PHYTOCHEMISTRY AND BIOACTIVE NATURAL PRODUCTS FROM *LANNEA ALATA*, *LANNEA RIVAE*, *LANNEA SCHIMPERI* AND *LANNEA SCHWEINFURTHII* (ANACARDIACEAE)

2014

OKOTH AKINYI DOROTHY
PHYTOCHEMISTRY AND BIOACTIVE NATURAL PRODUCTS FROM *LANNEA ALATA*,
*LANNEA RIVAE*, *LANNEA SCHIMPERI* AND *LANNEA SCHWEINFURTHII*
(ANACARDIACEAE)

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A thesis submitted to the School of Chemistry, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, for the degree of Doctor of Philosophy.

This Thesis has been prepared according to Format 4 as outlined in the guidelines from the College of Agriculture, Engineering and Science which states:

This is a thesis in which chapters are written as a set of discrete research papers, with an overall introduction and final discussion, where one (or all) of the chapters have already been published. Typically these chapters will have been published in internationally recognized, peer-reviewed journals.

As the candidate’s supervisor, I have approved this thesis for submission.

Supervisor: Prof. Neil A. Koorbanally

Signed: ----------------------------Name: -------------------------- Date: -------------
ABSTRACT

_Lannea_ plants belong to the family Anacardiaceae and are used in traditional medicine in the management of infectious diseases. Previous research on this genus focused on the biological activities of the plants. The Phytochemistry of most _Lannea_ species is not reported. **Lannea alata**, **Lannea rivae**, **Lannea schimperi** and **Lannea schweinfurthii** were selected for this study. Previous studies illustrated antiplasmodial, cytotoxicity, antiviral, antioxidant and acetylcholinesterase inhibition of **Lannea schweinfurthii** extracts. **Lannea schimperi** extracts demonstrated antiulcer, antibacterial, cytotoxic and antifungal activities. However, no investigations were conducted to determine the compounds responsible for the observed activities. No prior work on **Lannea alata** and **Lannea rivae** has been reported. The present study aimed to isolate the biologically active phytochemicals occurring in the plants. This is the first phytochemical report of these _Lannea_ species. In addition, pharmacological activities of the isolated compounds are discussed.

From the four _Lannea_ species seven known terpenes were isolated; sitosterol (**A6**), sitosterol glycoside (**B6**), taraxerol (**B8**), taraxerone (**B7**), lupeol (**A5**), lupenone (**D5**) and lutein (**B9**). Two novel prenylated flavonoids, lanneaflavonol (**A1**) and dihydrolanneaflavonol (**A2**), together with eight known flavonoids, myricetin (**B10**), myricetin-3-**O-α-**rhamnopyranoside (**A3**), myricetin-3-**O-α-**arabinofuranoside (**A4**), myricetin-3-**O-β-**galactopyranoside (**B11**), catechin (**D7**), epicatechin (**D6**), epicatechin gallate (**B12**) and rutin (**D8**) were also isolated.

Mixtures of phenolic lipids (cardanols) and its derivatives the alkyl cyclohexenols and alkyl cyclohexenones were isolated from **Lannea rivae**, **Lannea schimperi** and **Lannea schweinfurthii**. The non-isoprenyl aliphatic side chains of these compounds varied in length.
with odd carbon chains of between 13 to 23 carbons and were either fully saturated or contained one or two double bonds. The cardanols and the phenolic derivatives were not observed in *Lannea alata*, possibly indicating a more distant chemotaxonomic relationship with the other three species. Although the core structures of the cardanols have been reported previously, there were novel side chains attached to $B_1a$ from *Lannea rivae*, $C_1a$ and $C_1d$ from *Lannea schimperii* and $D_{1c-D1e}$ from *Lannea schweinfurthii*. Novel alkylated furanocyclohex-2-enone ($B_2$), trihydroxycyclohexanone ($B_3$), dihydroxycyclohex-2-enones ($B_4a$ and $B_4b$) and a trihydroxycyclohexane ($B_5$) were isolated from *Lannea rivae*. While the core structures of 4,5-dihydroxycyclohex-2-enones ($C_{2a}$ and $C_{2d}$) isolated from *Lannea schimperii* is known, the side chains in these molecules make them novel. Also novel from *Lannea schimperii* were the alkylated trihydroxycyclohexenes ($C_{3a-C3c}$) and the dihydroxycyclohexenes ($C_{4a-C4c}$). From *Lannea schweinfurthii*, the hydroxycyclohex-2- enones ($D_{2a-D2e}$) with a novel core structure were isolated as well as novel compounds with the core structures of $C_3$ and $C_4$ but with novel alkylated side chains ($D_{3a-D3b}$ and $D_{4a-Dg}$).

The flavonoids, myricetin ($B_{10}$), its glycosides ($B_{11}$ and $A_3$) and epicatechin gallate ($B_{12}$) showed good antioxidant activity with myricetin ($B_{10}$) and epicatechin gallate ($B_{12}$) exhibiting the best activity, comparable to the standards used. The novel dihydrolanneaflavonol ($A_2$), betmidin ($A_4$), myricetin ($B_{10}$) and epicatechin gallate ($B_{12}$) also showed good antibacterial activity. The pentacyclic triterpenes exhibited moderate to good cytotoxicity. The 5-[[alkenyl]-4,5-dihydroxycyclohex-2-enone mixture ($C_{2a-C2d}$) exhibited good *in vitro* cytotoxicity against the Chinese Hamster Ovarian mammalian cell-line. The furanocyclohex-2-enone ($B_2$) and the trihydroxycyclohexanone ($B_3$) showed good cytotoxic and antiplasmodial activity while the mixture of $B_{4a}$ and $B_{4b}$ also showed
antiplasmodial activity. The mixture where the core structure had a conjugated ketone (D2a-D2e) exhibited good antibacterial, antiplasmodial, larvicidal and cytotoxic activities.

Myricetin flavonoids are considered chemotaxonomic markers in the Anacardiaceae and their isolation in these *Lannea* species confirm their place within the Anarcadiaceae. The cardanols are also characteristic within the Anacardiaceae and their isolation like the myricetin flavonoids are expected in these species. The alkyl cyclohexenone and alkyl cyclohexenol derivatives of the types isolated in these *Lannea* species are however rare, only previously reported in *Lannea* and *Tapirira* and are not widespread in the Anarcadiaceae. Their occurrence within the species studied here points to the possibility of a unique set of enzymes only contained in the *Lannea* and *Tapirira* genera responsible for the biosynthesis of these secondary metabolites. These findings also suggest the possibility of a common evolutionary pathway in the *Lannea* and *Tapirira* genera. Further to this, isolation of similar cardanols, trihydroxycyclohexenes and dihydroxycyclohexenes from *Lannea schimperii* and *Lannea schweinfurthii* suggest a closer relationship between these two species.

The isolation of bioactive compounds from these four *Lannea* species indicates a rationale for their use in African traditional medicinal. These studies indicate that *Lannea* species are promising sources of new active phytochemicals that can be used as leads for the synthesis of potentially bioactive compounds.
List of isolated compounds

Novel compounds are indicated in bold

Chapter 2 Compounds from *Lannea alata*

A1  lanneaflavonol
A2  dihydrolanneaflavonol
A3  myricetin-3-\(\alpha\)-rhamnopyranoside
A4  myricetin-3-\(\alpha\)-arabinofuranoside (betmidin)
A5  lupeol
A6  sitosterol

Chapter 3\(^a\) Compounds from *Lannea rivae*

B1a  3-nonadec-14'-\(Z\)-enyl phenol
B1b  3-heptadec-12'-\(Z\)-enyl phenol
B1c  3-pentadec-10'-\(Z\)-enyl phenol
B1d  3-pentadecyl phenol
B2  4,5-dihydroxy-4,5-furan-2'-(16'-\(Z\)-18'-\(E\))-heneicosyldiene] cyclohex-2-enone
B3  2,4,5-trihydroxy-2-[16'-(Z)-heneicosenyl] cyclohexanone
B4a  4S,6R-dihydroxy-6-(12'-(Z)-heptadecenyl) 2-cyclohexenone
B4b  4S,6R-dihydroxy-6-(14'-(Z)-nonadecenyl) 2-cyclohexenone
B5  1,2,4-trihydroxy-4-[16'(Z)-heneicosenyl] cyclohexane
B6  sitosterol glucoside
B7  taraxerone
B8  taraxerol
B9  E-lutein
B10  myricetin
B11  myricetin-3-\(\beta\)-galactopyranoside
B12  epicatechin gallate

\(^a\) A3 (myricetin-3-\(\alpha\)-rhamnopyranoside) and A6 (sitosterol) were also featured in Chapter 3.
Chapter 4b Compounds from *Lannea schimperi*

C1a 3-[12'({E})-pentadecenyl] phenol
C1b 3-[14'({E})-heptadecenyl] phenol
C1c 3-[16'({E})-nonadecenyl] phenol
C1d 3-[18'({E})-heneicosenyl] phenol
C2a 5-[12'({E})-pentadecenyl] 4,5-dihydroxycyclohex-2-enone
C2b 5-[14'({E})-heptadecenyl] 4,5-dihydroxycyclohex-2-enone
C2c 5-[16'({E})-nonadecenyl] 4,5-dihydroxycyclohex-2-enone
C2d 5-[18'({E})-heneicosenyl] 4,5-dihydroxycyclohex-2-enone
C3a 1-[12'({E})-pentadecenyl] cyclohex-3-en-1,2,5-triol
C3b 1-[14'({E})-heptadecenyl] cyclohex-3-en-1,2,5-triol
C3c 1-[16'({E})-nonadecenyl] cyclohex-3-en-1,2,5-triol
C4a 1-[14'({E})-heptadecenyl] 4-cyclohex-4-en-1,3-diol
C4b 1-[16'({E})-nonadecenyl] 4-cyclohex-4-en-1,3-diol
C4c 1-[18'({E})-heneicosenyl] 4-cyclohex-4-en-1,3-diol

bA6 (sitosterol), (B7) taraxerone and (B8) taraxerol were also featured in Chapter 4
Chapter 5 Compounds from *Lannea schweinfurthii*

D1a 3-[tridecyl] phenol
D1b 3-[heptadecyl] phenol
D1c 3-[heptadec-12'(Z),14'(E)-diene] phenol
D1d 3-[nonadec-14'(Z),16'(E)-diene] phenol
D1e 3-[heneicos-16'(Z),18'(E)-diene] phenol
D2a 5-hydroxy-5-[tridecyl] cyclohex-2-enone
D2b 5-hydroxy-5-[pentadecyl] cyclohex-2-enone
D2c 5-hydroxy-5-[heptadecyl] cyclohex-2-enone
D2d 5-hydroxy-5-[pentadec-12'(E)-enyl] cyclohex-2-enone
D2e 5-hydroxy-5-[heptadec-14'(E)-enyl] cyclohex-2-enone
D3a 1-[tridecyl] cyclohex-3-en-1,2,5-triol
D3b 1-[heptadecyl] cyclohex-3-en-1,2,5-triol
D4a 1-[tridecyl] cyclohex-4-en-1,3-diol
D4b 1-[nonadecyl] cyclohex-4-en-1,3-diol
D4c 1-[heneicosyl] cyclohex-4-en-1,3-diol
D4d 1-[tricosyl] cyclohex-4-en-1,3-diol
D4e 1-[pentadec-12'(E)-enyl] cyclohex-4-en-1,3-diol
D4f 1-[nonadec-14'(Z),16'(E)-dienyl] cyclohex-4-en-1,3-diol
D4g 1-[heneicos-16'(Z),18'(E)-dienyl] cyclohex-4-en-1,3 diol
D5 lupenone
D6 epicatechin
D7 catechin
D8 Rutin

* A6, B6, B1d, B12, C1a-C1d, C3a, C3c, C4a-C4c were also featured in Chapter 5.
Structures of isolated compounds

Structures in subsequent chapters are not repeated

Chapter 2

A1

A2

A3: R = rhamnose
A4: R = arabofuranose

A5

A6
Chapter 3

Numbering system refers to B1a

B1a: n = 10
B1b: n = 8
B1c: n = 6

M⁺ 358
M⁺ 330
M⁺ 302

M⁺ 304

B2

B3: n = 12

B4a: n = 8
B4b: n = 10

B5: n = 12

B6 R = glucose

B7 R = =O
B8 R = -OH
Chapter 3 continued…

B9

B10 R = rhamnose
B11 R = galactose

Chapter 4

C1a n = 8
C1b n = 10
C1c n = 12
C1d n = 14

C2a n = 8
C2b n = 10
C2c n = 12
C2d n = 14

C3a n = 8
C3b n = 10
C3c n = 12

C4a n = 10
C4b n = 12
C4c n = 14
Chapter 5

D1a: n = 8  D1b: n = 12  
D1c: n = 8  D1d: n = 10  D1e: n = 12

D2a: n = 7  D2b: n = 9  D2c: n = 11  
D2d: n = 8  D2e: n = 10

D3a: n = 7  D3b: n = 11

D4a: n = 7  D4b: n = 13  
D4c: n = 15  D4d: n = 17

D4e: n = 9  
D4f: n = 10  D4g: n = 12
Chapter 5 continued…

D5

D6

D7

D8: R = rutinose
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{13}$C NMR</td>
<td>C-13 nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>br</td>
<td>broad</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CD$_3$OD</td>
<td>deuterated methanol</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>deuterated chloroform</td>
</tr>
<tr>
<td>COSY</td>
<td>correlated spectroscopy</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>dd</td>
<td>double doublet</td>
</tr>
<tr>
<td>DEPT</td>
<td>distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DMSO-d$_6$</td>
<td>deuterated dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond correlations</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>HREIMS</td>
<td>high resolution electron impact mass spectroscopy</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>LCMS</td>
<td>liquid chromatography- mass spectrometry</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>Me</td>
<td>methyl</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>Mp</td>
<td>melting point</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectroscopy</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NCAPD</td>
<td>national coordinating agency for population and development</td>
</tr>
<tr>
<td>NOESY</td>
<td>nuclear overhauser effect spectroscopy</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>td</td>
<td>triplet of doublets</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
DECLARATIONS
DECLARATION 1 – PLAGIARISM

I, Okoth Akinyi Dorothy declare that

1. The research reported in this thesis is my original research, except where otherwise indicated.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons’ data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed …………………………………………………………………………

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication)


In the above publications/manuscripts I carried out all the experimental work, interpreted the data and compiled the findings thereof. The co-authors contribution was to edit, check the scientific content and verify my interpretation of the data based on their areas of expertise.

Signed: ..................................
DEDICATION

This thesis is dedicated to my beloved Dad, Mr. Daniel Okoth Okwany and Son Neil-Antony Okoth
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I wish to express my sincere appreciation to my supervisor, Prof. N.A Koorbanally for his assistance and advice throughout my PhD studies. Your kindness and dedication to assist your students is highly regarded.

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Chapter 1  Introduction

1.1  An introduction to traditional medicine

Traditional medicine plays an important role in the primary health care of a number of developing countries. In Kenya, conventional medicine provides for only 30% of the population. Thus, more than two thirds of the population relies on traditional medicine to meet their primary health care needs. Due to the high cost of modern medicine and drugs, most Kenyans turn to traditional medical practitioners since they are a cheaper alternative to modern medicine, are more accessible and the majority of the population are confident in their ability to manage debilitating, incurable disease (NCAPD, 2008).

Many people use herbal concoctions with the assumption that the medicine is safe and effective. For people with chronic diseases, combining herbal medicine with conventional medicine is a common practice. It is expected that there may be synergistic effects between the conventional drugs and the herbal remedies. The use of indigenous medicinal plants is not based on science and is mostly unregulated and as such varies considerably from place to place. There is often no standard dosage and the safety of such practice remains unclear.

In traditional practice, several plants are often used in combinations and the activity may be a result of either additive or synergistic effects. Many medicinal plants are the focus of biological screening and the crude extracts are screened for in vitro or in vivo activity or both. In some cases single active constituents for a particular action have been successfully identified. Phytochemical and toxicological studies can identify toxic components in order to assess and possibly eliminate or reduce harmful side effects of the extracts (Nyika, 2009). There is still a great need for phytochemical and pharmacological research of medicinal
plants in order to improve the efficacy and reduce potentially harmful side effects that may be associated with it.

Interest in medicinal plants arises from the desire to provide a scientific rationale for the plant uses and the possibility of discovering novel compounds of pharmaceutical value. Historically, compounds containing novel structures from plant sources were a major source for the discovery and development of new drugs for several diseases. These include quinine from *Cinchona* species and artemisinin (Artemisinin®) from *Artemisia annua*. Quinine was used as a template for the synthesis of chloroquine, which was the main antimalarial drug until recently. Semi-synthetic derivatives of artemisinine such as atreether (Artemotil®), artemether (Artemether®) and sodium artesunate (Arinate®) are often used more frequently for malaria treatment. Lapachol (a prenylnaphthoquinone), an antimalarial drug from *Tabebuia species* (Bignoniaceae) was a template for the synthesis of the newest antimalarial drug atovaquinone (Malarone®) (Kaur et al., 2009; Newman and Cragg, 2007; Oliveira et al., 2009). Plant-derived antibacterials such as daptomycin (Cubicin®), fosfomycin tromentamol (Monuril®) and Isepamicin (Isepacin®) (isolated from plants) and Biapenem (Omegacin), cefuroxime axetil (Zinnat), dorifenem (Finibax®), Eritthromycin acistrate (Erasis®), Tigecyclin (Tyagcil®) (semi-synthetized from natural products) (Newnan and Cragg, 2007; 2012; Cragg and Newman, 2013) demonstrate the potential of finding new and better antibacterial agents with fewer side-effects, which could provide alternative antibacterial agents to resistant bacteria. Flavonoids such as epicatechin gallate, epigallocatechin gallate and gallicatechin gallate isolated from tea are examples of plant-derived compounds with potentially exploitable activities, including antibacterial activity (Hamilton-Miller and Shah, 2000), synergism with antibiotics (Shiota et al., 1999; Qin et al., 2009).
suppression of bacterial virulence (Shah et al., 2008) and suppression of resistance (Stapleton et al., 2004; 2007).

A number of plant extracts and compounds have demonstrated activity against malarial pathogens. Different classes of compounds (alkaloids, flavonoids, quinones, xanthones and terpenes) with antiplasmodial activity have been isolated (Batista et al., 2009; Caniato and Puricelli, 2003; Nogueira and Lopes, 2011; Oliveira et al., 2009). Some of the compounds like galloylated catechins and procyanidins are reported to inhibit or reverse resistance of the most virulent malarial parasite *Plasmodium falciparum* (Park et al., 2010; Ramanandralbe et al., 2008; Sannella et al., 2007; Tasdemir et al., 2006).

Anticancer agents derived from plants and their derivatives e.g. paclitaxel (Taxol®), vincristine (Oncovin®), vinorelbine (Navelbine®), teniposide (Vumon®) and camptothecin (e.g. Hycamtin®) have been proven to be effective for cancer prevention and therapeutics (Cragg et al., 2009; Cragg and Newman, 2013; Pezzuto, 1997). Phytochemicals are used for the treatment of cancer related symptoms due to their safety, low toxicity, and general availability. Many active phytochemicals are in human clinical trials but there is the possibility of discovering novel and more effective anticancer phytochemicals (Pratheeshkumar et al., 2012). Consuming phytochemicals as part of a daily diet have been shown to have cancer protective effects by inhibiting, delaying, or reversing carcinogenesis by inducing detoxifying and antioxidant enzyme systems, regulating inflammatory and proliferative signaling pathways, and inducing cell cycle arrest and apoptosis (Pratheeshkumar et al., 2012; Rajput and Mandal, 2012; Singh et al., 2002).
1.2 Traditional medicinal uses of the genus *Lannea*

The genus *Lannea* belongs to the family Anacardiaceae and consists of about 40 species of shrubs or trees native to tropical Africa. They are used as sources of timber, as a source of fruit and for a number of purposes in indigenous medicine. Table 1-1 shows a selection of the more popular *Lannea* species and their use in indigenous medicine. The medicinal use varies with the species and community or tribe involved. In general, *Lannea* species are used in the management of mental disorders, gastrointestinal disorders, bacterial infections, viral infections, fungal infections and fever (Table 1-1). The plants are used to manage an array of symptoms rather than a specific disease. It must be noted that the diagnosis is made by traditional healers with no scientific training. A disease symptom such as fever may be due to a number of causes such as bacterial or viral infections, malaria and food poisoning.

*Lannea* species are traditionally used in folk medicine as natural healing remedies with therapeutic effects such as the prevention of cardiovascular diseases such as hypertension, atherosclerosis and ischemia, inflammation disorders (Singh and Singh, 1994; 1996) such as asthma, gingivitis, rheumatoid arthritis, cellulites, gastroenteritis and inflammatory bowel disease (Kone *et al.*, 2004; 2011; Maiga *et al* 2006; Maregesi *et al.*, 2007; Singh and Singh, 1994), neurological diseases such as Alzheimer’s disease, Parkinson’s disease, memory loss and depression (Adewusi and Steenkamp, 2011) and pulmonary diseases such as chronic obstructive pulmonary diseases (Kerharo and Adams, 1974; Maiga *et al* 2006). They are also used to reduce the risk of cancer (Adewusi and Steenkamp, 2011; Atawodi, 2005).

A number of *Lannea* species have demonstrated antibacterial, anti-inflammatory, antioxidant and radical scavenging activities and lipoxygenase inhibition activities (Diallo *et al.*, 2001;
these activities have been associated with their polyphenol contents. The ability of some species to cure wounds, abrasions and sores are known (Agyare et al., 2013; Deji-Agboola and Olajubu 2010; Sathish, 2010). This could also be due to their polyphenol content since antioxidants are also involved in wound healing and tissue repairs (Sen et al., 2002; Shetty, 2013). The plants have also demonstrated their capacity to manage diabetes as well as hypertension and related symptoms (Deutschlander et al., 2009; Nyarko et al., 2005; Okine et al., 2005; Rahmatullah et al., 2012; Singh and Singh, 1996).

*Lannea* species are also used in the management of antibacterial, antifungal and viral diseases (HIV type 1 and II, herpes zoster and herpes simplex), which have been verified by pharmacological studies (Bationo et al., 2012; Diallo et al., 2001; Haule et al., 2012; Kisangau et al., 2007; 2009; Kone et al., 2004; 2011; Maregesi et al., 2010; Maregesi et al., 2007; Maregesi et al., 2008; Ouattara et al., 2011a; Ouattara et al., 2011b, Runyoro et al., 2006). These infections are often considered as HIV opportunistic infections. The use of *Lannea* species in the treatment of malaria and associated symptoms has been justified by the demonstrated antiplasmodial action of *Lannea disclor* and *Lannea schweinfurthii* extracts (Clarkson et al., 2004; Gathirwa et al., 2008; 2011; Maregesi et al., 2010). The antigiardial, vibriocidal, antibacterial and antidiarrheal activity of some species demonstrate the ability of the plants to manage diarrhea and related infections (Akinsinde and Olukoya, 1995; Etuk et al., 2009; Johns et al., 1995; Olatokunboh et al., 2010).

Acetylcholinesterase inhibition activity has been observed in some *Lannea* species (Adewusi and Steenkamp, 2011; Koné et al., 2011) and hence has the potential to be used for
Alzheimer's disease and other neurological disorders. Cytotoxicity and anticancer activity has also been observed in *L. acida*, *L. coromandelica*, *L. humilis*, *L. schweinfurthii*, *L. schimperi*, *L. welwitschii* and *L. nigratiana* (Akter et al., 2013; Fadeyi et al., 2013; Gathirwa et al., 2011; George et al., 2010; Groweiss et al., 1997; Kapche et al., 2007; Mothana et al., 2009; Nibret et al., 2010; Roy et al., 2011; Sowemimo et al., 2009). In most of the studies, no follow up isolation of the phytocompounds were carried out on the crude extracts, however these were done in a few cases where flavonoids, terpenes, alkyl phenols and their derivatives were identified as the compounds responsible for the activity.

1.3 Phytochemical studies of the genus Lannea

Phytochemical studies of the genus *Lannea* indicate various classes of compounds (Table 1-2) such as tetracyclic and pentacyclic triterpenes, flavonoids (flavones, flavonols, dihydroflavonols, flavanones, flavanols, isoflavans, anthocyanidins) and phenolic lipids and cyclohexene derivatives (Govindac et al., 1971; Groweiss et al., 1997; Islam and Tahara, 2000; Kapche et al., 2007; Nair et al., 1963; Picerno et al., 2006; Queiroz et al., 2003; Sankara and Nair, 1971; Sultana and Ilyas, 1986a; 1986b; Yun et al., 2012). Representative structures are shown in Figure 1-1 to Figure 1-5.

The most commonly isolated class of compounds found in the *Lannea* species are the flavonoids being found in seven of the eight species studied. These are *L. acida*, *L. coromandelica*, *L. edulis*, *L. grandis*, *L. microcarpa*, *L. nigratiana* and *L. velutina*. Examples of the flavonoids are quercetin (1) (flavonol), 7,2'-dimethoxy-4',5'-methylenedioxyflavone (2) (flavone), 6,7,2,2-dimethylchromeno-8γ,γ-dimethylallyl flavanone (flavanone) (3), 5-methoxyvestitol (4) (isoflavan), cyanidin 3-O-β-D-galactopyranoside (5) (anthocyanidin), (2R,3R)-(++)-4',5,7-trimethoxydihydroflavonol (6) (dihydroflavonol), epigallocatechin gallate
(7) (flavan) and physcion (8) (anthraquinone). This is followed by the triterpenes isolated from *L. grandis* and *L. coromandelica* species. These triterpenes have either tetracyclic or pentacyclic skeletons. Examples of tetracyclic triterpenes are lanosterol (9) β-sitosterol (10) stigmaster-4-ene-6β-ol-3-one (11) and 5α-stigmastane-3,6-dione (12). Taraxerone (13), taraxerol (14) and taraxeryl acetate (15) are pentacyclic triterpenes all isolated from *L. coromandelica*. In addition phytochemical studies of *Lannea edulis*, *Lannea nigratiana* and *Lannea welwitschii* led to the isolation of phenolic lipids (alkyl/alkenyl hydroquinones and cardanols/3-alkyl/alkenyl phenols) and their derivatives (the alkyl/alkenyl cyclohexenones and alkyl/alkenyl cyclohexenols). Lanneaquinol (16) and 2-(R)-hydroxylanneaquinol (17) are examples of alkyl hydroquinones isolated from *Lannea welwitschii* (Groweiss et al., 1997). The cardanols, 3-[14′E-nonadecenyl]-phenol (18) and 3-[16′E-heptadecenyl]-phenol (19) were reported in *Lannea edulis* (Queiroz et al., 2003). The alkyl cyclohexenones are represented by 4,5-dihydroxy-5-[14′(E)-heptadecenyl]-2-cyclohexenone (20) and the isomeric 4,5-dihydroxy-5-[8′(Z)-heptadecenyl]-2-cyclohexenones (21 and 22) (Groweiss et al. 1997; Queiroz et al., 2003). The alkylated cyclohexenetriol, lanneanol (23) was isolated from *Lannea nigratiana* (Kapche et al., 2007). The alkyl phenols and their derivatives isolated from the different species vary in the number, position and configuration of the double bonds along the side chain, the number of carbons on the side chains as well as the number and position of hydroxyl functional groups. Alkyl cyclohexenols and alkyl cyclohexenones have only been isolated from the *Lannea* and *Tapirira* genera (Correia et al., 2001; David et al., 1998; Roumy et al., 2009). They are proposed to be the biogenic precursors of the alkyl phenols in these species (Correia et al., 2001). The long chain phenols are characteristic of the Anacardiaceae. Some of these compounds have demonstrated antioxidant, antibacterial, antiplasmodial, wound healing ability and cytotoxicity.
Figure 1-1 Representative structures of flavonoids from *Lannea* species
Figure 1-2  Representative tetracyclic triterpenes from *Lannea* species

Figure 1-3  Pentacyclic triterpenes from *Lannea* species
Figure 1-4 Phenolic lipids from *Lannea* species

Figure 1-5 Representative structures of cyclohexene lipid derivatives from *Lannea* species
1.4 An introduction to the plants studied in this work

*Lannea schweinfurthii*, *Lannea alata*, *Lannea rivae* and *Lannea schimperi* are widely distributed in Kenya. In East Africa root and stem bark concoctions of *Lannea schweinfurthii* is used in the treatment of boils, paludism/ high fever and chills related to malaria, gastrointestinal diseases, stomach pain, edema, diarrhea, constipation, abscesses, cellulitis (localized or diffused inflammation of connective tissues), oral candidiasis, amoebiasis, venereal diseases (syphilis) and opportunistic infections associated with HIV like herpes zoster and herpes simplex (Egunyomi *et al.*, 2009; Gathirwa *et al.*, 2011; Geissler *et al.*, 2002; Johns *et al.*, 1995; Maregesi *et al.*, 2007; Mothana *et al.*, 2009; Tabuti *et al.*, 2003).

The stem, branch and trunk juice or boiled decoction of *Lannea schimperi* is used to treat chronic diarrhea, chest problems, stomach disorders, stomach pain, coughs, opportunistic diseases (herpes simplex and herpes zoster), skin infections, anemia, mental disorders, swelling and snake bites and used as purgatives, laxatives, carminatives and anthelmintics (Batista *et al.*, 2009; George *et al.*, 2010; Johns *et al.*, 1994; Kisangau *et al.*, 2009; Nogueira and Lopes, 2011; Yun *et al.*, 2012). The extracts from *Lannea schimperi* exhibited antiulcer, antibacterial, cytotoxicity and antifungal activities (Haule *et al.*, 2012; Kisangau *et al.*, 2009).

The bark decoction of *Lannea alata* is drunk for fever and malaria, whilst an infusion is taken for snake bite and a paste is applied on fractures, injuries and wounds (Pandey and Rizvi, 2009). It is also an excellent fruit tree for dry lands (Maundu *et al.*, 2005). The inner bark of *Lannea rivae* is chewed for its sweet taste and as a source of water. The inner bark is chewed to treat colds, fever, coughs and stomachache (Maundu *et al.*, 2005, Kokwaro *et al.*, 2009).
Plant extracts from *Lannea schweinfurthii* have previously demonstrated antiplasmodial, antimalarial, antitrypanosomal, antigiardial, antioxidant, antibacterial antifungal, cytotoxic, acetylcholinesterase inhibition, anti-HIV and used for Herpes simplex (Adewusi and Steenkamp, 2011; Gathirwa *et al.*, 2008; 2011; Johns *et al.*, 1995; Maregesi *et al.*, 2008; 2010; Nibret *et al.*, 2010).

There are no reports of the phytochemistry or biological activity of *Lannea rivae* and *Lannea alata* in the literature even though their indigenous medicinal uses suggest antioxidant, antibacterial and antimalarial activities. Even though *Lannea schweinfurthii* and *Lannea schimperi* demonstrated biological activity that validate their medicinal roles, no phytochemical studies was performed to isolate the chemical constituents responsible for the observed activity. The aim of this study was to validate some of the medicinal uses of these four plants and to determine the compounds that are responsible for the activities observed.

### 1.5 Methodology used in this study

*Lannea alata, Lannea rivae, Lannea schimperi,* and *Lannea schweinfurthii* were identified for this study. They were collected, identified and voucher specimens deposited in reputable herbariums. The different parts of the plant were extracted with solvents of varying degrees of polarity and each extract purified using column chromatography. The aim of the separation was to isolate the components in the extract in as pure a form as possible. Fats, oils, sugars and amino acids were regarded as primary metabolites and were not targeted for isolation. Once isolated and purified, the secondary metabolites (normally small organic molecules) were subject to a range of spectroscopic techniques, NMR (both 1D and 2D), mass spectrometry, IR and UV spectroscopy to determine the structures of the isolated compounds. The known compounds were verified and compared with the data in the literature and new
compounds were verified by usually more than one set of spectroscopic evidence. Usually 2D NMR, COSY, HSQC, HMBC and NOESY data coupled with Electron Impact Mass Spectrometry (EIMS) and High Resolution Mass Spectrometry (HRMS) was used for the unequivocal elucidation of the structure.

Biological studies were conducted on the isolated compounds and in some cases crude extracts based on the traditional use of the plant. In addition, the type of activity tested was also dependent on particular compound types, for instance flavonoids, are known to be good antioxidants and therefore the flavonoids isolated were evaluated for their antioxidant activity.

This thesis reports on the new and known compounds isolated from *Lannea alata*, *Lannea rivae*, *Lannea schimperi* and *Lannea schweinfurthii* and their biological activities. This is the first phytochemical investigation of these four *Lannea* species. Compounds responsible for antioxidant, antibacterial, antiplasmodial, larvicidal and cytotoxic activity in these plants is reported here.

### 1.6 Hypothesis

The four species of *Lannea* are reported to have many medicinal uses and are used by traditional healers in Kenya for a range of illnesses. It is therefore hypothesized that there must be active chemical constituents either acting alone or in synergy to produce these effects.
1.7 Objectives

- To isolate the secondary metabolites present in extracts of the four *Lannea* species under investigation.
- To determine the identity of the compounds isolated if known.
- To elucidate the structures of the compounds if novel including an attempt to assign the stereochemistry in the molecule if possible.
- To determine the biological activity of the extracts of each of the species in line with what they are used for ethnomedicinally.
- To determine the biological activity of the isolated compounds in line with both the class of the compound and with what the plants are used for medicinally.
- To provide a rationale for the use of the plants in traditional medicine.
- To provide lead compounds for drug development.
Table 1-1 Ethnobotanical uses and biological activities of *Lannea* species

<table>
<thead>
<tr>
<th>species</th>
<th>Plant part</th>
<th>Ethnobotanical uses</th>
<th>Biological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Engl.</td>
<td>bark, leaves</td>
<td>febrifuge, malaria, syphilis, cellulitis, abscesses, oral candidiasis, gingivitis, nasal ulcers, asthma, neurological disorders, anaemia, coughs</td>
<td>antiplasmodial, antimalarial, toxicity, antigiardial, inhibition of acetylcholinesterase, antioxidant</td>
<td></td>
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<tr>
<td><em>L. humilis</em> Oliv.</td>
<td>roots</td>
<td>Anaemia, stomach pains, nausea, general body weakness</td>
<td>Cytotoxicity, antitrypanosomal</td>
<td>Kokwaro, 2009; Maregesi <em>et al.</em>, 2007; Nibret <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><em>L. rivae</em></td>
<td>bark</td>
<td>colds, chewed for its sweet taste and as a source of water</td>
<td>none</td>
<td>Kokwaro, 2009</td>
</tr>
<tr>
<td><em>L. alata</em></td>
<td>Bark, roots</td>
<td>fever, malaria, snake bites, fractures and injuries</td>
<td>none</td>
<td>Maundu <em>et al.</em>, 2005</td>
</tr>
<tr>
<td><em>L. triphya</em></td>
<td>bark</td>
<td>Coughs, constipation, colds</td>
<td>none</td>
<td>Kokwaro, 2009</td>
</tr>
<tr>
<td><em>L. schimperi</em> (A.)</td>
<td>bark</td>
<td>Chronic diarrhea, pain, stomach</td>
<td>Antiulcer, antibacterial,</td>
<td>Chinsembu and Hedinbi, 2010;</td>
</tr>
<tr>
<td>Species</td>
<td>Parts Used</td>
<td>Uses</td>
<td>Activities</td>
<td>References</td>
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<tr>
<td><em>L. microcarpa</em></td>
<td>Leaves, bark, root</td>
<td>Conjunctivitis, stomatitis, gingivitis, dressing wounds, skin eruptions, stomachache, beriberi, schistosomiasis and haemorrhoids; mouth blisters, rheumatism, sore throat, dysentery, as a cathartic and as a dressing on boils</td>
<td>Anti-inflammatory effect, antidiarrhoeic activity, antioxidant</td>
<td>Bationo <em>et al.</em>, 2012; Marquet and Jansen, 2005; Lamien-Meda <em>et al.</em>, 2008; Ouattara <em>et al.</em>, 2011b; Picerno <em>et al.</em>, 2006; Tapsoba and Deschamps, 2006</td>
</tr>
<tr>
<td><em>L. velutina</em></td>
<td>Roots and bark</td>
<td>diarrhoea, rachitic children, wounds and strained muscles, respiratory diseases, oedema, paralysis, epilepsy and insanity</td>
<td>Antioxidant and radical scavenging activities, larvicidal, molluscicidal, lipoxygenase inhibition</td>
<td>Diallo <em>et al.</em>, 2001; Maiga <em>et al.</em>, 2006; 2007; Ouattara <em>et al.</em>, 2011b</td>
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<tr>
<td><em>L. acida</em></td>
<td>Stem bark, root</td>
<td>Diarrhea, stomach ache, gonorrhea, rheumatism, oral diseases, malaria</td>
<td>Antibacterial, antioxidant, vibriocidal, cytotoxicity</td>
<td>Akinsinde and Olukoya, 1995; Asase <em>et al.</em>, 2005; Etuk <em>et al.</em>, 2009; Kone <em>et al.</em>, 2004; Ouattara <em>et al.</em>, 2011a; 2011b; Sowemimo <em>et al.</em>, 2009; Tapsoba and Deschamps, 2006</td>
</tr>
<tr>
<td><em>L. edulis</em></td>
<td>Root bark</td>
<td>Diarrhea, sore eyes, boils, abscesses, diabetes, schistosomiasis (bilhazia), gonorrhea, pre-hepatic</td>
<td>Mutagenic effects, antioxidant</td>
<td>Deutschlander et al., 2009; Maroyi, 2011, 2013; Queiroz <em>et al.</em>, 2003; Sohni <em>et al.</em>, 1995; Segawa and</td>
</tr>
<tr>
<td>Species</td>
<td>Part Used</td>
<td>Symptoms</td>
<td>Other Activities</td>
<td>References</td>
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<tr>
<td><em>L. discolor</em></td>
<td>Bark, seeds</td>
<td>Malaria, fever, constipation, menorrhagia, infertility</td>
<td>antimalarial</td>
<td>Clarkson <em>et al.</em>, 2004; Kazembe <em>et al.</em>, 2012; Maroyi, 2013</td>
</tr>
<tr>
<td><em>L. barteri</em></td>
<td>bark</td>
<td>Wounds, rheumatic, diarrhea, gastritis, sterility, intestinal</td>
<td>Antibacterial, antifungal, antioxidant,</td>
<td>Allabi <em>et al.</em>, 2011; Jansen, 2005; Kone <em>et al.</em>, 2005; 2011; Adoum, 2009</td>
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<td><strong>L. transuta</strong></td>
<td>Bark, flowers,</td>
<td>Haemostatic for wounds, abrasion</td>
<td>Anticancer, antioxidant,</td>
<td>Mothana <em>et al.</em>, 2009</td>
</tr>
<tr>
<td></td>
<td>leaves</td>
<td>and sores</td>
<td>antimicrobial</td>
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<tr>
<td><strong>L. nigritana</strong></td>
<td>Stem and root</td>
<td>diarrhea, dysentery; pain-killers,</td>
<td>Cytotoxic, anticancer</td>
<td>Burkill, 1985; <em>Kapche et al.</em>, 2007</td>
</tr>
<tr>
<td></td>
<td>bark, leaves</td>
<td>pulmonary troubles; skin, mucosae,</td>
<td></td>
<td><em>Fadeyi et al.</em>, 2013; <em>Magassouba et al.</em>, 2007</td>
</tr>
<tr>
<td>Plant species</td>
<td>Isolated compounds</td>
<td>References</td>
<td></td>
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<td>---------------</td>
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<tr>
<td><em>L. acida</em></td>
<td>Flavonoids: <em>Flavanone</em>: 6,7,2,2-dimethylchromeno-8γ,γ-dimethylallyl &lt;br&gt; <em>Flavones</em>: Lanceolatin B and 7,2'-dimethoxy-4',5'-methyleneedioxyflavone</td>
<td>Sultana and Ilyas, 1986a;1986b</td>
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<td><em>L. coromandelica</em></td>
<td>Flavonoids: <em>Dihydraflavonols</em>: (2R,3S)-(+)3',5-dihydroxy-4',7-dimethoxydihydroflavonol, (2R,3R)-(+)4',5,7-trimethoxydihydroflavonol, (2R,3R)-(+)4',7-di-O-methylidihydroquercetin, (2R,3R)-(+)4',7-di-O-methylidihydrokaempferol and (2R,3R)-(+)4'-O-methylidihydroquercetin &lt;br&gt; <em>Flavanols</em>: quercetin, isoquercitrin, quercetin-3-O-arabinoside, quercetin-3-O-rutinoside, morin &lt;br&gt; <em>Leucoanthocyanidins</em>: leucodelphinin, leucocyanidin &lt;br&gt; <em>Anthraquinones</em>: physcion and physcion anthranol B &lt;br&gt; <em>Terpenes and terpenoids</em>: lanosterol, sitosterol, β-sitosterol glycoside, stigmast-4-ene-6-β-ol-3-one, 5-α-stig mastane-3-6-dione, taraxerone, taraxerol and taraxeryl acetate &lt;br&gt; <em>Other phenolic compounds</em>: 4-hydroxy-3-methoxybenzaldehyde</td>
<td>Islam and Tahara, 2000; Nair <em>et al</em>., 1963; Sankara and Nair, 1971; Subramanian and Nair, 1971; Yun, 2012</td>
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<td><em>L. grandis</em></td>
<td>Triterpenes: lanosterol &lt;br&gt; Flavonoids: epicatechin, quercetin, rutin, (+) leucocyanidin &lt;br&gt; Other phenolic compounds: cluytyl ferrulate</td>
<td>Govindac <em>et al</em>., 1971; Sulochana <em>et al</em>., 1967; 1970; Sulochana and Sastry, 1968</td>
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<td></td>
</tr>
</tbody>
</table>
| **L. microcarpa** | Flavonoids: **Flavonols**: 4’-methoxymyricetin-3-O-α-L-rhamnopyranoside, myricetin-3-O-α-L-rhamnopyranoside, myricetin-3-O-β-D-glucopyranoside  
**Flavones**: Vitexin, isovitexin  
**anthocyanidins**: cyanidin 3-O-(2-O-β-D-xylopyranosyl)-β-D-galactopyranoside and cyanidin 3-O-β-D-galactopyranoside | Pale *et al.*, 1998; Picerno *et al.*, 2006 |
| **L. nigratiana** | Flavonoids: **Flavanols**: epicatechin gallate, epigallocatechin gallate  
Phenolic lipids and their derivatives: gallic acid, 3,4,5-trimethoxy phenol, 3,4,5 trimethyl-phenol glucoside, 4-hydroxy-3-methoxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid, lanneanol (1,2,4 trihydroxy-4-[12’(Z)-heptadecenyl]cyclohexene-5-ene) | Kapche *et al.*, 2007 |
| **L. welwitschii** | Phenolic lipids and their derivatives; alkyl hydroquinines lanneaquinol and 2′(R)-hydroxyhydroquinones, alkyl cyclohexenones, 4,5-dihydroxy-5-[8’(Z)-heptadecenyl]-2-cyclohexenones | Groweiss *et al.*, 1997 |
| **L. velutina** | Catechin, procyanidin B-1 and a series of proanthocyanidins with different degrees of polymerization between 3-12 | Maiga *et al.*, 2007 |
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Chapter 2  Antibacterial and antioxidant activities of flavonoids from *Lannea alata* (Engl.) Engl. (Anacardiaceae)

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Abstract

Two new prenylated flavonoids, lanneaflavonol (A1) and dihydrolanneaflavonol (A2) together with the known compounds myricetin-3-0-α-rhamnopyranoside (myricitrin) (A3) and myricetin-3-0-α-arabinofuranoside (betmidin) (A4), lupeol (A5) and sitosterol (A6) were isolated from the roots of Lannea alata. Compounds A1-A4 exhibited good antibacterial and radical scavenging activity with the glycosides A3 and A4 showing better antioxidant activity than the aglycones A1 and A2 and betmidin (A4) showing the best antimicrobial activity followed by the aglycones A1 and A2. Betmidin (A4) with an arabinose moiety at the 3-O-position showed the best antibacterial activity against Gram-positive bacteria, followed by the prenylated dihydroflavonol (A2), whilst the prenylated linear flavonol (A1) showed limited activity against Gram-negative bacteria. The arabinofuranoside (A4) followed by the rhamnopyranoside (A3) showed the best antioxidant activity comparable to that of ascorbic acid. The biological activities justify the ethnomedicinal uses of the plant in the management of diseases associated with Gram-positive bacteria, such as being used to treat injuries and wounds.

Keywords: Lannea alata, prenylated flavonoids, lanneaflavonol, lanneadihydroflavonol, antibacterial activity, antioxidant activity.
2.1 Introduction

*Lannea alata* (Engl.) Engl. is a deciduous shrub about 1.5-4 m high, with drooping branches and a smooth grey bark. The leaves are clustered on short shoots and divided into tiny leaflets which are bluntly toothed towards the apex and the roots are covered by dense wool like hair (Maundu *et al.*, 1999). The bark decoction is drunk for fever and malaria, whilst an infusion is taken for snake bite and a paste is applied on fractures, injuries and wounds (Maundu *et al.*, 1999). It is also an excellent fruit tree for dry lands (Maundu *et al.*, 1999).

There is no literature on the chemistry of the plant or biological activities, but based on its ethnomedicinal uses it potentially has anti-plasmodial (being used for malaria) and antimicrobial (being used for injuries and wounds) activities. Although there have been no phytochemical reports on *L. alata* itself, other related species of *Lannea* have been reported to contain alkylated hydroquinones and cyclohexenones (Groweiss *et al.*, 1997), polyflavonoid tannins (Islam *et al.*, 2002), polysaccharides (Ramachandran and Joshi, 1968), cyanidins (Sulochana *et al.*, 1967), flavonoids (Sulochana and Sastry, 1968; Sultana and Ilyas, 1986), alkylphenols and dihydroalkylhexenones (Queiroz *et al.*, 2003).

The phytochemical investigations of *L. alata* stem and root extracts led to the isolation of four flavonoids (A1-A4) and two common triterpenes, sitosterol (A5) and lupeol (A6). Based on the traditional use of the plant and nature of the isolated compounds, antibacterial and antioxidant activity testing was carried out on the isolated compounds and is reported herein.

2.2 Results and Discussion

The ethyl acetate and n-hexane extracts yielded two novel prenylated flavonoids A1 and A2 as well as two known flavonoid glycosides, myricetin-3-α-L-rhamnopyranoside
(myricitrin) (A3) (Fossen et al., 1999) and myricetin-3-O-α-L-arabinofuranoside (betmidin) 
(A4) (Kim et al., 1994) as well as two ubiquitous triterpenoids, lupeol (A5) (Burns et al., 
2000) and sitosterol (A6) (Kovganko et al., 1999). The structures of A1 and A2 (Figure 2-1) 
were elucidated by a combination of spectroscopic methods while compounds A3-A6 were 
identified by comparison of their spectral data with that in the literature.

\[
\text{Figure 2-1 The structures of lanneaflavonol (A1) and dihydrolanneaflavonol (A2)}
\]

Compound A1 was isolated as a yellow solid. The IR spectrum exhibited absorption bands 
suggesting the presence of hydroxyl (3323 cm\(^{-1}\)), chelated carbonyl (1655 cm\(^{-1}\)), geminal 
dimethyl (1380 cm\(^{-1}\)), methoxy groups (2985 cm\(^{-1}\)) and aromatic rings (1585 and 1478 cm\(^{-1}\)). 
The UV spectrum showed absorption bands at 290 and 357 nm indicative of a flavonol 
skeleton (Mabry et al., 1970). The mass spectrum showed a molecular ion peak at \(m/z\ 398\) 
followed by the cleavage of two methyl groups, \(m/z\ 283 \ [M^+ - CH_3] \) and \(m/z \ 268 \ [M^+ - 2CH_3]\).

The \(^1\)H NMR spectrum showed the characteristic resonances of a cyclised prenyl group with 
a pair of doublets at \(\delta_H 5.79 \ (H-2'', J = 10.04 \text{ Hz}) \) and \(6.61 \ (H-1'', J = 10.04 \text{ Hz}) \) and a six- 
proton methyl resonance indicating two equivalent methyl groups at \(\delta_H 1.43 \ (2 \times CH_3-4'') \). In 
addition, a two-proton singlet aromatic resonance at \(\delta_H 7.19\), characteristic of phenyl protons
(H-2'/6') and a benzopyran aromatic resonance at $\delta_H$ 6.45 (H-8) was observed. A methoxy resonance at $\delta_H$ 3.74 (4'-OCH$_3$) was also present. Further to this, three exchangeable proton resonances at $\delta_H$ 12.83, 9.58 and 9.40 (2H) was present and ascribed to the phenolic protons 5-OH, 3-OH and 3'/5'-OH respectively.

The carbon spectrum consisted of seventeen carbon resonances with fourteen of these resonances being in the aromatic or double bond region and a chelated carbonyl resonance at $\delta_C$ 176.21, consistent with the structure of a flavonoid. Of the fourteen resonances, seven resonances were oxygenated between $\delta_C$ 136 and 158 with the resonance at $\delta_C$ 150.53 being due to two carbon resonances as indicated by the intensity of the resonance. These were attributed to the eight oxygenated carbon atoms, C-2, C-3, C-5, C-7, C-9, C-3'/5' and C-4'. A further six resonances in the olefinic and aromatic region between $\delta_C$ 94 and 129 were attributed to either the protonated or singlet carbon atoms of the olefinic bonds or the aromatic rings, C-6/10, C-8, C-1', C-2'/6', C-1'' and C-2''. The oxygenated carbon C-3'' occurred at $\delta_C$ 77.96, the methoxy carbon at $\delta_C$ 59.74 and the equivalent geminal dimethyl resonance occurred at $\delta_C$ 27.84.

Both the olefinic proton resonances of the cyclised prenyl group, H-1'' and H-2'' were seen coupled in the COSY spectrum. H-2'' showed HMBC correlations to C-4'', the methyl carbon at $\delta_C$ 27.84 and the oxygenated carbon of the prenyl group at $\delta_C$ 77.96 as well as to the singlet aromatic carbon at $\delta_C$ 104.02. The fact that this resonance was also seen coupled to the chelated hydroxyl proton of 5-OH prompted us to form a bond between C-1'' and C-6 rather than C-1'' and C-8, resulting in a linear pyranoflavone rather than an angular one. Furthermore, C-10 at $\delta_C$ 103.98 was very close to that of C-6 and both these resonances showed HMBC correlations to H-8 at $\delta_H$ 6.45. H-1'' showed HMBC correlations to C-6 ($\delta_C$
104.02) and C-5 at δC 155.24 and C-7 at δC 158.61. The H-8 resonance showed a HMBC
correlation to C-7 and C-9 at δC 154.67. The proton resonance at δH 7.19 was assigned to H-
2'/6' of the phenyl ring based on HMBC correlations to C-2 at δC 146.56. Furthermore, the
3'/5'-OH proton resonance showed HMBC correlations to both C-4' and C-2'/6', confirming
the substitution pattern on ring B. Finally, the resonance at δC 137.32 was assigned to C-4'
due to a HMBC correlation with the methoxy group at δH 3.75. The compound was thus
identified as 3,5,3',5'-tetrahydroxy-4'-methoxy-6,7-(2''',2'''-dimethylchromene)-flavonol and
accorded the trivial name lanneaflavonol.

The 1H NMR spectrum of compound A2 (a light yellow solid) was very similar to that of A1
with the proton resonances and splitting patterns of the 5-OH, 3'/5'-OH, H-8, H-2'/6', 4'-
OCH3, H-1", H-2" and 3H-4" all being very close to that of A1. However there were three
new resonances, two doublets and a double doublet, that appeared in the 1H NMR spectrum
of A2, at δH 5.88 (J = 6.28 Hz), which did not correspond to any carbon in the HSQC
spectrum, indicative of a hydroxyl proton resonance, and at δH 4.97 (J = 11.05 Hz) and δH
4.47 (J = 11.05, 6.20 Hz) which corresponded to aliphatic oxygenated carbon resonances at
δC 82.98 and 71.51 respectively. These two carbon resonances were also not present in the
13C NMR spectrum of compound A1. Furthermore, the C-2 and C-3 resonances of A1 were
absent in A2. This led to the conclusion that the Δ2 double bond in A1 had now been
saturated in A2 and the 2,3-dihydro derivative was now present. Based on their coupling
constants and correlations in the COSY spectrum, δH 5.88 was attributed to the 3-OH
resonance, δH 4.97 to H-2 and δH 4.47 to H-3. COSY correlations existed between H-2 and
H-3 and H-3 and 3-OH. The three carbon resonances at δC 83.02 (C-2), 71.55 (C-3) and
198.32 (C-4) were characteristic of 3-hydroxyflavanones (Agrawal, 1989). The molecular
ion peak at m/z 400 in the MS confirmed this and there was also a peak at m/z 382 [M+ - H2O] typical for an aliphatic hydroxyl group.

Based on the large coupling constant of H-2 and H-3 (J = 11.05 Hz), the configuration at C-2 and C-3 was assigned as trans diaxial and together with a positive optical rotation, the absolute stereochemistry was of the 2R, 3R configuration, consistent with dihydroflavanols with a positive optical rotation (Bohm, 1975). This was supported by the CD curve of A2, which showed four cotton effects in the order (+), (-), (+), (+) from 400 to 200 nm (Markham and Mabry, 1968; Ruangrungsi et al., 1981), and a positive cotton effect at [Θ]320 due to the n-π* transition and a negative effect at [Θ]300, due to the π - π* transition, consistent with that of the 2R:3R configuration (Gaffield, 1970; Slade et al., 2005; Cao et al., 2006).

Thus, A2 was identified as (2R,3R)-3,5,3′,5′-tetrahydroxy-4′-methoxy-6,7-(2′′,2′′-dimethylchromene)-dihydroflavonol and accorded the trivial name lanneadihydroflavonol.

Compound A3, identified as myricetin-3-O-α-L-rhamnopyranoside (myricetin) was previously isolated from Nyphaea caerulae (Fossen et al., 1999) and Lannea microcarpa (Picerno et al., 2006), while compound A4 was identified as the flavonol glycoside, myricetin-3-O-α-L-arabinofuranoside (betmidin), which has been previously isolated from the leaves of Polygonium aviculare (Kim et al., 1994).

**Antibacterial activity**

The crude methanol and ethyl acetate extracts showed good activity against Enterococcus faecium ATCC 19434 with inhibition zones of 18 and 16 mm respectively and the hexane extract showed intermediate activity with a 10 mm inhibition zone. However, none of the
isolated compounds showed activity to \textit{E. faecium} indicating that this activity must have either been due to some other compound or a synergistic effect of several compounds. Compound \textbf{A1} demonstrated activity comparable to that of tetracycline against both \textit{Pseudomonas} strains (Table 2-1). Interestingly the dihydroflavonol (A2) did not show the same activity, suggesting that the planar structure of the flavonol (A1) is more suited as a Gram-negative antibacterial agent and that the activity is probably associated with the 3-OH group which must be situated on a planar double bond.

All four isolated compounds demonstrated antimicrobial activity against several strains of Gram-positive bacteria, with compound \textbf{A4} (myricetin-3-\textit{O-α}-arabinofuranoside) being the most effective, followed by compounds \textbf{A2}, \textbf{A1} and \textbf{A3}. \textit{B. subtilis} ATCC 6633 demonstrated susceptibility to both compounds \textbf{A2} and \textbf{A4} and was intermediately susceptible to compound \textbf{A1}, while \textit{S. pyogenes} ATCC 19615 was susceptible to compound \textbf{A4}, and intermediately susceptible to compounds \textbf{A2} and \textbf{A3}. All the compounds were generally less active compared to the standard antimicrobial agents, ampicillin and tetracycline, although compound \textbf{A4} demonstrated activity against \textit{S. aureus} strains that were close to that of ampicillin. The activity of \textbf{A4} over \textbf{A3} indicates that the arabinose sugar attached to the 3-position has better activity than the rhamnose attached at the same position, demonstrating the importance that the type of sugar attached to the 3-oxygenated position has on antimicrobial activity. Results of the disc diffusion assay indicate that the presence of the double bond actually decreases activity against Gram-positive bacteria and that the chiral centres of C-2 and C-3 are important for activity. This contrasts with findings of Xu and Lee (2001), who demonstrated that quercetin (flavonol) with the $\Delta^2$ double bond displayed better activity than catechin (flavanol) (Xu and Lee, 2001). However, the inactivity of catechin could also be due to the absence of the carbonyl group at C-4.
### Table 2-1 Antimicrobial susceptibility: Mean zones of inhibition (mm) for isolated compounds 1-4 and standard antimicrobial agents, ampicillin and tetracycline

| Bacteria<sup>a</sup> | Compounds/Extracts<sup>b</sup> | A1 | A2 | A3 | A4 | H | E | M | A | T |
|----------------------|--------------------------------|----|----|----|----|---|---|---|---|---|---|
| Gram negative        |                                |    |    |    |    |   |   |   |   |   |   |
| P. aeruginosa ATCC 27853 |                            | 8<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 10<sup>(R)</sup> |
| P. aeruginosa ATCC 35032 |                            | 9<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 9<sup>(R)</sup> |
| Gram positive        |                                |    |    |    |    |   |   |   |   |   |   |
| B. subtilis ATCC 6633 |                            | 11<sup>(I)</sup> | 16<sup>(S)</sup> | 10<sup>(R)</sup> | 20<sup>(S)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 32<sup>(S)</sup> | 35<sup>(S)</sup> |
| E. faecium ATCC 19434 |                            | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 10<sup>(S)</sup> | 16<sup>(S)</sup> | 18<sup>(S)</sup> | 16<sup>(S)</sup> | 9<sup>(R)</sup> |
| S. aureus ATCC 29213 |                            | 8<sup>(R)</sup> | 15<sup>(S)</sup> | 9<sup>(R)</sup> | 20<sup>(S)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 22<sup>(S)</sup> | 26<sup>(S)</sup> |
| S. aureus ATCC 43300 |                            | 9<sup>(R)</sup> | 14<sup>(I)</sup> | 0<sup>(R)</sup> | 18<sup>(S)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 22<sup>(S)</sup> | 26<sup>(S)</sup> |
| S. epidermidis ATCC14990 |                        | 8<sup>(R)</sup> | 11<sup>(I)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 21<sup>(S)</sup> | 29<sup>(S)</sup> |
| S. saprophyticus ATCC 35552 |                      | 8<sup>(R)</sup> | 13<sup>(I)</sup> | 8<sup>(R)</sup> | 7<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 36<sup>(S)</sup> | 26<sup>(S)</sup> |
| S. sciuri ATCC 29062 |                            | 9<sup>(R)</sup> | 12<sup>(I)</sup> | 0<sup>(R)</sup> | 14<sup>(S)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 21<sup>(S)</sup> | 29<sup>(S)</sup> |
| S. xylosus ATCC 35033 |                            | 8<sup>(R)</sup> | 14<sup>(I)</sup> | 8<sup>(R)</sup> | 16<sup>(S)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 23<sup>(S)</sup> | 26<sup>(S)</sup> |
| S. agalactiae ATCC 13813 |                        | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 10<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 22<sup>(S)</sup> |
| S. pyogenes ATCC 19615 |                            | 10<sup>(R)</sup> | 14<sup>(I)</sup> | 12<sup>(S)</sup> | 22<sup>(S)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 0<sup>(R)</sup> | 26<sup>(S)</sup> | 32<sup>(S)</sup> |

<sup>a</sup>The Gram-positive Enterococcus faecalis ATCC 51299 and the Gram-negative Escherichia coli ATCC 25922 and ATCC 35218 and Klebsiella pneumoniae ATCC 700603 were resistant to compounds A1-A4 and the crude extracts and exhibited no zones of inhibition.

<sup>b</sup>Compounds/Extracts: lanneaflavonol (A1), dihydrolanneaflavonol (A2), myricetin-3-O-α-rhamnopyranoside (myricitrin) (A3), myricetin-3-O-α-arabinofuranoside (A4), hexane extract (H), ethyl acetate extract (E), methanol extract (M), Ampicillin AMP10 (A); Tetracycline TE30 (T).

(R) denotes resistance, (I) denotes intermediate susceptibility and (S) denotes complete susceptibility.

The MIC values of the four compounds were determined on three Staphylococcus spp. strains (S. aureus ATCC 29213, S. sciuri ATCC 29062 and S. xylosus ATCC 35033), based on the disc diffusion results. However, the compounds were only active in the mM range with compounds A3 and A4 showing slightly better activity than A1 and A2 (Table 2-2).
However the MIC values of the test compounds were not comparable to the standard antibiotics ampicillin and tetracycline which was active against the same bacterial species in the µM range.

**Table 2-2** Minimum inhibitory concentrations of compounds A1-A4 against selected Gram-positive bacterial species

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Compounds (mM)</th>
<th>Antibiotics (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>A2</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 29213</td>
<td>2.01</td>
<td>2.00</td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 29062</td>
<td>2.01</td>
<td>2.00</td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 35033</td>
<td>2.01</td>
<td>2.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard antibiotics Ampicillin and Tetracycline

**Antioxidant activity**

When tested individually, each flavonoid (A1-A4) exhibited dose dependent radical-scavenging activity in the presence of the DPPH radical. The percentage radical scavenging activity for the isolated flavonol glycosides and ascorbic acid for comparison are given in Table 2-3. The flavonoids exhibited radical scavenging activity in the order A4>A3>A1>A2. This is in keeping with the idea that the glycosides can generate extra hydrogen radicals due to the greater number of free hydroxyl groups, capable of quenching radicals better than the aglycones. Our studies are contrary to those reported in a review by Rice-Evans *et al.* (1996) which reports that blocking the 3-OH group with a glycoside reduces activity, however it must be noted that the compounds reported in the review by Rice-Evans *et al.* (1996) is based on compounds with free hydroxy groups at C-5 and C-7. Compounds A1 and A2 possess a cyclised prenyl group, which may be responsible for reduced antioxidant activity compared to the glycosides. Furthermore, the fact that the dihydroflavonol (A2) is a better antioxidant...
than the flavonol (A1) is also contrary to reports that the unsaturated $\Delta^2$ double bond is better for antioxidant activity (Rice-Evans et al., 1996). Since the two compounds A1 and A2 are the same in all respects except for the difference in saturation at C-2 and C-3, it must be concluded that this is specific for cyclised prenylated flavonols.

Table 2-3 Antioxidant activity of compounds isolated from *Lannea alata*: Mean % radical scavenging activity

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Concentrations</th>
<th>Mean Comp.</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.25 µg mL$^{-1}$</td>
<td>12.50 µg mL$^{-1}$</td>
<td>25.00 µg mL$^{-1}$</td>
</tr>
<tr>
<td>A1</td>
<td>23.80 ± 0.88</td>
<td>31.91 ± 1.74</td>
<td>48.86 ± 0.72</td>
</tr>
<tr>
<td>A2</td>
<td>46.07 ± 1.03</td>
<td>53.36 ± 1.17</td>
<td>72.36 ± 2.16</td>
</tr>
<tr>
<td>A3</td>
<td>62.68 ± 2.15</td>
<td>72.07 ± 1.42</td>
<td>80.46 ± 1.40</td>
</tr>
<tr>
<td>A4</td>
<td>65.49 ± 2.15</td>
<td>78.82 ± 1.95</td>
<td>86.72 ± 0.38</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>87.26 ± 1.72</td>
<td>94.04 ± 1.13</td>
<td>96.66 ± 0.57</td>
</tr>
<tr>
<td>Mean, Conc.$^*$</td>
<td>57.26$^a$</td>
<td>59.08$^b$</td>
<td>68.40$^c$</td>
</tr>
</tbody>
</table>

$^a$means that differ significantly have different letters, p < 0.05; mean conc. indicates the mean of all the compounds tested at a specific concentration; mean comp. indicates the mean of the compound tested at different concentrations.

The flavonoids myricetin-3-\(\text{O-}\alpha\text{-rhamnopyranoside (A3)}\) and myricetin-3-\(\text{O-}\alpha\text{-arabinofuranoside (A4)}\) displayed antioxidant activity slightly lower than ascorbic acid at lower concentrations but comparable to ascorbic acid at higher concentrations. These compounds (A3 and A4) are known to have strong antioxidant activity (Jayasinghe et al., 2012; Tung et al., 2009; Abd El-Kader et al., 2012; Yan et al., 2002).
2.3 Experimental

General Experimental Procedures

The \(^1\)H, \(^{13}\)C and all 2D NMR spectroscopy were recorded using a Bruker Avance III 400 MHz spectrometer at 400.22 MHz for \(^1\)H and 100.63 MHz for \(^{13}\)C. Chemical shifts (\(\delta\)) are reported in ppm and coupling constants (\(J\)) in Hz. The \(^1\)H and \(^{13}\)C NMR chemical shifts of the deuterated solvent were 7.24 and 77.0 referenced to the internal standard, TMS, respectively. IR spectra were recorded using a Perkin Elmer Universal ATR spectrometer. Optical rotations were measured at 20 °C on a Perkin Elmer™, Model 341 Polarimeter with a 10 cm flow tube. Circular dichroism was performed on a Chirosan Plus spectropolarimeter (Applied Photophysics). UV spectra were obtained on a Hewlett Packard UV-3600 Spectrophotometer. The melting points were determined on an Ernst Leitz Wetziar micro-hot stage melting point apparatus and are uncorrected. Merck silica gel 60 (0.040–0.063 mm) was used for column chromatography and Merck 20 cm \(\times\) 20 cm silica gel 60 F\(_{254}\) aluminium sheets were used for thin-layer chromatography. The TLC plates were analysed under UV (254 and 366 nm) before being sprayed and developed with a [1:2:97] anisaldehyde:concentrated sulphuric acid:methanol spray reagent and then heated.

Plant Material

The stem and roots of Lannea alata (Engl.) Engl. were collected in June 2011, at Wote (Makueni District) in Kenya and the specimen authenticated and deposited at the Maseno University, Botanic garden Herbarium in Kenya, voucher no MSU/ BG-3/13. The stem and roots of the plant were dried and ground using a Wiley mill.

Chromatographic Isolation

The powdered dry roots of Lannea alata (5 kg) were sequentially extracted with 10 L of hexane, ethyl acetate and methanol using an orbital shaker. For the root extraction, TLC of
the crude extracts before evaporation showed that the hexane and ethyl acetate extracts had a similar profile and these extracts were therefore combined. After evaporation of the solvents, 33.54 g of hexane-ethyl acetate extract and 43.43 g of methanol extract was obtained. The hexane-ethyl acetate extract (22.8 g) was chromatographed on silica gel with a hexane:ethyl acetate gradient mixture starting from 100% hexane and increasing the polarity stepwise by 10% after collection of every 1L of eluent, up to 100% ethyl acetate. In the last step, a 5% methanol in ethyl acetate mixture was used. Ten fractions of 100 mL were collected for each step and 120 fractions collected in total. Fractions were monitored on TLC plates using UV light and anisaldehyde spray reagent. Further purification of fraction 20-30 with 5% ethyl acetate in hexane led to the isolation of an amorphous white solid \textbf{A5} (35.42 mg) in fractions 6-13 and white crystals \textbf{A6} (98.56 mg) in fractions 16-20. Fraction 30-34 of the crude column was rechromatographed and eluted with 20% ethyl acetate in hexane. Fifteen fractions were collected of which fraction 7-10 yielded a yellow solid \textbf{A1} (379.80 mg). Fraction 41-48 was eluted with 30% ethyl acetate in hexane and resulted in a pale yellow solid \textbf{A2} (290.27 mg), which was eluted in fractions 19-26. TLC analysis of fractions 80-100 of the crude column indicated that it was made up of two compounds. Purification of this fraction with Sephadex LH20 using methanol as the solvent, resulted in two compounds \textbf{A3} (62.68 mg) and \textbf{A4} (29.50 mg) in fractions 7 and 10 respectively. The methanol extract of the roots (28.6 g) was chromatographed with 2L of ethyl acetate: methanol (95:5). Fraction 5-9 contained compounds \textbf{A3} and \textbf{A4}, which was purified as above yielding 239.6 mg of \textbf{A3} and 184.8 mg of \textbf{A4}.

The stem bark (1.58 kg) was extracted sequentially as above with hexane, ethyl acetate and methanol to yield 6.97 g of hexane, 10.15 g of ethyl acetate and 22.18 g of methanol extract. TLC analysis of the hexane and ethyl acetate extracts showed similar profiles and were
combined and separated as above, yielding 35.12 mg of A1, 72.13 mg of A2, 187.53 mg of A5 and 283.21 mg of A6. The methanol extract yielded 45 mg of A3 and 29.65 mg of A4. The compounds were isolated and purified as above.

3,5,3',5'-tetrahydroxy-4'-methoxy-6,7-(2'',2''-dimethylchromene)-flavonol (lanneaflavonol) (A1) yellow solid; m.p. 202-203 °C; UV (CH₃OH) λ_max (log ε) 290 (2.80), 357 (2.97) nm; IR ν_max (cm⁻¹) 3323 (O-H), 1655 (C=O), 1631, 1585, 1478, 1380, 1147, 1045; ¹H NMR (400 MHz, DMSO-d₆) δ 1.43 (6H, s, 2 x H-4''), 3.74 (3H, s, 4'-OCH₃), 5.80 (1H, d, J = 10.04 Hz, H-2''), 6.45 (1H, s, H-8), 6.61 (1H, d, J = 10.04 Hz, H-1''), 7.19 (2H, s, H-2'/6'), 9.40 (2H, s, 3'/5'-OH), 9.58 (1H, s, 3-OH), 12.83 (1H, s, 5-OH); ¹³C NMR (100 MHz, DMSO-d₆) δ 27.84 (2C, C-4''), 59.73 (4'-OCH₃), 77.96 (C-3''), 94.36 (C-8), 103.98 (C-10), 104.02 (C-6), 107.34 (2C, C-2'/6'), 114.44 (C-1''), 125.65 (C-1'), 129.00 (C-2''), 136.74 (C-3), 137.31 (C-4''), 146.56 (C-2), 150.53 (C-3'/5'), 154.67 (C-9), 155.24 (C-5), 158.61 (C-7), 176.21 (C-4); EIMS m/z (rel. int.): 398 [M⁺] (34), 383 [M⁺-CH₃] (100), 368 [M⁺ - (2 x CH₃)] (30); HREIMS m/z 398.1006 [M⁺] (calcd for C₂₁H₁₈O₈, 398.1002).

(2R,3R)-3,5,3',5'-tetrahydroxy-4'-methoxy-6,7-(2'',2''-dimethylchromene)-dihydroflavonol (lanneadihydroflavonol) (A2) light yellow solid; m.p. 208-209 °C; [α]_D^{20} +30.77° (c = 0.13, MeOH); CD (MeOH) [θ] (deg cm² decimole⁻¹): [θ]_{220} +3000, [θ]_{300} -12180, [θ]_{320} +9450, [θ]_{370} +9450; UV (MeOH) λ_max (log ε) 204 (4.94), 272 (4.89), 295 (4.42) nm; IR ν_max (cm⁻¹) 3571, 3428 (O-H), 3362 (O-H), 2977, 1651 (C=O), 1634, 1574, 1463, 1360, 1126, 1035; ¹H NMR (400 MHz, DMSO-d₆) δ 1.39 (6H, s, 2 x H-4''), 3.68 (3H, s, 4'-OCH₃), 4.47 (1H, dd, J = 11.04, 6.20 Hz, H-3), 4.97 (1H, d, J = 11.04 Hz, H-2), 5.65 (1H, d, J = 10.08 Hz, H-2''), 5.88 (1H, d, J = 6.28 Hz, 3'-OH), 5.91 (1H, s, H-8), 6.43 (2H, s, H-2'/6'), 9.16 (2H, s, 3'/5'-OH), 12.19 (1H, s, 5-OH); ¹³C NMR (100 MHz, DMSO-d₆) δ 27.90 (2 x C-4''), 59.60 (4'-
OCH₃), 71.51 (C-3), 78.26 (C-3''), 82.98 (C-2'), 95.56 (C-8), 101.15 (C-10), 102.18 (C-6), 107.11 (C-2'/6'), 114.37 (C-1''), 126.99 (C-2''), 132.04 (C-1''), 135.67 (C-4''), 150.42 (C-3'/5''), 157.22 (C-5), 161.19 (C-9), 161.73 (C-7), 198.28 (C-4). EIMS m/z (rel. int.): 400 [M⁺] (2), 382 [M⁺-H₂O] (23), 367 [M⁺-H₂O-CH₃] (7), 281 (21), 241 (21), 207 (48), 177 (100). HREIMS m/z 400.1162 [M⁺] (calcd for C₂₁H₂₀O₈, 400.1158).

Antibacterial Activity

Bacterial strains

Eleven Gram-positive strains: Bacillus subtilis ATCC 6633, Enterococcus faecium ATCC 19434, Enterococcus faecalis ATCC 51299, Staphylococcus aureus ATCC 29213 and ATCC 43300, Staphylococcus epidermidis ATCC 14990, Staphylococcus saprophyticus ATCC 35552, Staphylococcus sciuri ATCC 29062, Staphylococcus xylosus ATCC 35033, Streptococcus agalactiae ATCC 13813, Streptococcus pyogenes ATCC 19615 and five Gram-negative strains, Escherichia coli ATCC 25922 and ATCC 35218, Pseudomonas aeruginosa ATCC 27853 and ATCC 35032 and Klebsiella pneumoniae ATCC 700603 were used for the antibacterial assays. Isolates were maintained on brain heart infusion (BHI) agar or tryptic soy agar (TSA) plates at 4 °C and for long-term storage as 20% glycerol stocks at -70 °C.

Antimicrobial susceptibility testing

The antimicrobial susceptibility to the flavonoids isolated from L. alata was determined using the disc diffusion method (CLSI, 2007). A 20 mg mL⁻¹ stock solution of each compound and crude extracts was made using DMSO. Blank discs (6 mm; MAST, UK) were impregnated with 20 µL of each compound (400 µg mL⁻¹) and crude extracts (400 µg mL⁻¹) and allowed to dry. The bacterial isolates, grown overnight on BHI or TSA agar plates, were resuspended in sterile distilled water and the turbidity of cell suspensions adjusted equivalent
to that of a 0.5 McFarland standard. These were used to inoculate Mueller-Hinton (MH) agar plates by streaking swabs over the entire agar surface followed by the application of the respective phytochemical extract discs (CLSI, 2007). Plates were then incubated for 24 h at 37 °C. Testing was done in duplicate and tetracycline (TE30) and ampicillin (AMP10) discs (Oxoid, UK) were used as standard antimicrobial agent controls, while DMSO-impregnated discs were used as negative controls. Zone diameters were determined and averaged. The following zone diameter criteria were used to assign susceptibility or resistance to phytochemicals tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 11 – 14 mm, and Resistant (R) ≤ 10 mm. The criteria for assigning susceptibility or resistance to AMP10 was as follows: (S) ≥ 17 mm, (I) = 14 – 16 mm, (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) 15 – 18 mm, (R) ≤ 14 mm (CLSI, 2007).

Three Staphylococcus spp. strains (S. aureus ATCC 29213, S. sciuri ATCC 29062 and S. xylosus ATCC 35033) were selected for the determination of MICs based on their disc-diffusion susceptibility data. MICs of the four isolated compounds together with ampicillin and tetracycline were determined using the broth microdilution assay (Andrews, 2001). Cultures were grown overnight on TSA and diluted equivalent to a 0.5 McFarland standard (Andrews, 2001). Microtiter plate wells (final total volume of 200 µL), each containing 90 µL of Mueller-Hinton (MH) broth were inoculated with 10 µL of cell suspension and two-fold serial dilutions of compounds 1-4 (215.34 µM to 4.02 mM) and antimicrobial agents ampicillin and tetracycline (9.00 nM to 9.22 µM). The plates were incubated at 30 ºC for 24 h without shaking. The negative control wells contained MH broth only and the positive control wells contained the respective cell suspensions with no compound/antimicrobial agents added. This was done in triplicate (Andrews, 2001). The MIC was the lowest concentration of antimicrobial agent, which inhibited visible growth of organism.
Antioxidant Activity

A 10 mg mL\(^{-1}\) stock solution of each compound was made by dissolving the compounds in DMSO. Sample concentrations of 6.25, 12.50, 25.00, 50.00 and 100 µg mL\(^{-1}\) were made in methanol. The DPPH radical scavenging activity of the compounds was determined according to the method of Kumawat et al. (2012). Briefly, 1 mL of each compound was added to 1 mL of DPPH (1,1-diphenyl-2-picrylhydrazyl) solution (0.1 mM in methanol). The mixture was shaken and kept in darkness for 30 minutes at room temperature. The decrease of solution absorbance was determined at 517 nm. Vitamin C (ascorbic acid) was used as the positive control. The DPPH radical scavenging activity was calculated using the following formula:

\[
\text{DPPH Radical Scavenging Activity (\%) = \left[ \left( A_0 - A_1 / A_0 \right) \times 100 \right]},
\]

where \(A_0\) is the absorbance of the control, and \(A_1\) is the absorbance of the compound or standard sample.

The statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by the Tukey’s multiple range post-hoc test to separate the means. SPSS version 12 software was used for analysis.

2.4 Conclusion

The occurrence of the new flavonol and dihydroflavonol (A1 and A2), derived from myricetin add to the number of flavonoids already identified within Lannea species and in particular add prenylated flavonoids to the list. Compound A4 was effective against certain strains of Gram-positive bacteria, while both compounds A3 and A4 had antioxidant activity.
comparable to that of ascorbic acid. A prenylated flavanone (6,7[2",2"-dimethyl chromene]) has only once been reported in *Lannea acida* (Sultana and Ilyas, 1986). This is the second occurrence of prenylated flavonoids in a *Lannea* species and indicates a closer taxonomical link between *L. acida* and *L. alata*. The current finding justifies the ethno botanical use of the plant extracts to make a paste for the treatment of wounds and injuries as this paste may act as an antibacterial agent, preventing the wound from becoming infected and allowing it to heal.

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Chapter 3 Antibacterial, antioxidant, antiplasmodial and cytotoxic activities of *Lannea rivae* (chiov) Sacleux (Anacardiaceae)

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Abstract

Six novel compounds, 3-nonadec-14'-(Z)-enyl phenol (B1a); 4,5-dihydroxy-4,2'-epoxy-5-[16'Z-18'E-heneicosenylidene]-cyclohex-2-enone (B2), 2,4,5-trihydroxy-2-[16'Z-heneicosenyl]-cyclohexanone (B3), 4S,6R-dihydroxy-6-[12'Z-heptadecenyl]-cyclohex-2-enone (B4a); 4S,6R-dihydroxy-6-[14'Z-nonadecenyl]-cyclohex-2-enone (B4b); and 1,2,4-trihydroxy-4-[16'Z-heneicosenyl]-cyclohexane (B5) were isolated from the roots and stems of *Lannea rivae* in addition to the known compounds; cardanols B1b-B1d, sitosterol (A6), sitosterol glucoside (B6), taraxerone (B7), taraxerol (B8), E-lutein (B9), myricetin (B10), myricetin-3-O-α-rhamnopyranoside (A3), myricetin-3-O-β-galactopyranoside (B11), and (-)-epicatechin-3-O-gallate (B12). Myricetin (B10), its glycosides (B11 and A3) and epicatechin gallate (B12) showed good antioxidant activity, while B10 and B12 but not the glycosides showed good antibacterial activity. B1a-B1d, B4a-B4b and B5 were all relatively non-toxic, whilst B2 and B3 showed more toxicity than the others. These two toxic compounds, B2 and B3 also showed good antiplasmodial activity. The mixture of B4a and B4b, which was far less toxic than B2 and B3 also showed promising antiplasmodial activity and is a good lead for an antiplasmodial drug.

**Keywords**: *Lannea rivae*, alkenyl phenols, alkenyl cyclohexenones, alkenyl cyclohexenols, cytotoxicity, antiplasmodial, antibacterial, antioxidant.
3.1 Introduction

In sub-Saharan Africa where access to healthcare facilities and medicine is difficult, the majority of the population, especially those living in rural areas far from cities turn to traditional medicines for medical disorders and disease (WHO, 2008). Although the safety and efficacy of the traditional remedies are not verified, most of the population in need of these medicines has no alternative but to use them. The majority of the remedies is herbal, with many remedies containing different parts of more than a few plants and is administered as concoctions or poultices. The three main diseases that plague most countries in Africa are malaria, tuberculosis and HIV and whilst other diseases are also rampant on the African continent, the majority of the population seeks relief and cure for these three diseases. The fact that traditional healers are so popularly used is evidence enough that the remedies must contain compounds which are probably active against either the symptoms or the causes of their diseases. The phytochemical study of plants used in African traditional medicine is therefore a good lead to finding active compounds against these diseases.

*Lannea rivae* (Chiov) Sacleux is one such plant where the inner bark is applied in traditional medicine in the management of colds, fever, coughs and stomach ache (Kokwaro, 2009). The bark is also chewed for its sweet taste and as a source of water. The fruits of the plant are also edible (Maundu et al., 1999). To the best of our knowledge, this plant has not been studied previously for its phytochemical constituents as there are no accounts reported in the literature. Phytochemical studies of other *Lannea* species reported the isolation of flavonoids, some with antibacterial and antioxidant activity (Sultana and Ilyas, 1986; Islam et al., 2002; Picerno et al., 2006; Okoth et al., 2013), alkylphenols and dihydrocyclohexenones with antioxidant activity (Queiroz et al., 2003), and alkylated hydroquinones,
dihydrocyclohexenols and phenolic compounds with cytotoxic activity (Groweiss et al., 1997; Kapche et al., 2007).

Apart from these phytochemical investigations, extracts of other *Lannea* species were also reported to have antioxidant, lipoxygenase, anti-inflammatory, analgesic, acetylcholinesterase, anti-malarial, anti-HIV, antibacterial, antifungal and antiviral activity (Clarkson et al., 2004; Picerno et al., 2006; Maiga et al., 2006; 2007; Gathirwa et al., 2007; 2008; 2011; Maregesi et al., 2008; 2010; Deji-Agboola and Olajubu, 2010; Koné et al., 2011; Ouattara et al., 2011; Alam et al., 2012). In the present study, a phytochemical investigation of *Lannea rivae* was conducted and the isolated compounds assessed for their antioxidant, antibacterial, and antiplasmodial activity. Cytotoxicity studies are also reported for the isolated compounds.

### 3.2 Results and discussion

Purification of the leaf, root and stem bark extracts led to isolation of six new compounds, 3-nonadec-14'-(Z)-enyl phenol (*B1a*); 4,5-dihydroxy-4,2'-epoxy-5-[16'-Z-18'-E-heneicosenyldiene]-cyclohex-2-enone (*B2*), 2,4,5-trihydroxy-2-[16'-Z-heneicosenyl]-cyclohexanone (*B3*), 4S,6R-dihydroxy-6-[12'-Z-heptadecenyl]-cyclohex-2-enone (*B4a*); 4S,6R-dihydroxy-6-[14'-Z-nonadecenyl]-cyclohex-2-enone (*B4b*); and 1,2,4-trihydroxy-4-[16'-Z-heneicosenyl]-cyclohexane (*B5*) (Figure 3-1). The new compounds were elucidated mainly by 1D and 2D NMR, and mass spectral data.

In addition, several known compounds whose structures were determined from their $^1$H, $^{13}$C and 2D NMR spectroscopy were also isolated. Once identified, their structures were verified by comparison with the data from the literature. These were the alkenyl phenols (or
cardanols) 3-heptadec-12’-Z-enyl phenol (B1b) and 3-pentadec-10’-Z-enyl phenol (B1c) (Liu and Abreu, 2006), and the alkyl phenol, 3-pentadecyl phenol (B1d) (Lomonaco et al., 2009), which were isolated as a mixture along with B1a; the triterpenes sitosterol (A6) (Kovganko et al., 1999), sitosterol glucoside (B6) (Faizi et al., 2001), taraxerone (B7) (Sakurai et al., 1987), and taraxerol (B8) (Liu et al., 2010), a tetraterpene, trans lutein (B9) (Khachik and Chang, 2009) and the flavonoids myricetin (B10) (Liu et al., 2011), myricetin-3-O-α-L-rhamnopyranoside (A3) (Braca et al., 2003), myricetin-3-O-β-galactopyranoside (B11) (Lee et al., 2011) and (-)-epicatechin gallate (B12) (Braca et al., 2003).

![Numbering system refers to B1a](image1)

![Figure 3-1](image2)

**Figure 3-1** Novel compounds isolated from *Lannea Rivae*
The cardanols (B1a-B1d) were isolated as a mixture and despite several attempts could not be separated quantitatively. The NMR spectra of the mixture compare well with that in the literature for B1b-B1d (Liu and Abreu, 2006; Lomonaco et al., 2009). They were however qualitatively separated by GCMS, where the four compounds were identified as the novel cardanol B1a containing a C-19 side chain with M⁺ at m/z 358, two known monounsaturated cardanols containing a C-17 side chain (M⁺ at m/z 330) (B1b) and a C-15 side chain (M⁺ at m/z 302) (B1c) and a saturated cardanol with a C-15 side chain (M⁺ at m/z 304) (B1d). The mass spectra of all the compounds indicated an ion at m/z 108 due to the benzylic cleavage resulting in a 3-methylhydroxybenzene cation, confirming the presence of the aromatic substituent. The double bond in the monounsaturated long alkyl chain was characterized by the carbon resonances at δC 129.92 and 129.84 and the position of the double bond using B1a as an example was based on COSY correlations between H-13' and H-14' and between H-14' and H-15' as well as HMBC correlations between C-15' and H-17' and C-16' and H-17'. The fact that H-14' and H-15' appeared as overlapping resonances at δ 5.32 (t, J = 4.48 Hz) with corresponding allylic carbon resonances for C-13' and C-16' occurring at δ 26.91 and 27.20 indicate a Z configuration for the double bond in the side chain as the E configuration has slightly higher values for the allylic carbon resonances at approximately δ 32.0 (Roumy et al., 2009). The C-17 and C-15 alkylated phenols, 3-heptadec-12'-(Z)-enyl phenol (B1b) and 3-pentadec-10'-(Z)-enyl phenol (B1c) besides being isolated in Ozoroa insignus (Liu and Abreu, 2006) have also been reported in Ginko biloba (Sun et al., 2012) and Knema laurina (Akhtar et al., 2011). A C-19 (3-nonadec-16'-(E)-enyl phenol), different to B1a in the geometry and position of the double bond in the side chain has been reported in Lannea edulis (Queiroz et al. 2003).
Compound B2 showed IR absorption bands typical of a hydroxyl stretch at 3382 cm\(^{-1}\) and an \(\alpha,\beta\)-unsaturated carbonyl stretch at 1679 cm\(^{-1}\). The molecular ion peak at \(m/z\) 432 was evident in the mass spectrum. The \(^1\)H NMR spectrum showed the presence of six olefinic proton resonances, including two which overlapped at \(\delta_H\) 5.91. This indicated that three double bonds were present in the molecule. The H-2 and H-3 resonances occurred as a pair of doublets at \(\delta_H\) 5.91 and 6.71 (\(J = 10.2\) Hz), indicative of an isolated \textit{cis} double bond without adjacent protons. The other two pairs of olefinic protons were located on the alkyl chain with H-16', H-17' and H-18' all being \textit{cis} to each other as indicated by their coupling constants of approximately 10 Hz at \(\delta_H\) 5.24-5.32 (m), \(\delta_H\) 5.92 (dd) and \(\delta_H\) 6.27 (dd) with coupling constants of \(J_{16',17'} = 10.2\) Hz and \(J_{17',18'} = 10.8\) Hz. The H-19' proton was located \textit{trans} to H-18' at \(\delta_H\) 5.67 (dt, \(J = 15.1, 6.6\) Hz) due to \(J_{18',19'}\) being approximately 15 Hz. The double bonds on the alkyl chain were located at \(\Delta^{16'}\) and \(\Delta^{18'}\) as H-19' showed COSY correlations with H-20' which in turn showed COSY correlations with the terminal methyl group in the alkyl chain. The length of the alkyl chain was determined by the mass spectrum taking into account the mass of the rest of the molecule.

The resonance at \(\delta_H\) 6.71 showed HMBC correlations to the ketone peak at \(\delta_C\) 196.9 and the other olefinic peak at \(\delta_H\) 5.91 showed HMBC correlations to the methylene carbon peak at \(\delta_C\) 47.0, which corresponded to the two non-equivalent doublet resonances at \(\delta_H\) 2.82 and 2.68 (\(J = 16.0\) Hz). Thus, \(\delta_H\) 6.71, \(\delta_H\) 5.91, \(\delta_C\) 196.9 and \(\delta_C\) 47.0 were attributed to H-3, H-2, C-1 and C-6 respectively. The H-6 proton resonances of \(\delta_H\) 2.82 and 2.68 showed HMBC correlations to three other carbon resonances at \(\delta_C\) 43.6, 78.7 and 99.5, the latter two being oxygenated with the last resonance being much more deshielded than the others, indicating a hemiacetal carbon atom. These resonances were therefore assigned to C-1', C-5 and C-4 respectively. There was an additional methine oxygenated resonance at \(\delta_H\) 4.37-4.41 (m, H-
2'), which formed part of the furan ring fused to the cyclohexenone moiety. The stereochemistry at C-4, C-5 and C-2' could not be determined from the NOESY data available. Compound B2 was thus identified as 4,5-dihydroxy-4,5-furan-2'-[16'-(Z)-18'-(E)-heneicosenyldiene]-cyclohex-2-enone.

Compound B3 displayed an IR spectrum with a broad hydroxyl stretching absorption at 3384 cm⁻¹ and a carbonyl stretching frequency at 1724 cm⁻¹. The molecular mass of the compound was deduced from the mass spectrum, where even though the M⁺ ion was absent, an M⁺-H₂O ion was present, quite common for secondary and tertiary alcohols. This M⁺-H₂O peak was evident at m/z 420, for the compound with a molecular mass of 438 and a molecular formula of C₂₇H₅₀O₄.

The ¹H NMR spectrum indicated the presence of an alkenyl side chain with a cis double bond due to the triplet at δ_H 5.32 (J = 4.7 Hz) and the carbon resonances adjacent to the double bond being at a chemical shift of δ_C 27.2 and 26.9 (Roumy et al., 2009) similar to compounds B1a-B1c. The position of the double bond was ascertained by HMBC correlations between C-17' and H-19' as well as between H-19' and C-20', and C-20' and H-21'. Three pairs of non-equivalent proton resonances at δ_H 1.68 (td, J = 12.4, 4.1 Hz, H-1'a) and δ_H 1.94 (m, H-1'b); δ_H 2.16 (dd, J = 14.5, 4.0 Hz, H-3a) and δ_H 2.00 (m, H-3b); and δ_H 2.99 (dd, J = 13.9, 3.8 Hz, H-6a) and δ_H 2.62 (dd, J = 13.9, 5.7 Hz) as well as two deshielded oxygenated resonances at δ_H 4.09 (m, H-5) and δ_H 4.04 (m, H-4), the ketone resonance at δ_C 211.8 (C-1) and the oxygenated tertiary carbon resonance at δ_C 78.1 (C-2) make up the resonances of the cyclohexanone ring. Their positions on the cyclohexanone ring were established by COSY correlations between H-5 and H-6a and H-6b and between H-4 and H-3a and H-3b as well as the HMBC correlations of C-1 with H-5, H-6a, H-6b, H-3a, H-3b and
H-1’a and H-1’b; C-2 with H-4; and C-4 with H-6a and H-6b. Unfortunately, the stereochemistry again could not be established from NOESY correlations. Thus, compound B3 was named 2,4,5-trihydroxy-2-[16’-(Z)-heneicosenyl]-cyclohexanone.

Compounds B4a and B4b were obtained as a mixture, exhibiting molecular ions of $m/z$ 364 and 392 consistent with molecular formulae of $C_{23}H_{40}O_3$ and $C_{25}H_{44}O_3$ with C-17 and C-19 side chains respectively. In both cases, the $M^+\cdot H_2O$ peaks at $m/z$ 346 (for B4a) and $m/z$ 374 (B4b) are more prominent than the $M^+$ ions, typical for secondary and tertiary alcohols. The IR spectrum indicated the presence of hydroxyl groups (3392 cm$^{-1}$), aliphatic groups (2922 and 2852 cm$^{-1}$) and an $\alpha,\beta$-unsaturated carbonyl group (1678 cm$^{-1}$). The $^1H$ and $^{13}C$ NMR spectral data of B4a and B4b were similar to both 4S,6S-dihydroxy-6-(14’(Z)-nonadecenyl)-2-cyclohexenone (relative configuration) (de Jesus Correia et al., 2001) and 4R,6S-dihydroxy-4-(10’(Z)-heptadecenyl)-2-cyclohexenone (absolute configuration) (David et al., 1998). The difference between B4b and its isomer published in de Jesus Correia et al. (2001) is the difference in stereochemistry at C-6. The difference between B4a and its isomer in David et al. (1998) is in the position of the alkyl chain in the molecule, the side chain in B4a occurring at C-6 instead of C-4 and the position of the double bond in the side chain, B4a occurring at $\Delta^{12}$ and its isomer in David et al. (1998) occurring at $\Delta^{10}$.

The position of the alkyl chain in B4a and B4b was determined to be at C-6 due to HMBC correlations between C-1 and 2H-1’. The configuration of H-4 could be determined as pseudoaxial (4S) from the coupling constants of its neighbouring H-5 proton resonances ($\delta_H$ 2.22 and $\delta_H$ 2.23 had $J$ values of 4.6 and 5.3 Hz respectively) (Roumy et al., 2009). Irradiation of the H-3 resonance in a 1D NOE experiment did not show a positive correlation with the H-1’ resonances as seen in de Jesus Correia et al. (2001) and as such, the stereochemistry at this
position was made $6R$ in relation to the $4S$ stereocentre. Thus, compounds $B4a$ and $B4b$ had the relative configuration of $4S$, $6R$ and were identified as $4S,6R$-dihydroxy-$6$-$($12'($Z$)$)$-heptadecenyl)-2-cyclohexenone and $4S,6R$-dihydroxy-$6$-$($14'($Z$)$)$-nonadecenyl)-2-cyclohexenone respectively.

In comparison to $B3$, one of the oxygenated methine resonances in compound $B5$ moved more upfield to $\delta H 3.41$ (dd, $J = 10.5, 4.6$ Hz) and one pair of non-equivalent methylene resonances also moved more upfield in the region from $\delta H 1.5-2.0$ in comparison to $\delta H 2.62$ and $2.92$ in $B3$. There was also an additional methylene carbon resonance in the $^{13}C$ NMR spectrum at $\delta C 25.0$ with a concomitant disappearance of the carbonyl resonance which was evident in the $^{13}C$ NMR spectrum in $B3$ at $\delta 211.8$. The absence of the carbonyl stretching band was also noted in the IR spectrum. Further to this, the H-1' methylene carbon resonance was also shifted upfield to $\delta 32.0$ from $\delta 39.0$ in $B3$. These critical changes in the $^1H$ and $^{13}C$ NMR spectra were indicative that the carbonyl group at C-1 in compound $B3$ had now been reduced in compound $B5$ to a methylene group (C-5). The $^1H$ NMR resonances of the alkenyl side chain (H-2'-14', H15'/18', H-16'/17' and H-21') remained similar to that of $B3$ and was confirmed by mass spectrometry with a molecular ion peak at $m/z 424$. The structure was supported by HMBC correlations between C-4 and the two H-1' resonances and between C-1 and H-6. Similar to compound $B3$, the stereochemistry at C-1, C-3 and C-4 could not be determined from the NOESY data. Compound $B5$ was thus identified as 1,2,4-trihydroxy-4-[$16'($Z$)$)$-heneicosenyl]-cyclohexane.

**Antioxidant activity**

The antioxidant activity of the aromatic cardanols and the flavonoids were carried out and compared to ascorbic acid and was found to be in the order ascorbic acid $>$ epicatechin.
gallate (B12) > myricetin (B10) > myricetin-3-O-α-rhamnopyranoside (A3) = myricetin-3-O-β-galactopyranoside (B11) > cardanol (B1a-B1d) (g). In general the flavonoids had better activity than their glycosides. The cardanols had the worst activity. The most important features of flavonoids for optimum radical scavenging activity are the ortho-dihydroxy substitution of ring B, the Δ2 double bond conjugated to a C-4 carbonyl and the additional presence of both C-3 and C-5 hydroxyl groups (Croft, 1998). In addition to these myricetin has hydroxyl groups at C-5’ and C-7 which make it a good radical scavenger. The 3’,4’,5’-trihydroxy substitution pattern in ring B renders myricetin a more effective radical scavenging compound than other flavonoid aglycones with a different substitution pattern on ring B.

Table 3-1: Antioxidant activity of the flavonoids and flavonoid glycosides from Lannea rivae

<table>
<thead>
<tr>
<th>Compound</th>
<th>DPPH radical scavenging activity</th>
<th>Mean* Comp.</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc.</td>
<td>6.25 µg mL⁻¹</td>
<td>12.50 µg mL⁻¹</td>
<td>25.00 µg mL⁻¹</td>
</tr>
<tr>
<td>Cardanol (B1a-d)</td>
<td>13.18 ± 0.32</td>
<td>15.91 ± 0.08</td>
<td>26.63 ± 0.15</td>
</tr>
<tr>
<td>Myricetin-3-O-α-rhamnoside (A3)</td>
<td>30.26 ± 0.06</td>
<td>49.51 ± 0.51</td>
<td>61.74 ± 0.24</td>
</tr>
<tr>
<td>Myricetin-3-O-β-galactoside (B11)</td>
<td>31.26 ± 0.16</td>
<td>49.41 ± 0.41</td>
<td>60.84 ± 0.03</td>
</tr>
<tr>
<td>Myricetin (B10)</td>
<td>42.76 ± 0.54</td>
<td>62.40 ± 1.21</td>
<td>83.03 ± 0.02</td>
</tr>
<tr>
<td>Epicatechin gallate (B12)</td>
<td>45.93 ± 0.95</td>
<td>66.09 ± 0.88</td>
<td>86.72 ± 0.36</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>59.78 ± 0.41</td>
<td>73.77 ± 0.17</td>
<td>95.30 ± 0.06</td>
</tr>
<tr>
<td>Mean* Conc.</td>
<td>48.77a</td>
<td>53.90b</td>
<td>70.77c</td>
</tr>
</tbody>
</table>

*a* means that differ significantly have different letters, p < 0.05; mean conc. indicates the mean of all the compounds tested at a specific concentration; mean comp. indicates the mean of the compound tested at different concentrations.
Glycosidation of the C-3 hydroxyl group, as in the case of myricetin-3-O-α-rhamnoside and myricetin-3-O-β-galactoside lowered the antioxidant activity in comparison to the aglycones. This was consistent with the literature (Rice-Evans et al., 1996). Antioxidant activity of myricetin and its glycosides are known (Lee et al., 2011, Chaabia et al., 2008). Although epicatechin gallate lacks the Δ2 double bond and carbonyl group, the galloyl moiety at position 3 has a strong radical scavenging activity equal or superior to the ortho-dihydroxy group in the B-ring (Nanjo et al., 1996, Guo et al., 1999, Xu et al., 2004).

**Antibacterial activity**

Selected compounds including the flavonoids and flavonoid glycosides along with the crude hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) extracts of the stems and roots were tested for their antibacterial activity against both Gram positive and Gram negative bacterial strains. The hexane extracts exhibited intermediate antibacterial activity only against *E. faecalis* while the DCM extracts showed intermediate activity against both Gram positive bacteria *E. faecalis* and *S. aureus*, but no activity against gram negative bacteria (Table 3-2). The EtOAc and MeOH extracts demonstrated a broader spectrum of activity with better activity being observed with the gram positive bacteria.

Myricetin (B10) and epicatechin gallate (B12) exhibited good activity in both gram positive and gram negative bacteria, again with better activity against Gram positive bacteria. The activity of both B10 and B12 were comparable to the standard antibiotic erythromycin. B4a/B4b and B2 exhibited good antibacterial activity against the gram positive bacteria but weak activity against gram negative bacteria. None of the compounds or extracts tested demonstrated activity against *Salmonella typhimurium*. 
Table 3-2: Antibacterial activity (zones of inhibition in mm) of extracts and active compounds isolated from Lannea rivae

<table>
<thead>
<tr>
<th>Extract/ standard</th>
<th>Gram positive bacteria</th>
<th>Gram negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. faecalis ATCC 29212</td>
<td>S. aureus 25923</td>
</tr>
<tr>
<td>Hexane stem</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Hexane root</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Dichloromethane stem</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Dichloromethane root</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Ethyl acetate stem</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>Ethyl acetate root</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>Methanol stem</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Methanol root</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>B4a and B4b\a</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>B2\b</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>Myricetin (B10)</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td>Epicatechin gallate (B12)</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td>Penicillin</td>
<td>nd</td>
<td>34</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>nd</td>
<td>23</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>18</td>
<td>nd</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

\a 4,6-dihydroxy-6-(alkyl)-2-cyclohexenone mixture; \b 4,5-dihydroxy-4,5-furan-2'-[16'-(Z)-18'- (E)-heneicosyldiene]-cyclohex-2-enone; nd = not determined

The antibacterial properties of epicatechin gallate (B12) is known (Gibbons et al., 2004; Hamilton-Miller and Shah, 2000; Park et al., 2004; Sakanaka et al, 2000) and is associated with the presence of the gallic ester at C-3. There have been many reports of epicatechin gallate (B12) potentiating the effect of current antibiotics, especially β-lactams against multidrug resistant bacteria (Anderson et al., 2005; 2011; Gibbons, 2005; Park et al 2004; Shiota et al., 1999; Stapleton et al., 2004; 2006; 2007; Qin et al., 2013; Xiao et al., 2012). Galloylated catechins disrupts the bacterial membrane by intercalating within the hydrocarbon chains of the bacterial phospholipid palisade, thus affecting the physical properties of the membranes significantly (Shah et al., 2008; Catura et al., 2003). The
flavan-3-\textit{O}-gallates are more effective against gram positive than gram negative bacteria. The structure of the bacterial cell wall and different affinities of the catechin gallates with various cell wall components are responsible for the different susceptibilities (Ciu \textit{et al.} 2012, Yoda \textit{et al.} 2004).

Myricetin is active against several gram positive and gram negative bacteria, with MIC values ranging from 64-256 $\mu$g mL$^{-1}$ (D’Souza \textit{et al.}, 2010; Griep \textit{et al.}, 2007; Jayaraman \textit{et al.}, 2010; Liu and Matsuzaki, 1995; Xu and Lee, 2001). In the literature, myricetin was also shown to be inactive against \textit{Salmonella typhi} and \textit{Vibrio cholerae} (D’Souza \textit{et al.}, 2010). The good antibacterial activity is associated with the presence of a free B ring with a 3’,4’,5’-trihydroxy substitution pattern in addition to a free C-3 hydroxyl group. The mechanism of action of myricetin is proposed to involve inhibition of bacterial protein synthesis (Xu and Lee 2001).

\textbf{Cytotoxicity}

The cardanols (B1a-B1d) and the alkylated dihydroxycyclohexenones (B4a-B4b) did not show cytotoxicity at the concentrations tested i.e IC$_{50}$ > 100 $\mu$g mL$^{-1}$ (Table 3-3). The alkylated trihydroxycyclohexane (B5) and triterpenes taraxerone (B7) and taraxerol (B8) exhibited moderate cytotoxicity. However, 2,4,5-trihydroxy-2-[16’-(Z)-heneicosenyl]-cyclohexanone (B3) and 4,5-dihydroxy-4,5-furan-2’-[16’-(Z)-18’-(E)-heneicosenyldiene]-cyclohex-2-enone (B2) exhibited high cytotoxicity values. Cytotoxicity seems to be associated with the furan ring in B2 as the mixture of B4a and B4b although having an $\alpha$,\textbeta-unsaturated ketone moiety in the cyclohexenone ring similar to that in B2, did not show any toxicity at the concentrations tested. However, addition of water across the double bond in
**B4a** results in a more toxic compound **B3**, which could also be due to the added hydroxyl group.

**Table 3-3**: Cytotoxicity of *Lannea rivae* compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytotoxicity</th>
<th>IC$_{50}$ (µg/ml), n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardanol mixture (<strong>B1a-B1d</strong>)</td>
<td></td>
<td>&gt;100</td>
</tr>
<tr>
<td>4,5-dihydroxy-4,5-furan-2'-[16'-(Z)-18'-(E)-heneicosenyl]2-cyclohexeneone (<strong>B2</strong>)</td>
<td></td>
<td>1.93</td>
</tr>
<tr>
<td>2,4,5-trihydroxy-2-[16'-(Z)-heneicosenyl]-cyclohexanone (<strong>B3</strong>)</td>
<td></td>
<td>6.52</td>
</tr>
<tr>
<td>4,6-dihydroxy-6-(alkyl)-2-cyclohexenone mixture (<strong>B4a and B4b</strong>)</td>
<td></td>
<td>&gt;100</td>
</tr>
<tr>
<td>1,2,4-trihydroxy-4-[16'(Z)-heneicosenyl]-cyclohexane (<strong>B5</strong>)</td>
<td></td>
<td>56.5</td>
</tr>
<tr>
<td>Taraxerone (<strong>B7</strong>)</td>
<td></td>
<td>56.2</td>
</tr>
<tr>
<td>Taraxerol (<strong>B8</strong>)</td>
<td></td>
<td>42.2</td>
</tr>
<tr>
<td>Emetine</td>
<td></td>
<td>0.069</td>
</tr>
</tbody>
</table>

n = 3 indicates the number of replicates carried out.

Compounds related structurally to **B1a-B1d**, lanneaquinol and 2'('R)-hydroxylanneaquinol isolated from *Lannea welwitschii* exhibited modest cytotoxicity against human tumor cell lines (Groweiss *et al.*, 1997) and it may be worthwhile testing the mixture of **B1a-d** for anticancer activity. Compounds structurally similar to **B2** and **B4a and B4b**, 4,6,2'-trihydroxy-6-[10'(Z)-heptadecenyl]-1-cyclohexen-2-one (similar to **B4a-B4b**) and 1,4,6-trihydroxy-1,2'-epoxy-6-[10'(Z)-heptadecenyl]-2-cyclohexene (similar to **B2**) isolated from *Tapirira guianensis* (Roumy *et al.*, 2009) were also found to be cytotoxic and 4S,6S-dihydroxy-6-(14'-nonadecenyl)-2-cyclohexenone (an isomer of **B4b**) from *Tapirira obtusa* demonstrated cytotoxic activity against human cancer cell lines (de Jesus Correia *et al.*, 2001). The fact that **B4a-B4b** did not show toxicity must have to do with the specific stereochemistry of **B4a-B4b** or the fact that it is a mixture of the two compounds. It would be interesting to see whether the mixture **B4a-b** shows the same anticancer activity as that reported for the isomer of **B4b** in de Jesus Correia *et al.* (2001).
**Antiplasmodial activity**

Compounds containing the cyclohexenone and cyclohexanone moieties, \textbf{B2, B3} and \textbf{B4a-B4b}, as well as the flavonoids, myricetin (\textbf{B10}) and epicatechin gallate (\textbf{B12}) exhibited good antiplasmodial activity against both chloroquine sensitive and chloroquine resistant strains, with the cyclohexenone compounds \textbf{B2} and \textbf{B4a-B4b} showing better activity than the cyclohexanone compound (\textbf{B3}) and the flavonoids \textbf{B10} and \textbf{B12} (Table 3-4). The flavonoid glycosides, myricetin-3-\textit{O}-\textalpha{-rhamnoside (A3)} and myricetin-3-\textit{O}-\textbeta{-galactoside (B11)} did not exhibit any antiplasmodial activity. Even though \textbf{B2} and \textbf{B3} had good antiplasmodial activity, its cytotoxicity was higher than the other compounds, although it was not as toxic as the reference drug emetine. The most promising lead amongst these compounds is the mixture, \textbf{B4a-b}, which had good antiplasmodial activity as well as low cytotoxic values.

Compounds with structures closely resembling \textbf{B2} and \textbf{B4a-b} have also demonstrated good antiplasmodial activity (Roumy \textit{et al.}, 2009). The mode of activity of such compounds is not known but is thought to be related to the unsaturated ketone moiety, which acts as a Michael acceptor (Roumy \textit{et al.}, 2009). The antiplasmodial activity of epicatechin gallate (\textbf{B12}) against both chloroquine sensitive and resistant strains is consistent with that in the literature (Tasdemir \textit{et al.}, 2006; Sannella \textit{et al.}, 2007). This activity is associated with the galloyl ester substituent as catechin itself is not active. Myricetin's (\textbf{B10}) antiplasmodial activity is also consistent with the literature (Tasdemir \textit{et al.}, 2006; Lehane and Saliba, 2008). Hydroxy substitution at two or more sites on the phenyl ring B of a flavone structure increases the antiplasmodial activity with the pyrogallol moiety (3',4',5'-trihydroxy) in ring B increasing selectivity toward the FabI enzyme of \textit{P. falciparum} (Tasdemir \textit{et al.}, 2006).
Table 3-4: Antiplasmodial activity of active compounds from *Lannea rivae*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (µg/ml) D6 (CQ resistant clone)</th>
<th>IC$_{50}$ (µg/ml) W2 (CQ sensitive clone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,5-dihydroxy-4,5-furan-2'-[16'-(Z)-18'-(E)-heneicosenyldiene]-cyclohex-2-enone (B2)</td>
<td>0.484±0.054</td>
<td>0.430±0.084</td>
</tr>
<tr>
<td>2,4,5-trihydroxy-2-[16'-(Z)-heneicosenyl]-cyclohexanone (B3)</td>
<td>2.055±0.124</td>
<td>1.406±0.173</td>
</tr>
<tr>
<td>4S,6R-dihydroxy-6-(12'(Z)-heptadecenyl)-2-cyclohexenone (B4a/B4b) (10'(Z) isomer)</td>
<td>1.039±0.139</td>
<td>0.826±0.066</td>
</tr>
<tr>
<td>Myricetin (B10)</td>
<td>4.638±0.280</td>
<td>7.503±0.517</td>
</tr>
<tr>
<td>Epicatechin gallate (B12)</td>
<td>2.787±0.341</td>
<td>2.106±1.97</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.07626</td>
<td>0.00443</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>0.0374</td>
<td>0.01217</td>
</tr>
</tbody>
</table>

3.3 Experimental

General experimental procedures

Reagents and chemicals used in this study were purchased from Merck, South Africa and all organic solvents were redistilled and dried according to standard procedures before being used. Optical rotation was recorded using a Perkin Elmer™, Model 341 Polarimeter with a 10 cm flow tube. The melting points of the isolated compounds were recorded on an Ernst Leitz Wetziar micro-hot stage melting point apparatus and are uncorrected. NMR spectra were recorded using CDCl$_3$, CD$_3$OD or DMSO-d$_6$ on a Bruker Avance™ 400 MHz spectrometer at room temperature with chemical shifts (δ) recorded against tetramethylsilane (TMS), the internal standard. IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer with universal ATR sampling accessory. UV spectra were obtained on a Varian Cary UV-VIS Spectrophotometer in chloroform. For GC-MS analyses, the samples were analysed on an Agilent MS 5973 instrument connected to a GC 6890 equipped with a DB-5SIL MS (30 m x 0.25 mm i.d., 0.25 µm film thickness) fused-silica capillary column using helium as a carrier gas with a flow rate of 2 mL min$^{-1}$. The MS was operated in the EI mode at 70 eV. Chromatographic separations were carried out by column chromatography.
(CC) using silica gel 60 (40–63 µm, Merck 1.09385) and analytical TLC was performed on pre-coated silica gel 60 F254 plates (Merck 1.05554) and developed by spraying with anisaldehyde:H2SO4:MeOH (1:2:97 v/v) followed by heating.

**Plant material**

The leaves, roots and stem bark of *Lannea Rivae* (Chiov) Sacleux were collected from Wote town, Makueni District, Kenya, and was authenticated and deposited at Maseno University Botanic Gardens herbarium and assigned a voucher number MSU/BG-2/13. The plant material was air-dried under shade for 14 days and thereafter ground using a Wiley laboratory mill available at Kibos sugar company- Kisumu.

**Extraction and Isolation**

The n-hexane root extract (59.57 g) was packed on a silica gel open column (700 g, 80 mm column diameter) and eluted with a gradient solvent system from 100% n-hexane to 50:50 n-hexane:ethyl acetate, collecting 10 × 100 mL fractions before increasing the polarity by 10% at each stage. A total of 60 fractions were collected and combined based on their TLC profiles. Fraction 6-13 (356.91 mg) was repacked on a smaller column (40 mm diameter) and eluted with 500 mL of n-hexane followed by 5% ethyl acetate in hexane, collecting 50 mL fractions. Fraction 6-10 yielded a yellow liquid and contained a mixture of four cardanols (B1a-B1d) (167.93 mg), fraction 17-21 white crystals of taraxerone (B7) (112.91 mg), fractions 23-28 tiny crystals of taraxerol (B8) (131.20 mg) and fraction 30-45 white needle like crystals of sitosterol (A6 – see chapter 2) (77.89 mg). All these compounds were purified using 5% ethyl acetate in n-hexane on smaller columns. Despite several attempts on silica and sephadex, the cardanols B1a-B1d could not be separated. The cardanols (B1a-
B1d) (34.63 mg), taraxerone (B7) (18.95 mg), taraxerol (B8) (32.76 mg) and sitosterol (A6) (38.66 mg) were also obtained from the hexane extract (20.08 g) of the leaves.

The ethyl acetate extract (69.95 g) was chromatographed as above using a n-hexane:ethyl acetate step gradient increasing the polarity by 10% for each step until 100% ethyl acetate was reached. At each stage 10 × 100 mL fractions were collected. Sitosterol (A6) (345.65 mg) was isolated from fraction 32-41 of the ethyl acetate crude extracts and needed no further purification. Fraction 44-76 (419.68 mg) from the crude column was rechromatographed with 20% and 30% ethyl acetate in n-hexane respectively collecting 20 × 50 mL fractions at each stage. Fraction 24-30 yielded a reddish brown paste which contained a mixture of two compounds (224.67 mg) (B4a and B4b). This too, despite several attempts on silica and sephadex, could not be separated. Fraction 80-90 (173.34 mg) of the crude column was further purified with 2L of n-hexane:ethyl acetate (1:1) collecting 40 × 50 mL fractions. A reddish brown paste (90.11 mg) (B3) was obtained from fraction 13-16 and purified with the same solvent system on silica gel.

The methanol extracts of the roots (64.21g) was chromatographed with 2 L of ethyl acetate (20 × 100 mL) followed by 2 L of 5% methanol in ethyl acetate (20 × 100 mL) on a silica gel column. Fraction 6-15 (589.45 mg) of this crude separation was packed on a LH-20 sephadex (30 g) column and eluted with 100% methanol, collecting 10 mL fractions. Fraction 8-18 contained sitosterol glucoside (B6), a white amorphous solid (295.06 mg) and a yellow crystalline solid, myricetin rhamnopyranoside (A3 – see chapter 2) (111.18 mg). These two compounds (B6 and A3) were obtained similarly from the stem bark extracts.
The ethyl acetate extract (56.33 g) from the leaves was chromatographed similarly to the ethyl acetate root extract above. Based on their TLC profiles, the fractions were combined into three larger fractions 30-60 (464.56 mg), 70-80 (217.67 mg) and 80-88 (274.44 mg). Fraction 30-60 was rechromatographed on sephadex LH-20 and eluted with methanol, collecting 10 mL fractions. Fractions 6-10 and 13-14 yielded 13.14 mg of red solid, all E-lutein (B9) and 216.22 mg of a yellowish brown paste (B2). Fraction 70-80, which was also eluted on sephadex in a similar way yielded an amorphous white solid, sitosterol glucoside (B6) (78.55 mg) and two yellow solids, myricetin-3-\(\alpha\)-rhamnopyranoside (A3) (77.2 mg) and myricetin (B10) (98.92 mg) from fractions 16-24, 30-37 and 40-45 respectively. Purification of fraction 80-88 with sephadex resulted in the isolation of a reddish brown paste, myricetin-3-\(\beta\)-galactopyranoside (B11) (39.18 mg) and epicatechin gallate (B12) (149.89 mg).

3-[nonadec-14'(Z)-enyl]-phenol (B1a) isolated as a yellow liquid mixture with B1b-B1d; UV (MeOH) \(\lambda_{\text{max}}\) (log \(\varepsilon\)) 273, 216 nm; IR (\(\nu_{\text{max}}\)) (neat) cm\(^{-1}\) 3354, 2922, 2852, 1589, 1456, 1265, 1154; EIMS \(m/z\) (rel. int.): B1a 358 (24) [M\(^+\)], 121 (12), 120 (17), 108 (100) [C\(_7\)H\(_8\)O]; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta_H\) 7.11 (1H, t, \(J = 7.7\) Hz, H-5), 6.73 (1H, d, \(J = 7.7\) Hz, H-4), 6.63 (1H, s, H-2), 6.61 (1H, d, \(J = 2.1\) Hz, H-6), 5.33 (2H, t, \(J = 4.5\) Hz, H-14'/15'), 2.54 (2H, t, \(J = 5.6\) Hz, H-1'), 1.99 (4H, m, H-13'/16'), 1.57 (2H, t, \(J = 6.7\) Hz, H-2'), 1.29 (4H, m, H-17'/18'), 1.20-1.26 (20H, m, H-3'-12'), 0.86 (3H, t, \(J = 6.1\) Hz, H-19'); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta_C\) 155.5 (C-1), 145.0 (C-3), 129.9 (C-15\(^\text{a}\)), 129.8 (C-14\(^\text{a}\)), 129.3 (C-5), 120.9 (C-4), 115.3 (C-2), 112.5 (C-6), 35.8 (C-1'), 32.0 (C-17'), 31.3 (C-2'), 29.3-29.9 (C-3'-12'), 27.2 (C-13'), 26.9 (C-16'), 22.4 (C-18'), 14.0 (C-19'). \(^a\)Assignments may be interchanged.
4,5-dihydroxy-4,5-furan-2'-[16'(Z),18'(E)-nonadecadienyl]-cyclohex-2-enone (B2) yellowish oil; $[^{20}\alpha]D^2 -42.86^\circ$ (c = 1.05, CH$_2$Cl$_2$); UV (CHCl$_3$) $\lambda_{max}$ (log $\varepsilon$) 268 (1.28), 221 (3.02) nm. IR (v$_{max}$) (neat) cm$^{-1}$: 3383, 2923, 2853, 1679, 1456, 1373, 1130, 1063; EIMS m/z (rel. int.): 432 (4), 360 (33), 342 (8), 124 (100); $^1$H NMR (400 MHz, CDCl$_3$) $\delta_H$ 6.71 (1H, d, $J = 10.2$ Hz, H-3), 6.27 (1H, dt, $J = 15.1, 10.8$ Hz), 5.92 (1H, dd, $J = 10.8, 10.2$ Hz, H-17'), 5.91 (1H, d, $J = 10.2$ Hz, H-2), 5.69 (1H, dt, $J = 15.1, 6.6$ Hz, H-19'), 5.24-5.32 (1H, m, H-16'), 4.37-4.41 (1H, m, H-2'), 2.81 (1H, d, $J = 16.0$ Hz, H-6a), 2.66 (1H, d, $J = 16.0$ Hz, H-6b), 2.19 (1H, dd, $J = 13.2, 6.1$ Hz, H-1'a), 2.05-2.15 (4H, m, H-15'/20'), 1.66 (1H, dd, $J = 13.2, 9.4$ Hz, H-1'b), 1.57-1.61 (1H, m, H-3'a), 1.34-1.36 (1H, m, H-3'b), 1.20-1.40 (22H, m, H-4'-14'), 1.00 (3H, t, $J = 7.5$ Hz, H-21'); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta_C$ 196.9 (C-1), 146.1 (C-3), 136.1 (C-19'), 130.1 (C-16'), 128.6 (C-17'), 126.8 (C-2), 124.7 (C-18'), 99.5 (C-4), 78.7 (C-5), 77.0 (C-2'), 47.0 (C-6), 43.6 (C-1'), 36.0 (C-3'), 29.2-29.7 (C-4'-C13'), 27.7 (C-15'), 25.9 (C-20'), 25.6 (C-14'), 13.7 (C-21').

2,4,5-trihydroxy-2-[heneicos-16'(Z)-enyl]-cyclohexanone (B3) reddish brown residue; $[^{20}\alpha]D^2 +30.48^\circ$ (c = 1.05, CH$_2$Cl$_2$); UV (CHCl$_3$) $\lambda_{max}$ (log $\varepsilon$) 240 (3.28) nm. IR (v$_{max}$) (neat) cm$^{-1}$: 3384, 2918, 2850, 1724, 1465, 1078; EIMS m/z (rel. int.): 420 (10) [M-H$_2$O]$^+$, 402 (8) [M-2xH$_2$O]$^+$, 293 (18), 185 (30), 167 (34), 142 (76), 99 (100); $^1$H NMR (400 MHz, CDCl$_3$) $\delta_H$ 5.32 (2H, t, $J = 4.7$ Hz, H-16'/17'), 4.07-4.09 (1H, m, H-5), 4.02-4.05 (1H, m, H-4), 2.99 (1H, dd, $J = 13.9, 3.8$ Hz, H-6a), 2.62 (1H, dd, $J = 13.9, 5.7$ Hz, H-6b), 2.16 (1H, dd, $J = 14.5, 4.0$ Hz, H-3a), 2.00 (1H, m, H-3b), 1.91-2.04 (4H, m, H-15'/18'), 1.92-1.94 (1H, m, H-1'a), 1.68 (1H, td, $J = 12.4, 4.1$ Hz, H-1'b), 1.27-1.29 (4H, m, H-19'/20'), 1.21-1.30 (26H, m, H-2'-H-14'), 0.86 (3H, t, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta_C$ 211.8 (C-1), 129.9 (C-16'), 129.8 (C-17'), 78.1 (C-2), 74.2 (C-5), 69.8 (C-4), 42.0 (C-6), 41.4 (C-3), 38.9 (C-1'), 32.0
(C-19'), 29.3-29.9 (C-2'-13'), 27.2 (C-15'), 26.9 (C-18'), 23.2 (C-14'), 22.3 (C-20'), 14.0 (C-21'). Assignments may be interchanged.

4S,6R-dihydroxy-6-[12'(Z)-heptadecenyl]-cyclohex-2-enone (B4a); isolated as a reddish brown oily mixture with B4b; \([\alpha]^{20}_D + 30.95^\circ (c = 1.05, \text{CH}_2\text{Cl}_2); \text{UV (CHC}_{13}\) \(\lambda_{\text{max}} (\log \epsilon) 242 \text{ nm}. IR (\nu_{\text{max}}) \text{(neat)} \text{cm}^{-1}; 3393, 2922, 2852, 1678, 1464, 1377, 1033; \text{EIMS } m/z \text{(rel. int.): 364(5) [M^+]}, 346 [M^+\text{-H}_2\text{O}] (14), 84 (100); ^1\text{H NMR (400 MHz, CDCl}_3) \delta H 6.83 (1H, dd, \(J = 10.1, 3.7 \text{ Hz, H-3}), 6.01 (1H, dd, \(J = 10.1, 0.8 \text{ Hz, H-2}), 5.33 (2H, t, J = 4.5 \text{ Hz, H-12'/13'}), 4.65-4.67 (1H, m, H-4), 2.22 (1H, dd, \(J = 6.2, 4.7 \text{ Hz, H-5}), 1.95-2.03 (4H, m, H-11'/14'), 1.73-1.77 (2H, m, H-1'), 1.27-1.32 (4H, m, H-15'/16'), 1.21-1.29 (18H, m, H-2'-H-10'), 0.86 (3H, t, J = 7.0 \text{ Hz, H-17'}); ^13\text{C NMR (100 MHz, CDCl}_3) \delta C 201.2 (C-1), 149.2 (C-3), 129.9 (C-12'), 129.8 (C-13'), 126.7 (C-2), 74.6 (C-6), 64.2 (C-4), 41.1 (C-5), 39.2 (C-1'), 32.0 (C-15'), 29.3-29.9 (C-1'-C-10'), 27.2 (C-11'a), 26.9 (C-14'a), 23.0 (C-2'), 22.3 (C-16'), 14.0 (C-17'). The assignments refer to the numbering system of B4a; Assignments may be interchanged.

1,2,4-trihydroxy-4-[16'(Z)-heneicosenyl]-cyclohexane (B5); reddish brown residue; \([\alpha]^{20}_D + 2.85^\circ (c = 1.05, \text{CH}_2\text{Cl}_2); \text{UV (CHC}_{13}\) \(\lambda_{\text{max}} (\log \epsilon) 229 (2.12) \text{ nm}. \text{IR (}\nu_{\text{max}}) \text{(neat) cm}^{-1}; 3382, 2921, 2852, 1457, 1056; \text{EIMS } m/z \text{(rel. int.): 424 (13) [M^+]}, 393 (8), 365 (100), 187 (42), 108 (57); ^1\text{H NMR (400 MHz, CDCl}_3) \delta H 5.32 (2H, t, J = 5.0 \text{ Hz, H-16'/17'}), 4.01 (1H, bs, H-2), 3.41 (1H, dd, \(J = 10.5, 4.6 \text{ Hz, H-1}), 1.98-2.02 (4H, m, H-15'/18'), 1.96-1.98 (1H, m, H-3), 1.86 (1H, m, H-5), 1.84 (1H, m, H-1'), 1.62 (1H, m, H-5), 1.53 (1H, m, H-6), 1.48 (1H, m, H-1'), 1.45 (1H, m, H-3), 1.20-1.30 (30H, m, H-2'-H-14', H-19'/20'), 0.86 (3H, t, J = 6.8 Hz); ^13\text{C NMR (100 MHz, CDCl}_3) \delta C 129.84 (C-16'a), 129.90 (C-17'a), 75.4 (C-4), 72.8 (C-1), 66.9 (C-2), 25.0 (C-6), 38.8 (C-6), 38.3 (C-3), 32.0 (C-1'), 31.2 (C-19'), 29.3-30.2 (C-19').
2'-C-13'), 27.2 (C-15'), 26.9 (C-18'), 25.0 (C-5), 23.4 (C-14'), 22.3 (C-20'), 14.0 (C-21').

*aAssignments may be interchanged.

**Antioxidant activity**

A 10 mg mL⁻¹ stock solution of selected compounds (Table 3-1) thought to have the potential to have antioxidant activity was made by dissolving the compounds in DMSO. Sample concentrations of 6.25, 12.50, 25.00, 50.00 and 100.00 µg mL⁻¹ were made in methanol. The DPPH radical scavenging activity of the compounds was determined according to the method of Kumawat *et al.*, (2012). Briefly, 1 mL of each compound was added to 1 mL of DPPH (1,1-diphenyl-2-picrylhydrazyl) solution (0.1 mM in methanol). The mixture was shaken and kept in the dark for 30 minutes at room temperature. The decrease in absorbance was determined at 517 nm. Vitamin C (ascorbic acid) was used as the positive control. The DPPH radical scavenging activity was calculated using the following formula:

\[
\text{DPPH Radical Scavenging Activity (\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100,}
\]

where \(A_0\) is the absorbance of the control and \(A_1\) is the absorbance of the compound or standard sample.

All the tests were performed in triplicate. The results were given as means ± S.D. analysis of variance and significant differences among means were tested by two way ANOVA, using SPSS (Version 12.0 for Windows, SPSS Inc., Chicago, IL, USA). When significant main effects existed, differences were tested by Duncans test at 95% confidence. The \(IC_{50}\) values were calculated from dose-response curves, using non-linear dose-response curve fitting analysis GraphPad Prism (Version 5) software.
Disc diffusion antibacterial assays.

The antibacterial activity was conducted by the agar diffusion assay on all isolated compounds as well as the extracts of different parts of the plant. Briefly, filter paper discs (6 mm in diameter) impregnated with sample solutions were placed on Mueller Hinton agar plates (BBL, Becton Dickinson and Co., Cockeysville, MD), which have been inoculated with test organisms (Staphylococcus aureus ATCC 25923, Enterococcus faecalis 29212, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853 and Salmonella typhimurium ATCC 13311) according to the standard protocol described by the National Committee of Clinical Laboratory Standards (2008). An amount of 100 µg of sample was tested on each disc. The plates were incubated at 35 °C and the diameters of the inhibition zones were measured after 24 h. Filter paper discs containing DMSO without any test compound served as a control and no inhibition was observed. Commercial penicillin (10 units), erythromycin (15 µg), cefuroxime (30 µg), nalidixic acid (30 µg), vancomycin (30 µg) and ciprofloxacin discs (5 µg) were included as a positive control.

SYBR Green I antiplasmodial assay

The SYBR Green I assay was used to screen the antiplasmodial activity of all isolated compounds against two Plasmodium falciparum strains, chloroquine sensitive (D6) from Sierra Leone and chloroquine resistant (W-2) from Vietnam. The cultures were maintained at the US Army Medical Research Unit in the Malaria Resistance Laboratories at the Kenya Medical Research Institute (KEMRI), Kisian-Kisumu, according to protocols in the literature (Smilkstein et al., 2004). Mefloquine and chloroquine reference drugs were used as a positive control. The culture medium was prepared as described by Johnson et al. (2007). Mefloquine was dissolved in 70% ethanol while the compounds and chloroquine were dissolved in 100% DMSO, to an initial concentration of 1 mg mL⁻¹. The P. falciparum
cultures were adjusted to 2% haematocrit and 1% parasitaemia for the assay (Akala et al. 2011), a modification of Desjardins et al. (1979) and Trager and Jensen (1976). After 72 h incubation, 100 µL of lysis buffer containing SYBR Green I dye was added to the 96-well plates prior to 1 h incubation in the dark. Five replicates were conducted for each test compound. The fluorescence was read using a Genios Tecan® micro-plate reader. IC₅₀ values were then calculated by Graphpad Prism (Graphpad Prism for Windows, version 5.0; Graphpad Software, Inc., San Diego, CA).

Cytotoxicity assay

In vitro cytotoxicity of compounds B1-B5, B7 and B8 were tested against a mammalian cell-line, Chinese Hamster Ovarian (CHO). Only these compounds were chosen for this assay as compounds similar in structure demonstrated activity in similar cytotoxicity assays (Chaturvedula et al., 2004; David et al., 1998; Gachet et al., 2011; Groweiss et al., 1997; Kapche et al., 2007; Roumy et al., 2009). This was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays (Mosmann et al., 1983 and Rubinstein et al., 1990). The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The test samples were tested in triplicate on one occasion. The test samples were prepared to a 20 mg mL⁻¹ stock solution in 100% DMSO and were tested as a suspension if not properly dissolved. Test compounds were stored at -20 ºC until used. Emetine was used as the reference drug in all experiments. The initial concentration of test samples was 100 µg mL⁻¹. This was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 1 ng mL⁻¹. The same dilution technique was applied to all the test samples. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability. The
50% inhibitory concentration (IC\textsubscript{50}) values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

3.4 Conclusions

The \textit{in vitro} activity of the isolated compounds and some crude extracts reported here support the reports by traditional healers that \textit{Lannea rivae} extracts are effective in disease management. Nevertheless \textit{in vivo} studies are recommended to further justify these claims. Six new cytotoxic compounds are reported here with \textbf{B1a} occurring in a mixture along with known compounds and \textbf{B4a} and \textbf{B4b}, two of the novel compounds, also being isolated as a mixture with each other. The cyclohexane moiety of compounds \textbf{B3}, \textbf{B4a-b} and \textbf{B5} can all be chemically transformed into each other, \textbf{B3} being dehydrated to \textbf{B4a-b} and reduced to \textbf{B5}. Some of the novel compounds showed good antibacterial and antiplasmodial activity in conjunction with low cytotoxicity and could be good lead compounds for antibiotics and antiplasmodial drugs. These types of novel compounds have only been reported in the genus \textit{Tapirira}, which shows a close biosynthetic link between \textit{Lannea} and \textit{Tapirira}.

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Chapter 4 Cardanols, long chain cyclohexenones and cyclohexenols from *Lannea schimperi* (Hochst. Ex. A. Rich.) (Anacardiaceae)

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Abstract

Cardanols (C1a-C1d), alkenyl cyclohexenones (C2a-C2d) and alkenyl cyclohexenols (C3a-C3c and C4a-C4c) were isolated from the stem bark and root of Lannea schimperi. The cardanols C1a and C1d and the alkenyl cyclohexenols (C2a and C2d) have side chains which have not been reported previously in combination with the core skeletal structures. In addition, compounds C3a-C3c and C4a-C4c are all novel cyclohexenols. Also isolated were the triterpenes, sitosterol (A6), taraxerone (B7) and taraxerol (B8). The suite of compounds isolated (cyclohexenones and cyclohexenols) make up a nice biosynthetic pathway to the cardanols. The 5-[alkenyl]-4,5-dihydroxycyclohex-2-enone mixture (C2a-C2d) exhibited good in vitro cytotoxicity against the Chinese Hamster Ovarian mammalian cell-line. The compounds were identified by mainly GC-MS and NMR spectroscopic techniques.

Key words: Lannea schimperi, cardanols, alkenyl cyclohexenones, alkenyl cyclohexenols, taraxerol, taraxerone, sitosterol, cytotoxicity.
4.1 Introduction

The family Anacardiaceae is widely known for causing skin irritations, inflammation and blistering in sensitive individuals. Members of the family that are important in dermatology are the *Toxicodendron* genus (common poison ivy, poison oak, poison sumac), *Mangifera indica* L. (mango), *Toxicodendron vernicifluum* (lacquer tree) and *Anacardium occidentale* L. (cashew nut) (Rosen and Fordice, 1994; Hamilton and Zug, 1998; Oka *et al*., 2004; Gladman, 2006; Hirao *et al* 2008; Thoo and Freeman 2008). The active ingredients in these plants are mixtures of homologous long chain phenolic compounds collectively known as ‘urushiol’. They are mono- or dihydroxybenzene derivatives of phenol (cardanols, catechols or resorcinols) or salicylic acid with a long alkyl or alkenyl carbon side chain (Evans and Schmidt, 1980; Stahl *et al*., 1983). The phenols vary in the number and position of hydroxyl groups, in their alkyl chain length and in the number and position of double bonds in this chain. Phenolic lipids are also potentially useful in the treatment of cancer and skin diseases (Stasiuk and Kozubek, 2010). They can also be used as starting materials in the semi-synthesis of compounds for various biological activities such as long lasting hydrophobic anti-inflammatory drugs or analogues of cannabinoids (Kozubek and Tyman 1999).

Cardanol is used in a wide range of technological applications such as in friction dusts for brake lining and clutch facings, and in polymer chemistry to form soft resins that are resistant to acids and bases. Derivatives of cardanol, cardol and anacardic acids have novel applications in dyes, pharmaceutical antioxidants and monomers for polymerization (Tyman, 1979). Plant species within the Anacardiaceae synthesize these toxic phenols to interact with their environment and ward off insect pests and microbes, to deter herbivores grazing from their leaves or to prevent seeds of other species from germinating in their proximity (Grayer, 2005).
*Lannea schimperi* occurs in tropical Africa. The bark of the roots and stem as well as the leaves of the plant are used medicinally to clean teeth and manage toothache, for diarrhea, chest infections, stomach pains, mental disorders, epilepsy, snake bites, tuberculosis, skin infections, herpes simplex, herpes zoster and other opportunistic infections resulting from HIV/AIDS (Verzar and Petrii, 1987; Ruffo, 1991; Kisangau *et al*., 2007; Jeruto *et al*., 2008; Lulekal *et al*., 2008; Kokwaro, 2009). Crude methanol and water extracts of the plant exhibited cytotoxic and antifungal activity (Moshi *et al*., 2006; Kisangau *et al*., 2009). No phytochemical studies appear to have been done on this plant. Therefore, the stem and root bark were analyzed for their phytochemical constituents.

4.2 Results and discussion

The hexane and ethyl acetate extracts led to isolation of the cardanols (C1a-C1d), the dihydroxycyclohexenones (C2a-C2d), the cyclohexene triols (C3a-C3c) and the cyclohexene diols (C4a-C4c) (Table 4-1; Figure 4-1). All the compounds were alkylated on the cyclohexane ring and contained a double bond three bonds away from the end of the chain. Of these alkylated cyclohexenes and cardanols, only the cardanols C1b and C1c and the dihydroxycyclohexenones (C2b and C2c) were known (Queiroz *et al*., 2003). All the other cyclohexenes and cardanols were novel. Also isolated from the plant were the known triterpenes sitosterol (A6), taraxerol (B8) and taraxerone (B7). The known triterpenes were identified by comparison of their spectral data with those in the literature (Sakurai *et al*., 1987; Kovganko *et al*., 1999; Mejin, 2009).
Compounds C1a-C1d all contained the same phenol structural moiety but differed in the length and nature of the alkyl chain. These compounds were isolated as a mixture of four compounds, with chain lengths varying from C-15 to C-21. Compounds C1a-C1d contained monounsaturated C-15, C-17, C-19 and C-21 alkyl chains. The structural elucidation was briefly discussed in Franke et al. (2001) and the $^1$H and $^{13}$C NMR data are the same as that published in Queiroz et al. (2003) for C1b and C1c isolated from Lannea edulis, however we have interchanged the assignments of C-18' and C-15' based on a HMBC correlation between C-18' and the methyl group H-19' (the numbering system refers to C1c). The C-18' resonance is now at $\delta_C$ 25.6 and the C-15' resonance at $\delta_C$ 32.6. We have also confirmed the assignments of the aromatic ring by HMBC correlations between H-1' and C-2, C-3 and C-4. The H-5, H-6 and H-2 proton resonances also showed HMBC correlations to C-1. The H-5 resonance, which appeared as a triplet ($J = 7.7$ Hz) due to coalescing of the dd resonance, was
placed between H-4 (d, J = 7.7 Hz) and H-6 (overlapping doublet with H-2) because of their splitting patterns. The names of the compounds, their molecular formulae and their molecular ion peaks as identified in the mass spectrum are contained in Table 4-1. Both C1a and C1d, the pentadecenyl and the heneicosenyl derivative respectively are novel and have not been reported previously.

Compounds C2a-d occurred as a mixture of four 4-alkylated-4,5-dihydroxycyclohex-2-enones with C-15, C-17, C-19 and C-21 side chains. All the side chains had a double bond situated three bonds away from the terminal methyl group, since the olefinic carbon resonance in the side chain had a HMBC correlation with the terminal methyl group. Compounds C2a-C2d are listed in Table 4-1 and were able to be identified based on their molecular ion peaks as detected in the EIMS. The $^1$H and $^{13}$C NMR data for the mixture of compounds are the same as that published in Queiroz et al. (2003), where the structural elucidation is also discussed. We have however swopped around the assignments of H-2 and H-3 (now 6.88 for H-2 and 6.01 for H-3) as well as C-2 (now 152.8) and C-3 (now 125.8) based on HMBC correlations between C-2 and H-6$_{ax}$/H-6$_{eq}$ as well as between C-5 and H-3. Compounds C2a and C2d are novel and have not been reported previously.

The $^1$H NMR spectrum of compounds C3a-C3c showed a pair of olefinic resonances at $\delta_H$ 5.81 (dd, $J = 10.2, 1.6$ Hz) and 5.57 (dd, $J = 10.2, 2.0$ Hz) indicating a cis double bond. Two other oxygenated methine protons could be seen as broad singlets at $\delta_H$ 4.02 and $\delta_H$ 4.46 respectively and corresponded to the oxygenated methine carbon resonances at $\delta_C$ 65.5 and 70.0. There was also an additional oxygenated carbon resonance at $\delta_C$ 74.3. The double bond and the three oxygenated carbons takes up five carbon atoms of the cyclohexene ring with the last carbon in the ring being a methylene carbon atom at $\delta_C$ 40.8. This corresponded
to two non-equivalent proton resonances at $\delta_H$ 2.24 (dd, $J = 5.5$, 13.4 Hz, H-6a) and $\delta_H$ 1.42 (dd, $J = 9.3$, 13.2 Hz, H-6b).

**Table 4-1** Compounds isolated from *Lannea schimperi*

<table>
<thead>
<tr>
<th></th>
<th>Name</th>
<th>Molecular formula</th>
<th>EIMS (m/z (rel. int.))</th>
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<tbody>
<tr>
<td>C1a</td>
<td>3-[12'($E$)-pentadecenyl]phenol</td>
<td>C$<em>{21}$H$</em>{34}$O</td>
<td>302 [M$^+$] (11), 120 (16), 108 (100)</td>
</tr>
<tr>
<td>C1b</td>
<td>3-[14'($E$)-heptadecenyl]phenol</td>
<td>C$<em>{23}$H$</em>{38}$O</td>
<td>330 [M$^+$] (19), 147 (7), 120 (19), 108 (100)</td>
</tr>
<tr>
<td>C1c</td>
<td>3-[16'($E$)-nonadecenyl]phenol</td>
<td>C$<em>{25}$H$</em>{42}$O</td>
<td>358 [M$^+$] (17), 147 (6), 133 (5), 120 (18), 108 (100)</td>
</tr>
<tr>
<td>C1d</td>
<td>3-[18'($E$)-heneicosenyl]phenol</td>
<td>C$<em>{27}$H$</em>{46}$O</td>
<td>386 [M$^+$] (19), 147 (5), 133 (4), 120 (14), 108 (100)</td>
</tr>
<tr>
<td>C2a</td>
<td>5-[12'($E$)-pentadecenyl]-4,5-dihydroxycyclohex-2-enone</td>
<td>C$<em>{21}$H$</em>{36}$O$_3$</td>
<td>336 [M$^+$] (4), 318 (13), 237 (11), 123 (11), 95 (19), 84 (100)</td>
</tr>
<tr>
<td>C2b</td>
<td>5-[14'($E$)-heptadecenyl]-4,5-dihydroxycyclohex-2-enone</td>
<td>C$<em>{23}$H$</em>{40}$O$_3$</td>
<td>364 [M$^+$] (4), 346 (21), 265 (16), 123 (14), 95 (18), 84 (100)</td>
</tr>
<tr>
<td>C2c</td>
<td>5-[16'($E$)-nonadecenyl]-4,5-dihydroxycyclohex-2-enone</td>
<td>C$<em>{25}$H$</em>{44}$O$_3$</td>
<td>392 [M$^+$] (4), 374 (21), 293 (14), 123 (14), 95 (18), 84 (100)</td>
</tr>
<tr>
<td>C2d</td>
<td>5-[18'($E$)-heneicosenyl]-4,5-dihydroxycyclohex-2-enone</td>
<td>C$<em>{27}$H$</em>{48}$O$_3$</td>
<td>420 [M$^+$] (2), 402 (10), 346 (20), 265 (7), 179 (13), 12 (46), 95 (48), 84 (100)</td>
</tr>
<tr>
<td>C3a</td>
<td>1-[12'($E$)-pentadecenyl]-cyclohex-3-en-1,2,5-triol</td>
<td>C$<em>{21}$H$</em>{38}$O$_3$</td>
<td>320 [M$^+$-H$_2$O] (7), 237 (5), 111 (16), 95 (15), 86 (100)</td>
</tr>
<tr>
<td>C3b</td>
<td>1-[14'($E$)-heptadecenyl]-cyclohex-3-en-1,2,5-triol</td>
<td>C$<em>{23}$H$</em>{42}$O$_3$</td>
<td>348 [M$^+$-H$_2$O] (8), 265 (6), 111 (18), 95 (22), 86 (100)</td>
</tr>
<tr>
<td>C3c</td>
<td>1-[16'($E$)-nonadecenyl]-cyclohex-3-en-1,2,5-triol</td>
<td>C$<em>{25}$H$</em>{46}$O$_3$</td>
<td>376 [M$^+$-H$_2$O] (10), 293 (7), 111 (19), 95 (22), 86 (100)</td>
</tr>
<tr>
<td>C4a</td>
<td>1-[14'($E$)-heptadecenyl]-4-cyclohex-4-en-1,3-diol</td>
<td>C$<em>{23}$H$</em>{42}$O$_2$</td>
<td>332 [M$^+$-H$_2$O] (7), 314 (5), 265 (11), 104 (33), 95 (100)</td>
</tr>
<tr>
<td>C4b</td>
<td>1-[16'($E$)-nonadecenyl]-4-cyclohex-4-en-1,3-diol</td>
<td>C$<em>{25}$H$</em>{46}$O$_2$</td>
<td>360 [M$^+$-H$_2$O] (7), 342 (6), 293 (11), 123 (6), 104 (30), 95 (100)</td>
</tr>
<tr>
<td>C4c</td>
<td>1-[18'($E$)-heneicosenyl]-4-cyclohex-4-en-1,3-diol</td>
<td>C$<em>{27}$H$</em>{50}$O$_2$</td>
<td>388 [M$^+$-H$_2$O] (8), 370 (6), 321 (11), 104 (31), 95 (100)</td>
</tr>
</tbody>
</table>

*H and $^{13}$C NMR data in Queiroz et al. (2003)
The two *cis* coupled olefinic protons showed strong coupling in the COSY spectrum. Another coupled system was seen between the two H-6 resonances and the oxygenated methine resonance at $\delta_H 4.46$ (H-5). In the NOESY spectrum, this H-5 resonance was also seen coupled to the H-4 olefinic resonance at $\delta_H 5.81$ and the other oxygenated methine resonance at $\delta_H 4.02$ (H-2) showed NOESY correlations to the other olefinic doublet at $\delta_H 5.57$ (H-3). The substitution pattern on the cyclohexene ring was supported by the HMBC correlations of H-4 with C-2, H-3 with C-5 and the other fully substituted oxygenated carbon resonance at $\delta_C 74.3$ (C-1) as well as H-6a and H-6b showing HMBC correlations to C-1, C-2, C-4 and C-5.

The position of the alkyl chain was deduced from HMBC correlations between H-1' with C-1, C-2 and C-6. The double bond in the side chain was located three bonds away from the terminal methyl group since there was a HMBC correlation between one of the olefinic double bonds and the terminal methyl group of the alkyl chain. In the case of the heptadecenyl side chain, this was seen between C-15' and H-17'. The relative stereochemistry of H-2 and the alkyl chain was taken to be *cis*, since there was a NOESY correlation between H-2 and 2H-1'. However, due to a lack of NOESY correlations and coupling constants of H-5, the stereochemistry at C-5 could not be determined.

GCMS data of compound **C3a-C3c** (Table 4-1) indicated that it was a mixture of three compounds, each with a double bond three bonds away from the terminal methyl group and with C-15, C-17 and C-19 alkyl chains. The molecular ion peaks for the three compounds were negligibly detected, but a more pronounced $M^+ - H_2O$ ion as is common for most secondary and tertiary alcohols is present in all three compounds. These ions together with
the molecular formulae and names of 3a-C3c are given in Table 4-1. Thus, 3a-C3c were identified as their various 1-alkylated-1,2,5-trihydroxy-3-cyclohexenes.

In comparison to compound C3a-C3c, the 1H NMR spectrum of C4a-C4c showed three changes; the multiplicity of the olefinic resonance H-5 changed to a multiplet and was shifted slightly downfield to $\delta_H$ 5.65-5.69, the oxymethine resonance at $\delta_H$ 4.00 disappeared and the 2-hydroxy resonance at $\delta_H$ 2.18 also disappeared. In the 13C NMR spectrum, the oxymethine resonance at $\delta_C$ 70.0 disappeared and an additional methylene resonance at $\delta_C$ 37.4 was seen. Apart from these changes, all the other resonances of compound C4a-C4c were similar to that of C3a-C3c. Thus, apart from the resonances of the alkylated olefinic side chain, compound C4a-C4c had two hydroxyl groups, indicated by secondary and tertiary oxygenated carbon resonances at $\delta_C$ 66.0 and 72.4 respectively and two methylene groups at $\delta_C$ 42.9 and 37.4 in the 13C NMR spectrum. The oxygenated methine proton was present as a broad singlet at $\delta_H$ 4.46. The 1H NMR resonances of the methylene groups were present as non-equivalent resonances at $\delta_H$ 2.09 (dd, $J = 12.8, 6.0$ Hz, H-2a) and $\delta_H$ 1.39 (dd, $J = 12.8, 9.6$ Hz, H-2b). The other pair of non-equivalent resonances for the second methylene group appeared at $\delta_H$ 2.16 (dd, $J = 18.3, 2.7$ Hz, H-6a) and a multiplet at $\delta_H$ 1.97 (H-6b). In addition, an olefinic double bond indicated by the doublet at $\delta_H$ 5.77 and the multiplet at $\delta_H$ 5.65-5.69 in the 1H NMR spectrum was present. The olefinic carbon resonances were present at $\delta_C$ 130.6 and 125.9.

The positions of these groups on the cyclohexene ring were established by COSY correlations between H-2a and H-2b and H-3, and between H-6a and H-6b and H-5. This was supported by HMBC correlations; C-6 with H-4 and H-5; C-4 with H-2a and H-2b; C-3 with H-5; and C-1 with H-2a and H-2b. The position of the alkyl chain at C-1 was supported
by HMBC correlations between 2H-1' with C-1, C-2 and C-6 and the double bond on the side chain located three bonds away from the terminal methyl group due to a correlation between the terminal methyl proton resonance and the olefinic carbon resonance. Unfortunately, there were no NOESY correlations to determine the relative stereochemistry at C-1 and C-3.

Analysis of the GCMS data showed that compound C4a-C4c was a mixture of three compounds, each with a different alkyl chain length; C-17 (C4a); C-19 (C4b); and C-21 (C4c). Although the molecular ion peaks were not present in the mass spectra of the compounds, a prominent M+H2O peak is seen for all three compounds, typical for tertiary alcohols. The names of C4a-c are presented in Table 4-1.

Compounds C2a-C2d, C3a-C3c and C4a-C4c are biogenic precursors of the cardanols C1a-C1d (Walters et al., 1990). Reduction of C2a-C2d would lead to the formation of C3a-C3c and dehydroxylation of C3a-C3c would result in C4a-C4c. Dehydration of C4a-C4c results in the cardanols (Figure 4-1). The 5-alkenyl cyclohexenone mixture C2a-C2d exhibited good in vitro cytotoxicity activity (IC50 = 7.95 µg mL⁻¹) against the Chinese Hamster Ovarian mammalian cell-line. The cardanols (C1a-C1d), the alkenyl cyclohexene triols (C3a-C3c) and the alkenyl cyclohexene diol mixtures (C4a-C4c) did not exhibit any cytotoxic activity (IC50 > 100 µg mL⁻¹). The standard drug emetine had an IC50 of 0.069 µg mL⁻¹. Alkenyl cyclohexenones have also previously demonstrated cytotoxicity (Correia et al., 2001., David et al., 1998, Goweiss et al., 1997, Roumy et al., 2009) and their activity is possibly related to the conjugated ketone, a Michael acceptor, often involved in pharmacological activities (Roumy et al., 2009).

Alkyl phenols that differ in chain length, degree of unsaturation and position of the double bonds occur in several genera within the Anacardiaceae family (Himejima and Kubo, 1991;
Correia et al., 2001; Franke et al., 2001; Masuda et al., 2002; Queiroz et al., 2003; Liu and Abreu, 2006; Saitta et al., 2009). The side chain usually has an odd number of carbons atoms ranging from C-15 to C-29, the most common chain length being C-15 and C-17 (Correia et al., 2006). The cardanols C1b and C1c with C-17 and C-19 side chains respectively were reported previously in *Lannea edulis* and *Rhus thyrsiflora* (Queiroz et al., 2003; Franke et al., 2001). Furthermore, the dihydroxy alkylated cyclohexenones (C2b and C2c) were also isolated in *L. edulis* (Queiroz et al., 2003). As such, the isolation of these compounds in *Lannea schimperi* as well suggests a close chemotaxonomic relationship between both *L. edulis* and *L. schimperi*.

Compounds similar to C2a-C2d and C3a-C3c with the same cyclohexenone and cyclohexenol skeletons but with the functional groups arranged differently and the position of the double bond in the side chain being different, were isolated in *Lannea welwitschii* (Groweiss et al., 1997), *Lannea nigritana* (Kapche et al., 2007) and from *Tapirira guianensis* (David et al. 1998; Roumy et al., 2009). This also suggests a chemotaxonomic link between the *Lannea* and *Tapirira* genera. The triterpenes taraxerol (B8) and taraxerone (B7) were also isolated in *Lannea rivae*.

4.3 Experimental

Plants material

The root and stem bark of *Lannea schimperi* (Engl.) Engl. were collected from Wote town, Makueni District, Kenya and were authenticated at the Maseno University Botanic garden Herbarium. This was assigned a voucher number (MSU/BG-3/13) and a sample specimen deposited at the museum for future reference. The plant material was air-dried under shade...
for fourteen days and ground using a Wiley laboratory mill available at Kibos Sugar Company-Kisumu.

**General experimental procedures**

NMR spectra were recorded in CDCl₃ or CD₃OD on a Varian 400 MHz spectrometer. EIMS data were recorded on an Agilent MS 5973 instrument connected to a GC 6890. Ultraviolet absorption spectra were obtained on a Varian DMS 300 UV/visible spectrophotometer and Infrared spectra were recorded using a Nicolet Impact 400D Fourier-Transform Infra-Red (FT-IR) spectrometer. For column chromatography (CC), silica gel 60 (40–63 µm, Merck 1.09385) was used as the stationary phase. Analytical TLC was performed on precoated silica gel 60 F₂₅₄ plates (Merck 1.05554) and was developed by spraying with anisaldehyde:H₂SO₄:MeOH (1:2:97 v/v) followed by heating. GC-MS data were recorded on an Agilent GC-MSD apparatus equipped with a DB-5SIL MS (30 m x 0.25 mm i.d., 0.25 µm film thickness) fused silica capillary column. He (2 mL min⁻¹) was used as a carrier gas and acetone or methanol (MeOH) was used to dissolve the sample. The injector was kept at 250 °C and the transfer line at 280 °C. The column temperature was held at 50 °C for 2 min, and then ramped to 280 °C at 20 °C min⁻¹ where it was held for 15 min. The MS was operated in the EI mode at 70 eV.

**Extraction and Isolation**

Two kilograms of the root was extracted in the cold on an orbital shaker sequentially with hexane and ethyl acetate for 72 hrs each to give crude extracts of 14.17 g and 34.56 g respectively. The TLC profiles of the extracts indicated that similar compounds were extracted by both solvents and therefore the extracts were combined. The crude extracts were
subjected to open column chromatography over silica gel and eluted with a stepwise gradient of n-hexane:ethyl acetate (1:0 to 0:1, increasing the amount of ethyl acetate gradually), followed by ethyl acetate:methanol (19:1). Fractions of 100 mL each were collected and monitored by TLC. TLC plates were observed under UV light at wavelengths of 254 nm and 365 nm as well as anisaldehyde spraying reagent. Similar fractions were combined and concentrated under vacuum using a Buchi rotary evaporator R-124.

Fractions 1-6 obtained from the crude column with hexane:ethyl acetate (95:5) was purified further with the same solvent system and resulted in a red liquid (4.44 g, cardanols C1a-C1d). The mixture could not be separated further and further attempts only resulted in the compounds co-eluting. Fractions 8-15 of the crude column was chromatographed with hexane:ethyl acetate (9:1), where the triterpenes, taraxerol (B8) (598 mg, fine colourless crystals) and taraxerone (B7) (799 mg, colourless crystals) were obtained from fractions 7-10 and 12-14 respectively. Sitosterol (A6) (1.69 g) was purified from fractions 19-30 of the crude column with 20% ethyl acetate in hexane. Fractions 30-39 from the crude column was separated with hexane:ethyl acetate (6:4 and 5:5) to yield two mixtures, alkenyl cyclohexenones (C2a-C2d) (34.60 mg) and alkenyl cyclohexene diols (C4a-C4c) (74.68 mg). The final mixture isolated was purified from fraction 45-55 from the crude column with ethyl acetate:hexane (6:4) to yield the alkenyl cyclohexene triols (C3a-C3c) (63.33 mg).

The stem bark extract was separated in a similar manner to the roots above where the same compounds were isolated from similar fractions.

Cytotoxicity assay

The MTT-assay was used as a colorimetric assay for cellular growth and survival. The method compares well with other assays (Mosmann, 1983; Rubinstein et al., 1990). The
tetrazolium salt MTT was used to measure all growth and chemosensitivity. The test samples were tested in triplicate.

The test samples were prepared to a 20 mg mL\(^{-1}\) stock solution in 100% DMSO. Test compounds were stored at -20 °C until used. Emetine was used as the reference drug in all experiments. The initial concentration of test samples was 100 µg mL\(^{-1}\), which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg mL\(^{-1}\). The same dilution technique was applied to the all test samples. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability (data not shown). The 50% inhibitory concentration (IC\(_{50}\)) values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

1-[12’(E)-pentadecenyl]-cyclohex-3-en-1,2,5-triol (C3a), 1-[14’(E)-heptadecenyl]-cyclohex-3-en-1,2,5-triol (C3b), 1-[16’(E)-nonadecenyl]-1,2,5-triol (C3c) reddish brown liquid mixture; \([\alpha]\)\(^{20}_D\) 49.67° (c 0.25, CH\(_2\)Cl\(_2\)); UV (CH\(_2\)Cl\(_2\)) \(\lambda_{\text{max}}\) 288 nm; IR \(\nu_{\text{max}}\) cm\(^{-1}\): 3364, 2920, 2851, 1461, 1282, 1017, 964, 833, 720 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta\) 5.81 (1H, dd, \(J = 10.2, 1.6\) Hz, H-4), 5.57 (1H, dd, \(J = 10.2, 2.0\) Hz, H-3), 5.33-5.42 (2H, m, H-14’, H-15’), 4.46 (1H, bs, H-5), 4.02 (1H, bs, H-2), 2.38 (bs, 2-OH), 2.24 (1H, dd, \(J = 13.4, 5.5\) Hz, H-6a), 2.18 (1H, bs, OH), 1.90-2.01 (4H, m, H-13’, H-16’), 1.83 (bs, OH), 1.57 (2H, m, H-1’), 1.42 (1H, dd, \(J = 13.2, 9.3\) Hz, H-6b), 1.23-1.30 (m, H-2’-H-12’), 0.93 (3H, t, \(J = 7.4\) Hz, CH\(_3\)); \(^{13}\)C NMR (CDCl\(_3\), 100 MHz) \(\delta\) 132.7 (C-4), 131.8 (C-15’), 129.6 (C-3), 129.4 (C-14’), 74.3 (C-1), 70.0 (C-2), 65.5 (C-5), 40.8 (C-6), 39.4 (C-1’), 32.6 (C-13’), 29.2-29.7 (C-3’-C-12’), 25.6 (C-16’), 23.6 (C-2’), 14.0 (C-17’); The numbering system refers to C3b. EIMS see Table 4-1.
1-[14'(E)-heptadecenyl]-cyclohex-4-en-1,3-diol (4a), 1-[16'(E)-nonadecenyl]-cyclohex-4-en-1,3-diol (4b), 1-[18'(E)-heneicosenyl]-cyclohex-4-en-1,3-diol (4c) reddish brown liquid mixture, \([\alpha]_{D}^{20} = 14.20^\circ\) (c 0.27, CH\(_2\)Cl\(_2\)); UV (CH\(_2\)Cl\(_2\)) \(\lambda_{\text{max}}\) 289 nm, IR \(\nu_{\text{max}}\) cm\(^{-1}\): 3291, 2916, 2849, 1468, 1309, 1015, 963; \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta\) 5.77 (1H, d, \(J = 10.0\) Hz, H-4), 5.67 (1H, m, H-5), 5.35-5.42 (2H, m, H-14', H-15'), 4.47 (1H, bs, H-3), 2.16 (1H, dd, \(J = 18.3, 2.7\) Hz, H-6a), 2.09 (1H, dd, \(J = 12.8, 6.0\) Hz, H-2a), 1.96-1.99 (4H, m, H-13', H-16'), 1.97 (1H, m, H-6b), 1.48 (2H, m, H-1'), 1.39 (1H, dd, \(J = 12.8, 9.6\) Hz, H-2b), 1.23-1.31 (m, H-2'-H-12'), 0.94 (3H, t, \(J = 7.4\) Hz, H-17'); \(^13\)C NMR (CDCl\(_3\), 100 MHz) \(\delta\) 131.8 (C-15'), 130.6 (C-4), 129.4 (C-14'), 125.9 (C-5), 72.4 (C-1), 66.0 (C-3), 43.3 (C-1'), 42.9 (C-2), 37.4 (C-6), 32.6 (C-13'), 29.2-29.7 (C-3'-C-12'), 25.6 (C-16'), 23.0 (C-2'), 14.0 (C-17').
Numbering system refers to C-4a. EIMS see Table 4-1.

### 4.4 Conclusion

The isolation of the cardanols (C1a-C1d), cycohexenones (C2a-C2d) and cyclohexenols (C3a-C3c and C4a-C4c) confirm *Lannea schimperi*’s place in the Anacardiaceae and its close relationship with *Lannea edulis*. Furthermore, the isolation of the cyclohexenols (C3a-C3c and C4a-C4c) also indicate its similarity to *Lannea welwitschii* and *Lannea nigritana* and to *Tapirira guianensis*, which also contained similar compounds albeit with different substitution patterns on the cyclohexenone and cyclohexenol rings. The isolation of taraxerone (B7) and taraxerol (B8) from *Lannea schimperi*, which we have also recently isolated from *Lannea rivae* indicate a further chemotaxonomic marker for the *Lannea*. The demonstrated cytotoxicity of the \(\alpha,\beta\)-unsaturated cyclohexenone (C2a-C2d) indicates that this class of compound could be a lead compound for the discovery of anticancer compounds.
ACKNOWLEDGEMENTS

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4.5 References


Chapter 5 Alkyl phenols, alkyl cyclohexenols and alkyl cyclohexenones isolated from Lannea schweinfurthii (Engl.) Engl. and their bioactivity

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Key words: Lannea schweinfurthii, Anacardiaceae, alkyl phenols, alkyl cyclohexenols, alkyl cyclohexenone, antioxidant, cytotoxicity, larvicidal
ABSTRACT

We have isolated six mixtures from the roots and stem bark of *Lannea schweinfurthii* which consisted of alkylated phenols (cardanols), cyclohexenones and cyclohexenols. Each of the mixtures contained novel compounds, which was novel by virtue of a novel alkyl side-chain with the core structure being isolated previously with other side chains with the exception of mixture 3 (D2a-D2e), which contained a novel α,β-unsaturated ketone moiety within the cyclohexenone structure. Among the novel compounds are unique conjugated diunsaturated side chains (D1c-D1e and D4f-D4g) which take on a *cis* and *trans* configuration. In addition, we have also isolated the known triterpenoids, sitosterol (A6), sitosterol glucoside (B6) and lupenone (D5), and flavonoids, epicatechin (D6), epicatechin gallate (B12), catechin (D7) and rutin (D8). The novel mixture 3 (D2a-D2e) showed good antibacterial, antiplasmodial, larvicidal and cytotoxic activity, while epicatechin gallate (B12) also showed good antibacterial, antiplasmodial and antioxidant activity.
5.1 Introduction

*L. schweinfurthii* (Engl.) Engl. (Anacardiaceae) is a tree which grows to 15 m high and has a rounded usually dense crown. These trees are widespread in East Africa. Its fruits are reddish brown when ripe and although edible, are not used as a food source. Like most other *Lannea* species, the bark is soft and fleshy and is generally used for tea and medicine, with the dye obtained from it being used to decorate baskets (Maundu *et al*., 1999). The stem and root bark decoction is traditionally used for stomach ailments such as stomach pain, gastrointestinal diseases, diarrhea and constipation, and to treat headaches and boils (Johns *et al*., 1990; 1995; Geissler *et al*., 2002; Arwa *et al*., 2010). In Tanzania, the Sukuma of the Bunda district boil the stem bark to treat abscesses, oral candidiasis and syphilis as well as being used to reduce cellulite (Maregesi *et al*., 2007). The bark infusion is also used in Bumalogi, Uganda to cure sterility (Tabuti *et al*., 2003).

The methanol extract of the stem bark was found to have anti-HIV-1 and 2 activity (Maregesi *et al*., 2010) as well as antigiardial activity (Johns *et al*., 1995). In addition, the aqueous extracts of the plant demonstrated cytotoxicity and antiplasmodial activities (Gathirwa *et al*., 2008; 2011). In our ongoing study of the genus *Lannea* and because of its medicinal use and bioactivity of the methanol and aqueous extracts, we have worked on the phytochemistry of the plant in order to determine what compounds were present and whether or not the isolated compounds were active in some of the assays in which the extracts of the plant were tested.

The phytochemistry of *L. schweinfurthii* has to our knowledge not been documented and this is the first report on the plant’s chemistry. Previous studies of other *Lannea* species has led to isolation of alkylated hydroquinones (Groweiss *et al*., 1997), alkyl phenols and dihydroakylhexenones (Queiroz *et al*., 2003), a dihydroakylcyclohexenol (Kapche *et al*., 2010).
and flavonoids (Soluchana and Sastry, 1968; Sankara and Nair, 1971; Sultana and Ilyas 1986a; 1986b; Islam and Tahara, 2000). We report here on the phytochemistry of the root and stem bark of *L. schweinfurthii* and the antioxidant, cytotoxicity and larvicidal activity of the compounds isolated as well as include a discussion on the chemotaxonomic significance of these findings within the Anacardiaceae.

### 5.2 Results and discussion

The roots, stems and leaves of *Lannea schweinfurthii* (Engl.) Engl. led to the isolation of six mixtures of compounds, cardanols, cyclohexenones or cyclohexenols with either saturated, monounsaturated or diunsaturated alkyl side chains; **D1a-D1b** and **C1a-C1c** (mixture 1); **D1a-D1e**, **B1d** and **C1a-C1d** (mixture 2); **D2a-D2e** (mixture 3); **D3a-D3b**, **C3a** and **C3c** (mixture 4); **D4a**, **D4e** and **C4a-C4b** (mixture 5); and **D4b-D4d**, **D4f-D4g** and **C4b-C4c** (mixture 6). In addition to this, the known triterpenes, sitosterol (**A6**), sitosterol glucoside (**B6**), lupenone (**D5**), and four known flavonoids, epicatechin (**D6**), epicatechin gallate (**B12**), catechin (**D7**) and rutin (**D8**) were also isolated. The known compounds were identified from their $^1$H and $^{13}$C NMR spectra as well as their 2D NMR spectra and their structures confirmed by comparison of their spectral data with those published in the literature (Queiroz, *et al.*, 2003; Sakurai *et al.*, 1987; Mahato and Kundu, 2004; Biabani *et al.*, 2002; Kovganko *et al.*, 1999; Faizi *et al.*, 2001; Galotta *et al.*, 2008; Fang and Ye, 2008; Demirezer *et al.*, 2006).

Mixture 1 contained five similar cardanols, **D1a-D1b**, *meta* alkylated phenols with saturated C-13 and C-17 chains respectively and **C1a-C1c** with C-15, C-17 and C-19 monounsaturated alkyl chains where the double bond is situated three bonds away from the terminal methyl group. The structural elucidation of **C1a-C1c** was discussed in Chapter 4. Compounds **D1a-**
**D1b** contained the same aromatic moiety compared to **C1a-C1c** in Chapter 4, however the GC-MS spectrum indicated two additional compounds, **D1a**, C_{19}H_{32}O with a saturated C-13 side chain and which indicated a molecular ion peak at M' 276 amu, and **D1b**, C_{23}H_{40}O with a saturated C-17 side chain indicated by a molecular ion peak at M' 332 amu in the mass spectrum. Compounds **D1a** and **D1b** have been reported previously (Franke et al., 2001; Liu and Abreu, 2006).

The $^1$H and $^{13}$C NMR spectra of mixture 2 (**D1a-D1e, B1d** and **C1a-C1d**) showed resonances that were similar to the spectra in mixture 1 and **C1a-C1c** in Chapter 4. In particular, the aromatic resonances of H-5, H-4, H-2 and H-6 were all the same as well as H-1' (a triplet at $\delta_H$ 2.53, $J = 7.6$ Hz) and H-2' (a multiplet at $\delta_H$ 1.55-1.59) and the olefinic resonances of the double bond in the monounsaturated side chain, a multiplet at $\delta_H$ 5.36-5.41. However, there were four additional olefinic resonances in the $^1$H NMR spectrum, a multiplet at $\delta_H$ 5.25-5.35 (H-14'), a doublet of triplets at $\delta_H$ 5.68 (1H, $J = 15.1, 6.7$ Hz, H-17'), a triplet (actually a dd which resembles a triplet due to the same coupling constant values for each of the coupled protons) at $\delta_H$ 5.93 ($J = 10.9$ Hz, H-15') and a double doublet at $\delta_H$ 6.28 (1H, $J = 15.0, 10.9$ Hz, H-16'). The coupling constants of these resonances indicated that H-14', H-15' and H-16' were all *cis* to each other and that H-17' was *trans* to H-16' and formed part of a conjugated double bond system that was three bonds away from the terminal methyl group, due to a HMBC correlation between H-19' and C-17' (this refers to the numbering system of **D1d**). The $^1$H NMR spectrum also indicated that three different chains were present by having three different methyl triplet resonances, two were the monounsaturated and diunsaturated chain methyl groups at $\delta_H$ 1.00 (3H, t, $J = 7.5$ Hz, terminal methyl group of the diunsaturated chain), $\delta_H$ 0.94 (3H, t, $J = 7.4$ Hz, terminal methyl group of the monounsaturated chain) and
δ_H 0.86 (3H, t, J = 7.0 Hz, terminal methyl group of the saturated chain). The NMR data for D1d is shown in Table 5-1.

From the GCMS analysis, ten compounds could be identified in this mixture, three of which had saturated side chains (D1a, D1b and B1d, all isolated earlier), four had monounsaturated side chains (C1a-C1d, also isolated earlier) and D1c-D1e, novel compounds with diunsaturated side chains with molecular masses at M^+ 328, M^+ 356 and M^+ 384 respectively. In all cases, the mass spectra displayed a base peak at m/z 108 due to benzylic cleavage of the 1-hydroxy-3-alkyl-benzene. The ions at m/z 77, 91 and 108 suggested the presence of a benzene ring with a phenolic hydroxyl and the ions at m/z 133, 147, 161, 175, and 206, 220 and 234 suggested the presence of an alkyl chain.

The ^1H NMR spectrum of the mixture 3 (D2a-D2e) contained a pair of doublets at δ_H 6.07 (1H, d, J = 10.1 Hz, H-2) and δ_H 6.83-6.87 (1H, m, H-3). Both these resonances were coupled to each other in the COSY spectrum and to the aliphatic methylene resonance at δ_H 2.47-2.50 (2H, H-4). The fact that H-2 was a doublet with 10.1 Hz indicated that it was part of a cis double bond. In addition, H-3 showed HMBC correlations to the carbonyl resonance at δ_C 198.5 (C-1), which in turn showed HMBC correlations to another methylene resonance at δ_H 2.53 (1H, s, H-6). The H-3 proton resonance also showed HMBC correlations to another oxygenated tertiary carbon resonance at δ_C 74.0. The remainder of the ^1H NMR resonances was assigned to the aliphatic side chain. Thus, at the tertiary carbon atom (C-5) of the α,β unsaturated cyclohexenone ring, both a hydroxy group and a long aliphatic side chain was placed.
Table 5-1 \(^1\text{H}\) and \(^{13}\text{C}\) NMR data of the cardanol D1d, the cyclohexenone D2e and the cyclohexenediol D4f (400 MHz, CDCl\(_3\)) (\(J\) values in Hz are in parenthesis)

<table>
<thead>
<tr>
<th>Pos.</th>
<th>D1d (^1\text{H})</th>
<th>(^{13}\text{C})</th>
<th>D2e (^1\text{H})</th>
<th>(^{13}\text{C})</th>
<th>D4f (^1\text{H})</th>
<th>(^{13}\text{C})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>155.5</td>
<td>-</td>
<td>198.5</td>
<td>-</td>
<td>72.4</td>
</tr>
<tr>
<td>2</td>
<td>6.64 s</td>
<td>115.3</td>
<td>6.07 (dt) (10.1, 2.0)</td>
<td>129.4</td>
<td>2.10-2.15 (m)</td>
<td>42.9</td>
</tr>
<tr>
<td></td>
<td>1.42 (dd) (12.7, 9.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>145.0</td>
<td>6.83-6.87 (m)</td>
<td>146.7</td>
<td>4.45-4.49 (m)</td>
<td>66.0</td>
</tr>
<tr>
<td>4</td>
<td>6.74 (d) (7.6)</td>
<td>120.9</td>
<td>2.47-2.50 (m)</td>
<td>38.2</td>
<td>5.78 (d) (10.2)</td>
<td>130.6</td>
</tr>
<tr>
<td>5</td>
<td>7.12 (t) (7.6)</td>
<td>129.4</td>
<td>-</td>
<td>74.0</td>
<td>5.67-5.72 (m)</td>
<td>125.9</td>
</tr>
<tr>
<td>6</td>
<td>6.61 (d) (2.4)</td>
<td>112.5</td>
<td>2.53 (s)</td>
<td>50.3</td>
<td>2.17-2.20 (m)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2.08-2.12 (m)</td>
<td></td>
</tr>
<tr>
<td>1'</td>
<td>2.53 (t) (7.6)</td>
<td>35.8</td>
<td>1.53-1.57 (m)</td>
<td>42.0</td>
<td>1.45-1.50 (m)</td>
<td>43.3</td>
</tr>
<tr>
<td>2'</td>
<td>1.55-1.59 (m)</td>
<td>31.3</td>
<td></td>
<td></td>
<td>22.7</td>
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<tr>
<td>3'- 12'</td>
<td>1.23-1.33 (m)</td>
<td>29.2-29.8</td>
<td>1.22-1.36 (m)</td>
<td>29.2-29.9</td>
<td>1.22-1.33 (m)</td>
<td>29.3-29.7</td>
</tr>
<tr>
<td>13'</td>
<td>2.08-2.15 (m)</td>
<td>27.8</td>
<td>1.91-1.98 (m)</td>
<td>32.6</td>
<td>2.10-2.15 (m)</td>
<td>27.7</td>
</tr>
<tr>
<td>14'</td>
<td>5.25-5.35 (m)</td>
<td>130.2</td>
<td>5.36-5.41 (m)</td>
<td>129.4</td>
<td>5.24-5.34 (m)</td>
<td>130.2</td>
</tr>
<tr>
<td>15'</td>
<td>5.93 (t) (10.9)</td>
<td>128.6</td>
<td>5.36-5.41 (m)</td>
<td>131.9</td>
<td>5.92 (t) (10.9)</td>
<td>128.5</td>
</tr>
<tr>
<td>16'</td>
<td>6.28 (dd) (15.1, 10.9)</td>
<td>124.7</td>
<td>1.91-1.98 (m)</td>
<td>25.4</td>
<td>6.27 (dd) (15.1, 10.9)</td>
<td>124.7</td>
</tr>
<tr>
<td>17'</td>
<td>5.68 (dt) (15.1, 6.7)</td>
<td>136.1</td>
<td>0.94 (t) (7.4)</td>
<td>14.0</td>
<td>5.63-5.71 (m)</td>
<td>136.1</td>
</tr>
<tr>
<td>18'</td>
<td>2.08-2.15 (m)</td>
<td>25.9</td>
<td></td>
<td>2.08-2.12 (m)</td>
<td>25.9</td>
<td></td>
</tr>
<tr>
<td>19'</td>
<td>1.00 (t) (7.5)</td>
<td>13.7</td>
<td></td>
<td>1.00 (t) (7.4)</td>
<td>13.7</td>
<td></td>
</tr>
</tbody>
</table>

The \(^1\text{H}\) NMR spectrum contained a mixture of compounds with the same core skeleton and the two-proton multiplet resonance at \(\delta_{\text{H}}\) 5.36-5.41 indicated that some of the side chains contained a double bond. This double bond (H-14'/15') had a \textit{trans} configuration and was coupled to another aliphatic multiplet resonance at \(\delta_{\text{H}}\) 1.91-1.98 (4H, \(m\), H-13'/16'). The numbering refers to D2e. One of the terminal methyl groups at \(\delta_{\text{H}}\) 0.94 (3H, \(t\), \(J = 7.4\) Hz, H-
17') showed an HMBC correlation to C-15' indicating that the double bond was three bonds away from the terminal methyl group. The other triplet at $\delta_{\text{H}}$ 0.85 ($J = 7.0$ Hz) was assigned to C-17#, the methyl group of the fully saturated alkyl chain. The multiplet at $\delta_{\text{H}}$ 1.53-1.57 was attributed to the methylene group (H-1') as this resonance was seen coupled to C-4, C-5 and C-6 in the HMBC spectrum.

From the GCMS spectrum it was possible to identify five major compounds, D2a, D2b and D2c, with the same core skeleton and saturated side chains with C-13, C-15 and C-17 carbon atoms respectively. These compounds showed ion fragment peaks at $M^+ - \text{H}_2\text{O}$, $m/z$ 276, $M^+ - \text{H}_2\text{O}$, $m/z$ 304 and $M^+ - \text{H}_2\text{O}$, $m/z$ 332 amu respectively as expected in compounds with tertiary alcohols. Two further monounsaturated C-15 and C-17 side chains, D2d and D2e were also identified in the mixture with $M^+ - \text{H}_2\text{O}$, $m/z$ 302 and $M^+ - \text{H}_2\text{O}$, $m/z$ 330 respectively. The saturated and unsaturated derivatives with C-15 side chains co-eluted at almost the same time as did the saturated and unsaturated derivatives with C-17 side chains.

The $^1\text{H}$ and $^{13}\text{C}$ NMR spectra of mixture 4 (D3a-D3b, C3a and C3c) showed resonances similar to that of the mixture that contained C3a-C3c. In particular, the cis olefinic proton resonances at $\delta_{\text{H}}$ 5.81 (H-4) and $\delta_{\text{H}}$ 5.57 (H-3), each double doublets, the broad singlets at $\delta_{\text{H}}$ 4.02 (H-2) and $\delta_{\text{H}}$ 4.46 (H-5) and the non-equivalent double doublet resonances of H-6 at $\delta_{\text{H}}$ 2.24 and $\delta_{\text{H}}$ 1.42 were the same as in the mixture of C3a-C3c discussed in Chapter 4. The monounsaturated side chain resonances at $\delta_{\text{H}}$ 5.33-5.42 (2H, m), the methylene resonances attributed to the methylene groups on either side of the double bond at $\delta_{\text{H}}$ 1.90-2.01 (4H, m) and the terminal methyl group of the monounsaturated methyl group at $\delta_{\text{H}}$ 0.93 were also all the same as that for C3a-C3c. The $^1\text{H}$ NMR spectrum however indicated that another alkyl chain was present $\delta_{\text{H}}$ 0.86 (3H, t, $J = 6.9$ Hz, terminal methyl group of the saturated chain).
This was supported by the analysis of the GCMS data which indicated the presence of four compounds, two saturated, \textbf{D3a}, with a C-13 saturated alkyl chain (M$^+$ - H$_2$O at m/z 294) and \textbf{D3b} with a C-17 saturated alkyl chain (M$^+$ at 368 amu), and two unsaturated, \textbf{C3a} (M$^+$ - H$_2$O at m/z 320) and \textbf{C3c} (M$^+$ at 394 amu).

The $^1$H and $^{13}$C NMR spectra of the mixture 5 (\textbf{D4a}, \textbf{D4e} and \textbf{C4a-C4b}) were very similar to that of the mixture of \textbf{C4a-C4c} in chapter 4. The only differences were the extra triplet methyl resonance at $\delta_H$ 0.83-0.87 indicating a saturated methyl group and in the $^{13}$C NMR spectrum, two methylene resonances, one and two bonds away from the terminal methyl group marked as C-11'# and C-12'# using the numbering system for \textbf{D4a}. Thus, this mixture contained a higher proportion of the core skeleton with a saturated aliphatic side chain than the mixture \textbf{C4a-C4c} from \textit{Lannea schimperi}. GCMS analysis indicated the presence of four compounds with this core skeleton, one with a saturated C-13 aliphatic side chain \textbf{D4a} with M$^+$ - H$_2$O at 278 amu and three with a monounsaturated side chain, \textbf{D4e} with a C-15 side chain and M$^+$ - H$_2$O at 304 amu, \textbf{C4a} with a C-17 side chain (M$^+$- H$_2$O at 332 amu) and \textbf{C4b} with a C-19 side chain (M$^+$ - H$_2$O at 360 amu).

The $^1$H and $^{13}$C NMR spectrum of mixture 6 (\textbf{D4b-D4d}, \textbf{D4f-D4g} and \textbf{C4b-C4c}) was very similar to mixture 5 and the mixture of \textbf{C4a-C4c} in chapter 4 in that it had the same H-4 and H-5 proton resonances of the double bond, the H-3 methine resonance and the non-equivalent H-2 and H-6 methylene resonances. The $^{13}$C NMR spectrum also indicated the same olefinic C-4 and C-5 resonances, the oxygenated C-1 and C-3 resonances and the methylene C-2 and C-6 resonances. With regard to the side chain, the monounsaturated olefinic protons were the same as that for the other mixtures with a multiplet at $\delta_H$ 5.36-5.41. The olefinic carbon resonances of the monounsaturated aliphatic chain were also the same on either side of the C-
4 resonance at $\delta_C$ 129.4 and $\delta_C$ 131.9. The olefinic region of the $^1$H NMR spectrum also indicated four other olefinic resonances, two multiplets at $\delta_H$ 5.24-5.34 (H-14') and $\delta_H$ 5.63-5.71 (H-17') overlapping with the H-5 resonance and two more distinct olefinic resonances at $\delta_H$ 5.92 (H-15'), a double doublet appearing as a triplet due to the coalescing of resonances since the same coupling was observed to each proton on either side of it. This $J$ value was 10.9 Hz indicating a cis relationship to H-14' and H-16'. The numbering system refers to D4f. The remaining olefinic resonance at $\delta_H$ 6.27 (1H, dd, $J = 15.1, 10.9$ Hz, H-16') was cis to H-15' and trans to H-17' as indicated by the coupling constants of the double doublet. Thus, another unsaturated side chain with two conjugated double bonds where H-14', H-15' and H-16' had a cis relationship and H-16' and H-17' had a trans relationship was present.

The $^1$H NMR spectrum of the mixture 6 also indicated that three types of side chains were present since three terminal methyl group resonances could be seen at $\delta_H$ 0.99 (3H, t, $J = 7.5$ Hz, terminal methyl group of the diunsaturated chain), $\delta_H$ 0.93 (3H, t, $J = 7.4$ Hz, terminal methyl group of the monounsaturated chain) and $\delta_H$ 0.85 (3H, t, $J = 7.0$ Hz, terminal methyl group of the saturated chain). From the GCMS analysis of the mixture, seven compounds were identified, three with saturated C-19 (D4b, M$^+$ - H$_2$O at 362), C-21 (D4c, M$^+$ - H$_2$O at 390) and C-23 (D4d, M$^+$ - H$_2$O at 418) side chains, two with monounsaturated C-19 (C4b, M$^+$ - H$_2$O at 360) and C-21 (C4c, M$^+$ - H$_2$O at 388) side chains which were also isolated in Lannea schimperi (Chapter 4) and two with diunsaturated C-19 (D4f, M$^+$ - H$_2$O at 358) and C-21 (D4g, M$^+$ - H$_2$O at 386) side chains. The mass spectral data and molecular formulae of the novel compounds are indicated in Table 5-2 and the structures of the novel compounds are shown in Figure 5-1. Novel compounds discussed in other chapters do not appear in Figure 5-1.
Figure 5-1 Novel alkylated cardanols, cyclohexenones and cyclohexenols isolated from
*Lannea schweinfurthii* (*D1a* and *D1b* has been isolated previously (Franke *et al.*, 2001))
Table 5-2 Mass spectral data for novel compounds isolated from *Lannea schweinfurthii*

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular formula</th>
<th>EIMS (m/z rel. int.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1c</td>
<td>3-[heptadec-12'(Z),14'(E)-dienyl] phenol</td>
<td>C(<em>{23})H(</em>{36})O</td>
</tr>
<tr>
<td>D1d</td>
<td>3-[nonadec-14'(Z),16'(E)-dienyl] phenol</td>
<td>C(<em>{25})H(</em>{40})O</td>
</tr>
<tr>
<td>D1e</td>
<td>3-[heneicos-16'(Z),18'(E)-dienyl] phenol</td>
<td>C(<em>{27})H(</em>{44})O</td>
</tr>
<tr>
<td>D2a</td>
<td>5-hydroxy-5-[tridecyl] cyclohex-2-enone</td>
<td>C(<em>{19})H(</em>{34})O</td>
</tr>
<tr>
<td>D2b</td>
<td>5-hydroxy-5-[pentadecyl] cyclohex-2-enone</td>
<td>C(<em>{21})H(</em>{38})O</td>
</tr>
<tr>
<td>D2c</td>
<td>5-hydroxy-5-[heptadecyl] cyclohex-2-enone</td>
<td>C(<em>{23})H(</em>{42})O</td>
</tr>
<tr>
<td>D2d</td>
<td>5-hydroxy-5-[pentadec-12'(E)-enyl] cyclohex-2-enone</td>
<td>C(<em>{25})H(</em>{46})O</td>
</tr>
<tr>
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<td>5-hydroxy-5-[heptadec-14'(E)-enyl] cyclohex-2-enone</td>
<td>C(<em>{23})H(</em>{40})O</td>
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<tr>
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<td>D3b</td>
<td>1-[heptadecyl] cyclohex-3-en-1,2,5-triol</td>
<td>C(<em>{23})H(</em>{44})O(_3)</td>
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<tr>
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<td>C(<em>{27})H(</em>{48})O(_2)</td>
</tr>
</tbody>
</table>
**Antibacterial activity**

The hexane extract showed a narrow spectrum of activity against *E. faecium* and *E. faecalis* only (Table 5-3). A broader spectrum of activity was observed with the methanol extracts of both the stem and the roots, with the highest activity being recorded by the methanol extract of the root against *S. aureus* and *E. faecium* (15 mm each). A previous study indicated that the water and methanol extracts of the same plant had moderate activity against other *S. aureus* strains (Maregesi et al. 2008). Lower activities of the methanol extracts were observed against *P. aeruginosa* and *S. typhimurium* (8 mm each), while no activity was seen with *E. coli*. Of all the mixtures tested, only mixture 3, which contained the alkylated (both saturated and monounsaturated) hydroxycylohexenones (D2a-D2e) was active against *S. aureus, E. faecium* and *E. faecalis* with zones of inhibition of 10 mm for each of the strains. This indicates the importance of the α,β-unsaturated ketone with regard to antibacterial activity as this was the only mixture that contained compounds with this core skeleton.

The flavonoids epicatechin (D6), catechin (D7), rutin (D8) and the triterpenoid sitosterol glucoside (B6) were also inactive. This was also indicated in previous studies (Kajiya et al., 2004). Epicatechin gallate (B12) had the best and broadest spectrum of antibacterial activity. Its activity toward *S. aureus* is comparable to erythromycin, a well known antibiotic. This compound is known to be active against *S. aureus* and several other bacterial strains and enhances the activity of antibiotics even against resistant strains (Hamilton-Miller and Shah, 2000; Stapleton et al., 2004; Hatano et al., 2005; Anderson et al., 2011; Qin et al., 2013). It is also known to inhibit bacterial DNA enzymes and cell wall synthesis and to disrupt virulence related proteins (Stepleton et al., 2007; Shah et al., 2008).
The plant is traditionally used to treat dysentery and severe diarrhea but none of the mixtures or compounds were active against *E. coli* except for epicatechin gallate, which had moderate activity against *E. coli*. Moderate activity was also seen with the methanol extracts and epicatechin gallate (B12) against *S. typhimurium*. This may imply that the plant could be treating diarrhea and dysentery resulting from other parasites such as amoeba, giardia or even viruses. Johns *et al.* (1995) demonstrated anti-giardial activity of *L. schweinfurthii* extracts.

**Table 5-3** Antibacterial activity of extracts, mixtures and compounds isolated from *Lannea schweinfurthii*

<table>
<thead>
<tr>
<th>Sample</th>
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</thead>
<tbody>
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<td></td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>hexane root extract</td>
<td>0</td>
</tr>
<tr>
<td>Methanol stem extract</td>
<td>12</td>
</tr>
<tr>
<td>Methanol root extract</td>
<td>15</td>
</tr>
<tr>
<td>Mixture 3: D2a-D2e</td>
<td>10</td>
</tr>
<tr>
<td>Epicatechin gallate (B12)</td>
<td>22</td>
</tr>
<tr>
<td>Penicillin</td>
<td>34</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>23</td>
</tr>
<tr>
<td>vancomycin</td>
<td>-</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>-</td>
</tr>
<tr>
<td>Ciproflacin</td>
<td>-</td>
</tr>
<tr>
<td>Chloraphenicol</td>
<td>-</td>
</tr>
</tbody>
</table>

Data reported as an average of three readings; "-" indicates no testing was carried out

**Antiplasmodial activity**

The methanol and water extracts of *L. schweinfurthii* were not tested in this work, but demonstrated good antiplasmodial and antimalarial activity previously (Gathirwa *et al.*, 2007; 2008; Maregesi *et al.*, 2010). All isolated compounds were tested for their antiplasmodial
activity, however only mixture 3 (D2a-D2e) and epicatechin gallate (B12) showed antiplasmodial activity (Table 5-4). The mixture D2a-D2e was moderately active whilst epicatechin gallate (B12) showed good antiplasmodial activity with IC$_{50}$ values of 2.79 and 2.11 µg mL$^{-1}$ against the chloroquine sensitive D6 and chloroquine resistant W2 Plasmodium falciparum strains respectively. This is indicative of the importance of the galloyl moiety of flavan-3-ol compounds (Ramanandralbe et al., 2008, Gibbons et al., 2004). This compound also inhibits important plasmodium enzymes (Tasdemir et al. 2006).

Table 5-4 Antiplasmodial activity of the active mixtures and compounds isolated from Lannea schweinfurthii

<table>
<thead>
<tr>
<th>Sample</th>
<th>D6 IC$_{50}$ (µg mL$^{-1}$)</th>
<th>W2 IC$_{50}$ (µg mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mixture 3: D2a-D2e</td>
<td>30.00 ± 1.34</td>
<td>24.12 ± 0.96</td>
</tr>
<tr>
<td>epicatechin gallate (B12)</td>
<td>2.79 ± 0.34</td>
<td>2.11 ± 1.9</td>
</tr>
<tr>
<td>chloroquine</td>
<td>0.0762</td>
<td>0.00443</td>
</tr>
<tr>
<td>mefloquine</td>
<td>0.0374</td>
<td>0.0122</td>
</tr>
</tbody>
</table>

Antioxidant activity

The antioxidant activity of the hexane and ethyl acetate extracts were not determined as the hexane extract contained mainly fatty acids and the ethyl acetate extract was reported to have low antioxidant potential (Adewusi and Steenkamp, 2011). The methanol extract and the flavonoids, known to have good antioxidant activity were tested using the DPPH radical scavenging method. At lower concentrations (6.25-25.0 µg mL$^{-1}$), only epicatechin gallate (B12) showed antioxidant activity comparable to ascorbic acid (
Table 5-5. Epicatechin (D6), catechin (D7) and rutin (D8), as well as the methanol extract of the roots all showed moderate antioxidant activity at low concentrations, but good activity at higher concentrations (50.0-100 µg mL\(^{-1}\)). At higher concentrations the best activity was displayed by epicatechin gallate (B12) with antioxidant activity comparable to ascorbic acid. The gallate substitution at C-3 is responsible for the higher activity of epicatechin gallate. The antioxidant properties of phenolic compounds are due to their ability to donate an electron, quenching free radicals. The resultant radical anion is stabilized by the aromatic system of the phenols. In the same manner, phenols are also capable of donating hydrogen atoms to quench radicals. Studies have shown that the use of antioxidants in combination with chloroquine at the first signs of cerebral malaria prevents both inflammatory and vascular changes in the tissues of the brain as well as development of cognitive damage (Reis et al., 2010). This was seen in the use of artesunate, which when taken in conjunction with antioxidants was effective in the treatment of cerebral malaria and in preventing subsequent cognitive impairment in mice (Zimmerman and Castro, 2010; Percario et al., 2012).

<table>
<thead>
<tr>
<th>sample</th>
<th>DPPH radical scavenging activity</th>
<th>Mean Comp.*</th>
<th>IC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.25 µg mL(^{-1})</td>
<td>12.5 µg mL(^{-1})</td>
<td>25.0 µg mL(^{-1})</td>
</tr>
</tbody>
</table>
Table 5-5  Antioxidant activity of mixtures and compounds isolated from *Lannea schweinfurthii*

*means that differ significantly have different letters, p < 0.05; mean conc. indicates the mean of all the compounds tested at a specific concentration; mean comp. indicates the mean of the compound tested at different concentrations

| Compound                  | LC<sub>50</sub> at 95% confidence limit (µg mL<sup>-1</sup>) | Mean Conc. | * |
|---------------------------|----------------------------------------------------------|------------|
| Rutin (D8)                | 24.88 ± 0.85                                             | 36.49<sup>a</sup> |  |
| Epicatechin (D6)          | 32.80 ± 0.42                                             | 47.41<sup>b</sup> |  |
| Catechin (D7)             | 30.26 ± 0.07                                             | 68.54<sup>c</sup> |  |
| Epicatechin gallate (B12) | 45.93 ± 0.95                                             | 81.23<sup>d</sup> |  |
| Methanol extract (roots)  | 28.33 ± 0.82                                             | 86.20<sup>e</sup> |  |
| Ascorbic acid             | 59.78 ± 0.41                                             |            |  |
| Mean Conc.*               | 36.49<sup>a</sup>                                        |            |  |

Larvicidal activity

All the mixtures 1-6 showed good larvicidal activity (between 6.34 - 16.04 µg mL<sup>-1</sup>) in comparison to the standard, pylarvex (3.12 µg mL<sup>-1</sup>) (Table 5-6). The best activity was seen by mixture 3, the α,β-unsaturated cyclohexenones (D2a-D2e) (6.34 µg mL<sup>-1</sup>), followed closely by the cardanol mixture 2 (D1a-D1e, B1d, C1a-C1d) (8.17 µg mL<sup>-1</sup>) and then the cyclohexene triol mixture 4 (D3a, D3b, C3a, C3c) (9.66 µg mL<sup>-1</sup>). The high activity of mixture 3 could be attributed to the α,β-unsaturated cyclohexenone moiety. The cardanol mixture 1 was not as active as the cardanol mixture 2, possibly because mixture 1 (16.04 µg mL<sup>-1</sup>) contained more cardanols with a saturated alkyl chain than cardanols with an unsaturated alkyl chain.

Table 5-6  Larvicidal activity of extracts and mixtures isolated from *Lannea schweinfurthii*

<table>
<thead>
<tr>
<th>Extracts</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; at 95% confidence limit (µg mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Mixtures</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; at 95% confidence limit (µg mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>22.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epicatechin</td>
<td>59.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catechin</td>
<td>59.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Conc.</td>
<td>74.42&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>7.30</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>Activity (µg/mL) ± Standard Deviation</th>
<th>Mixture Composition</th>
<th>Activity (µg/mL) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves (hexane)</td>
<td>51.57 ± 2.16</td>
<td>mixture 1: D1a, D1b, C1b and C1c</td>
<td>16.04 ± 0.28</td>
</tr>
<tr>
<td>Leaves (EtOAc)</td>
<td>47.89 ± 2.94</td>
<td>mixture 2: D1a-D1e, B1d, C1a-C1d</td>
<td>8.17 ± 0.86</td>
</tr>
<tr>
<td>Leaves (MeOH)</td>
<td>240.37 ± 1.31</td>
<td>mixture 3: D2a-D2e</td>
<td>6.34 ± 0.51</td>
</tr>
<tr>
<td>root (hexane)</td>
<td>73.36 ± 1.57</td>
<td>mixture 4: D3a, D3b, C3a and C3c</td>
<td>9.66 ± 1.07</td>
</tr>
<tr>
<td>root (EtOAc)</td>
<td>73.66 ± 1.59</td>
<td>mixture 5: D4a, D4e, C4a and C4b</td>
<td>11.19 ± 1.03</td>
</tr>
<tr>
<td>root (MeOH)</td>
<td>147.09 ± 1.11</td>
<td>mixture 6: D4b-D4d, D4f, D4g, C4b and C4c</td>
<td>14.34 ± 1.01</td>
</tr>
<tr>
<td>stem (hexane)</td>
<td>46.04 ± 1.32</td>
<td>Pylarvex</td>
<td>3.12 ± 0.26</td>
</tr>
<tr>
<td>stem (EtOAc)</td>
<td>59.66 ± 0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stem (MeOH)</td>
<td>139.06 ± 2.90</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different superscript letters indicate significant differences in activity in the compounds (p < 0.05)

Larvicidal activity of cardanols is known (Lomonaco et al., 2009; Costa Oliveira et al., 2011; Souza et al., 2012). A higher degree of unsaturation was also shown to increase lipophilicity, which contributed to increased activity (Lomonaco et al., 2009). The cyclohexene diol mixtures 5 (D4a, D4e, C4a and C4b) and 6 (D4b-D4d, D4f, D4g, C4b and C4c), were the least active of the cyclohexenes. In comparison to the mixtures, the crude extracts had no significant larvicidal activity (Table 5-6). The mode of action of many insecticides is by inhibition of the enzyme acetylcholinesterase (Finkelstein et al., 2002). The ethyl acetate crude root extract of *Lannea schweinfurthii* has been shown to have acetylcholinesterase inhibitory properties (Adewusi and Steenkamp, 2011) and hence this could be due to the alkylated cyclohexene derivatives. Furthermore, this must also be the mode of action for larvicidal activity of these compounds.

**Cytotoxicity**

Mixtures 1, 2, 4 and 5 were non-toxic with IC\textsubscript{50} values of between 80.5 to >100 µg mL\textsuperscript{-1} relative to the standard emetine (0.069 µg mL\textsuperscript{-1}) and hence could be considered safe to use for medicinal purposes (Table 5-7). However, mixtures 3 (the α,β-unsaturated
cyclohexenones) and 6 (cyclohexene diols) could be considered as potentially toxic with IC$_{50}$ values of 5.11 and 2.12 $\mu$g mL$^{-1}$ respectively. In a structure-activity relationship study of lupane triterpenes on the induction of B16 cell 2F2 cell differentiation and apoptosis, it was demonstrated that the keto function at C-3 enhanced their differentiation inducing activities (Hata et al., 2003). Lupenone (D5) also showed cytotoxic activity with an IC$_{50}$ value of 1.03 $\mu$g mL$^{-1}$. Previous studies have shown lupenone to have moderate cytotoxic activity (Gachet et al., 2011; Villareal et al., 2013). Mixtures 5 and 6 contain compounds with the same core structure, both cyclohexene diols and the fact that mixture 6 is quite toxic whereas mixture 5 is non-toxic could have to do with either the degree of unsaturation in the side chain (mixture 6 having compounds with a diunsaturated alkyl chain) or the particular Z and E configuration of the conjugated double bond in conjunction with the core structure. Previous cytotoxicity studies on Lannea schweinfurthii leaf extracts showed them to be cytotoxic against MT-4 cells and Vero E6 cells (Maregesi et al., 2010; Gathirwa et al., 2011). Moderate cytotoxicity of phenolic lipid derivatives isolated from Lannea welwitschii and Lannea nigritana were reported (Groweiss et al. 1997; Kapche et al., 2007). The mixtures 3 and 6 isolated from Lannea schweinfurthii could be leads to alternative antiproliferative agents for future development as anti-cancer drugs.

**Table 5-7** Cytotoxicity of the mixtures and some of the compounds isolated from Lannea schweinfurthii

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC$_{50}$ ($\mu$g mL$^{-1}$) n = 3</th>
<th>Sample</th>
<th>IC$_{50}$ ($\mu$g mL$^{-1}$) n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>mixture 1: D1a, D1b, C1b and C1e</td>
<td>100</td>
<td>taraxerol (B8)</td>
<td>42.2</td>
</tr>
<tr>
<td>mixture 2: D1a-D1e, B1d, C1a-C1d</td>
<td>80.5</td>
<td>taraxerone (B7)</td>
<td>56.2</td>
</tr>
<tr>
<td>mixture 3: D2a-D2e</td>
<td>5.11</td>
<td>lupenone (D5)</td>
<td>1.03</td>
</tr>
<tr>
<td>mixture 4: D3a, D3b, C3a, C3c</td>
<td>&gt; 100</td>
<td>emetine</td>
<td>0.069</td>
</tr>
<tr>
<td>mixture 5: D4a, D4e</td>
<td>&gt; 100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chemotaxonomic significance of the study

Cardanols, alkylcyclohexenones, alkylcyclohexenols, triterpenes and flavonoids are widely distributed in the Anacardiaceae family. The alkylcyclohexenones are thought to be the biosynthetic precursors of cardanols and have been previously isolated from *Lannea edulis*, *Lannea welwitschii*, *Tapirira guianensis* and *Tapirira obtusa*, the genus *Tapirira* also belonging to the Anacardiaceae (Queiroz et al., 2003, Roumy et al., 2009, Groweiss et al., 1997, David et al., 1998, Correia et al., 2001). A dihydroxyalkylcyclohexenol and a trihydroxyalkylcyclohexenol were isolated from *Lannea nigratina* and *Tapirira guianensis* respectively (Kapche et al., 2007; Roumy et al., 2009). In addition, a benzene derivative of a hydroxycyclohexenol was reported from *Tapirira obtusa* (Correia et al., 2001). Therefore, the occurrence of the cardanols, alkylcyclohexenols and alkylcyclohexenones confirm *Lannea schweinfurthii*'s place in the Anacardiaceae and its close relationship to the other *Lannea* species and to the genus *Tapirira*.

Several cardanols where the alkyl chain differs in length, degree of unsaturation and position of double bonds occur in several genera within the Anacardiaceae family. Usually, the side chain is with an odd number of carbons C-15 to C-29, the most common chain length being C-15 and C-17. Cardanols with C-19 and C-17 side chains were reported in *Lannea edulis* and *Rhus thyrsiflora* (Franke et al., 2001; Queiroz et al., 2003; Liu and Abreu, 2006). We also report compounds with rare C-13 alkyl chains previously reported in *Ozoroa insignis* (Liu and Abreu, 2006).
The triterpenes, sitosterol (A6) and sitosterol glucoside (B6) are ubiquitous within the Plant Kingdom, however, the suite of compounds comprising sitosterol glucoside (B6), taraxerone (B7), taraxerol (B8) and epicatechin gallate (B12) were also reported in *Lannea rivae* (Chapter 3), indicating an even closer relationship between these two plant species than the other *Lannea* species. The flavonoid rutin (D8) has been reported previously in *Lannea grandis* (Soluchana and Sastry, 1968) while the flavonoids catechin (D7), epicatechin (D6) and epicatechin gallate (B12) reported here have also been reported to occur in *Lannea velutina, Lannea microcarpa* and *Lannea nigratina* respectively (Kapche et al., 2007; Maiga et al., 2007; Picerno et al., 2006). This is the first report of lupenone (D5) in the genus *Lannea*.

5.3 Materials and methods

*Plant collection, identification and preparation*

The plant material was collected at Seme- Kit Mikayi, Kombewa division, Kisumu West district in Kenya in June, 2010 and a voucher specimen deposited at the Maseno University Botanic Garden Herbarium (MSU/BG-1/13). The leaves, roots and stem bark of the plant were air dried away from direct sunlight for two weeks followed by grinding to a fine powder using a Wiley mill at Kibos Sugar Research Institute in Kisumu.

*General experimental procedures*

$^1$H and $^{13}$C NMR spectra were recorded in either CDCl$_3$ or CD$_3$OD on a Bruker Avance$^{\text{III}}$ 400 MHz spectrometer at room temperature. GC-MS analysis was carried out using a Hewlett-Packard GC 5890 and a Hewlett-Packard 5970 mass selective detector on an HP-1 ultra-2 column, where the temperature was programmed from 50 to 250 °C at 10 °C min$^{-1}$ and
maintained at 250 °C for 30 min. Optical rotations were recorded using a Perkin Elmer model 341 Polarimeter. UV absorption spectra were obtained on a UV/VIS 3600 Shimadzu spectrophotometer and Infrared spectra were recorded using a Perkin Elmer Universal Attenuated Total Reflectance (ATR) 100 series spectrophotometer. Chromatographic separations were achieved by column chromatography (CC) using silica gel 60 (40–63 µm, Merck 1.09385) and analytical TLC was performed on precoated silica gel 60 F254 plates (Merck 1.05554) and was developed by spraying with anisaldehyde:H2SO4:MeOH (1:2:97) followed by heating.

**Extraction and isolation**

The powdered root material (5 kg) was exhaustively extracted with n-hexane for 72 hours followed by ethyl acetate (EtOAc) and methanol (MeOH) and concentrated in vacuo to give 27.0 g of hexane extract, 25.8 g of EtOAc extract and 60.9 g of MeOH extract. The hexane extract was fractionated by open column chromatography on silica gel with a stepwise gradient of hexane:EtOAc (19:1 to 4:1). Sixty 100 mL fractions were collected. The following fractions were combined based on their TLC analysis: 1-8, 10-14, 20-26, 30-40, 50-56. Fraction 1-8 (3.78 g) was rechromatographed on a silica gel column (2cm diameter) and eluted with 19:1 hexane:EtOAc, collecting 20 mL fractions to yield a red liquid (2.07 g), a mixture of cardanols (mixture 1: D1a, D1b, C1b and C1c) from fraction 18-34. Fraction 10-14 (3.06 g) was rechromatographed on a silica gel column and eluted with 9:1 and 8:2 hexane:EtOAc. Thirteen × 20 mL fractions were collected. Fractions 6-10 and 12-14 of this column yielded 343 mg of taraxerone (B7) (white plates) and 193 mg of taraxerol (B8) (fine white crystals) respectively. Fraction 20-26 was purified with hexane:EtOAc (8:2) where a colourless paste 160.2 mg (mixture 3: D2a-D2e) was obtained from fractions 6-13. Purification of fraction 30-40 with hexane:EtOAc (7:3) yielded 1.04 g of sitosterol (A6).
(white needlelike crystals). Fraction 50-56 (6.87 g) was purified on a silica gel column and eluted with 7:3 and 6:4 hexane:EtOAc. Fractions of 20 mL were collected. Fraction 5-19 resulted in a yellow amorphous solid (4.23 g) (mixture 5: D4a, D4e, C4a and C4b).

The EtOAc extract of the roots was subject to the same chromatographic procedure, but with a stepwise gradient of hexane:dichloromethane (DCM) (19:1 to 100 % DCM). A total of 140 fractions of 100 mL each were collected. From these, four sets of fractions were combined (3-20, 50-60, 85-100, 106-139) based on similarities in their TLC profiles. Purification of fractions 3-20 with 19:1 hexane:EtOAc yielded more of mixture 1 (56.3 mg), the cardanol (D1a, D1b, C1b and C1c). Fraction 50-60 was purified with 30% EtOAc in hexane and a light yellow paste was obtained from fractions 10-29 (34.6 mg) (more of mixture 3: D2a-D2e). A 1:1 ethyl acetate:hexane was used to purify fraction 85-100. A yellow solid (45.3 mg) (mixture 5: D4a, D4e, C4a and C4b) was obtained from fractions 14-18. Fractions 106-139 was purified with 6:4 hexane:EtOAc and yielded a reddish brown solid (67.8 mg) (mixture 4: D3a, D3b, C3a and C3c) from fractions 17-37.

The methanol extracts of the roots, stems and leaves were combined and separated as above with a stepwise gradient of DCM:MeOH (19:1 to 7:3) to give three fractions, 50-56, 60-76 and 80-86. Fraction 50-56 was separated on a LH-20 sephadex column using 1:1 methanol:acetone to yield a reddish brown solid, and pale white solid, catechin (D7) (56.9 mg) and epicatechin (D6) (30.5 mg) respectively. Using 100% methanol, fraction 60-76 was purified on a LH-20 sephadex column to yield a reddish brown solid, epicatechin gallate (B12) (72.4 mg). A similar column was used to purify sitosterol glucoside (B6) (93.4 mg) (amorphous white solid) and rutin (D8) (45.1 mg) (yellow solid) from fraction 80-86.
The hexane stem bark extract was subjected to the same protocol as the hexane root extracts and yielded the same compounds as that contained in the root. In addition, the ethyl acetate extract which was separated with hexane:DCM (19:1) yielded a further mixture of cardanol (82.7 mg) (mixture 2: D1a-D1e, B1d, C1a-C1d) which was purified from fraction 85-105. Lupenone (D5) (38.4 mg) was obtained from fraction 70-76 as a colourless solid. Fraction 180-188 resulted in a yellow solid mixture (56.8 mg) (mixture 6: D4b-D4d, D4f, D4g, C4b and C4c). These were purified with hexane:EtOAc solvent mixtures (9:1 to 100% ethyl acetate) as in the root above.

**mixture 1:** 3-[tridecyl] phenol (D1a), 3-[heptadecyl] phenol (D1b), 3-[heptadec-14'(E)-enyl] phenol (C1b) and 3-[nonadec-16'(E)-enyl] phenol (C1c) red liquid; UV $\lambda_{max}$ (MeOH) 273, 220 nm; IR $\nu_{max}$ cm$^{-1}$: 3369, 2922, 2852, 1588, 1485, 1265, 1154. EIMS see chapter 4; $^1$H and $^{13}$C NMR see Queiroz et al. (2003).

**mixture 2:** 3-[tridecyl] phenol (D1a), 3-[heptadecyl] phenol (D1b), 3-[heptadec-12'(Z),14'(E)-diienyl] phenol (D1c), 3-[nonadec-14'(Z),16'(E)-diienyl] phenol (D1d), 3-[heneicos-16'(Z),18'(E)-diienyl] phenol (D1e), 3-[pentadecyl] phenol (B1d), 3-[12'(E)-pentadecenyl]phenol (C1a), 3-[14'(E)-heptadecenyl]phenol (C1b), 3-[16'(E)-nonadecenyl]phenol (C1c), 3-[18'(E)-heneicosenyl]phenol (C1d) colorless oil; UV $\lambda_{max}$ (MeOH) 310, 273 nm; IR $\nu_{max}$ cm$^{-1}$: 3371, 2922, 2852, 1589, 1456,1268, 1154; EIMS see chapters 3 and 4 and Table 5-2; $^1$H and $^{13}$C NMR see Queiroz et al. (2003) and Table 5-1 for D1c, D1d and D1e.

**mixture 3:** 5-hydroxy-5-[tridecyl] cyclohex-2-enone (D2a), 5-hydroxy-5-[pentadecyl] cyclohex-2-enone (D2b), 5-hydroxy-5-[heptadecyl] cyclohex-2-enone (D2c), 5-hydroxy-5-
[pentadec-12(E)-enyl] cyclohex-2-enone (D2d), 5-hydroxy-5-heptadec-14(E)-enyl cyclohex-2-enone (D2e) colourless paste; $[\alpha]_{D}^{20} -7.62$ (c = 1.05, CHCl$_2$); UV $\lambda_{\text{max}}$ (CH$_2$Cl$_2$) 273, 231 nm; IR $\nu_{\text{max}}$ cm$^{-1}$: 3390, 2921, 2851, 1668, 1463, 1387, 1255; EIMS see Table 5-2; $^1$H and $^{13}$C NMR see Table 5-1.

**mixture 4:** 1-[tridecyl] cyclohex-3-en-1,2,5-triol (D3a), 1-[heptadecyl] cyclohex-3-en-1,2,5-triol (D3b), 1-[12(E)-pentadecenyl]-cyclohex-3-en-1,2,5-triol (C3a), 1-[16(E)-nonadecenyl]-cyclohex-3-en-1,2,5-triol (C3c) reddish brown paste; $[\alpha]_{D}^{20} +30.48$ (c = 1.05, CHCl$_2$); UV $\lambda_{\text{max}}$ (CH$_2$Cl$_2$) 232 nm; IR $\nu_{\text{max}}$ cm$^{-1}$: 3390, 2919, 2851, 1465, 1376, 1277; EIMS see chapter 4 and Table 5-2; $^1$H and $^{13}$C NMR see chapter 4.

**mixture 5:** 1-[tridecyl] cyclohex-4-en-1,3-diol (D4a), 1-[pentadec-12(E)-enyl] cyclohex-4-en-1,3-diol (D4e), 1-[14(E)-heptadecenyl]-cyclohex-4-en-1,3-diol (C4a), 1-[16(E)-nonadecenyl]-cyclohex-4-en-1,3-diol (C4b) yellow solid; $[\alpha]_{D}^{20} +17.14$ (c = 1.05, CHCl$_2$); UV $\lambda_{\text{max}}$ (CHCl$_3$) 239 nm; IR $\nu_{\text{max}}$ cm$^{-1}$: 3269, 2916, 2850, 1468, 1310; EIMS see chapter 4 and Table 5-2; $^1$H and $^{13}$C NMR see chapter 4.

**mixture 6:** 1-[nonadecyl] cyclohex-4-en-1,3-diol (D4b), 1-[heneicosyl] cyclohex-4-en-1,3-diol (D4c), 1-[tricosyl] cyclohex-4-en-1,3-diol (D4d), 1-[nonadec-14(Z),16'(E)-dienyl] cyclohex-4-en-1,3-diol (D4f), 1-[heneicos-16'(Z),18'(E)-dienyl] cyclohex-4-en-1,3-diol (D4g), 1-[16'(E)-nonadecenyl]-4-cyclohex-4-en-1,3-diol (C4b), 1-[18'(E)-heneicosenyl]-4-cyclohex-4-en-1,3-diol (C4c) colourless solid; $[\alpha]_{D}^{20} +20.07$ (c = 1.05, CHCl$_2$); UV $\lambda_{\text{max}}$ (CH$_2$Cl$_2$) 207, 242 nm; IR $\nu_{\text{max}}$ cm$^{-1}$: 3314, 2918, 2850, 1467, 1380, 1310; EIMS see chapter 4 and Table 5-2. $^1$H and $^{13}$C NMR see Table 5-1.
Antibacterial assay

Microorganisms used in this study were isolated from clinical samples at CDC Kombewa, Kenya. Commercially available antibiotic diffusion discs were used. The bacteria tested were *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 19434, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923.

The stock solutions of compounds and extracts were dissolved in 10% dimethylsulfoxide (DMSO) in water to a final concentration of 100 µg mL⁻¹. The sterile discs (6 mm) were impregnated with 10 µL of the compounds (1 µg). Petri plates were prepared with 20 mL of a base layer of molten Mueller Hinton agar. The inoculum of ATCC used was adjusted to 0.5 McFarland standard (10⁶ CFU mL⁻¹). The plates were incubated for 24 h at 37 °C (CLSI, 2007). Negative controls were prepared using discs impregnated with 10% DMSO in water and commercially available antibiotic diffusion discs, penicillin (10 units), erythromycin (15 µg mL⁻¹), vancomycin (30 µg mL⁻¹) and cefuroxime (30 µg mL⁻¹) were used as positive reference standards. The diameters of the inhibition zones were evaluated in mm. The extracts or compounds inducing inhibition zones of ≥ 9 mm were considered as potential antibacterial compounds. All tests were performed in triplicate and the zone of inhibition (bacterial activity) was expressed as an average of the three readings in mm.

Cytotoxicity assay

*In vitro* cytotoxicity tests of the compounds were performed on a Chinese Hamster Ovarian mammalian cell-line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays (Mosmann *et al.*, 1983; Rubinstein *et
al., 1990). The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The samples were tested in triplicate on one occasion.

The test samples were prepared to a 20 mg mL\(^{-1}\) stock solution in 100% DMSO and were tested as a suspension if not properly dissolved. Test compounds were stored at -20 °C until used. Emetine was used as the reference drug in all experiments. The initial concentration of test samples was 100 µg mL\(^{-1}\), which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg mL\(^{-1}\). The same dilution technique was applied to all the test samples. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability (data not shown). The 50% inhibitory concentration (IC\(_{50}\)) values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

Larvicidal assays

The compounds were solubilized in dimethyl sulphoxide (DMSO, analytical reagent, Lobarchemi) and diluted to the required concentration with spring water. The concentration of DMSO was kept below 1%. The bioassay experiments were conducted according to standard procedure (WHO, 2005) with slight modifications. The bioassays were conducted at the Kenya Medical Research Institute (KEMRI), Insect Unit, Kisumu, Kenya, where the insects were reared in plastic and enamel trays in spring river water. They were maintained and all experiments were carried out at 26 ± 3 °C and the humidity ranged between 70% and 75%. The bioassays were performed with late third and early fourth instar larvae of *A. gambiae* and carried out in triplicate using 25 larvae for each replicate assay according to established procedures (WHO, 2005). The replicates were run simultaneously yielding a final total of 75 larvae for each dosage. The larvae were placed in 40 mL disposable plastic
cups containing 20 mL of test solution and fed on tetramin fish feed during all testing. Mortality and survival was established after 24 h of exposure. Larvae were considered dead if they were unresponsive within a period of time, even when gently prodded. The dead larvae in the three replicates were recorded after the 24 hr exposure. The negative control was 1% DMSO in spring river water while the positive control was the pyrethrum based larvicide pylarvex® (Pyrethrum Board of Kenya). The probit analysis of the concentration mortality data was conducted using IBM SPSS statistics version 19 to obtain LD$_{50}$ values and the associated 95% confidence limits.

**SYBR Green I assay antiplasmodial assay**

The plant extracts were screened against two *Plasmodium falciparum* strains, chloroquine sensitive (D6) from Sierra Leone and chloroquine resistant (W2) from Vietnam. The cultures were maintained at the US Army Medical Research Unit—Kenya, Malaria Resistance Laboratories at the Kenya Medical Research Institute (KEMRI), Kisian-Kisumu, according to literature (Smilkstein et al., 2004). Mefloquine and chloroquine reference drugs were used as a positive control. The culture medium was prepared as described by Johnson et al. (2007). Mefloquine was dissolved in 70% ethanol while the compounds and chloroquine were dissolved in 100% DMSO to an initial concentration of 1 mg mL$^{-1}$. The *P. falciparum* cultures were adjusted to 2% haematocrit and 1% parasitemia for the assay (Akala et al., 2011) a modification of Desjardins et al. (1979) and Trager and Jensen (1976). After 72 h incubation, 100 µL of lysis buffer containing SYBR Green I dye was added to the 96-well plates prior to 1 h incubation in the dark. The fluorescence was then measured using a Genios Tecan® micro-plate reader. IC$_{50}$ values were then calculated by Graphpad Prism (Graphpad Prism for Windows, version 5.0; Graphpad Software, Inc., San Diego, CA).
Antioxidant assay

A 10 mg mL\(^{-1}\) stock solution of each compound was made by dissolving the compounds in DMSO. Sample concentrations of 6.25, 12.50, 25.00, 50.00 and 100 \(\mu\)g mL\(^{-1}\) were made in methanol. The DPPH radical scavenging activity of the compounds was determined according to the method of Kumawat et al. (2012). Briefly, 1 mL of each compound was added to 1 mL of DPPH (1,1-diphenyl-2-picrylhydrazyl) solution (0.1 mM in methanol). The mixture was shaken and kept in the darkness for 30 minutes at room temperature. The decrease of solution absorbance was determined at 517 nm. Vitamin C (ascorbic acid) was used as the positive control. The DPPH radical scavenging activity was calculated using the following formula:

\[
\text{DPPH Radical Scavenging Activity (\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100},
\]

where \(A_0\) is the absorbance of the control, and \(A_1\) is the absorbance of the compound or standard sample.

All the tests were performed in triplicate. The results were given as means ± S.D. Analysis of variance and significant differences among means were tested by two way ANOVA, using SPSS (Version 12.0 for Windows, SPSS Inc., Chicago, IL, USA). When significant main effects existed, differences were tested by Duncans test at 95% confidence. The IC\(_{50}\) values were calculated using dose-response curves, using a non-linear dose-response curve fitting analysis GraphPad Prism (Version 5) software.

5.4 Conclusion

We have found six mixtures in the roots and stem bark of \(L.\) \textit{schweinfurthii}, one which had a novel core structure with an \(\alpha,\beta\)-unsaturated ketone moiety (mixture 3; \textbf{D2a-D2e}) and which was found to be active in antibacterial, antiplasmodial, larvicidal and cytotoxicity assays along with epicatechin gallate (\textbf{B12}), which did not show larvicidal activity, but
showed antioxidant activity in addition to the antibacterial, antiplasmodial and cytotoxic activity. It is highly likely that the medicinal uses and bioactivity of the extracts is a result of these phytochemical constituents present in the plant. While epicatechin gallate (B12) has been studied extensively, the novel structures in mixture 3 can provide leads for antibacterial, antiplasmodial, larvicidal and anti-cancer drugs. The fact that mixture 6, which is more cytotoxic is not active in the any of the assays above indicates that the toxicity of mixture 3 is not responsible for the antibacterial, antiplasmodial and larvicidal activity, but rather the α,β-unsaturated ketone moiety could play a role in the activity of the compounds in mixture 3.

Acknowledgements
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5.5 References


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Chapter 6  Conclusion

This thesis describes the phytochemistry of *Lannea alata*, *Lannea rivae*, *Lannea schweinfurthii* and *Lannea schimperi* and the bioactivities of the extracts, mixtures and compounds from these four species. The plants are used in traditional medicine to treat a variety of disease symptoms like fever, malaria, diarrhea and wounds.

Prior to this study, *Lannea alata* was only known to be used to treat fever, malaria, fractures, wounds and snakebites. This investigation led to the isolation of two known and two new flavonoids (myricetin-3-*O-*α-rhamnopyranoside (A3), myricetin-3-*O-*α-arabinofuranoside (betmidine) (A4), lanneaflavonol (A1) and lanneadihyroflavonol (A2)), along with lupeol (A5) and sitosterol (A6). The flavonoids were shown to exhibit antibacterial and antioxidant activities and lupeol (A5) is reported to have numerous biological activities including antibacterial, antifungal, antiplasmodial, anti-trypanosomal, antitumor/anticancer, anti-inflammatory and anti-arthritic activities. Sitosterol (A6) is most commonly known for its immune boosting properties. The antibacterial and antiplasmodial activity of the compounds isolated from *L. alata* could therefore be responsible for the antipyretic and antimalarial action of the plant extracts and its use to treat snakebites, wounds and fractures, could be due to the anti-inflammatory action of lupeol (A5). The immunostimulant properties of sitosterol (A6) may also contribute generally to the plant being used for medicinal purposes. The new flavonoids, lanneaflavonol (A1) and dihydroflavonol (A2) could be leads for the development of antibacterial and antioxidant pharmaceuticals.

*Lannea rivae* is a medicinal plant used for the treatment of colds, coughs, fever and stomachache. Its medicinal uses suggest the presence of antibacterial compounds in its
extracts. The phytochemical investigation led to isolation of the triterpenes, sitosterol (A6), sitosterol glucoside (B6), taraxerone (B7) and taraxerol (B8), the flavonoids, myricetin (B10), myricetin-3-O-α-rhamnopyranoside (A3), myricetin-3-O-β-galactopyranoside (B11), (-)-epicatechin-3-O-gallate (B12), and a mixture of four cardanols (B1a-B1d), a furanocyclohex-2-enone (B2), a trihydroxycyclohexanone (B3), a mixture of dihydroxycyclohex-2-enones (B4a and B4b) and a trihydroxycyclohexane (B5). A tetratraterpene, trans lutein (B9) was also isolated. Myricetin (B10), its glycosides (B11 and A3) and epicatechin gallate (B12) showed good antioxidant activity and myricetin (B10) and epicatechin gallate (B12) showed good antibacterial activity. The furanocyclohex-2-enone (B2) and trihydroxycyclohexanone (B3) showed good cytotoxic activity as well as good antiplasmodial activity. The mixture of B4a and B4b was much less toxic than B2 and B3 and showed promising antiplasmodial activity, suggesting a good lead for a non-toxic antiplasmodial drug. The isolation of these active compounds in the plant extracts provides a rationale for the use of the plant to treat coughs, colds, fever and stomachache. The novel compounds B2, B3 and B4a-B4b could be pursued as leads for antiplasmodial and anticancer drugs.

The bark of the roots and stem as well as the leaves of Lannea schimperi is used medicinally to clean teeth and manage toothache, for diarrhea, chest infections, stomach pains, mental disorders, epilepsy, snake bites, tuberculosis, skin infections, herpes simplex, herpes zoster and other opportunistic infections from HIV/AIDS. This study led to the isolation of moderately cytotoxic triterpenoids (taraxerol (B8) and taraxerone (B7)) as well as cardanols (C1a-C1d), dihydroxycyclohex-2-enones (C2a-C2d), 1,2,5-trihydroxycyclohex-3-enes (C3a-C3c) and 1,3-dihydroxycyclohex-4-enes (C4a-C4c). The 5-[alkenyl]-4,5-dihydroxycyclohex-2-enone mixture (C2a-C2d) exhibited good in vitro cytotoxicity. We were not able to test these particular extracts for antibacterial activity, however D2a-D2e
isolated from Lannea schweinfurthii below, with structures similar to C2a-C2d showed moderate antibacterial activity. The compounds in the other mixtures C3a-C3c and C4a-C4c were also present in the mixtures from L. schweinfurthii below, but showed no demonstrable antibacterial activity. Future work could involve the reisolation of the mixture C2a-C2d to see if the antibacterial activity is better than D2a-D2e. Although the isolates from L. schimperii do not necessarily support the reported medicinal uses of the plant, the mixture of C2a-C2d could be a good lead for an anticancer drug.

Previous studies on Lannea schweinfurthii crude extracts indicated moderate cytotoxicity, antioxidant, antiplasmodial and acetylcholinesterase inhibition activities but no active constituents were reported. The stem and root bark decoction is traditionally used for stomach ailments such as stomach pain, gastrointestinal diseases, diarrhea and constipation, and to treat headaches and boils. We report the isolation of antioxidant flavonoids (epicatechin (D6), epicatechin gallate (B12), catechin (D7) and rutin (D8)) from the plant. Of these only epicatechin gallate (B12) demonstrated good antibacterial and antiplasmodial activity. Six mixtures of alkylated cardanols, cyclohexenones and cyclohexenols were also isolated. Of these mixtures, mixture 3 (D2a-D2e) showed good antibacterial, antiplasmodial, larvicidal and cytotoxic activity. In addition, the triterpenoids, sitosterol (A6), sitosterol glucoside (B6) and lupenone (D5) was also isolated. Sitosterol (A6) and sitosterol glucoside (B6) are known immune boosters and could well contribute to the many medicinal uses of the plant. The many compounds and mixtures, each with their own bioactivity could play a role in the bioactivity of the extracts of the plant as well as the medicinal effects of the plant extracts administered as traditional medicine.
Phenolic lipids are derivatives of mono and dihydroxyphenols, namely catechol, resorcinol and hydroquinones. Plants within the Anacardiaceae are the main source of phenolic lipids. The cardanols were isolated from the three *Lannea* species, *alata*, *schimperi* and *schweinfurthii*. The alkyl/alkenyl cyclohexenones and alkyl/alkenyl cyclohexenols have only been isolated from *Tapirira* (*Tapirira obtusa, Tapirira guainensis*) and *Lannea* (*Lannea welwitschii, Lannea nigratina* and *Lannea edulis*) within the Anacardiaceae family. They are thought to be precursors in the biosynthetic pathway of phenolic lipids. Isolation of these kinds of compounds in three out of four *Lannea* species studied is indicative of the close phylogenetic relationship between these three *Lannea* species and the two species from *Tapirira*. These findings agree with the classification of both *Lannea* and *Tapirira* in the Spondieae tribe of the Anacardiaceae family as proposed by Engler. Thus, phenolic lipids can be considered as chemotaxonomic markers of the *Lannea* and *Tapirira* genera. Phenolic lipids are also known to exhibit antibacterial, antifungal, antigenotoxic, antioxidant, cytostatic, cytotoxic and antiprotozoal activities. These activities are related to the ability of phenolic lipids to interact with proteins on cell membranes. Phenolic lipids are potentially useful for the treatment of cancer, viral and skin diseases. The *Lannea* genus promises to be a source of new bioactive substances.

**Limitations of the study and recommendations for future work**

Attempts to isolate the individual cardanols, cyclohexenonenones and cyclohexenols using silica gel, sephadex, and silica gel coated with silver nitrate packed columns were unsuccessful. Future work could involve the investigation of how to separate these compounds with very similar structures and polarity and which only differ in the size and nature of the side chain present. Preparative silver ion HPLC can be explored as one alternative and derivatisation, followed by separation as another alternative, however it must
be possible to convert the derivatised compounds back to their original compounds so that they could be tested in its original form.

The absolute stereochemistry of some compounds could not be established since they were isolated as mixtures. It would be good to explore possibilities of determining the absolute stereochemistry of the compounds. One could try to derivatise the molecule without affecting the stereochemical centres and then try to crystallise the derivatised compounds and get an X-ray structure of the molecule.

Alkyl phenols and their derivatives have previously demonstrated cytotoxicity against tumor cells. In this study, their cytotoxicity was evaluated against a mammalian cell-line, Chinese Hamster Ovarian (CHO). We did not test the isolates from the *Lannea* species against tumor cell lines as we did not have access to these cell lines. Future work could involve testing of the compounds against tumor cell lines to identify possible leads for anticancer chemotherapy. Some of the compounds that were cytotoxic also exhibited good antiplasmodial and antibacterial activities. A further study could assess the cytotoxic selectivity of these compounds i.e whether they are more toxic towards infected cells compared to normal cells.

*In vitro* activity was carried out in all the bioassays undertaken in this work. *In vivo* experiments need to be carried out on the active compounds to determine whether or not they have the potential to be developed into drugs.
The MTT assay used to evaluate cytotoxicity. Though fast and reliable, it does not differentiate whether these compounds kill cells or merely inhibit their growth. The mode of action of these compounds could be determined by clonogenic assays.

This thesis demonstrated some biological activities of individual compounds isolated from *Lannea* species. Studies using combinations of the isolated compounds can be carried out to determine their synergistic or antagonistic effects.

In general, we have successfully carried out the phytochemistry of four *Lannea* species and the compounds isolated exhibited cytotoxicity, antibacterial, antiplasmodial, larvicidal and antioxidant activities and supported their use in traditional medicine.
Appendix 1

PHYTOCHEMISTRY AND BIOACTIVE NATURAL PRODUCTS FROM LANNEA ALATA, LANNEA RIVAE, LANNEA SCHIMPERI AND LANNEA SCHWEINFURTHII (ANACARDIACEAE)

2014

OKOTH AKINYI DOROTHY
$^1$H NMR spectrum of A1
$^{13}$C NMR spectrum of A1
COSY spectrum of A1
NOESY spectrum of A1
HSQC spectrum of Al

KIT 30-34 7-10 in DMSO HSQC
HMBC spectrum of A1
IR spectrum of A1
UV Spectrum of Al

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- Volume: 10 µg
- Sample Preparation Properties: None
- Measurement Properties: None
- Scan Mode: Single
- Wavelength Interval: 0.0 nm
- Scanning Interval: 0.1 nm
- Measurement Properties: None

![UV Spectrum Graph]

Data Set: K2 30-34 7-10 D 5 spc - Store 135929
MS spectrum of A1
$^1$H NMR spectrum of A2 expanded (4-7 ppm)
NOESY spectrum of A2
HMBC spectrum of A2
IR spectrum of A2
UV Spectrum of A2
CD spectrum of A2
$^1$H NMR spectrum of A3
$^{13}$C NMR spectrum of A3
COSY spectrum of A3
HSQC spectrum of A3
HMBC spectrum of A3
MS spectrum of A3
$^1$H NMR spectrum of A4
$^{13}$C NMR spectrum of A4
IR spectrum of A4
$^1$H NMR spectrum of A5
$^1$H NMR spectrum of A5 expanded (0-3.5 ppm)
$^{13}$C NMR spectrum of A5
$^{13}$C NMR spectrum of A5 expanded (0-60 ppm)
DEPT spectrum of A5
DEPT spectrum of A5 expanded (0-60 ppm)
HSQC spectrum of A5
HSQC spectrum of A5 expanded (F1, 0-60 ppm, F2, 0-2 ppm)
HMBC spectrum of A5
MS spectrum of A5
$^1$H NMR spectrum of A6
$^{13}$C NMR spectrum of A6 (expanded 0–72 ppm)
COSY spectrum of A6
HSQC spectrum of A6
HSQC spectrum of A6 (expanded 0-25 ppm)
HMBC spectrum of A6
\(^1\)H NMR spectrum of B1 (mixture a, b, c and d)
$^1$H NMR spectrum of B1 expanded 0.6-2.5 ppm (mixture a, b, c and d)
$^1$H NMR spectrum of B1 expanded 6.4-7.3 ppm (mixture a, b, c and d)
$^{13}$C NMR spectrum of B1 (mixture a, b, c and d)
$^{13}$C NMR spectrum of B1 expanded 110-156 ppm (mixture a, b, c and d)
$^{13}$C NMR spectrum of B1 expanded 13.8-36 ppm (mixture a, b, c and d)
DEPT spectrum of B1 (mixture a, b, c and d)
COSY spectrum of B1 (mixture a, b, c and d)
COSY spectrum of B1 expanded section F2 6.5-7.3 ppm (mixture a, b, c and d)
COSY spectrum of B1 expanded section F2 0.6 - 2.6 ppm (mixture a, b, c and d)
HSQC spectrum of B1 (mixture a, b, c and d)
HSQC spectrum of B1 expanded F2 5.2-7.3 ppm (mixture a, b, c and d)
HSQC spectrum of B1 expanded F2 0.6 - 2.6 ppm (mixture a, b, c and d)
HMBC spectrum of B1 (mixture a, b, c and d)
HMBC spectrum of B1 expanded F2 6.4 – 7.3 ppm (mixture a, b, c and d)
HMBC spectrum of B1 expanded F2 0.7 – 2.7 ppm (mixture a, b, c and d)
IR spectrum of BI (mixture a, b, c and d)
UV spectrum of B1 (mixture a, b, c and d)
MS spectrum of B1 (a)
MS spectrum of B1 (c)
MS spectrum of B1 (d)
$^1$H NMR spectrum of B2
1H NMR spectrum of B2 expanded (0.8-2.9 ppm)
1H NMR spectrum of B2 expanded (4.3-6.7 ppm)
DEPT spectrum of B2 ($^{13}$C NMR, DEPT 90 and 135)
DEPT spectrum of B2 (DEPT 90 and 135)
NOESY spectrum of B2
NOESY spectrum of B2 expanded (F1 0-3.1, F2 0-2.9 ppm)
HMBC spectrum of B2
UV spectrum of B2
File: C:\msdchem\1data\dorothy\MULF 4 B.D
Operator: Dorothy
Acquired: 27 Feb 2013 13:16 using AcqMethod NATPRODUCTS MANUAL INJ SPLIT.M
Instrument: 5973N
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Misc Info: Vial Number: 1

Scan 1889 (21.055 min): MULF 4 B.D\data.ms

MS spectrum of B2
$^1$H NMR spectrum of B3
\(^1\)H NMR spectrum of B3 expanded (0.6-2.4 ppm)
\[13\text{C NMR spectrum of B3}\]
DEPT spectrum of B3
COSY spectrum of B3
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IR spectrum of B3
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- Sampling Interval: 1.0
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- Scan Mode: Single

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- Measuring Mode: Absorbance
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- Time Constant: 0.1 sec
- Source Lamp: Auto
- Light Source Change Wavelength: 310.00 nm
- Detector Unit: Direct
- Detector Change Wavelength: 830.00 nm 1600.00 nm
- Grating Change Wavelength: 720.00 nm
- S/R Exchange: Normal
- Detector Lock: Auto
- Slit Program: Normal
- Beam Mode: Double
- Stair Correction: Disable

**Attachment Properties**
- Attachment: None

**Sample Preparation Properties**
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- Volume:
- Dilution:
- Path Length:
- Additional Information:

**UV spectrum of B3**
MS spectrum of B3
$^1$H NMR spectrum of B4 (mixture of a and b)
\[ ^1H \text{ NMR spectrum of B4 expanded 4.6-6.9 ppm (mixture of a and b)} \]
$^1$H NMR spectrum of B4 expanded 0.6-2.3 ppm (mixture of a and b)
$^{13}$C NMR spectrum of B4 (mixture of a and b)
DEPT spectrum of B4 (mixture of a and b)
COSY spectrum of B4 (mixture of a and b)
NOESY spectrum of B4 (mixture of a and b)
HSQC spectrum of B4 (mixture of a and b)
HMBC spectrum of B4 (mixture of a and b)
IR spectrum of B4 (mixture of a and b)
UV spectrum of B4 (mixture of a and b)
MS spectrum of B4 (a)
$^1$H NMR spectrum of B5
$^1$H NMR spectrum of BS expanded (F1 0.6-2.1 ppm)
\text{13C NMR spectrum of B5}
$^{13}$C NMR spectrum of B5 expanded (10-44 ppm)
COSY spectrum of B5 expanded (F1, F2 0.6 - 4.1 ppm)
HSQC spectrum of B5
HMBC spectrum of B5
IR spectrum of B5
**Measurement Properties**

- **Wavelength Range (nm):** 200.00 to 800.00
- **Scan Speed:** Medium
- **Sampling Interval:** 1.0
- **Auto Sampling Interval:** Disabled
- **Scan Mode:** Single

**Instrument Properties**

- **Instrument Type:** UV-3600 Series
- **Measuring Mode:** Absorbance
- **Slit Width:** 8.0 nm
- **Time Constant:** 0.1 sec.
- **Source Lamp:** Auto
- **Light Source Change Wavelength:** 310.00 nm
- **Detector Unit:** Direct
- **Detector Change Wavelength:** 630.00 nm 1800.00
- **Grating Change Wavelength:** 720.00 nm
- **S/R Exchange:** Normal
- **Detector Lock:** Auto
- **Silt Program:** Normal
- **Beam Mode:** Double
- **Stair Correction:** Disable

**Attachment Properties**

- **Attachment:** None

**Sample Preparation Properties**

- **Weight:**
- **Volume:**
- **Dilution:**
- **Path Length:**
- **Additional Information:**

---

**UV spectrum of B5**

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MS spectrum of B5
$^1$H NMR spectrum of B6 expanded (0-2.5 ppm)
$^1$H NMR spectrum of B6 expanded (1.0-4.4 ppm)
$^{13}$C NMR spectrum of B6 expanded (10-46 ppm)
DEPT spectrum of B6 expanded (10-46 ppm)
NOESY spectrum of B6
HSQC spectrum of B6
HSQC spectrum of B6 (F1 10-64 ppm, F2 0.0-2.6 ppm)
IR spectrum of B6
UV spectrum of B6
Scan 3180 (21.754 min): MULFD1.D

M⁺ = 576
M - Sugar = 444
LC-MS spectrum of B6

$M^+ = 576$

$M^+ + Na = 599$
$^1$H NMR spectrum of B7
1H NMR spectrum of B7 expanded (0.6-1.2 ppm)
1H NMR spectrum of B7 expanded (0.6-1.2 ppm)
$^{13}$C NMR spectrum of B7
13C NMR spectrum of B7 expanded (14.0-56.0 ppm)
DEPT spectrum of B7 expanded (14.0-56.0 ppm)
kithoona 5 in odc13 HSQC

HSQC spectrum of B7
HSQC spectrum of B7 (F1 10-56 ppm, F2 0.7-2.6 ppm)
HSQC spectrum of B7 (F1 10-35 ppm, F2 0.7-1.25 ppm)
HMBC spectrum of B7
HMBC spectrum of B7 expanded (F1 0-158 ppm, F2 0-2.7 ppm)
IR spectrum of B7
UV spectrum of B7
MS spectrum of B7
$^1$H NMR spectrum of B8 expanded (0.6-1.2 ppm)
$^{13}$C NMR spectrum of B8 expanded (14.0-42.0 ppm)
$^{13}$C NMR spectrum of B8 expanded (14.0-56.0 ppm)
COSY spectrum of B8

kithoon 30-65 6-7 in cdcl3 COSY
HSQC spectrum of B8 (F1 17-56 ppm, F2 0.7-2.1 ppm)
HMBC spectrum of B8
HMBC spectrum of B8 expanded (F1 14-60 ppm, F2 0.7-1.3 ppm)
Spectrum Peak Pick Report

Data Set: kithoon 5.spc - Storage 180228

Not Good

Measurement Properties
- Wavelength Range (nm.): 200.00 to 400.00
- Scan Speed: Medium
- Sampling Interval: 1.0
- Auto Sampling Interval: Disabled
- Scan Mode: Single

Instrument Properties
- Instrument Type: UV-3600 Series
- Measuring Mode: Absorbance
- Slt Width: 2.0 nm
- Time Constant: 0.1 sec.
- Source Lamp: Auto
- Light Source Change Wavelength: 310.00 nm
- Detector Unit: Direct
- Detector Change Wavelength: 850.00 nm 1800.00 nm
- Grating Change Wavelength: 720.00 nm
- S/R Exchange: Normal
- Detector Lock: Auto
- Slt Program: Normal
- Beam Mode: Double
- Stair Correction: Disable

Attachment Properties
- Attachment: None

Sample Preparation Properties
- Weight:
- Volume:
- Dilution:
- Path Length:
- Additional Information:

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MS spectrum of B8
$^1$H NMR spectrum of B9
$^1H$ NMR spectrum of B9 expanded (3.7-6.8 ppm)
$^1$H NMR spectrum of B9 expanded (0.7-2.2 ppm)
$^{13}$C NMR spectrum c. δ9
$^{13}$C NMR spectrum of B9 expanded (124-139 ppm)
DEPT spectrum of B9
DEPT spectrum of B9 expanded (0-70 ppm)
DEPT spectrum of B9 expanded (124-139 ppm)
HSQC spectrum of B9 (F1 10-54 ppm, F2 0.5-2.6 ppm)
HSQC spectrum of B9 (F1 124-139 ppm, F2 5.4-6.6 ppm)
HMBC spectrum of B9 expanded (F1 10-75 ppm, F2 0.6-2.5 ppm)
HMBC spectrum of B9 expanded (F1 124-140 ppm, F2 1.0-2.4 ppm)
HMBC spectrum of B9 expanded (F1 120-143 ppm, F2 5.2-6.8 ppm)
IR spectrum of B9
Display Report - All Windows All Analyses

Operator: Operator
Instrument: LC-MS-Trap-VL
Print Date: 7/27/2012 2:33:13 PM

$M^+ = 568$

$M^+ + Na = 591$

$M^+Na - 18(H_2O) = 573$

LC-MS spectrum of B9
MS spectrum of B9
$^1$H NMR spectrum of B10
$^{13}$C NMR spectrum of B10
DEPT spectrum of B10
HMBC spectrum of B10
Expanded
LC-MS spectrum of B10
The image shows an NMR spectrum labeled \(^1\)H NMR spectrum of B11. The spectrum includes chemical shifts and peaks corresponding to different protons in the molecule. The molecule structure is also shown, with labeled atoms and protons. The chemical shifts are indicated near the peaks on the spectrum.
\[ \text{13C NMR spectrum of B1} \]
COSY spectrum of B11
NOESY spectrum of B11
HSQC spectrum of B11
HMBC spectrum of B11
**Spectrum Peak Pick Report**

Data Set: myricetin galactoside batter.spc - Storage 102515

![UV Spectrum](image)

**Measurement Properties**
- Wavelength Range (nm.): 200.00 to 400.00
- Scan Speed: Medium
- Sampling Interval: 1.0
- Auto Sampling Interval: Disabled
- Scan Mode: Single

**Instrument Properties**
- Instrument Type: UV-3600 Series
- Measuring Mode: Absorbance
- Slit Width: 1.0 nm
- Time Constant: 1.0 sec.
- Source Lamp: Auto
- Light Source Change Wavelength: 310.00 nm
- Detector Unit: Direct
- Detector Change Wavelength: 630.00 nm 1600.00 nm
- Grating Change Wavelength: 720.00 nm
- S/R Exchange: Normal
- Detector Lock: Auto
- Slit Program: Normal
- Beam Mode: Double
- Stair Correction: Disable

**Attachment Properties**
- Attachment: None

**Sample Preparation Properties**
- Weight: 
- Volume: 
- Dilution: 
- Path Length: 
- Additional Information: 

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UV spectrum of B11
$M^+ = 480$

$M^+ + 23 \left( N_a \right) = 503$
$^{1}H$ NMR spectrum of B12
$^{13}$C NMR spectrum of B12
NOESY spectrum of B12
HSQC spectrum of B12
HMBC spectrum of B12
HMBC spectrum of B12 (expanded F1 95-175 ppm, F2 6.4-7.2 ppm)
IR spectrum of B12
UV spectrum of B12
LC-MS spectrum of B12
$^1$H NMR spectrum of C-1 (a-d)
C NMR spectrum of C-1 (a-d)
COSY spectrum of C-1 (a-d)
NOESY spectrum of C-1 (a-d)
IR spectrum of C-1 (a-d)
UV spectrum of C-1 (a-d)
Scan 2039 (20.997 min): KITOONA .D

MS spectrum of C-1 c
Scan 2428 (23.842 min): KITHOONA.D

MS spectrum of C-1 d
$^1$H NMR spectrum of C-2 (a-d)
$\text{^13C NMR spectrum of C-2 (a-d)}$

- 202.0155
- 152.7913
- 131.8504
- 129.3971
- 125.8263

$2a, n = 8, 2b = 10^*$
$2c, n = 12$
$2d, n = 14$

* used for spectral assignment

- 75.8884
- 66.1830

- 44.4723
- 37.1554
- 32.5742
- 29.7610
- 29.6932
- 29.6649
- 29.6301
- 29.6093
- 29.5349
- 29.4672
- 29.1922
- 25.5987
- 23.0113
- 14.0079
DEPT spectrum of C-2 (a-d)
NOESY spectrum of C-2 (a-d)
HMBC spectrum of C-2 (a-d)
IR spectrum of C-2 (a-d)
UV spectrum of C-2 (a-d)
MS spectrum of C-2 a
MS spectrum of C-2 b
Abundance

Scan 2364 (23.374 min): Kithoon4.D

MS spectrum of C-2 c
MS spectrum of C-2 d
klothoins 45-55 17-23 (4-10) in cdc13

3a: n = 8  3b: n = 10  3c: n = 12
* used for spectral assignments

1H NMR spectrum of C-3 (a-c)
The image shows a 13C NMR spectrum of C-3 (a-c). The spectrum includes a structural diagram and assignments for the carbon nuclei. The chemical shifts are indicated on the right side of the spectrum. The structures are labeled with numbers corresponding to specific carbon positions. The assignments include the following: 3a: n = 8, 3b: n = 10, 3c: n = 12. The asterisk indicates that 3c is used for spectral assignments.
COSY spectrum of C-3 (a-c)
NOESY spectrum of C-3 (a-c)

3a: n = 8  3b: n = 10  3c: n = 12
* used for spectral assignments
Spectrum Peak Pick Report

Data Set: kithoona 45-55 17-23 (4-10).spc - Storage 174127

Measurement Properties
Wavelength Range (nm.): 200.00 to 400.00
Scan Speed: Medium
Sampling Interval: 1.0
Auto Sampling Interval: Disabled
Scan Mode: Single

Instrument Properties
Instrument Type: UV-3600 Series
Measuring Mode: Absorbance
Slt Wdth: 2.0 nm
Time Constant: 0.1 sec.
Source Lamp: Auto
Light Source Change Wavelength: 310.00 nm
Detector Unit: Direct
Detector Change Wavelength: 830.00 nm 1800.00 nm
Grating Change Wavelength: 720.00 nm
S/R Exchange: Normal
Detector Lock: Auto
Slt Program: Normal
Beam Mode: Double
Stair Correction: Disable

Attachment Properties
Attachment: None

Sample Preparation Properties
Weight:
Volume:
Dilution:
Path Length:
Additional Information:

UV spectrum of C-3 (a-c)
IR spectrum of C-3 (a-c)
Scan 1807 (19.299 min): KITHOONA2.D

M+ - H2O = 320

MS spectrum of C-3 a
MS spectrum of C-3 b

$M^+ - H_2O = 348$
MS spectrum of C-3 c
\[ \text{\(^1\text{H NMR spectrum of C-4 (a-e)}}] \]
$^{13}\text{C}$ NMR spectrum of C-4 (a-c)
DEPT spectrum of C-4 (a-c)
COSY spectrum of C-4 (a-c)
NOESY spectrum of C-4 (a-c)
HSQC spectrum of C-4 (a-c)

4a: n = 10
4b: n = 12
4c: n = 14

* used for spectral assignments
HMBC spectrum of C-4 (a-c)
IR spectrum of C-4 (a-c)

- 4a: n = 10
- 4b: n = 12
- 4c: n = 14

* used for spectral assignments
Spectrum Peak Pick Report

Data Set: kithoona 30-39 B 45-55 (17-23) 4-10.spc - Storage 174453

Measurement Properties
Wavelength Range (nm.): 200.00 to 400.00
Scan Speed: Medium
Sampling Interval: 1.0
Auto Sampling Interval: Disabled
Scan Mode: Single

Instrument Properties
Instrument Type: UV-3600 Series
Measuring Mode: Absorbance
Slt Width: 2.0 nm
Time Constant: 0.1 sec.
Source Lamp: Auto
Light Source Change Wavelength: 310.00 nm
Detector Unit: Direct
Detector Change Wavelength: 830.00 nm  1800.00 nm
Grating Change Wavelength: 720.00 nm
S/R Exchange: Normal
Detector Lock: Auto
Slt Program: Normal
Beam Mode: Double
Stair Correction: Disable

Attachment Properties
Attachment: None

Sample Preparation Properties
Weight:
Volume:
Dilution:
Path Length:
Additional Information:

UV spectrum of C-4 (a-c)

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* used for spectral assignments
$M^+ - 18 = 332$
$M^+ - 13 = 360$
MS spectrum of C-4 c
\(^1\)H NMR spectrum of a mixture of D1a, D1b, C1a, C1b and C1c

* compounds used for spectral assignments
$^{13}$C NMR spectrum of a mixture of D1a, D1b, C1a, C1b and C1c
DEPT spectrum of a mixture of D1a, D1b, C1a, C1b and C1c
COSY spectrum of a mixture of D1a, D1b, C1a, C1b and C1c
NOESY spectrum of a mixture of D1a, D1b, C1a, C1b and C1c
HSQC spectrum of a mixture of D1a, D1b, C1a, C1b and C1c
HMBC spectrum of a mixture of D1a, D1b, C1a, C1b and C1c
UV spectrum of a mixture of D1a, D1b, C1a, C1b and C1c
IR spectrum of a mixture of D1a, D1b, C1a, C1b and C1c
GC chromatogram of a mixture of D1a, D1b, Cla, C1b and Clc
$^1$H NMR spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
$^1$H NMR spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
'H NMR spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
'H NMR spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
\(^{13}\)C NMR spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
$^{13}$C NMR spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
$^{13}$C NMR spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
DEPT spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
DEPT spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
DEPT spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
COSY spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
NOESY spectrum of a mixture of Dla, Dlb, Dle, Dld, Del, Dla, Cia, and Cid.
HSQC spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
HSQC  spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
HSQC spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
HMBC spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
HMBC spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
HMBC spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
HMBC spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
HMBC spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
HMBC spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d (expanded)
IR spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
UV spectrum of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
GC chromatogram of a mixture of D1a, D1b, D1c, D1d, D1e, B1d, C1a, C1b, C1c and C1d
Scan 2531 (25.697 min): LSS3.D

Mass spectrum of D1b
Scan 2164 (22.562 min): LSS3.D

Mass spectrum of B1d
Scan 2520 (25.603 min): LSS3.D

Mass spectrum of C1b
Scan 2651 (26.722 min): LSS3.D

Mass spectrum of C1d
Scan 2445 (24.962 min): LSS3.D

Mass spectrum of D1c
Scan 1822 (19.640 min): LSS3.D

Mass spectrum of D1d

Abundance
'H NMR spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
$^1$H NMR spectrum of a mixture of D2a, D2b, D2c, D2d and D2e (expanded)
\(^1\)H NMR spectrum of a mixture of D2a, D2b, D2c, D2d and D2e (expanded)
13C NMR spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
$^{13}$C NMR spectrum of a mixture of D2a, D2b, D2c, D2d and D2e (expanded)
DEPT spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
COSY spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
NOESY spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
HSQC spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
HSQC spectrum of a mixture of D2a, D2b, D2c, D2d and D2e (expanded)
HSQC spectrum of a mixture of D2a, D2b, D2c, D2d and D2e (expanded)
HMBC spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
HMBC spectrum of a mixture of D2a, D2b, D2c, D2d and D2e (expanded)
IR spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
UV spectrum of a mixture of D2a, D2b, D2c, D2d and D2e
GC chromatogram of a mixture of D2a, D2b, D2c, D2d and D2e
Scan 1772 (13.491 min): EALSRTD7.D

Mass spectrum of D2a
Mass spectrum of D2b
Mass spectrum of D2c
Scan 1937 (14.460 min): EALSRTD7.D

Mass spectrum of D2d
Mass spectrum of D2e
"1H NMR spectrum of a mixture of D3a, D3b, C3a, and C3c"
\[ ^{13}\text{C} \text{NMR spectrum of a mixture of D3a, D3b, C3a, and C3c} \]
DEPT spectrum of a mixture of D3a, D3b, C3a, and C3c
COSY spectrum of a mixture of D3a, D3b, C3a, and C3c
NOESY spectrum of a mixture of D3a, D3b, C3a, and C3c
HSQC spectrum of a mixture of D3a, D3b, C3a, and C3c
HSQC spectrum of a mixture of D3a, D3b, C3a, and C3c (expanded)
HMBC spectrum of a mixture of D3a, D3b, C3a, and C3c
HMBC spectrum of a mixture of D3a, D3b, C3a, and C3c (expanded)
IR spectrum of a mixture of D3a, D3b, C3a, and C3c
UV spectrum of a mixture of D3a, D3b, C3a, and C3c
GC chromatogram of a mixture of D3a, D3b, C3a, and C3c
Scan 1866 (14.161 min): EARTD11.D

Mass spectrum of D3a
Mass spectrum of D3b
Scan 2096 (15.364 min): EARTD11.D

Mass spectrum of C3a
Scan 1995 (14.801 min): EARTD11.D

Mass spectrum of C3c
$^1$H NMR spectrum of a mixture of D4a, D4e, C4a, and C4b
$^{13}$C NMR spectrum of a mixture of D4a, D4e, C4a, and C4b
DEPT spectrum of a mixture of D4a, D4e, C4a, and C4b
COSY spectrum of a mixture of D4a, D4e, C4a, and C4b
NOESY spectrum of a mixture of D4a, D4e, C4a, and C4b
HSQC spectrum of a mixture of D4a, D4e, C4a, and C4b
HSQC spectrum of a mixture of D4a, D4e, C4a, and C4b (expanded)
HMBC spectrum of a mixture of D4a, D4e, C4a, and C4b
HMBC spectrum of a mixture of D4a, D4e, C4a, and C4b (expanded)
IR spectrum of a mixture of D4a, D4e, C4a, and C4b
Data Set: LS EA RT 851.spc - Storage 111641

Measurement Properties
- Wavelength Range (nm): 200.00 to 800.00
- Scan Speed: Medium
- Sampling Interval: 1.0
- Auto Sampling Interval: Disabled
- Scan Mode: Single

Instrument Properties
- Instrument Type: UV-3500 Series
- Measuring Mode: Absorbance
- Slit Width: 8.0 nm
- Time Constant: 0.1 sec.
- Source Lamp: Auto
- Light Source Change Wavelength: 310.00 nm
- Detector Unit: Direct
- Detector Change Wavelength: 830.00 nm, 1600.00 nm
- Grating Change Wavelength: 720.00 nm
- S/R Exchange: Normal
- Detector Look: Auto
- Silt Program: Normal
- Beam Mode: Double
- Stair Correction: Disable

Attachment Properties
- Attachment: None

Sample Preparation Properties
- Weight:
- Volume:
- Dilution:
- Path Length:
- Additional Information:

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UV spectrum of a mixture of D4a, D4e, C4a, and C4b

* compounds used for spectral assignments
GC chromatogram of a mixture of D4a, D4e, C4a, and C4b
Mass spectrum of D4a
Scan 3157 (27.173 min): DOROTHY7.D

Mass spectrum of C4a
Scan 3578 (30.253 min): DOROTHY7.D

Mass spectrum of C4b
$^1$H NMR spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
$^1$H NMR spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
$^1$H NMR spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
$^{13}$C NMR spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
$^{13}$C NMR spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
DEPT spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
DEPT spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
DEPT spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
COSY spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
NOESY spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
HSQC spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
HSQC spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
HSQC spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
HMBC spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
HMBC spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
HMBC spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c (expanded)
UV spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
IR spectrum of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
GC chromatogram of a mixture of D4b, D4c, D4d, D4f, D4g, C4b and C4c
Mass spectrum of D4b
Scan 3825 (32.060 min): DOROTHY3.D

Mass spectrum of D4c
File: C:\MSDCHEM1\DATA\DOROTHY GCMS TWO\DOROTHY GCMS TWO\DOROTHY3.D
Operator: dorothy
Acquired: 27 Nov 2011 14:33 using AcqMethod DOROTHY
Instrument: Instrument
Sample Name: LSS 180-188 34-48B
Misc Info:
Vial Number: 1

Mass spectrum of C4c

Scan 4286 (35.432 min): DOROTHY3.D

[Diagram showing molecular structure]
Scan 3728 (31.350 min): DOROTHY3.D

Mass spectrum of D4f
Mass spectrum of D4g
$^1$H NMR spectrum of D5 (expanded 0-1.7 ppm)
$^{13}$C NMR spectrum of D5
$^{13}$C NMR spectrum of D5 (expanded 0-55 ppm)
HSQC spectrum of D5 (expanded F1 0-2.5 ppm)
HMBC spectrum of D5 (expanded F1 0-2.5 ppm)
HMBC spectrum of D5 (expanded 0-1.8 ppm)
### Measurement Properties
- **Wavelength Range (nm.):** 200.00 to 800.00
- **Scan Speed:** Medium
- **Sampling Interval:** 1.0
- **Auto Sampling Interval:** Disabled
- **Scan Mode:** Single

### Instrument Properties
- **Instrument Type:** UV-3600 Series
- **Measuring Mode:** Absorbance
- **Silt Width:** 8.0 nm
- **Time Constant:** 0.1 sec.
- **Source Lamp:** Auto
- **Light Source Change Wavelength:** 310.00 nm
- **Detector Unit:** Direct
- **Detector Change Wavelength:** 830.00 nm, 1800.00 nm
- **Grating Change Wavelength:** 720.00 nm
- **S/R Exchange:** Normal
- **Detector Lock:** Auto
- **Silf Program:** Normal
- **Beam Mode:** Double
- **Stair Correction:** Disable

### Attachment Properties
- **Attachment:** None

### Sample Preparation Properties
- **Weight:** 
- **Volume:** 
- **Dilution:** 
- **Path Length:** 
- **Additional Information:** UV spectrum of D5
MS spectrum of D5
$^1$H NMR spectrum of D6
$^{13}$C NMR spectrum of D6
COSY spectrum of D6
NOESY spectrum of D6
HSQC spectrum of D6
HMBC spectrum of D6
UV spectrum of D6
IR spectrum of D6
$^1$H NMR spectrum of D7
NOESY spectrum of D7
HSQC spectrum of D7
HMBC spectrum of D7
IR spectrum of D7
UV spectrum of D7
MS spectrum of D7
$^1$H NMR spectrum of D8
$^1$H NMR spectrum of D8 (expanded 4.0-8 ppm)
$^{13}$C NMR spectrum of D8 (expanded 67-79 ppm)
COSY spectrum of D8
HSQC spectrum of D8 (expanded F1 3.2-3.9 ppm)
HMBC spectrum of D8
UV spectrum of D8