalyptus* in large concentrations,[78] including terpenoids, flavonoids, alkaloids and tannins.[79] Leaves, fruits, buds and bark preparations have been reported to be used for their antibacterial, antiseptic, antioxidant, anti-inflammatory, and anticancer activities, and for the treatment of respiratory diseases, common cold, influenza, and sinus congestion.[80, 81]

The leaves of *Eucalyptus* were reported to contain essential oils possessing antimicrobial properties.[82, 83] Hence, *Eucalyptus* essential oils have been approved as food additives and preservatives in Japan.[84] In the United States of America, formulations based on essential oils have been approved and included on the list of generally recognized, safe foodstuffs.[85] In Europe, plant essential oils and their products, such as eugenol and carvone are industrially produced and used in households to inhibit the growth of storage pathogens.[85] Furthermore, essential oils are known to be less likely associated with the development of resistance by fungi, as compared to synthetic fungicides, and are less hazardous to the environment and to human health.[86]

The *Eriosema* genus (Leguminosae) is composed of more than 140 species distributed in tropical areas.[87, 88] Most of these species are used in traditional medicine in different parts of the world.[89] They are used for the treatment of various health conditions, such as female sterility, acceleration of delivery during childbirth, erectile dysfunction, male impotence, diarrhea, orchitis, hydrophobia, cough and skin diseases.[87, 90-92]

In Rwanda, the leaf decoction of *E. montanum* (Figure 9) is used for the treatment of conjunctivitis, cough and snake bite.[93] Studies on its ethanolic leaf extract showed anti-HIV and anti-inflammatory activities.[94] In addition, its root extract exhibited anti-asthma activity.[12] *Eriosema* species are known for containing bioactive flavonoids and their derivatives.[87, 89, 95, 96]

Figure 9. E. montanum, Nyaruguru, Rwanda.

The *Senecio* genus belongs to the Senecioneae tribe in the family Asteraceae (Compositae). It is comprised of more than 1500 species distributed all over the globe, making it the largest and the most complex genus in the family.[97] *Senecio* species have been used in traditional medicine for the treatment of

wounds as well as antiemetic, anti-inflammatory, and vasodilatory preparations among other uses.[98] It is also used to treat epilepsy, cancer, pneumonia, cough, typhoid, microbial and fungal diseases.[99-101] In Rwanda, *S. mannii* (Figure 10) is involved in the treatment of poisoning, wounds, hemorrhoids and malaria.[102-104]

Despite their ethnopharmacological uses, some species have been reported to be poisonous and responsible for the death of livestock. These paradoxical biological activities have motivated the investigation of the chemical composition of *Senecio* plant parts including roots, stem barks, leaves, flowers and whole plant. The isolated metabolites include alkaloids, terpenoids, flavonoids and coumarins. Some of the isolates exhibited antibacterial, antitubercular, antiviral, and cytotoxic activities.[105-113]

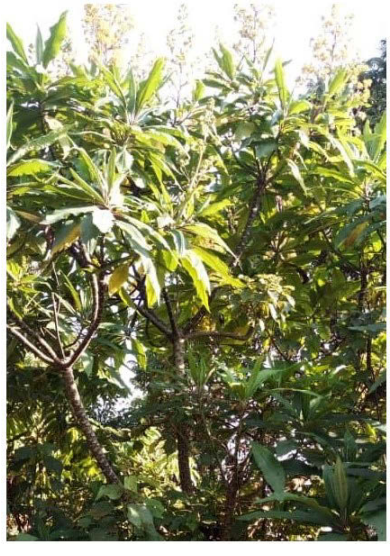


Figure 10. S. mannii, Nyungwe, Rwanda.

Clerodendrum (Lamiaceae) is a large and diversified genus with as many as 450 identified species.[114, 115] It is widely distributed in tropical and subtropical regions of the world and comprises small trees, shrubs and herbs. The genus *Clerodendrum* has been used in traditional medicine to treat various ailments around the world over many eras.[116-119] The extracts from roots and leaves of *C. indicum*, *C. phlomidis*, *C. serratum*, *C. trichotomum*, *C. chinense* and *C. petasites* have been used for the treatment of rheumatism, asthma and other inflammatory diseases.[120-125] *C. phlomidis* and *C. myricoides* (Figure 11) have been used as an astringent and also in the treatment of diarrhea and gonorrhea.[126, 127]

Preparations of the leaves, roots, and stems of *Clerodendrum* species have been used in folk medicine to treat different diseases such as malaria, cancer,

catarrhal affections of the lungs, human immunodeficiency virus (HIV), fever, inflammation, skin diseases and asthma.[125, 128, 129] Different secondary metabolites have been isolated from *Clerodendrum* species: including alkaloids, terpenoids, flavonoids and phenylethanoid glycosides.[130-132]

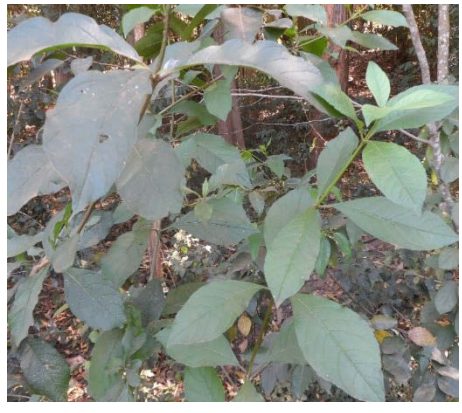


Figure 11. C. myricoides, Shyembe, Rwanda.

3.2 Plant material collection, extraction and purification

Plant species were selected based on the information about their ethnomedicinal use provided by Rwandese traditional healers along with previously published data. With the assistance of a botanist and experienced traditional healers, medicinal plants were identified, recorded, and collected from natural habitat. Samples were collected from *E. melliodora*, *(*Myrtaceae), *E. anceps* (Myrtaceae), *E. montanum* (Fabaceae), *C. myricoides* (Lamiaceae), and *S. mannii* (Asteraceae). Their specimens were deposited in the herbarium of the national industrial research and development agency (NIRDA), Rwanda with the following reference codes: UMDAN: 001-2018 (*E. melliodora*), UMDAN: 002-2018 (*E. anceps*), UMDAN: 001-2019 (*E. montanum*), UMDAN: 010-2019 (*C. myricoides*), and UMDAN: 007-2019 (*S. mannii*).

3.2.1 Crude extract preparation

In phytochemistry, extraction aims at separating the soluble plant metabolites from the insoluble residue.[133] Hence, it is usually used as the first step to isolate the target natural product (analyte) from the complex crude plant material.[134] The extraction of natural products usually involves steam or hydrodistillation, maceration, percolation, infusion, decoction and reflux extraction.[25, 135, 136]

Hydro / Steam distillation

Hydrodistillation and steam distillation are techniques for the extraction of volatile plant components, also known as essential oils.[134] In the hydrodistillation procedure, the mixture of plant material and water is boiled in a round bottomed flask, the essential oil is collected after condensation and is separated from water by decantation. The traces of moisture are further removed by passing the essential oil over anhydrous sodium sulphate prior to determining the yield and constituents.[137] In steam distillation, the plant material is not heated with water but the steam passes through the plant material and takes the essential oil with it which is subsequently condensed, collected and separated from water by decantation.[138]

Maceration

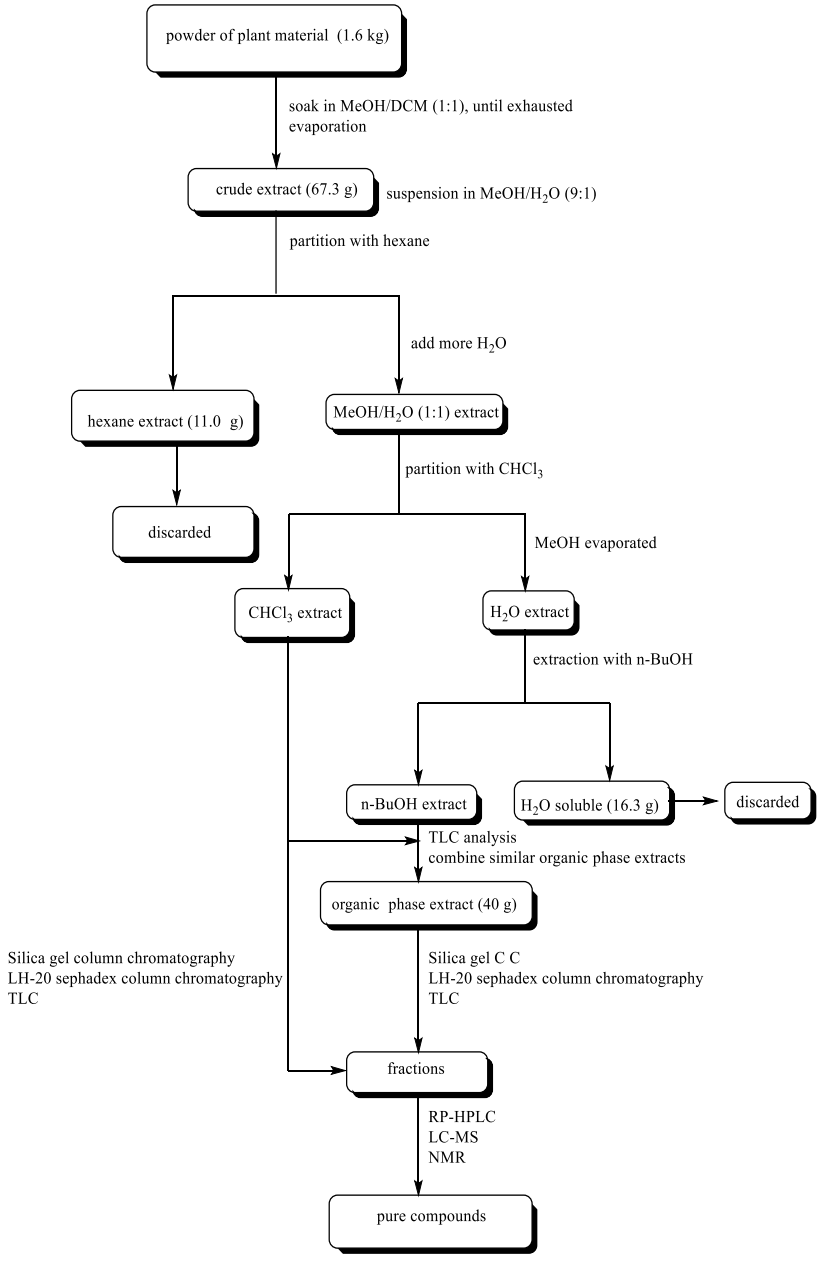
The ground plant material is placed in a container with the solvent, at room temperature for a period of 2 to 3 days. To facilitate the dissolution of the analyte, the mixture is frequently agitated during maceration. The solution, after filtration to remove any traces of solid particles, is dried in vacuum while the marc is discarded.[135, 136]

3.2.2 Fractionation, isolation, and purification

The crude extract prepared by the extraction procedure described in section 3.2.1 consisted of a complex mixture of natural products. Due to the large differences in their physicochemical properties, it is not practical to apply a single separation technique to isolate individual components from such a crude mixture. Therefore, the crude extract was fractionated based on polarity or molecular size of the constituents. These fractions were generated by different chromatographic or extraction techniques, such as column chromatography (CC), vacuum liquid chromatography (VLC), size-exclusion chromatography (SEC), liquid-liquid extraction or solid-phase extraction. For an efficient fraction collection, thin layer chromatography with an ultraviolet (UV) detection was used to monitor the fraction similarities which might result in fraction combinations. If necessary, the staining reagent was applied to the TLC plate to make spots more visible.[139-141]

In summary, the crude extract of each plant part was separately prepared by macerating its dried powder. Maceration was performed by using different organic solvents including dichloromethane, methanol, ethanol and ethyl acetate. Prior to the preliminary phytochemical screening to detect the presence of compound classes, the extracts were concentrated by a rotary evaporator at 40 ºC. To obtain pure constituents, the crude extract was subjected to fractionation, isolation and purification. Finally, the purified compounds were characterized by spectrometric and spectroscopic methods as described in section 3.3. These isolates and their corresponding crude extracts were assayed for

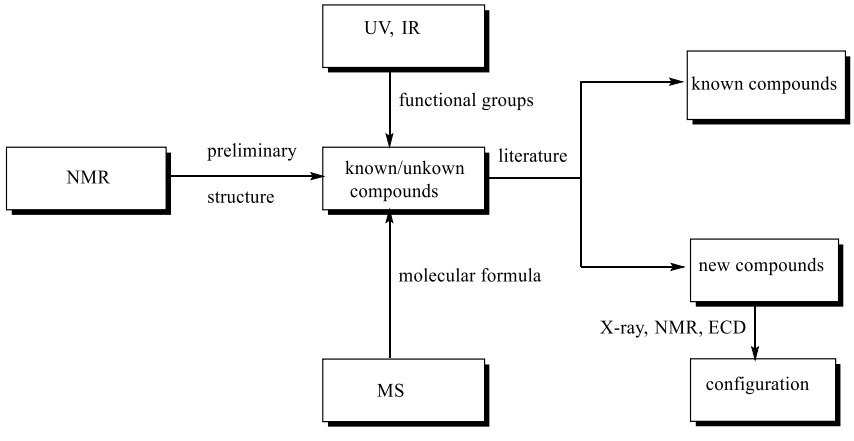
biological activity. The important steps applied in the investigation of *E. montanum* secondary metabolites were summarized in Scheme 6.



Scheme 6. Key steps for the isolation of secondary metabolites from plants.

3.3 Structure characterization

The structures of the isolated compounds were elucidated using High Resolution Electrospray Ionisation Mass Spectrometry (HRESIMS), X-ray Crystallographic, Infrared (IR), Ultraviolet-Visible (UV-VIS), Optical Rotary Dispersion (ORD), and Nuclear Magnetic Resonance (NMR) spectroscopic techniques. The HRESIMS was used to determine the molecular masses of isolates whereas IR spectroscopy helped to identify functional groups of the compounds. UV-VIS spectroscopy was applied to detect conjugated systems of the isolates. The ORD experiments were performed to record the optical activity, for determination of absolute configuration. X-ray crystallography was used to elucidate structures and to assign absolute configurations of crystallized compounds. Scheme 7 presents the process for structure elucidation of isolated natural compounds.



Scheme 7. Procedure of natural product structure elucidation.

NMR in combination with high resolution mass spectrometry has become the principal structure determination technique in natural product chemistry.[142] When placed in a strong magnetic field, NMR active nuclei resonate at a characteristic frequency of the electromagnetic spectrum. Slight variations in this resonance frequency give detailed information on the molecular environment in which the atoms reside.[143]

During the analysis of NMR data, three phenomena have been exploited. *Through bond interactions:* scalar spin-spin coupling (*J*) via electrons in covalent bonds; *through space interactions:* the NOE mediated through dipole–dipole relaxation; and *chemical exchange:* the physical exchange of one spin for another at a specific location.[142] Therefore, to fully assign the NMR spectra and hence to elucidate the structures of isolated compounds, this study made use of 1D (^{1}H and ^{13}C NMR) and 2D (COSY, HMBC, HSQC, TOCSY and NOESY) NMR experiments as shown in Table 1.

Table 1. Fundamental NMR experiments for chemical structure elucidation as adapted from [142]

Procedure	Technique	Information
1D ^{1}H spectrum	1D	Information from chemical shifts, coupling constants, integrals.
2D ^{1}H–^{1}H correlation	COSY	Identify *J*-coupling relationships between protons.
1D ^{13}C spectrum	1D	Carbon count and multiplicity determination (C, CH, CH_2, CH_3)
2D ^{1}H–^{13}C one-bond correlation	HSQC	Carbon assignments transposed from proton assignments.
2D ^{1}H–^{13}C long-range correlation	HMBC	Correlations identified over two and three bonds. Fragments of structure pieced together.
2D ^{1}H–^{1}H correlations	TOCSY	Relayed *J* couplings within a coupled spin system.
Through-space NOE correlation	2D NOESY	Stereochemical analysis: configuration and conformation.

3.3.1 NAMFIS analysis of senaetnine (**67**)

NOE-build-up derived interproton distances

To obtain quantitative interproton distances of **67** in $CDCl_3$, an NOE-build-up analysis was performed by recording seven individual NOESY experiments with 100, 200, 300, 400, 500, 600 and 700 ms mixing times. Experiments were recorded in random order to counteract systematic errors. Spectra were acquired at 25 °C on a 500 MHz Bruker Avance Neo NMR spectrometer equipped with a TXO cryogenic probe (CRPHe TR-^{13}C/^{15}N/^{1}H 5mm-Z). The acquired spectral size was 512 × 4096 (F1 × F2) complex points with a spectral width of 7800 Hz. The recycle relaxation delay (d1) was set to 2.5 sec.

Conformational sampling using MCMM

A Monte Carlo conformational search was performed to generate the conformational ensemble for NAMFIS analysis. Since the relative stereochemistry of the 3 stereocenters is unknown, we sampled 4 individual diastereomers

namely the RRR, RRS, RSR, RSS of carbons 7, 12 and 13, respectively. The corresponding enantiomers (SSS, SSR, SRS, SRR) were excluded derived from the fact that solution state NMR does not allow for the determination of absolute configuration. 50000 Monte Carlo steps using intermediate torsion sampling and a RMSD cut-off at 2.0 Å followed by Molecular Mechanics energy minimization using Macromodel as implemented in the Schrödinger Maestro package (v. 13.0.135). To ensure that the entire conformational space available for the molecule was sampled, for each diastereoisomer independent conformational searches were carried out utilizing OPLS3e, OPLS and AMBER* as force fields and the implicit solvation model GB/SA for water and $CHCl_3$. A Polak-Ribière type conjugate gradient (PRCG) algorithm was performed for energy minimization with 50000 iterative steps. All conformers within 42 kJ mol^{-1} from the global minimum were saved. After conformational searches including constrained searches all ensembles were combined and redundant conformer elimination (RCE) was performed by comparison of heavy atom coordinates. The RMSD cut-off was set to 0.8 Å resulting in the four final input ensembles to evaluate the relative stereochemistry of **67**.

NMR analysis of molecular flexibility in solution (NAMFIS)

The preliminary geometries for the four diastereomers of **67** (Figure 19), RRR, RRS, RSR, and RSS, were drawn in Schrödinger Maestro and corresponding conformers were sampled by MCMM conformational searches. Different force fields and solvation models were implemented to obtain complete conformational coverage. Conformers resulting from MCMM runs for individual diastereomers were combined, followed by elimination of redundant conformers (RMSD cut-off at 2.0 Å) to obtain input theoretical ensembles for NAMFIS. Interproton distances were quantified experimentally by NOESY-build-up analysis. The NAMFIS algorithm was implemented to deconvolute the experimental data into each of the four conformer ensembles. Additional runs were performed permutating the assignment of distances to diastereotopic protons on C-9 and C-14. The overall lowest RMSD, that is the best fit of experimental to theoretical data, was found for the RRR diastereomer (0.18, RRS: 0.28; RSR: 0.32; RSS: 0.33). This optimized fit was obtained selecting 4 conformers from the theoretical ensembles, that contained 23 conformers, with molar fractions of 5%, 10%, 18%, and 67%.

3.4 Biological activity assays

The samples were subjected to a number of experiments to evaluate their biological activities. The essential oils were assayed for antifungal potency. Their minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were measured against fungi strains namely, *Rhizopus nigricans*,

Aspergillus flavus, *Aspergillus niger*, *Fusarium oxysporum*, and *Penicillium digitatum* as described in paper I. The anti-aflatoxin efficacy was assessed using *A. flavus* and *A. parasiticus* as detailed in paper I.[144, 145]

The antibacterial and cytotoxic activities of samples from *E. montanum* and *S. mannii* were determined against *Escherichia coli* and *Bacillus subtilis*, and human MCF-7 cell line as described in papers II and IV.[146, 147]

The antiviral and cytotoxic activity experiments for the samples of *C. myricoides* were performed against human *respiratory syncytial virus (RSV)* in human laryngeal epidermoid carcinoma *(HEp-2)* cells (paper III).[148, 149]

4 Results and discussion

4.1 Antifungal essential oil of *E. melliodora* and *E. anceps* (paper I)

The *Eucalyptus* genus (Myrtaceae) is well known for its bioactive essential oils that have various medicinal and technical applications. They are used as folk medicine due to their anaesthetic, anodyne, antiperiodic, antiphlogistic, antiseptic, astringent, deodorant, diaphoretic, disinfectant, expectorant, febrifuge, fumigant, hemostat, inhalant, insect repellent, rubefacient, sedative, suppurative, tonic, and vermifuge properties.[150, 151]

However, there are only a few reports discussing the biological activity of essential oils from *Eucalyptus* species grown in Rwanda, and their potential use in foodstuff preservation. Therefore, we have extracted and analysed essential oils from *E. anceps* and *E. melliodora*, determined their effect on the growth of food spoilage fungi *Rhizopus sp., Aspergillus sp., Penicillium sp.* and *Fusarium sp.*, and tested their inhibitive capacity for aflatoxin production by *A. parasiticus* and *A. flavus.*

The essential oil was extracted by steam distillation from fresh and dried leaves of both *Eucalyptus* species. *E. anceps* gave a lower yield (0.80%) than *E. melliodora* (0.92%). Slight yield deviations have been observed between these results and those previously reported.[152, 153] These deviations could be attributed to the age of the leaves and other environmental factors.

GC-MS was used to identify the major components of the essential oil mixture. As presented in paper I (Tables 1 and 2), the essential oil composition did not deviate from the general trend that the predominant constituents of plant essential oils are terpenoids.[154] Thus, the main components, in both species, were monoterpenes but some sesquiterpenes and their alcohol derivatives were also observed, Figure 12. Terpenes are biosynthetically produced, through a series of enzymatic reactions, from the isomeric pyrophosphate esters isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), as shown in Scheme 4.

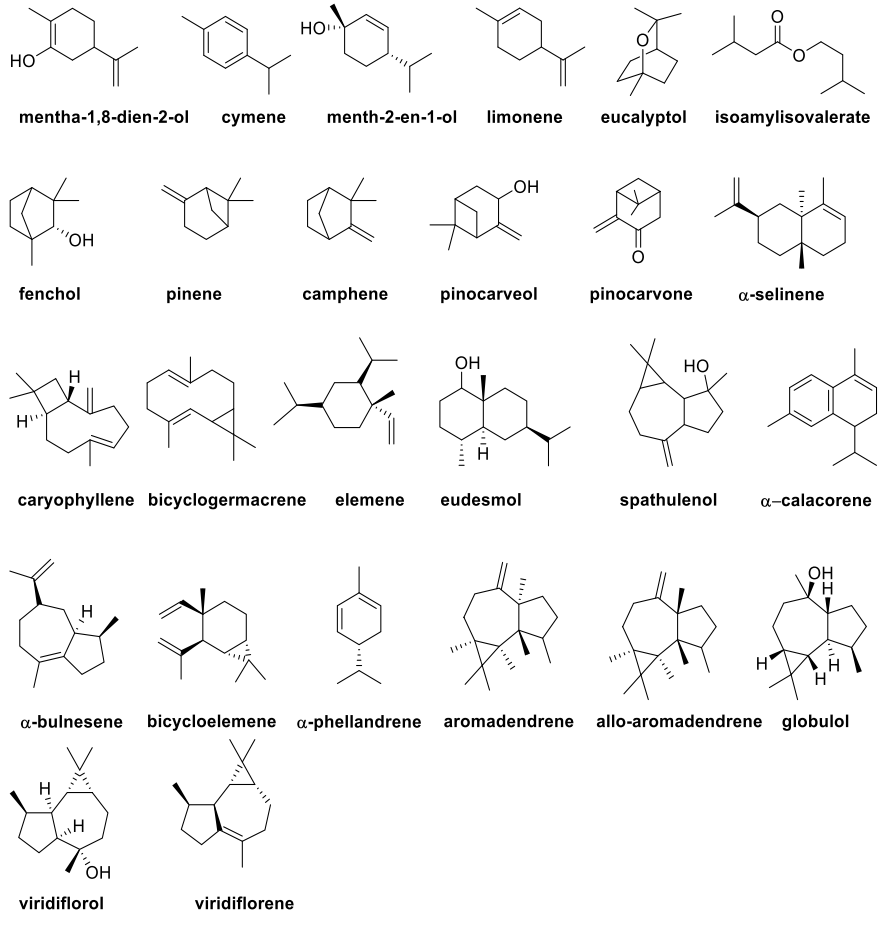


Figure 12. Components of essential oils reported in paper I.

The antifungal activity assay (paper I, Tables 3 and 4) showed that the *E. melliodora* essential oil was more efficient than the *E. anceps* essential oil with minimum inhibition concentration (MIC) values varying from 3.50 to 8.10 mg/mL. The minimum fungicidal concentration (MFC) followed a similar pattern yet with higher concentrations, ranging from 8.80 to 18.00 mg/mL. Furthermore*, E. melliodora* essential oil had a higher aflatoxin inhibition potential. The aflatoxin production was completely inhibited at a concentration of 6 µL/mL for *A. parasiticus* and 7 µL/mL for *A. flavus.* Therefore, these essential oils may be considered as good candidates for food preservation against aflatoxin production.[155]

4.2 Prenylated dihydrochalcones from *E. montanum* (paper II)

E. montanum Baker f. (Leguminosae) is a shrub locally known in Kinyarwanda as "Umupfunyantoki". This plant is commonly used in Rwandese traditional medicine. It is involved in the treatment of conjunctivitis, cough, snake bite, HIV, and asthma.[12, 93, 94] With regard to the role of *E. montanum* in Rwandese traditional medicine and due to the inexistence of any previous report on its chemical composition, this study has been conducted to investigate its constituents. Hence, as summarized in Figure 13, twenty compounds including two new prenylated dihydrochalcones (**36, 37**) along with eighteen known metabolites (**38-55**) were isolated and characterized from the root, the stem bark and the leaf extracts.

Most of the compounds isolated from *E. montanum* were polyketides with phenylpropanoid moiety or flavonoids. This class of compounds are biosynthesized via the combination of shikimic acid and polyketide pathways (Scheme 3).

The structures of the isolated secondary metabolites were elucidated based on their NMR, IR, UV spectroscopic and mass spectrometric data, as described in paper II. The sum formulae of the new compounds (**36, 37**) were determined by HRESIMS to be $C_{26}H_{32}O_5$ and $C_{27}H_{34}O_5$, respectively. IR spectroscopy indicated the presence of hydroxy and carbonyl (ketone) groups.

UV and NMR data revealed that both compounds were dihydrochalcone derivatives.

Compounds **36** and **37** were structurally similar (paper II), with the only difference being the presence of an additional methoxy group in **37**. Therefore, they were given the trivial names of montachalcone A and B, corresponding to 2',4',6'-trihydroxy-4-methoxy-3,5-diprenyldihydrochalcone and 2',6'-dihydroxy-4,4'-dimethoxy-3,5-diprenyldihydrochalcone, respectively.

Eriosema species have been reported to be a rich source of biologically active compounds including terpenoids, isoflavones and chalcones.[89, 90, 92] Therefore, the crude extracts and the isolates were assayed for antibacterial and cytotoxic activities. Compounds **36–39** and **45** were active against *B. subtilis* with a MIC value ranging from 3.1 to 8.9 μM and montachalcone A (**36**) was also cytotoxic with an EC value of 7.0 μM (paper II). The biological activity of compounds **36–39** and **45** corroborates previously reported activities of chalconoids of *Eriosema* species.[87, 156]

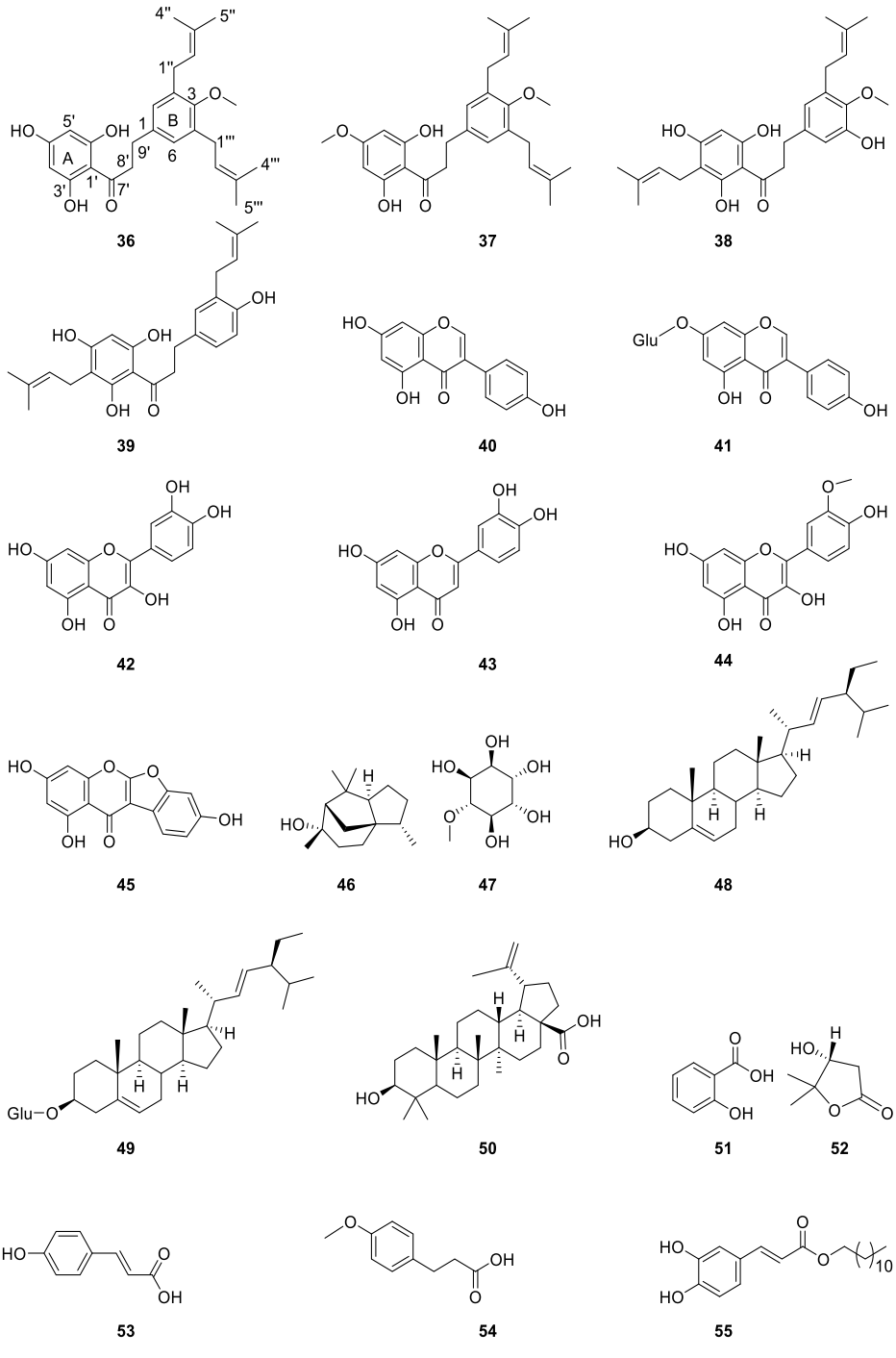


Figure13. Compounds isolated from E. montanum.

4.3 Antiviral iridoid glycosides from *C. myricoides* (paper III)

C. myricoides, locally known in Kinyarwanda as "Umukuzanyana", is a shrub characterized by ovate and alternate leaves that often crowd near the ends of branches. As a medicinal plant, it is used to treat human and livestock diseases such, as liver diseases, dysentery, diarrhea, impotence, sterility, spleen enlargement, cough and East Coast fever in cattle, as well as snake bite.[102] The importance of *C. myricoides* in Rwandese traditional medicine raised the interest to conduct a phytochemical investigation on the root extract and hence to identify its active constituents.

The compounds isolated from *C. myricoides* belong to the iridoid subclass of natural products. Iridoids represent a large group of monoterpenoids characterized by a 4,8-dimethylcyclopenta[*c*]pyran skeleton (Figure 14). They occur naturally as constituents of a considerable number of animal and plant families, usually as glycosides. If the C_7-C_8 bond of the cyclopentane ring opens, they are referred to as seco-iridoids. [157, 158]

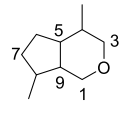
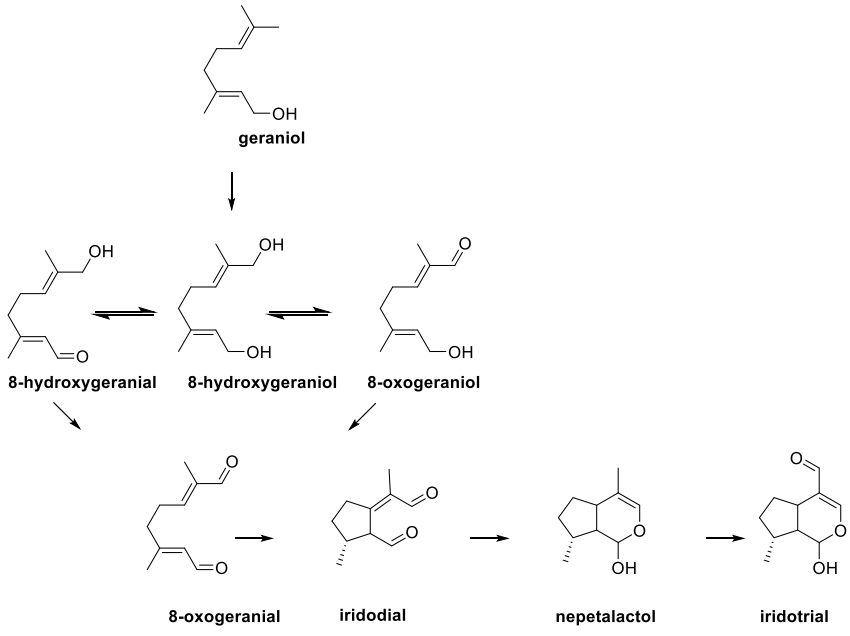


Figure 14. Iridoid skeleton.

Biosynthetically, iridoids are geraniol derivatives. Through a series of reactions catalysed by multiple enzymes including geraniol-8-hydroxylase, 8-hydroxygeraniol oxidoreductase, iridoid synthase, and iridoid oxidase, geraniol produces nepetalactol and iridotrial which subsequently generate diverse iridoid structures, Scheme 8.[159, 160]



Scheme 8. Iridoid biosynthesis.[159, 160]

Iridoids exhibit a wide spectrum of bioactivities. For example, antihepatotoxic, immunomodulatory, and choleretic effects of picroliv have been reported.[161, 162] Geniposidic acid, geniposide, aucubin, geniposide and catalpin showed antitumor activity. Furthermore, antiviral activity was observed on some iridoids, such as arbortristosides A and C, oleuropein, lucidumoside C, oleoside dimethylester, and ligustroside. Crude extract from *Gardenia jasmonoides* that contains geniposide had purgative effects.[163-166] Neuroprotective properties of catalpol, picrosides and jasminoidin have also been documented.[163-166] Some iridoids including isoplumericin, plumericin, galioside and gardenoside were found to possess antibacterial activity. Other iridoids, such as picroside, and kutkoside, oleuropein, gentiopicroside, and genipin have been proven to be antioxidant. The *in vitro* studies on *Gentiana lutea* spp. s*ymphyandra* extract and its constituents swertiamarin, sweroside and gentiopicroside were reported to have wound healing activities.[163-166]

The separation of the methanol extract components gave three new iridoid glycosides myricoidoside A (**56**), myricoidoside B (**57**), and myricoidoside C (**60**), along with two known ones, namely euphroside (**58**),[167] and 7-O-cinnamoyl-5-hydroxygardaloside (**59**).[168] Structures of isolated compounds, as presented in Figure 15, were elucidated based on spectroscopic and mass spectrometry data.

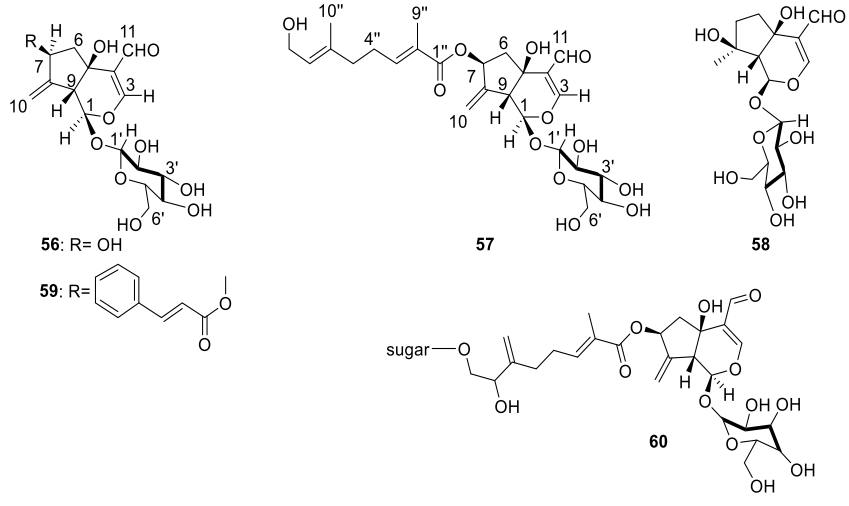


Figure 15. Compounds isolated from C. myricoides.

Compound **56** was assigned the molecular formula $C_{16}H_{22}O_{10}$ based on HRESIMS data analysis. The IR absorption bands suggested the presence of hydroxy and aldehyde functional groups. NMR data analysis revealed the presence of a C_9-type iridoid skeleton, an exocyclic methylene group and a glucose moiety (paper III, Table 1 and Supporting Information). Hence, this new compound **56**, with the trivial name myricoidoside A, was found to be a gardaloside derivative [169] and thus was characterized as 5-hydroxygardaloside.

The molecular formula of compound **57**, $C_{26}H_{36}O_{12}$, was determined by HRESIMS and NMR data analyses. It showed similar spectroscopic features to that of **56** with some additional signals observed in in the ^{1}H and ^{13}C NMR spectra. A careful analysis of those signals showed that they were assignable to the presence of a foliamenthoyl moiety.[170, 171] Therefore, this new compound **57**, myricoidoside B, was characterized as 7-*O*-foliamenthoyl-5-hydroxygardaloside. (paper III, Table 2 and Supporting Information)

Compound **60** displayed similar spectroscopic characteristics to **57**. However, compound **60** showed signals characteristic of terminal olefinic protons in the foliamenthoyl moiety. In addition, signals attributable to the presence of a second sugar moiety were also observed.[172, 173] This sugar moiety was placed at carbon C-8" due to the HMBC cross-peaks of H-8" to the anomeric carbon of the sugar moiety. However, due to the insufficient amount and instability of the sample, the sugar moiety was not unequivocally characterized (paper III, Supporting Information). An antiviral activity assay showed that **57** inhibited the human *respiratory syncytial virus (RSV)* at 10 µg/ml by 41.9% while it was weakly cytotoxic at 100 µg/ml.

4.4 Pyrrolizidine alkaloids and silphiperfolanol angelate esters from *S. mannii* (paper IV)

S. mannii (Asteraceae), locally known in Rwanda as "Umutagara", is a small and branched tree with conspicuous pale leaf scars. It is widespread in tropical African countries.[174] Plants of the *Senecio* genus are known for their high content in pyrrolizidine alkaloids. These alkaloids exhibit strong hepatotoxic, genotoxic, cytotoxic, tumorigenic, and neurotoxic activities.[98, 175-177]

Motivated by the importance of *S. mannii* in folk medicine and by its potential toxicity, we conducted a phytochemical investigation and evaluated the antimicrobial, and cytotoxic activities of its root and stem crude extracts. Hence, isolation and characterization of ten compounds including a new rare tricyclic sesquiterpene silphiperfolanol angelate ester (**61**), two new unsaturated macrocyclic pyrrolizidine alkaloids (**62**, **63**), two new 2-angeloyloxy-5,8-dihydroxypresilphiperfolane derivatives (**64**, **65**), and five known secondary metabolites (**66**-**70**) (Figure 16) along with their biological activities were reported in paper IV.

The isolated compounds include mostly the pyrrolizidine alkaloids and presilphiperfolane derivatives. As presented in Scheme 5, pyrrolizidine alkaloids are biosynthesized from ornithine via putrescine and homospermidine as symmetrical intermediates. Ornithine generates arginine, which is decaboxylated to afford agmatine. The latter is transformed into *N*-carbamoylputrescine, the precursor of putrescine. Putrescine is converted into spermidine which generates the PA necine base. On the other hand, presilphiperfolane derivatives, like other terpenoids, are biosynthesized from two isomeric five-carbon precursors namely isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), Scheme 4.

The structures of the isolated secondary metabolites were elucidated based on their NMR, IR spectroscopic and mass spectrometric data. The molecular formula of the new compound **61** was established to be $C_{20}H_{32}O_4$ based on HRESIMS data analysis. The IR data suggested the presence of hydroxy and carbonyl functionalities (paper IV, Supporting Information). Its chemical structure as presented in Figure 16, was deduced from the detailed analysis of 1D and 2D NMR data (paper IV, Table 1, and Supporting Information).

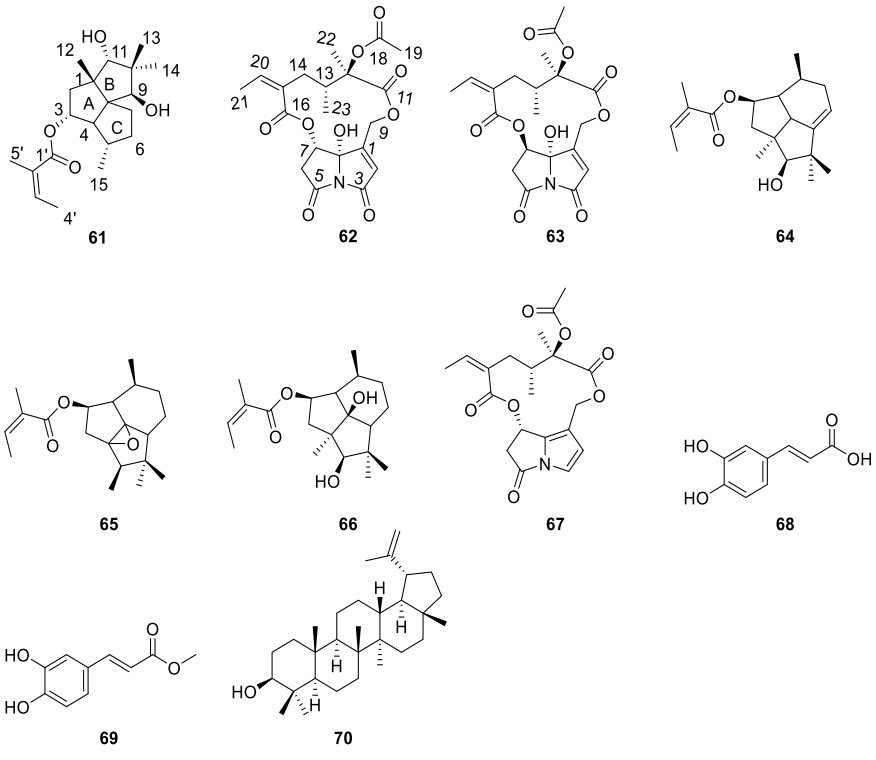


*Figure 16. Compounds isolated from S. mannii (**61-63**, **66-70**) and two synthetic derivatives (**64**, **65**) of **66**.*

Thus, the new silphiperfolanol angelate ester (**61**), in reference to the plant's name, was given the trivial name umutagarananol, which corresponds to 3-angeloyloxy-9,11-dihydroxysilphiperfolane.

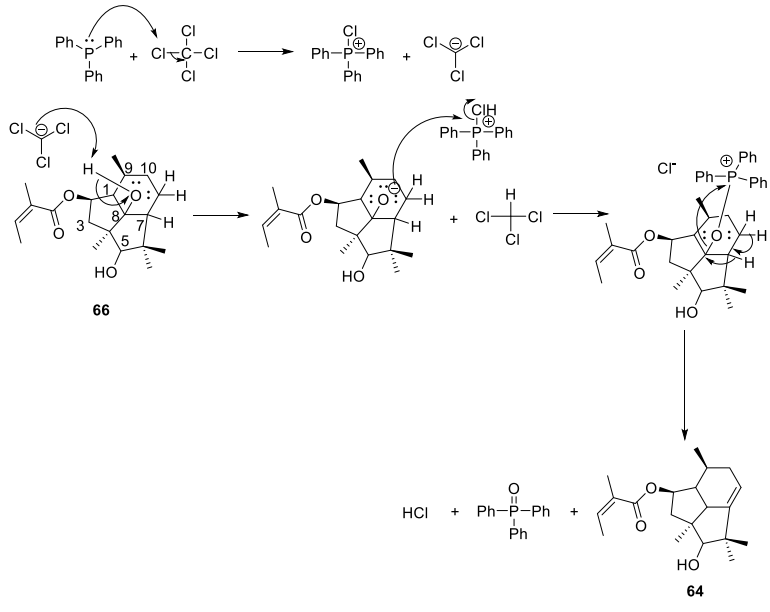
In a similar procedure, the experimental data of compound **62** were analysed and the molecular formula $C_{20}H_{23}NO_9$ was assigned based on HRESIMS data analysis. IR absorption bands suggested the presence of a hydroxy group, alkyl CH bonds, and carbonyl functionalities (paper IV, Supporting Information). The chemical structure of **62**, as presented in Figure 16, was elucidated through an extensive analysis of 1D and 2D NMR spectra (paper IV, Table 2, and Supporting Information). Therefore, this twelve-membered macrocyclic pyrrolizidine alkaloid (**62**), was characterized as 8-hydroxy-3-oxosenaetnine and given the trivial name umutagarinine A.

Compound **63** showed spectroscopic data similar to compound **62**, except for some chemical shift differences observed for H-7, OH-8 and H-9 and their corresponding C atoms in ^{1}H and ^{13}C NMR spectra (Table 2 and 3, Supporting Information). The non-protonated carbon C-8 chemical shift moved from δ_C 99.0 to δ_C 93.7 ppm, δ_C of C-3 moved from 167.2 to 163.3 ppm, δ_C of C-7 changed from 73.7 to 72.2 ppm and δ_C of C-9 changed from 56.8 to 60.6 ppm. These minor chemical shift differences suggest that these two compounds are

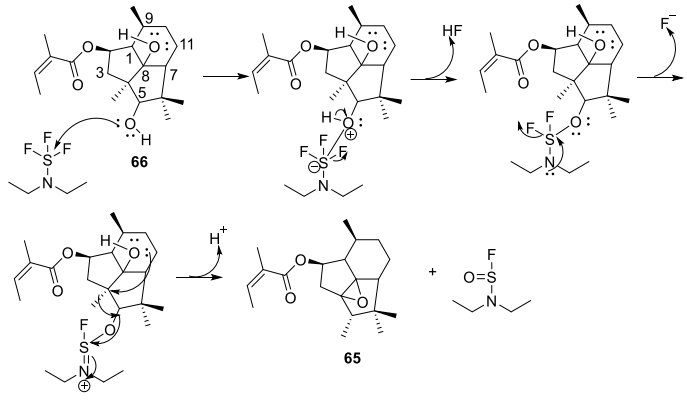
structurally similar and might be stereoisomers. The relative configurations of **62** and **63** were established based on NOESY correlations (paper IV, Supporting Information).

Compound **64** was isolated in an attempt to convert compound **66** into its chlorinated derivatives. Thus, compound **66** was treated with triphenylphosphine in carbon tetrachloride solution at 70 ^{o}C for 5 h. Consumption of starting material indicated full transformation. However, the chlorinated product could not be detected. Instead, compound **64** was obtained, most likely via dehydration followed by rearrangement. Loss of the hydroxyl group at C-8, followed by a hydride shift from C-7 to C-8, and finally deprotonation at C-11 resulted in the formation of a double bond between C-7 and C-11. The putative mechanism of dehydration and rearrangement is presented in Scheme 9.

Compound **65** was isolated in an attempt to produce fluorinated derivatives of **66** which was performed with diethylaminosulfur trifluoride (DAST) in dichloromethane, at room temperature, for 2 h. However, similar to the attempted chlorination of **66**, the fluorination conditions resulted in dehydration, rearrangement and was followed by the formation of an oxirane ring involving C-4 and C-8 as revealed by HRESIMS and NMR data (paper IV, Supporting Information), and further confirmed by X-ray diffraction analysis, Figure 17. The obtained product suggests a loss of the hydroxyl group at C-5, forming a carbocation, which was subjected to a rearrangement process where the methyl group at C-4 shifted to C-5 and the resulting tertiary carbocation formed the observed epoxide with O-8. The plausible mechanism of dehydration, rearrangement and epoxide formation is shown in Scheme 10.



*Scheme 9. Putative reaction mechanism for the formation of **64**.*



*Scheme 10. Suggested reaction mechanism for the formation of **65**.*

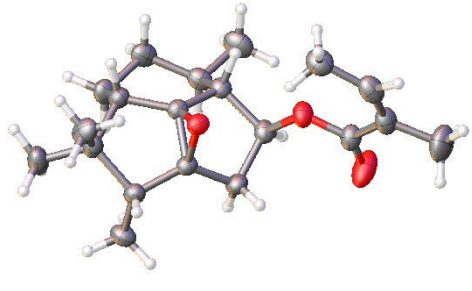


*Figure 17. The X-ray crystal structure of **65**.*

Compound **67** was subjected to NMR analysis to determine its relative stereochemistry since crystallization attempts were unsuccessful. Given the flexibility of the macrocycle, the relative configuration of its three stereocenters cannot be determined by qualitative assessment of NOEs in their proximity. As the configuration of **67** influences its overall conformational freedom and solution geometry,[178 179] the quantitative analysis of NOEs was expected to reveal the relative configuration. Hence, a NOESY build-up analysis provided 15 quantitative interproton distances covering the entire macrocycle, as presented in Table 2 and Figure 18. The data were processed using the NAMFIS algorithm.

NAMFIS deconvolutes the time-averaged experimental NMR data into individual solution conformations. It has been implemented successfully for the conformational analysis of macrocycles, natural product,[180, 181] peptides and drug candidates[182-184] as well as for the elucidation of relative chiralities.[185-187] Subjecting theoretical conformer ensembles of the four diastereomers of **67** namely: RRR, RRS, RSR and RSS to NAMFIS revealed a significantly better fit of the experimental NMR data to the conformer ensemble of the RRR-diastereomers, Figure 19. Whereas this approach is powerful for rigid molecules, the interpretation of NMR observables is not trivial for flexible molecules.

Table 2. Interproton distances in **67**

Position		Distance	RRR	RSR	RRS	RSS
H-1	**H-2**	**Exp.**	**Calc.**	**Calc.**	**Calc.**	**Calc.**
2	9b	2.56	2.74	2.71	2.83	2.71
7	9a	3.36	3.44	3.31	3.28	3.09
14b	21	2.53	2.67	2.61	2.73	2.56
13	18	2.85	3.10	2.61	2.89	2.82
14a	18	4.89	4.78	4.54	4.50	4.48
9b	18	5.92	5.15	5.08	5.44	5.63
9a	18	5.18	5.51	5.32	5.26	5.22
14b	18	3.48	3.94	4.05	3.20	3.52
13	21	3.25	3.25	3.43	3.02	3.34
7	14a	4.58	4.92	4.65	4.60	4.61
20	19	5.36	5.39	5.22	4.88	5.50
7	9b	4.20	4.68	4.62	4.43	4.50
9a	14b	5.18	5.15	5.20	5.13	5.20
9b	14a	5.21	5.17	5.18	5.21	5.21
14b	18	3.36	2.84	3.18	2.85	3.20
RMSD			**0.18**	**0.32**	**0.28**	**0.33**

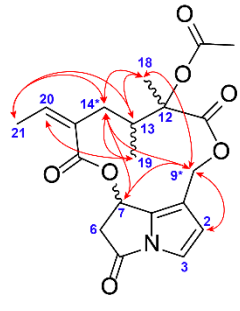


Figure 18. Interproton distances derived from NOE build-up analysis depicted as red arrows. (*): diastereotopic protons involved in the analysis.

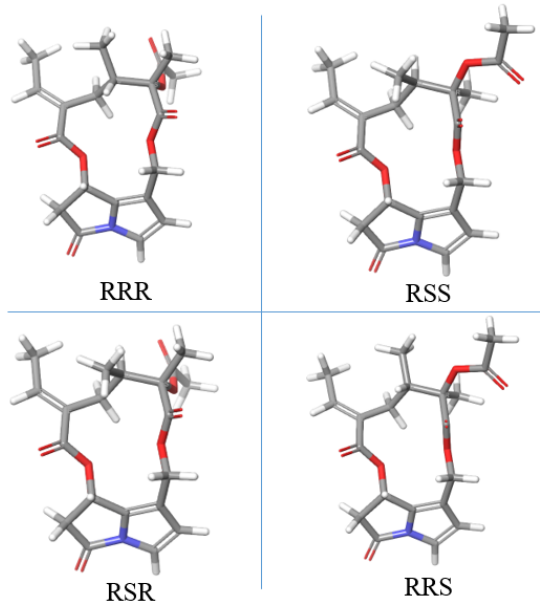


*Figure 19. Four diastereomers of **67**.*

We found that compound **67** adopts four major conformations in $CDCl_3$ solution with a 67% prevalence of the most abundant one. Upon overlap of all found conformers, an extensive flexibility at the side of the macrocycle opposite to the necine base was evident, Figure 20.

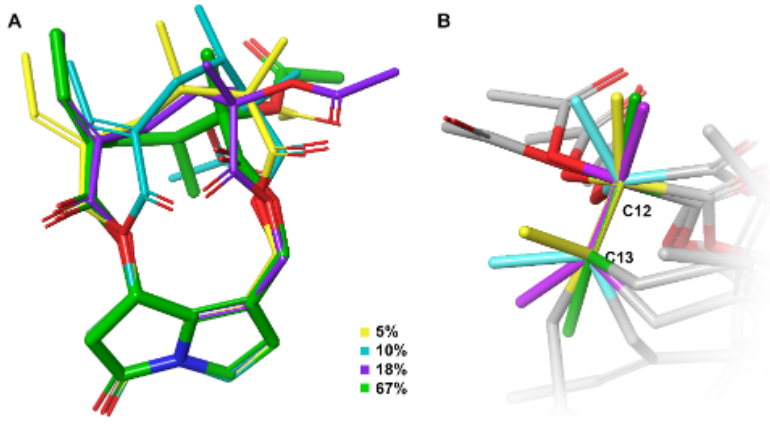


Figure 20. A. Superimposed conformers, and their corresponding populations. B. Orientation of substituents at C-12 and C-13.

An almost full rotation around the C-12 to C-13 bond is observed, which is in agreement with the NOE data. Although senaetnine (**67**) has been previously isolated and described,[180] the relative configurations at C-12 and C-13 were

only assumed based on its biosynthetic precursor, necic acid, and furthermore solely based on analogy for C-7. In this study, in combination with the findings of Bohlman *et al.*[180] evidence for compound **67**, senaetnine, to have a rel-(7R, 12R, 13R) configuration is presented.

The compounds isolated from *S. mannii* exhibited moderate antibacterial activity with IC_{50} values ranging 2.8-13.3 mM for *E. coli* and 0.74-13.3 mM for *B. subtilis*. They also displayed cytotoxic activity against a MCF-7 cell line with IC_{50} values ranging from 0.012 to 1.110 mM (paper IV).

4.5 Ethnopharmacological significance of the new secondary metabolites

The interest in plant-based drugs has been increasing in recent years not only due to their pharmacological potency on human diseases, such as cancer, diabetes, infectious, inflammatory, cardiovascular and parasitic diseases, and nervous system disorders, but also to their limited adverse effects.[181]

Plants containing chalcones such as *Eriosema*, *Piper*, *Angelica*, *Glycyrrhiza*, and *Milletia* genera have been identified mainly in the Fabaceae family and used by different communities as remedies for many years.[182-186] Chalcones are among the most prominent plant-derived medicines because they have shown to be pharmacologically active. They exhibit anticancer activity against a variety of cancer cell lines, antibacterial, anti-protozoal activities and various others. Some chalcones such as metochalcone, sofalcone and hesperidin methylchalcone have been clinically tested and approved as drugs.[187] Furthermore, licochalcone A has been proven to be a potent inhibitor of pro-inflammatory cytokine in different clinical trials.[188, 189] Moreover, the chalconoid skeleton exhibits some advantages for the development and discovery of new drugs. In various clinical studies, the chalcones' potency was not associated with adverse effects.[10] In addition, the chalconoid backbone is relatively more accessible by total synthesis or by derivatization of precursors to afford a variety of biologically active compounds.[190, 191]

Based on the antibacterial activity of the dihydrochalcones evaluated in this study, and on the previously reported bioactivities for similar compounds, the dihydrochalcones are the likely active constituents responsible for the efficacy and versatility of *E. montanum* in Rwandese traditional medicine against various diseases, including malaria, asthma and HIV infections.[12, 183, 192, 193] Although dihydrochalcones rarely occur naturally, they were previously reported to be one of the constituents of *Eriosema* genus.[194] Hence, further studies on these rare *E. montanum* constituents should be focused on determination of their cytotoxicity, selective index and eventually chemical structure modification leading to new drug development.

Pyrrolizidine alkaloids (PAs) form a large group of secondary metabolites naturally occurring in numerous and widespread plant genera. The main plant families include Asteraceae, Boraginaceae, and Leguminosae.[74, 195] The occurrence of PAs in Asteraceae is dominated by senecionine (**35**) type alkaloids, characteristic of the tribe Senecioneae to which belongs *S. mannii*, one of the plant species investigated in this project.[196]

Senecio species have been reported to synthesize PAs in roots and translocate them to aerial parts such as stem, leaves and flowers.[196] PAs, especially those with a 1,2-double bond in their necine base are known to be responsible for chronic liver diseases in animals and humans.[197] PA contamination usually occurs due to consumption of PA-containing plants or related products.[198] Herbal preparations or extracts from medicinal plants have been shown to be the source of PA poisoning.[199, 200]

Despite the advances of modern medicine, herbs remain in use in most African countries, often without having been thoroughly tested for efficacy and safety.[195] Therefore, the isolation of PAs with a 1,2-double bond in their necine base from *S. mannii*, a well-known traditional medicinal plant not only in Rwanda but also in most parts of East Africa, raised concern over its safety. Further animal model toxicity studies of this plant should determine whether *S. mannii* is useful as a medicinal plant, or if its toxicity should discourage its use. Silphiperfolane-type sesquiterpenoids are derived from caryophyllane and are rarely found in nature. They are part of volatile fractions of some higher plants' essential oils, and can also be obtained from fungal isolates and from marine organisms.[201-203] They exhibit antibacterial, cytotoxic, antifungal and anti-feedant properties.[203, 204] Sesquiterpenoid derivatives isolated from *S. mannii* have not yet been fully investigated. Hence, they offer new opportunities for structural modifications to enhance their pharmacological activity, and for the development of novel anti-feedants.

5 Conclusion and outlook

This project aimed at the phytochemical investigation of selected Rwandese medicinal plants. Five plant species belonging to four different genera were studied: *E. melliodora, E. anceps, E. montanum*, *S. mannii* and *C. myricoides*. Terpenes and terpenoids, flavonoids, and alkaloids were found among the isolates. Some of the isolated natural products showed antifungal, cytotoxic, antibacterial and antiviral activities.

In paper I, the essential oils of E. *melliodora* and *E. anceps* were analysed and the antifungal activity of the isolates was established. The main components of the essential oils were mono- and diterpenes, and their alcohol derivatives.

In paper II, the phytochemical investigation of *E. montanum* yielded 20 compounds. Most secondary metabolites of *E. montanum* were polyphenols with some terpenoids including two new prenylated dihydrochalcones (**36**, **37**) and eighteen known natural products (**38-55**). Compounds **36-39** and **45** showed strong activity against the Gram-positive bacterium *Bacillus subtilis*. Compounds **36** and **39** showed the highest cytotoxicity with EC_{50} values of 3.1 and 7.3 μM, respectively.

In paper III, the analysis of *C. myricoides* extract resulted in the isolation of three new (**56**, **58** and **60**) and two known (**57**, **59**) iridoid glycosides. The antiviral activity assay showed that compound **57** inhibited the human *respiratory syncytial virus (RSV)* at 10 μg/ml by 41.9% while it was cytotoxic at 100 μg/ml.

In paper IV, the study of *S. mannii* extract afforded one new silphiperfolanol angelate ester (**61**), two new macrocyclic pyrrolizidine alkaloids (**62**, **63**), and five known secondary metabolites (**66-70**). The chemical structural modification of **66** yielded two new derivatives **64** and **65**. The relative configuration of **67** was confirmed to be 7R, 12R, and 13R. While most of *S. mannii* constituents showed weak antibacterial activity, compounds **64**, **63** and **62** exhibited strong cytotoxicity against the MCF-7 human breast cancer cell line with IC_{50} values of 12.0, 21.5 and 33.2 μM, respectively.

The work described in this thesis aimed at discovering new bioactive natural products. Historically, the most successful phytochemical investigations have resulted in isolation of drug molecules sufficiently potent without additional structure modification, such as morphine, paclitaxel, and quinine. Some

compounds isolated in other studies possessed promising bioactivity, not sufficient active to be used as drugs directly but presenting valuable lead compounds; these were therefore subjected to structure modifications to enhance their bioactivity.

The new compounds isolated in this study have shown interesting biological activities so far, and thus it should be worthwhile to pursue their potential use as lead compounds for drug discovery. Therefore, there are several opportunities for continuing this work. For example, further studies on montachalcones, the rare *E. montanum* constituents should be focused on determination of their cytotoxicity, selective index and eventually chemical structure modification triggering to new drug development. Similarly, structural modification of sesquiterpenoids isolated from *S. mannii* would enhance their pharmacological activity, followed by a thorough structure-activity relationship study against emerging diseases and drug resistant pathogens. In addition, since toxic PAs were identified in *S. mannii*, further research would deal with the animal model toxicity studies to determine whether this plant is useful as a medicinal plant.

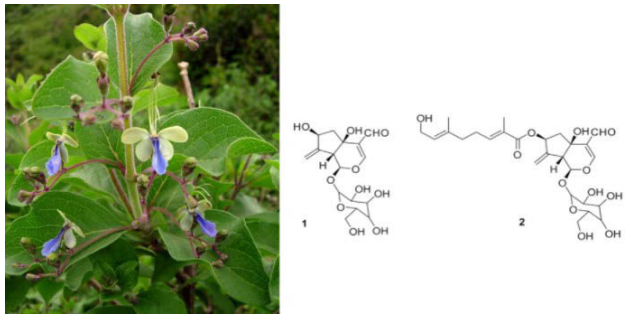
6 Sammanfattning på svenska

Traditionell medicin bygger på användningen av naturprodukter för att behandla sjukdomar. Världshälsoorganisationen uppskattar att 80% av jordens befolkning är beroende av traditionell medicin inom primärvården, först och främst i utvecklingsländerna. Växt-baserade läkemedel är de mest använda inom traditionell medicin över hela världen. I Rwanda (Figur 1) använder folkläkare medicinska växter på grund av deras tillgänglighet och effektivitet. Syftet med den här studien var att isolera och karakterisera de aktiva beståndsdelarna i utvalda rwandiska medicinalväxter. Dessutom utvärderades deras cytotoxiska, antifungala, antibakteriella och antivirala aktivitet.

Figur 1. Karta över Rwanda.

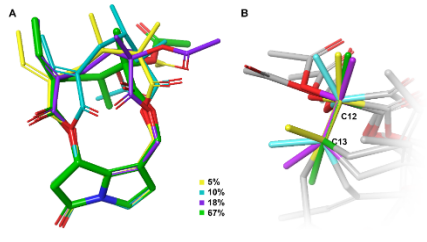
De undersökta växterna valdes ut baserat på information från lokala folkläkare och från literaturen. Vi undersökte *Eucalyptus melliodora*, *Eucalyptus anceps* (Myrtaceae), *Eriosema montanum* (Fabaceae), *Clerodendrum myricoides* (Lamiaceae) och *Senecio mannii* (Asteraceae). Studien använde kromatografiska, spektrometriska och spektroskopiska metoder för isolering och karakterisering av deras lågmolekylära beståndsdelar, dvs olika sekundära metaboliter.

Majoriteten av de isolerade föreningarna utgör terpenoider, polyfenoler och pyrrolizidinalkaloider. De eteriska oljorna från *E. melliodora* och *E. anceps* visade antifungala aktivitet, polyfenolerna av *E. montanum* och beståndsdelarna i *S. mannii* uppvisade antibakteriell aktivitet medan iridoidglykosider av *C. myricoides* (Figur 2) uppvisade antiviral aktivitet.



Figur 2. Nya iridoidglykosider (1, 2) isolerade från växten C. myricoides (till vänster).

Senaetnin (**67**), en av de makrocykliska pyrrolizidinalkaloiderna isolerade från *S. mannii*, utsattes för en mer detaljerad struktur analys för att bestämma dess relativa stereokemi eftersom kristallisationsförsök misslyckades. Den relativa konfigurationen av dess tre stereocentra visade sig vara rel-(7R, 12R, 13R), Figur 3.



Figur 3. A. Konformerpopulationer överlappade, B. Orientering av substituenter vid C-12 och C-13.

Eftersom de undersökta växterna innehöll bioaktiva substanser förklarar vår studie varför växterna användnings i traditionell medicin. Ytterligare studier rekommenderas för att minimera risken när dessa växter används som läkemedel. Den biologiska aktiviteten av isolerade föreningar kan utnyttjas i utvecklingen av nya läkemedel.

7 Acknowledgements

I am glad and privileged to have this opportunity to thank all of you who have made this PhD training such a successful experience. The project could not have been accomplished without the support and encouragement I benefitted throughout this wonderful journey.

First and foremost, I wish to express my deepest and heartfelt gratitude to my supervisors: Professor Máté Erdélyi, Professor Adolf Gogoll and Professor Théoneste Muhizi. I have been honoured to have you as my guides in this PhD adventure. Your science full of optimism, kindness and constructive criticism enlightened my way not only during this PhD training but also throughout the rest of my professional career.

I would like to humbly thank Professor Peter Sundin for your support, advice and understanding from the early step of this PhD journey.

I am deeply indebted to Dr. Yoseph Atilaw Assefa for your friendship and assistance. I will forever remember your regular visits to my fume hood with our scientific discussions spiked with humour. I would like to thank Dr. Thobias Mwalingo Kalenga for constructive discussions.

I wish to thank Hossein Aminaey, Peter Roth, Ulrika Kolsmyr and other staff members of International Science Programme for making all the paperwork smooth, you made my life easy in Uppsala.

I am grateful to Professor Helena Grennberg for your science and guidance, I would not have finished my training on time if I had not benefitted from your follow-up as FUAP.

Let me also appreciate the working environment in our lab. Thanks to my past and present colleagues in the halogen bonding group for having created such a pleasant workplace. I will always remember our invaluable discussions about science, culture and different life experiences.

I am glad to express my appreciations to all co-authors of the papers included in this thesis for your invaluable contributions.

The professional support obtained from my colleagues in the department of chemistry, University of Rwanda is gladly appreciated. Similarly, I would like to appreciate the support provided by the department of chemistry – BMC, Uppsala University.

The financial support from the International Science Programme, without which this project would not have been possible, through the collaboration with the department of chemistry of the University of Rwanda is appreciatively acknowledged. The study leave authorization and financial support granted by the University of Rwanda are gratefully recognized.

I am very grateful to my parents, sisters and brothers who have been supporting me from my childhood. I would not have enjoyed this wonderful experience if my brother Jacques Habyalimana had not provided me with all necessary resources and confidence from my earlier school time.

I would like to finally thank my family: my wife Valentine, our children Daniella and Danick for your unconditional and unreserved love, patience and support for all time.

Thank you sincerely.

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