REPUBLIQUE DU CAMEROUN
Paix – Travail – Patrie
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UNIVERSITE DE YAOUNDE I
FACULTE DES SCIENCES
DEPARTEMENT DE CHIMIE ORGANIQUE
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CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES, TECHNOLOGIES ET GEOSCIENCES
LABORATOIRE DES SUBSTANCES NATURELLES ET VALORISATION

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Peace – Work – Fatherland
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UNIVERSITY OF YAOUNDE I
FACULTY OF SCIENCE
DEPARTMENT OF ORGANIC CHEMISTRY
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POSTGRADUATE SCHOOL FOR SCIENCES, TECHNOLOGY AND GEOSCIENCES
LABORATORY OF NATURAL PRODUCTS AND THEIR VALORIZATION

CHEMICAL STUDIES AND EVALUATION OF SOME PHARMACOLOGICAL ACTIVITIES OF PROPOLIS FROM THREE GEOGRAPHYCAL REGIONS OF CAMEROON

Thesis
Submitted and defended for the award of Doctorat/ PhD

Par : **Alfred NGENGE TAMFU**
DIPES I&II, MSc

Sous la direction de
JOSEPH TANYI MBAFOR
Professor
TALLA Emmanuel
Associate Professor , UN

Année Académique : 2018

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UNITE DE RECHERCHE DE FORMATION DOCTORALE EN CHIMIE ET APPLICATIONS

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THE UNIVERSITY OF YAOUNDE I
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FACULTY OF SCIENCES
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POST GRADUATE SCHOOL OF SCIENCE, TECHNOLOGY AND GEOSCIENCES
****
DOCTORAL RESEACH UNIT FOR CHEMISTRY AND APPLICATIONS

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CHEMICAL STUDIES AND EVALUATION OF SOME PHARMACOLOGICAL ACTIVITIES OF PROPOLIS FROM THREE GEOGRAPHICAL REGIONS OF CAMEROON

Thesis submitted and defended in public in view of the award of a PhD/doctorate degree

OPTION: ORGANIC CHEMISTRY

By

Alfred NGENGE TAMFU
(DIPES I&II, MSc.)
Registration number: 06T664

Under the co-supervision of

Joseph Tanyi Mbafor (Professor, UYI)
Talla Emmanuel (Associate Professor, UN)
Vassya Bankova, DSc (Professor, IOCCP, BAS)

YEAR: 2018

UNIVERSITE DE YAOUNDE I

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THE UNIVERSITY OF YAOUNDE I

FACULTY OF SCIENCE

DEPARTEMENT DE CHIMIE ORGANIQUE

DEPARTMENT OF ORGANIC CHEMISTRY

ATTESTATION OF CORRECTION OF Ph. D/DOCTORATE THESIS OF Alfred NGENGE TAMFU, REGISTRATION NUMBER 06T664

We the undersigned, **Professor Tchuendem Kenmogne M. H.,** examiner and **Professor Dongo Etienne,** president of jury, attest that the Ph. D thesis in Organic Chemistry entitled: *« Chemical study and evaluation of some pharmacological activities of propolis from three geographical regions of Cameroon»* presented and defended in public by **Alfred NGENGE TAMFU,** registration number **06T664,** on the 26th of July 2018 has been corrected according to the recommendations of the jury.

This document is issued to serve the purpose for which it deserves.

EXAMINER

TCHUENDEM KENMOGNE Marguerite
Associate Professor, The University of Yaoundé 1

PRESIDENT

DONGO Etienne
Professor, The University of Yaoundé 1

Dongo Etienne
Professeur

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DEPARTMENT OF ACADEMIC AFFAIRS AND COOPERATION

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06 JUIL. 2018

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LE RECTEUR

A
Monsieur le Doyen de la
Faculté de Sciences
A
L'attention du Coordonnateur du CRFD/STG

Réf : 0000961/UYI/CFRD/URFD/STG/18 du 26 juin 2018
Objet : **Demande d'autorisation de soutenance de la thèse de Doctorat/Ph.D de Monsieur Alfred NGENGE TAMFU, Matricule 06T664.**

Suite à votre correspondance de référence dont l'objet est ci-dessus repris,

J'ai l'honneur d'autoriser **Monsieur Alfred NGENGE TAMFU, Matricule 06T664,** étudiant au Département de Chimie Organique, à soutenir sa thèse de Doctorat/Ph.D intitulée: **«Phytochemical studies and evaluation of some pharmacological activities of propolis from three geographical regions of Cameroon»**, devant le jury constitué ainsi qu'il suit :

Président :	**DONGO Etienne,** Professeur	**Université de Yaoundé I;**
Rapporteurs :	**MBAFOR TANYI Joseph,** Professeur	**Université de Yaoundé I;**
	VASSYA BANKOVA, Professeur	**Bulgarian Academy of Science, Sofia Bulgaria;**
	TALLA Emmanuel, Maître de Conférences	**Université de Ngaoundéré ;**
Membres :	**NJAMEN Dieudonné,** Professeur	**Université de Yaoundé I;**
	TCHUENDEM KENMOGNE Marguerite, Maître de Conférences	**Université de Yaoundé I;**
	TENE Mathieu, Maître de Conférences	**Université de Dschang.**

Le Recteur de l'Université de Yaoundé I

Maurice Aurélien SOSSE

Ampliations:
-VREPDTIC
-DAAC
-CRFD/STG
- Intéressé
- Chrono/Archives

LIST OF PERMANENT TEACHING STAFF

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LISTE DES ENSEIGNANTS PERMANENTS		**LIST OF PERMANENT TEACHING STAFF**

ACADEMIC YEAR: 2017/2018
UPDATED 10TH MARCH 2018

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Vice Dean / DPSAA **:** **DONGO Etienne (Professor)**

Vice Dean / DSSE**:** **OBEN Julius ENYONG (Professor)**

Vice Dean / DRC**:** **MBAZE MEVA'A Luc Leonard (Associate Professor)**

Head of Academic / Student Affairs and Research**: ABOSSOLO Monique (Associate Professor)**

Head of Administrative and Financial Division**: NDOYE FOE Marie C. F. (Associate Professor)**

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N°	NAME AND SURNAME	GRADE	OBSERVATIONS
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2	MBACHAM FON Wilfried	Professor	Full time
3	MOUNDIPA FEWOU Paul	Professor	**Head of Department**
4	NINTCHOM PENLAP V. épse BENG	Professor	Full time
5	OBEN Julius ENYONG	Professor	Full time
6	ATOGHO Barbara Mma	Associate Professor	Full time
7	BELINGA née NDOYE FOE M. C. F.	Associate Professor	Head of DAF / FS
8	BIGOGA DIAGA Jude	Associate Professor	Full time
9	BOUDJEKO Thaddée	Associate Professor	Full time
10	EFFA NNOMO Pierre	Associate Professor	Full time
11	FOKOU Elie	Associate Professor	Full time
12	KANSCI Germain	Associate Professor	Full time
13	NANA Louise épouse WAKAM	Associate Professor	Full time
14	NGONDI Judith Laure	Associate Professor	Full time
15	NGUEFACK Julienne	Associate Professor	Full time
16	NJAYOU Frédéric Nico	Associate Professor	Full time
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18	DEMMANO Gustave	Senior Lecturer	Full time
19	DJOKAM TAMO Rosine	Senior Lecturer	Full time
20	DJUIDJE NGOUNOUE Marcelline	Senior Lecturer	Full time
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22	EVEHE BEBANDOUE Marie-Solange	Senior Lecturer	Full time
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26	MBONG ANGIE M. Mary Anne	Senior Lecturer	Full time
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29	Palmer MASUMBE NETONGO	Senior Lecturer	Full time
30	TCHANA KOUATCHOUA Angèle	Senior Lecturer	Full time
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32	DONGMO LEKAGNE Joseph Blaise	Senior Lecturer	Full time
33	FONKOUA Martin	Senior Lecturer	Full time
34	BEBOY EDZENGUELE Sara Nathalie	Senior Lecturer	Full time
35	DAKOLE DABOY Charles	Senior Lecturer	Full time
36	MANANGA Marlyse Joséphine	Senior Lecturer	Full time
37	MBOUCHE FANMOE Marceline J.	Assistant Lecturer	Full time
38	BEBEE Fadimatou	Assistant Lecturer	Full time
39	TIENTCHEU DJOKAM Leopold	Assistant Lecturer	Full time

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2	DIMO Théophile	Professor	Full time
3	DJIETO LORDON Champlain	Professor	Full time
4	ESSOMBA née NTSAMA MBALA	Professor	*Vice dean/FMSB/UYI*
5	FOMENA Abraham	Professor	Full time
6	KAMTCHOUING Pierre	Professor	Full time
7	NJAMEN Dieudonné	Professor	Full time
8	NJIOKOU Flobert	Professor	Full time
9	NOLA Moïse	Professor	Full time
10	TAN Paul VERNYUY	Professor	Full time
11	TCHUEM TCHUENTE Louis Albert	Professor	*Prog. Coord.. MINSANTE*
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13	DZEUFIET DJOMENI Paul Désiré	Associate Professor	Full time
14	FOTO MENBOHAN Samuel	Associate Professor	Full time
15	KAMGANG René	Associate Professor	*C.S. MINRESI*
16	KEKEUNOU Sévilor	Associate Professor	Full time
17	MEGNEKOU Rosette	Associate Professor	Full time
18	MONY Ruth épse NTONE	Associate Professor	Full time
19	TOMBI Jeannette	Associate Professor	Full time
20	ZEBAZE TOGOUET Serge Hubert	Associate Professor	Full time
21	ALENE Désirée Chantal	Senior Lecturer	Full time
22	ATSAMO Albert Donatien	Senior Lecturer	Full time
23	BELLET EDIMO Oscar Roger	Senior Lecturer	Full time
24	BILANDA Danielle Claude	Senior Lecturer	Full time
25	DJIOGUE Séfirin	Senior Lecturer	Full time

26	DONFACK Mireille	Senior Lecturer	Full time
27	GOUNOUE KAMKUMO Raceline	Senior Lecturer	Full time
28	LEKEUFACK FOLEFACK Guy B.	Senior Lecturer	Full time
29	MAHOB Raymond Joseph	Senior Lecturer	Full time
30	MBENOUN MASSE Paul Serge	Senior Lecturer	Full time
31	MOUNGANG LucianeMarlyse	Senior Lecturer	Full time
32	MVEYO NDANKEU Yves Patrick	Senior Lecturer	Full time
33	NGOUATEU KENFACK Omer Bébé	Senior Lecturer	Full time
34	NGUEGUIM TSOFACK Florence	Senior Lecturer	Full time
35	NGUEMBOK	Senior Lecturer	Full time
36	NJATSA Hermine épse MEGAPTCHE	Senior Lecturer	Full time
37	NJUA Clarisse Yafi	Senior Lecturer	**HOD/UBa**
38	NOAH EWOTI Olive Vivien	Senior Lecturer	Full time
39	TADU Zephyrin	Senior Lecturer	Full time
40	YEDE	Senior Lecturer	Full time
41	ETEME ENAMA Serge	Assistant Lecturer	Full time
42	KANDEDA KAVAYE Antoine	Assistant Lecturer	Full time
43	KOGA MANG DOBARA	Assistant Lecturer	Full time
3- DEPARTMENT OF BIOLOGY AND PLANT PHYSIOLOGY (BPV) (26)			
1	AMBANG Zachée	Professor	Head of Division/UYII
2	BELL Joseph Martin	Professor	Full time
3	YOUMBI Emmanuel	Professor	**Head of Department**
4	MOSSEBO Dominique Claude	Professor	Full time
5	BIYE Elvire Hortense	Associate Professor	Full time
6	DJOCGOUE Pierre François	Associate Professor	Full time
7	KENGNE NOUMSI Ives Magloire	Associate Professor	Full time
8	MALA Armand William	Associate Professor	Full time
9	NDONGO BEKOLO	Associate Professor	*CE / MINRESI*
10	NGONKEU MAGAPTCHE Eddy L.	Associate Professor	Full time
11	ZAPFACK Louis	Associate Professor	Full time
12	MBARGA BINDZI Marie Alain	Associate Professor	Tech. Adv./Univ Dschang
13	MBOLO Marie	Associate Professor	Full time
14	ANGONI Hyacinthe	Senior Lecturer	Full time
15	MAHBOU SOMO TOUKAM. Gabriel	Senior Lecturer	Full time
16	ONANA JEAN MICHEL	Senior Lecturer	Full time
17	GOMANDJE Christelle	Senior Lecturer	Full time
18	NGODO MELINGUI Jean Baptiste	Senior Lecturer	Full time
19	NGALLE Hermine BILLE	Senior Lecturer	Full time
20	NGOUO Lucas Vincent	Senior Lecturer	Full time
21	NSOM ZAMO Annie C. épse PIAL	Senior Lecturer	*National Expert /UNESCO*
22	TONFACK Libert Brice	Senior Lecturer	Full time
23	TSOATA Esaïe	Senior Lecturer	Full time
24	DJEUANI Astride Carole	Assistant Lecturer	Full time
25	MAFFO MAFFO Nicole Liliane	Assistant Lecturer	Full time
26	NNANGA MEBENGA Ruth Laure	Assistant Lecturer	Full time
4- DEPARTMENT OF INORGANIC CHEMISTRY (CI) (33)			
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2	ELIMBI Antoine	Professor	Full time
3	Florence UFI CHINJE épouse MELO	Professor	*RECTOR Univ. Ngaoundere*
4	GHOGOMU Paul MINGO	Professor	*Director of Cabinet PM*
5	LAMINSI Samuel	Professor	Full time
6	NANSEU Njiki Charles Péguy	Professor	Full time
7	NDIFON Peter TEKE	Professor	*IS1 MINRESI/**Head of Department***
8	NENWA Justin	Professor	Full time
9	NGAMENI Emmanuel	Professor	*DEAN FS Univ. Dschang*
10	BABALE née DJAM DOUDOU	Associate Professor	*Head of Missions P.R.*
11	DJOUFAC WOUMFO Emmanuel	Associate Professor	Full time
12	KEMMEGNE MBOUGUEM Jean C.	Associate Professor	Full time
13	KONG SAKEO	Associate Professor	*Head of Missions at P. M.*
14	NDIKONTAR Maurice KOR	Associate Professor	*Vice-Dean UBa*
15	NGOMO Horace MANGA	Associate Professor	*VC/UB*
16	NJIOMOU C. épse DJANGANG	Associate Professor	Full time
17	YOUNANG Elie	Associate Professor	Full time
18	ACAYANKA Elie	Senior Lecturer	Full time
19	EMADACK Alphonse	Senior Lecturer	Full time
20	KAMGANG YOUBI Georges	Senior Lecturer	Full time
21	NDI NSAMI Julius	Senior Lecturer	Full time
22	NJOYA Dayirou	Senior Lecturer	Full time
23	PABOUDAM GBAMBIE A.	Senior Lecturer	Full time
24	TCHAKOUTE KOUAMO Hervé	Senior Lecturer	Full time
25	BELIBI BELIBI Placide Désiré	Senior Lecturer	Full time
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27	NYAMEN Linda Dyorisse	Senior Lecturer	Full time
28	KENNE DEDZO GUSTAVE	Senior Lecturer	Full time
29	KOUOTOU DAOUDA	Senior Lecturer	Full time
30	MAKON Thomas Beauregard	Senior Lecturer	Full time
31	MBEY Jean Aime	Senior Lecturer	Full time
32	NCHIMI NONO KATIA	Senior Lecturer	Full time
33	NEBA nee NDOSIRI Bridget N.	Senior Lecturer	Full time
5- DEPARTMENT OF ORGANIC CHEMISTRY (CO) (34)			
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4	NGADJUI TCHALEU Bonaventure	Professor	Full time
5	NGOUELA Silvère Augustin	Professor	Full time
6	NKENGFACK Augustin Ephraïm	Professor	**Head of Department**
7	NYASSE Barthélemy	Professor	*Director/UN*
8	PEGNYEMB Dieudonné Emmanuel	Professor	*Director/ MINESUP*
9	WANDJI Jean	Professor	Full time
10	Alex de Théodore ATCHADE	Associate Professor	*DEPE/ Rectorate/UYI*
11	FOLEFOC Gabriel NGOSONG	Associate Professor	*Full time*
12	KEUMEDJIO Félix	Associate Professor	Full time
13	KOUAM Jacques	Associate Professor	Full time
14	MBAZOA née DJAMA Céline	Associate Professor	Full time
15	NOUNGOUE TCHAMO Diderot	Associate Professor	Full time

16	TCHOUANKEU Jean-Claude	Associate Professor	*VR/ UYII*
17	YANKEP Emmanuel	Associate Professor	Full time
18	TIH née NGO BILONG E. Anastasie	Associate Professor	Full time
19	MKOUNGA Pierre	Associate Professor	Full time
20	NGO MBING Joséphine	Associate Professor	Full time
21	TABOPDA KUATE Turibio	Associate Professor	Full time
22	KEUMOGNE Marguerite	Associate Professor	Full time
23	AMBASSA Pantaléon	Senior Lecturer	Full time
24	EYONG Kenneth OBEN	Senior Lecturer	Full time
25	FOTSO WABO Ghislain	Senior Lecturer	Full time
26	KAMTO Eutrophe Le Doux	Senior Lecturer	Full time
27	NGONO BIKOBO Dominique Serge	Senior Lecturer	Full time
28	NOTE LOUGBOT Olivier Placide	Senior Lecturer	Chief of Service/MINESUP
29	OUAHOUO WACHE Blandine M.	Senior Lecturer	Full time
30	TAGATSING FOTSING Maurice	Senior Lecturer	Full time
31	ZONDENDEGOUMBA Ernestine	Senior Lecturer	Full time
32	NGOMO Orléans	Senior Lecturer	Full time
33	NGNINTEDO Dominique	Assistant Lecturer	Full time
6- DEPARTEMENT OF COMPUTER SCIENCE (IN) (25)			
1	ATSA ETOUNDI Roger	Professor	**Head of Department**
2	FOUDA NDJODO Marcel Laurent	Professor	*HOD ENS/ I.G.A.MINESUP*
3	NDOUNDAM Réné	Associate Professor	Full time
4	KOUOKAM KOUOKAM E. A.	Senior Lecturer	Full time
5	CHEDOM FOTSO Donatien	Senior Lecturer	Full time
6	MELATAGIA YONTA Paulin	Senior Lecturer	Full time
7	MOTO MPONG Serge Alain	Senior Lecturer	Full time
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9	TSOPZE Norbert	Senior Lecturer	Full time
10	WAKU KOUAMOU Jules	Senior Lecturer	Full time
11	TAPAMO Hyppolite	Senior Lecturer	Full time
12	ABESSOLO ALO'O Gislain	Assistant Lecturer	Full time
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15	DOMGA KOMGUEM Rodrigue	Assistant Lecturer	Full time
16	EBELE Serge	Assistant Lecturer	Full time
17	HAMZA Adamou	Assistant Lecturer	Full time
18	KAMDEM KENGNE Christiane	Assistant Lecturer	Full time
19	KAMGUEU Patrick Olivier	Assistant Lecturer	Full time
20	KENFACK DONGMO Clauvice V.	Assistant Lecturer	Full time
21	MEYEMDOU Nadège Sylvianne	Assistant Lecture	Full time
22	MONTHE DJIADEU Valery M.	Assistant Lecturer	Full time
23	JIOMEKONG AZANZI Fidel	Assistant Lecturer	Full time
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2	BITJONG NDOMBOL	Professor	*Full time*
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4	AYISSI Raoult Domingo	Associate Professor	**Head of Department**
5	EMVUDU WONO Yves S.	Associate Professor	*CD/ MINESUP*

6	NKUIMI JUGNIA Célestin	Associate Professor	Full time
7	NOUNDJEU Pierre	Associate Professor	Full time
8	TCHAPNDA NJABO Sophonie B.	Associate Professor	Director/AIMS Rwanda
9	AGHOUKENG JIOFACK Jean Gérard	Senior Lecturer	Head of Unit/ MINPLAMAT
10	CHENDJOU Gilbert	Senior Lecturer	Full time
11	FOMEKONG Christophe	Senior Lecturer	Full time
12	KIANPI Maurice	Senior Lecturer	Full time
13	KIKI Maxime Armand	Senior Lecturer	Full time
14	MBAKOP Guy Merlin	Senior Lecturer	Full time
15	MBANG Joseph	Senior Lecturer	Full time
16	MBEHOU Mohamed	Senior Lecturer	Full time
17	MBELE BIDIMA Martin Ledoux	Senior Lecturer	Full time
18	MENGUE MENGUE David Joe	Senior Lecturer	Full time
19	NGUEFACK Bernard	Senior Lecturer	Full time
20	POLA DOUNDOU Emmanuel	Senior Lecturer	Full time
21	TAKAM SOH Patrice	Senior Lecturer	Full time
22	TCHANGANG Roger Duclos	Senior Lecturer	Full time
23	TCHOUNDJA Edgar Landry	Senior Lecturer	Full time
24	TETSADJIO TCHILEPECK M. E.	Senior Lecturer	Full time
25	TIAYA TSAGUE N. Anne-Marie	Senior Lecturer	Full time
26	DJIADEU NGAHA Michel	Assistant Lecturer	Full time
27	MBIAKOP Hilaire George	Assistant Lecturer	Full time
28	NIMPA PEFOUNKEU Romain	Assistant Lecturer	Full time
29	TANG AHANDA Barnabé	Assistant Lecturer	Director/MINTP
8- DEPARTMENT OF MICROBIOLOGY (MIB) (13)			
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2	ETOA François Xavier	Professor	**Head of Department** Rector University of Douala
3	NWAGA Dieudonné M.	Associate Professor	Full time
4	NYEGUE Maximilienne Ascension	Associate Professor	Full time
5	SADO KAMDEM Sylvain Leroy	Associate Professor	Full time
6	BOYOMO ONANA	Associate Professor	Full time
7	RIWOM Sara Honorine	Associate Professor	Full time
8	BODA Maurice	Senior Lecturer	Full time
9	BOUGNOM Blaise Pascal	Senior Lecturer	Full time
10	ENO Anna Arey	Senior Lecturer	Full time
11	ESSONO OBOUGOU Germain G.	Senior Lecturer	Full time
12	NJIKI BIKOÏ Jacky	Senior Lecturer	Full time
13	TCHIKOUA Roger	Senior Lecturer	Full time
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4	NJOMO Donatien	Professor	Full time
5	PEMHA Elkana	Professor	Full time
6	TABOD Charles TABOD	Professor	*Dean UBa*
7	TCHAWOUA Clément	Professor	Full time
8	WOAFO Paul	Professor	Full time

9	EKOBENA FOUDA Henri Paul	Associate Professor	*Head of Division. UN*
10	NJANDJOCK NOUCK Philippe	Associate Professor	*Sub Director/ MINRESI*
11	BIYA MOTTO Frédéric	Associate Professor	**DG/HYDRO Mekin**
12	BEN- BOLIE Germain Hubert	Associate Professor	HOD/ENS/UN
13	DJUIDJE KENMOE épouse ALOYEM	Associate Professor	Full time
14	NANA NBENDJO Blaise	Associate Professor	Full time
15	NOUAYOU Robert	Associate Professor	Full time
16	SIEWE SIEWE Martin	Associate Professor	Full time
17	ZEKENG Serge Sylvain	Associate Professor	Full time
18	EYEBE FOUDA Jean sire	Associate Professor	Full time
19	FEWO Serge Ibraïd	Associate Professor	Full time
20	HONA Jacques	Associate Professor	Full time
21	OUMAROU BOUBA	Associate Professor	Full time
22	SAIDOU	Associate Professor	Sub Director/MINRESI
23	SIMO Elie	Associate Professor	Full time
24	BODO Bernard	Senior Lecturer	Full time
25	EDONGUE HERVAIS	Senior Lecturer	Full time
26	FOUEDJIO David	Senior Lecturer	Full time
27	MBANE BIOUELE	Senior Lecturer	Full time
28	MBINACK Clément	Senior Lecturer	Full time
29	MBONO SAMBA Yves Christian U.	Senior Lecturer	Full time
30	NDOP Joseph	Senior Lecturer	Full time
31	OBOUNOU Marcel	Senior Lecturer	Ass. Dir/Inter-state Univ
32	TABI Conrad Bertrand	Senior Lecturer	Full time
33	TCHOFFO Fidèle	Senior Lecturer	Full time
34	VONDOU Derbetini Appolinaire	Senior Lecturer	Full time
35	WOULACHE Rosalie Laure	Senior Lecturer	Full time
36	ABDOURAHIMI	Senior Lecturer	Full time
37	ENYEGUE A NYAM épse BELINGA	Senior Lecturer	Full time
38	WAKATA née BEYA Annie	Senior Lecturer	*Sub Director/ MINESUP*
39	MVOGO ALAIN	Senior Lecturer	Full time
40	CHAMANI Roméo	Assistant Lecturer	Full time
41	MLI JOELLE LARISSA	Assistant Lecturer	Full time
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2	BITOM Dieudonné	Professor	*Dean / FASA / UDs*
3	NZENTI Jean-Paul	Professor	Full time
4	KAMGANG Pierre	Professor	Full time
5	MEDJO EKO Robert	Professor	*Technical Adviser/UYII*
6	FOUATEU Rose épse YONGUE	Associate Professor	Full time
7	NDAM NGOUPAYOU Jules-Remy	Associate Professor	Full time
8	NGOS III Simon	Associate Professor	DAAC/UMa
9	NJILAH Isaac KONFOR	Associate Professor	Full time
10	NKOUMBOU Charles	Associate Professor	Full time
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15	MOUNDI Amidou	Associate Professor	*Head of Div. MINIMDT*
16	ONANA Vincent	Associate Professor	Full time
17	TCHOUANKOUE Jean-Pierre	Associate Professor	Full time
18	ZO'O ZAME Philémon	Associate Professor	*DG/ART*
19	MOUNDI Amidou	Associate Professor	*Tech. Adviser / MINIMDT*
20	BEKOA Etienne	Senior Lecturer	Full time
21	BISSO Dieudonne	Senior Lecturer	*Director/ Memve'ele Dam project*
22	ESSONO Jean	Senior Lecturer	Full time
23	EKOMANE Emile	Senior Lecturer	Full time
24	FUH Calistus Gentry	Senior Lecturer	*Sec. of State/MINMIDT*
25	GANNO Sylvestre	Senior Lecturer	Full time
26	LAMILEN BILLA Daniel	Senior Lecturer	Full time
27	MBIDA YEM	Senior Lecturer	Full time
28	MINYEM Dieudonné-Lucien	Senior Lecturer	*HOD/UMa*
29	MOUAFO Lucas	Senior Lecturer	Full time
31	NGO BELNOUN Rose Noël	Senior Lecturer	Full time
32	NGO BIDJECK Louise Marie	Senior Lecturer	Full time
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34	NYECK Bruno	Senior Lecturer	Full time
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36	METANG Victor	Senior Lecturer	Full time
37	NOMO NEGUE Emmanuel	Senior Lecturer	Full time
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39	TEHNA Nathanaël	Senior Lecturer	Full time
40	TEMGA Jean Pierre	Senior Lecturer	Full time
41	MBESSE CECILE OLIVE	Senior Lecturer	Full time
42	ELISE SABABA	Senior Lecturer	Full time
43	EYONG JOHN TAKEM	Assistant Lecturer	Full time
44	ANABA ONANA Achille Basile	Assistant Lecturer	Full time

Departmental allotment of permanent Lecturers in the Faculty of Science, The University of Yaoundé I

	Number of Lecturers				
DEPARTEMENT	**Professors**	**Associate professors**	**Senior Lecturers**	**Assistant Lecturers**	**Total**
B.C.	5 (1)	10 (5)	20 (10)	3 (1)	**39 (16)**
B.P.A.	11 (1)	9 (3)	20 (8)	3 (5)	**43 (17)**
B.P.V.	4 (0)	9(2)	10 (2)	4 (4)	**27 (8)**
C.I.	9(1)	8(2)	16 (4)	0 (2)	**33 (9)**
C.O.	8 (0)	13 (3)	8 (2)	1 (0)	**30 (5)**
I.N.	3 (0)	1 (0)	8 (0)	12 (3)	**24 (3)**
M.A.	3 (0)	5 (0)	18 (1)	4 (0)	**30 (1)**
M.B.	2 (0)	5 (2)	6 (2)	0 (0)	**13 (4)**
P.H.	8 (0)	17 (0)	15 (2)	2 (1)	**42 (3)**
S.T.	5 (0)	15 (2)	22 (3)	2 (0)	**44 (5)**
Total	**58 (3)**	**92(19)**	**144 (33)**	**31(16)**	**325 (71)**

Giving a total of 325 (71) as follows:

- **Professors** **58 (3)**
- **Associate Professors** **92 (19)**
- **Senior Lecturers** **144 (33)**
- **Assistant Lecturers** **31 (16)**

( ) = Number of females in parenthesis

DECLARATION

The experimental work presented in this thesis was conducted in the Department of Organic Chemistry, Faculty of Science, The University of Yaoundé I while the author was enrolled for the degree of Doctor of Philosophy in the Post-graduate school. This work also involved collaboration with the Bulgarian Academy of Science Sofia, the International Center for Chemical and Biological Sciences Pakistan and also the University of Mons in Belgium where spectral analysis where done.

All experimental work and results are entirely my own original work. Where use has been made of outside sources, proper attributions have been made in the text and the authors cited.

I therefore guarantee the authenticity of the material and declare the above statement to be true.

Candidate…….

Alfred Ngenge Tamfu

DEDICATION

I dedicate this work to my parents

Pa Tamfu Samari Emmanuel and Wirba Salamatou Nkumseka

My brothers and sisters

Yvonne, Taah, Elvis, Violet, Emile, I. Ndi, Sangnyuy, Chanceline,

Theodora, Desmond, Burinyuy

Armel Florian Tamfu and Stefanova Kila Tamfu

And finally, to

The Almighty God Who gave the dream and made the way
Where there seemed to be no way in the darkest moments of my life

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- The list of people who have contributed to this work or who have helped me is inexhaustible. Names not mentioned above are reserved for greater works in future.
- I am grateful to TWAS-ICCBS for the Post-Graduate fellowship offered to me at the end of my Ph. D research.

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%FCC	: Percentage Ferrous Ion Chelating Capacity
%TIC	: Percentage of Total Ion Current
^{13}C NMR	: Carbon-13 Nuclear Magnetic Resonance
1D-NMR	: One-dimensional Nuclear Magnetic Resonance
^{1}H NMR	: Proton Nuclear Magnetic Resonance
2D-NMR	: Two-dimensional Nuclear Magnetic Resonance
AcOEt	: Ethyl acetate
APT	: Attached proton test
BHT	: Butylated hydroxytoluene
brs	: broad singlet
BSTFA	: *N*, *O*-bis (trimethylsilyl) trifluoroacetamide
CC	: Column Chromatography
COSY	: Correlation spectroscopy
d	: Doublet
dd	: Doublet of doublet
DEPT	: Distortionless enhancement by polarization transfer
DMAP	: Dimethyl aminopyridine
DMSO	: Dimethylsulfoxide
DNA	: Deoxyribonucleic acid
DPPH	: 2,2-diphenyl-1-picrylhydrazylhydrazyl
EDTA	: ethylenediamine tetraacetate
EI-MS	Electronic impact-mass spectrum
ESI-TOF MS	: Electro Spray Ionization Time of Flight Mass Spectrometry
FAB	: Fast Atom Bombardment
GC-MS	: Gas Chromatography-Mass Spectrometry
GSH	: Glutathione
Hex	: Hexane
HIV	: Human Immuno-Deficiency Virus
HMBC	: Heteronuclear Multiple Bond Correlation
HSQC	: Heteronuclear Single Quantum Coherence
Hz	: Hertz
IC_{50}	: Concentration causing 50% inhibition
IR	: Infra-Red

ITMS	: Ionization transfer mass spectroscopy
J (Hz)	: Coupling constant (NMR) in Hertz
LD_{50}	: Lethal dose 50 or dose causing 50% deaths
m	: Multiplet
MALDI	: Matrix Assisted Laser Disorption Ionization
MBC	: Minimal bactericidal concentration
MDA	: Malondialdehyde
MHz	: Megahertz
MIC	: Minimal inhibitory concentration
min.	: Minute
mpt	: Melting point
NMR	: Nuclear Magnetic Resonance
NOESY	: Nuclear Overhauser Enhanced Spectroscopy
NSAID	: non-steroidal anti-inflammatory drugs
PCC	:Pyridinium chlorochromate
ppm	: parts per million
RDA	: Retro Diels-Alder
RT	: Retention Time
s	: Singlet
SEM	: Standard Error of the Mean
t	: Triplet
TIC	: Total Ion Chromatogram
TLC	: Thin Layer Chromatography
TMS	: Trimethylsilane
UV	: Ultraviolet
WHO	: World Health Organization
δC (ppm)	: Carbon chemical shift in ppm
δH (ppm)	: proton chemical shift in ppm

ABSTRACT

As part of an ongoing investigation of bioactive metabolites from natural sources, extracts of propolis samples from different regions of Cameroon were prepared and screened for their anti-ulcer, antioxidant and antimicrobial properties. Some secondary metabolites were isolated and their structures elucidated.

Thirty-two natural products were isolated: ten compounds and a triterpene fraction from the acetone extract of Nkambe propolis; ten from the ethanol extract of Foumban propolis; six from the hexane extract of Ndian propolis and six from the ethyl acetate extract of Ndian propolis using routine separation and chromatographic techniques. The structures of twenty-four of these compounds were elucidated by interpretation of their physical and spectroscopic data (IR, MS, melting point, 1D & 2D NMR) and by comparison of the latter with similar data reported in literature and due to similarity between some compounds, the number reduced to 20 distributed as follows:

- 16 triterpenes, two of which are novel derivatives namely: 3β,27-dihydroxycycloart-24-en-26-oic acid methyl ester (138) and 3-oxo-6,22-dihydroxy-4,4,14-trimethylspinast-7,23-dien-29-al (133). The 14 known triterpenoids are: lupeol acetate (82), betulinic acid (140), oleanolic acid (139), lupenone (81), 27-hydroxymangiferonic acid (135), mangiferonic acid (78), betulin (136), 3β-hydroxylanostan-9,24-dien-21-oic acid (137), 3β-hydroxycycloart-12,25-diene (86), 3β-O-acetylbetulin (144), lup-12,20(29)-dien-3-ol (142), and a mixture of lupeol and stigmasterol (88 and 134), 24-methylenecycloartenol and stigmasterol (141 and 134), lupeol and β–amyrine (88 and 143).
- 1 new mono-ether of glycerol: 1'-O-eicosanyl glycerol (145)
- 2 fatty acid esters: heptadecyl butanoate (148) and eicosanyl butanoate (146)
- 1 fatty alcohol: n-heptatriacontanol (147)

It is observed that, lupane and oleanane type triterpenoids could be possible chemical markers of Cameroonian propolis.

Also, six benzylic esters (three of lupeol and three of β-amyrine) were synthesized. It resulted from these syntheses that the yield depended on the activating effect of the methoxyl substituents on the benzene ring of the benzoylchloride used. The greater the number of methoxyl groups, the greater the inductive effect and the greater the yield. β-amyrine was also oxidized to amyrenone.

A triterpene fraction was isolated and analysed by GC-MS as a mixture of lanosterol, α-amyrine, 28-norolean-12-en-3-ol, Cycloartenol, 3-epi-α-amyrine, lupeol and 24-methylenecycloartenol.

GC-MS profiles of 13 extracts from different samples of propolis were established and revealed the presence of over 50 compounds belonging to different structural groups but containing mainly triterpenes as would be expected of propolis from tropical and subtropical zones. Characteristically, GC-MS profiles of Cameroonian propolis revealed alkenyl phenols and resorcinols, fatty acids, triterpenes and sugars. The presence of some of

these compounds indicated that *Mangifera indica* is the major plant from which bees collect propolis. Samples from Northern part of Cameroon were richer in triterpenes, void of alkenyl phenols and resorcinols and had averagely higher antimicrobial activity while alkenyl phenols and resorcinols and some phenolic compounds were found in samples of North-West and Western regions which showed averagely higher antiradical activity on DPPH. This difference is explained by the variation in vegetation and plants foraged by bees.

The methanol, acetone and hexane extracts of Nkambe propolis (200-600 mg/kg) dose-dependently prevented the formation of ethanol-induced gastric lesions (% inhibition, 61, 54 and 55%, respectively, at the dose of 600 mg/kg). Increasing doses of the extracts inhibited pylorus ligation–induced lesions by 64.5, 73.1 and 16.2 %, respectively, for the highest dose but none of them showed antisecretory activity as compared with controls. The most further significantly ($P<0.01$) reduced HCl/ethanol-induced ulcer indices from 4.33 ± 0.32 in cytoprotective (acetone) extract (56.6-73.1 % inhibition under highly acidic gastric environments) to 1.25 ± 0.53 and 0.6 ± 0.04 at the dose of 400 and 600 mg/kg, respectively (% inhibition: 71-86%). Furthermore, upon pretreatment of the rats with indomethacin prior to HCl/ethanol, the acetone extract significantly ($P<0.001$) decreased ulcer index from 5.55 ± 0.73 in the controls to 1.89 ± 0.15 at the dose of 600 mg/kg. Although pretreatment with indomethacin reduced the protective effect of the acetone extract by 23 to 27%, cytoprotection remained high (62-66% inhibition). The cytoprotective action of the most active (acetone) extract may involve the mediation of endogenous prostaglandins.

The order of decreasing radical scavenging activity was Hexane extract of Foumban propolis (IC_{50} = 5.6 mg/mL), Hexane extract of Ndian propolis (IC_{50} = 4.00 mg/mL), Ethyl acetate extract of Ndian propolis (IC_{50} = 1.65 mg/mL), Ethyl acetate extract of Foumban propolis (IC_{50} = 1.40 mg/mL), 3β-hydroxylanostan-9,24-dien-21-oic acid (IC_{50} = 1.22 mg/mL), mangiferonic acid (IC_{50} = 1.09 mg/mL), Methanol extract of Foumban propolis (IC_{50} = 1.07 mg/mL),methyl-3β,27-dihydroxycycloart-24-en-26-oate (IC_{50} = 0.98 mg/mL), 1'-O-eicosanyl glycerol (IC_{50} = 0.93 mg/mL), Vitamin C (IC_{50} = 0.80 mg/mL) and Gallic acid (IC_{50} = 0.30 mg/mL). Although none of the samples showed antiradical activity greater than that of the standards, their activities remained nevertheless closer to those of the standard antioxidants Gallic acid and vitamin C. It is observed that, all the pure compounds showed higher $DPPH^{\bullet}$ radical scavenging activity than the extracts except for methanol extract of Foumban propolis that was more active than mangiferonic acid and 3β-hydroxylanostan-9,24-dien-21-oic acid.

Keywords: Propolis, GC-MS, Triterpenes, 1'-O-eicosanyl glycerol, esterification, DPPH scavenging, anti-ulcer activity, antimicrobial activity.

RESUME

En tant qu'une contribution à la recherche des métabolites secondaires bioactives de source naturelle, la propolis des différentes régions du Cameroun a été extraites et leurs propriétés antiulcéreuse, antioxydante et antimicrobienne étudiées. La séparation par différentes techniques chromatographiques nous a permis d'isoler plusieurs composés dont leurs structures ont été caractérisées.

Trente-deux composés ont été isolés au total: dix composés de l'extrait à l'acétone de la propolis de Nkambe, dix composés de l'extrait à l'éthanol de la propolis de Foumban, six composés de l'extrait à l'hexane de la propolis de Ndian et six autres composés issus de l'extrait à l'acétate d'éthyle de la propolis de Ndian.Les structures de vingt-et-quartres composés ont été élucidées par interprétation de leurs propriétés physiques et les données spectroscopiques (IR, SM, point de fusion, RMN 1D et 2D) et par la comparaison des données décrites dans la littérature. En effet, la similitude entre quelques-uns de ces composés a réduit ce nombre à 20 à savoir :

- 16 triterpènes: 2 dérivés nouveau : 3β, 27-dihydroxycycloart-24-èn-26-oate de méthyl (138) et 3-oxo-6,22-dihydroxy-4,4,14-triméthylspinast-7,23-dièn-29-al (133) et 14 composés connus; l'acétate de lupéol (82), l'acide bétulinique (140), l'acide oléanolique (139), lupénone (89), l'acide 27-hydroxymangiféronique (135), l'acide mangiféronique (78), bétuline (136), l'acide 3β-hydroxylanostan-9,24-dién-21-oique (137), 3β-hydroxycycloart-12,25-diène (86), 3β-O-acétylbétuline (144), lup-12,20(29)-dién-3-ol (142), et un mélange de lupéol et stigmastérol (88 et 134), 24-méthylenecycloartenol et stigmastérol (141 et 134) et lupéol et β-amyrine (88 et 143).
- 1 nouveau mono-éther de glycérol : 1'-O-eicosanyl glycérol (145)
- 2 esters d'acide gras : butanoate d'heptadécanol (148) et butanoate d'eicosanol (146)
- 1 alcool gras: n-heptatriacontanol (147)

En plus, six esters benzyliques (trois de lupéol et trois de β-amyrine) ont été synthétisés. Au vu de rendement de ces réactions de synthèse, nous pouvons dire que ce rendement dépend de l'effet inductif des substituants qui sont les méthoxyles sur le noyau aromatique du chlorure de benzoyl utilisé. Plus le nombre de groupements méthoxylés augmente, plus l'effet inductif est grand et plus le rendement est grand. β-amyrine a également été oxydé en amyrenone.

Une fraction de triterpène analysée par CPG-SM comme mélange de lanostérol, α-amyrine, 28-noroléan-12-èn-3-ol, cycloarténol, 3-epi-α-amyrine, lupéol et 24-méthylènecycloarténol.

Des profils CPG-SM de 13 extraits des différents échantillons de la propolis ont été établis et montrent la présence de plus de 50 composés appartenant à différentes familles de composé. Elles sont riches en triterpènes comme toute propolis des zones tropicales et sous-tropicales. Caractéristiquement, les profils CPG-SM de la propolis camerounaise contiennent les lipides phénoliques, les résorcinols, les acides gras, les triterpènes et les sucres. Ceci indique que *mangifera indica* est l'une des plantes principales utilisées par les abeilles pour fabriquer

la propolis. Les échantillons de la propolis du grand Nord Cameroun étaient plus riches en triterpènes, ne contient pas les lipides phénoliques et résorcinols. Elles ont une activité antimicrobienne plus élevée tandis que les lipides phénoliques et résorcinols et quelques composés phénoliques ont été trouvés dans les échantillons de la région du Nord-Ouest et de l'Ouest qui ont une activité antiradicalaire plus élevée. Cette différence s'explique par la variation de la végétation et des plantes que les abeilles butinent.

Les extraits au méthanol, à l'acétone et à l'hexane de la propolis de Nkambe (200-600 mg/kg) ont empêché la formation des lésions gastriques induites par éthanol absolu de manière dose-dépendante (61 %, 54 % et 55 % d'inhibition, respectivement, à la dose de 600 mg/kg). Les doses croissantes des extraits ont empêché les lésions induites par la ligature du pylore par 64,5 ; 73,1 et 16,2 % respectivement, pour la dose la plus élevée mais aucun d'eux n'a montré une activité anti-sécrétoire comparativement aux contrôles positive. Le plus actif (extrait l'acétone) a réduit les indices d'ulcère de manière significative ($P<0,01$) induit par HCl/éthanol de 4,33 ± 0,32 à 1,25 ± 0,53 et 0,6 ± 0,04 à la dose de 400 (56,6-73,1 % d'inhibition dans les environnements gastriques fortement acides) et 600 mg/kg (71-86% d'inhibition) respectivement. Lors du prétraitement des rats avec de l'indométacine avant HCl/éthanol, l'extrait à l'acétone a diminué de manière significative ($P<0,001$) l'indice d'ulcère de 5,55 ± 0,73 dans les contrôles à 1,89 ± 0,15 à la dose de 600 mg/kg. Bien que le prétraitement avec de l'indométacine ait réduit l'effet protecteur de l'extrait à l'acétone de 23 à 27%, la cytoprotection est restée élevée (inhibition 62-66%). L'action cytoprotectrice de l'extrait à l'acétone peut comporter la médiation des prostaglandines endogènes.

L'ordre décroissant de l'activité antiradicalaire est comme suit; l'extrait à l'hexane de la propolis de Foumban (IC_{50} = 5,6 mg/mL), l'extrait à l'hexane de la propolis de Ndian (IC_{50} = 4,00 mg/mL), l'extrait à l'acétate d'éthyle de la propolis de Ndian (IC_{50} = 1,65 mg/mL), l'extrait à l'acétate éthyle de la propolis de Foumban (IC_{50} = 1,40 mg/mL), acide 3β-hydroxylanostan-9,24-dièn-21-oique (IC_{50} = 1,22 mg/mL), l'acide mangiféronique (IC_{50} = 1,09 mg/mL), l'extrait au méthanol de la propolis de Foumban (IC_{50} = 1,07 mg/mL), méthyl-3β, 27-dihydroxycycloart-24-èn-26-oate (IC_{50} = 0,98 mg/mL), 1'-O-eicosanyl glycérol (IC_{50} = 0,93 mg/mL), vitamine C (IC_{50} = 0,80 mg/mL) et acide gallique (IC_{50} = 0,30 mg/mL). Bien qu'aucun des échantillons n'ait montré une activité antiradicalaire plus grande que les standards, leurs activités sont néanmoins plus près de ceux des antioxydants standards comme acide gallique et la vitamine C. On observe que, les composés purs ont montré une activité antiradicalaire plus grande que les extraits mais l'extrait au méthanol de la propolis de Foumban était plus actif que l'acide mangiféronique et l'acide 3β-hydroxylanostan-9,24-dièn-21-oique.

Mots-clés : Propolis, CPG-SM, triterpènes, 1'-O-eicosanyl glycérol, estérification, activité antiradicalaire, activité antiulcéreuse, activité antimicrobienne.

GENERAL INTRODUCTION

Peptic ulcers are a deep gastrointestinal erosion disorder that involves the entire mucosal thickness, penetrating the muscular mucosa. An estimated 15.000 deaths occur each year as a consequence of peptic ulcer diseases (**Valle *et al.*, 2005**) and the prevalence of this disease increases over time. Some researchers reported that peptic ulcers are caused when the balance between aggressive factors (such as acid and pepsin) and defense mechanisms (such as mucus, bicarbonate, blood flow and mucosal turnover) are shifted in favour of the former (**Lima *et al.*, 2006**). Exogenous aggressive factors such as cigarette smoke, non-steroidal anti-inflammatory drugs (NSAIDs), alcohol, stress, fatty foods and *Helicobacter pylori* infections trigger tissue necrosis through mucosal ischemia, free radical generation and cessation of nutrient delivery. Although histamine H_2-receptor blockers (for example, ranitidine and famotidine), proton-pump inhibitors (for example omeprazole and lansoprazole), antibiotics (for example metronidazole, amoxicillin, clarithromycin, and tetracycline) and other drugs are extensively used in the management of peptic ulcers, there are reports of adverse effects and relapse within one year (**Wolfe and Sachs, 2000**) and also a number of side effects. Drugs for the treatment of gastric ulcers might be very expensive and unaffordable by many since a patient will have to use a tri-therapy involving antacids, antibiotics and cytoprotectors (**Tan *et al.*, 1996**). In addition to being economical, plant sources are effective and relatively less toxic and extensive research is currently being carried out in the search for potent antiulcer agents of natural products (**Vinay *et al.*, 2005; Srivastava *et al.*, 2011; Lakshmi *et al.*, 2013**). Traditionally, plants have not only provided food and shelter for mankind, but have also been used to cure many different ailments (**Gilani and Rahman, 2005**). Currently, the World Health Organization (WHO) estimates that approximately 80% of the inhabitants of the planet have recourse to traditional medicines, using medicinal plants for primary health care (**Kone, 2009**). Hence, interest in the pharmacological action of natural products has grown and found significant popular acceptance. Natural products are used directly in the pharmaceutical industry, which is growing rapidly in Europe and North America, as well as in traditional medicine programs being incorporated into the primary health care systems of many countries. The use of herbal drugs is once again becoming more popular in the form of food supplements, nutraceuticals, complementary and alternative medicine.

Natural products include bee products that have been used by man either as food or as a cure for himself for example honey, propolis, pollen and royal jelly. These valuable products of bees have

fascinated man who gradually learned how to raise and look after bees (apiculture) with *Apis mellifera* being the most interesting species in bee keeping. It is estimated that there were at least 20,000 beekeepers in Cameroon in 2009. The principal honey production zones are Adamawa, producing more than 3.3 million liters annually, valued at around 2 billion FCFA. At least 92,843 liters originate from the English-speaking Northwest Region, and 48,900 liters are produced in the West Region. About 235 tons of wax was produced in 2006, primarily for export within Central Africa, with an estimated value of 530 million FCFA. Other apicultural products such as propolis added about 1.5 million FCFA to total revenues per year (**Ingram and Njikeu, 2011**). The numbers of hives per beekeeper also varies widely per region: the average is 11, ranging from 45 in Adamawa, 16 in the Northwest, to 3 in the Southwest and West. Based on the fact that bees gather averagely 300 g of propolis per colony each year, Cameroon therefore have a potential of producing 66 tons (11 x 20000 x 300g) of propolis per year, a call for concern for the valorization of this highly medicinal product. It is called '*Nlaa-nfuu'* or '*Mbihdong'* in Limbum language and '*Dhatche-Nyaki'* by the 'Bororo'. Other popular local names include '*Kilei'* in Oku, Bui division of the North-West region and *'Ndaki-goro'* by the 'Gbayas' of the Adamawa region amongst others where the use of propolis is becoming very popular. In many localities in Cameroon, propolis is used by local sculptors for ornamental works and mending of calabashes. It is also exploited for its medicinal uses to treat tooth ache, stomach disorders, gastritis and sore throat by chewing directly. Its aqueous extract is used in treating wounds, skin rashes, boils and burns (**Talla *et al.*, 2014; Tamfu *et al.*, 2016**).

Among the bee products, propolis has been highlighted due to its wide spectra of biological activities and its applicability. Propolis (bee glue) is a sticky dark-colored material that honeybees collect from plants and use it in the hive. They apply it to seal the walls, to strengthen the borders of combs, to embalm dead invaders (**Burdock, 1998**). Propolis is considered as one of the most promising natural products that have therapeutic and preventive actions and it contains more than 300 compounds belonging to different structural groups (**El-Mahalaway *et al.*, 2015**). This chemical composition and biological properties of propolis vary according to the geographic location and the different plant sources (local flora) of the site of collection (**Markham *et al.*, 1996; Sforcin *et al.*, 2005**). It is clear that comparing propolis samples from different regions might be the same as comparing extracts of two plants that belong to different plant families (**Bankova, 2005**). As a result, almost every publication on biological activity of propolis includes

some kind of chemical characterization of the propolis and relating it to its biological activities (**Bankova, 2005**). However, in order to be accepted officially into the main stream of the healthcare system, propolis needs chemical standardization that guarantees its quality, safety and efficacy.

This research was motivated by the following:

- The inaccessibility to conventional medicine in certain localities and its high cost making it unavailable to a large population
- The valorization of the use of plant-derived products for medicinal purposes in view of the preparation of an ameliorated traditional medication.
- Provision of alternative sources of natural products other than plant species, a solution to nature protection and promotion of beekeeping for exploitation of bee products.
- Exploitation of the potential pharmacological properties of propolis. Problem of chemical variability of propolis depending on its geographical and botanical origins. Propolis of areas that have not yet been studied seems to be a promising source of bioactive molecules (**Petrova *et al*., 2010**).

In our project we intend to solve the problem of inaccessibility to modern medicine by seeking alternative solutions to peptic ulcer diseases from natural sources other than medicinal plants which could involve protected plant species and destruction of ecosystems. Also, a study of the chemical variability in propolis composition and biological activities is necessary in order to validate the use of propolis in traditional medicine. The choice of our substance based on a plant-derived and environmentally friendly substance with rich chemical diversity and wide pharmacological activities is well justified. In order to realize this project, a number of objectives were envisaged.

General objective

- Carryout chemical and antimicrobial, antiulcer and antiradical studies on propolis from different regions of Cameroon.

Specific objectives

Phytochemistry

- Prepare extracts from the different raw propolis samples collected
- Establish GC-MS profiles of the studied propolis samples
- Purify the extracts by CC and preparative TLC to obtain pure compounds

- Characterize the pure compounds using physical and spectroscopic methods

Pharmacology

- Evaluate antiulcer, antimicrobial and antiradical properties of extracts and compounds from propolis.

Society

- Sensitize population on alternative natural resources which can be exploited for medicinal purposes (propolis) to avoid exploitation of endangered plants and destruction of ecosystems.
- Promote the use of propolis in traditional medicine

To attain these objectives, a general **research question** will be applied thus: Does propolis contains bioactive compounds that can be used in managing peptic ulcers? Since peptic ulcer is a complex health problem involving inflammation, oxidation and bacterial infection, our hypotheses to address this wuestion will be formulated and verified as stated below.

Hypotheses:

- Propolis possesses antiulcer, antioxidant and antimicrobial activities.
- The compounds isolated from propolis are responsible for these pharmacological properties which differs from one sample to another.

The aforementioned aspects are handled in our thesis which is structured as follows. The first part of this research involves a literature review which makes a survey of propolis and bee products, plant souces of propolis, chemical compounds previously isolated from propolis of temperate and tropical regions as well as some biological activities of propolis. An overview of triterpenes was made as most of our compounds belong to this class. The second part involves the presentation of the results obtained and the discussion arising thereof. The methods used are given in the third chapter while the bibliography, documents and articles cited in the text are collectively listed in alphabetical order as references at the end.

CHAPTER I: LITERATURE REVIEW

I-1: BEE PRODUCTS

The products resulting from the work of this small insect *Apis mellifera* have been used since thousands of years by many civilizations. Formidably, bees are well organized in colonies and representing one the interesting areas of studies that gives pleasure, health, happiness and generates incomes throughout the whole world. Indeed, bee products are becoming more and more significant in natural medicines and often complement conventional medicines. Honey, royal jelly, propolis, pollen, wax and bee venom find applications in therapeutics. Apitherapy refers to the medical use of these products of the hive and has become the subject of several scientific studies which remain however very few or incomplete due to the regional variations. However, it is a form of medicine which evolves year in year out depending on the plants that are being foraged, the specie of bee as well as the climatic conditions making them more effective as compared to synthetic molecules like antibiotics for example which may encounter resistance from some pathogens.

I-1-1: Propolis

Propolis is a chemically complex product obtained by bees from the resinous exudates of leaf buds, shoots, and petioles of leaves of different plants present around the hive, which is mixed with wax and salivary secretions. The term “propolis” comes from the Greek origin: 'pro' meaning in front or in defense of, and 'polis' meaning ‘city’. Therefore, it is a substance used by bees in front of the city, that is, the hive to partially close the access roads to their community or city and protect the hive against intruders and natural phenomena. In the hive it has a multifunctional role as a material for construction, maintenance, and defense (**Burdock, 1998**) and also to maintain a high humidity within the hive, preventing sudden changes either during the rainy season or dry periods, and to varnish the brood combs and the walls of the hive. A significant number of papers dealing with propolis chemistry were also published and researchers began to understand that its chemical composition was highly variable and depended on the local flora at the site of collection, seasonality, altitude, collector type, food availability, and activity developed during propolis exploitation (**Marcucci, 1995; Bankova *et al.*, 2000**). Characteristically, it is a lipophilic material, hard and brittle when cold, but soft, flexible and very sticky when warm. It has a characteristic odour and shows adhesive properties of oils and interacts strongly with skin proteins. The colour

of propolis varies from yellowish green to dark brown, depending on location - savannah, tropical forests, and desert, coastal and mountainous regions where it is produced. Its taste is slightly sweet when you begin chewing but gradually becomes bitter with prolonged chewing. Although the biological activity of bee glue and especially its activity against microorganisms were always present in samples from different geographic and climatic zones, this activity results from completely different chemical composition (**Kujumgiev *et al.*, 1999**). The term propolis is not characterizing with respect to the chemical composition, unlike the term bee venom for example (**Bankova, 2005**), such that the biological studies on propolis must be carried out accompanied by its botanical sources and chemical composition as well. Propolis has been used as a popular remedy in folk medicine, in apitherapy, as a constituent of biocosmetics, health foods and for other numerous purposes (**Bankova *et al.*, 2000; Banskota *et al.*, 2001; Kalogeropoulos *et al.*, 2009**). It is still one of the most frequently used remedies in the Balkan states (**Wollenweber *et al.*, 1990**), applied for treatment of wounds and burns, sore throat, stomach ulcer, etc. It was noticed that it can be more effective and less toxic than certain compounds. Although reports of allergic reactions are not uncommon, propolis is relatively non-toxic, with a non-observed effect level of 1400 mg/kg body weight/day in a mouse study (**Kalogeropoulos *et al.*, 2009**). Since social insects generally live in large groups of constantly interacting, related individuals, there is an increased risk of disease outbreaks and evolution of specialized parasites (**Schmid-Hempel and Ebert, 2003**). It is believed that the interior of the hive and the bodies of the bees are covered with a protective coating which prevents and counters proliferation of bacteria and thus the hive is a milieu that is practically sterile due to bee products such as propolis. Propolis is the most important chemical weapon of bees against pathogen microorganisms.

Figure 1: Propolis sample and extracts (Tamfu, 2014)

I-1-2: Honey

Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature. Honey originating from a single flower species is called mono-floral honey, such as kapok honey, banana honey or coffee honey. If the nectar of more than one species is collected, then it is called multi-floral or poly-floral honey. Honey has been a symbol of immortality in ancient Egypt, presenting a great religious value during Antiquity and remains significant in certain birth and death rituals in Africa (**Domerego, 2001**). Honey is mostly composed of different sugars, fructose and glucose being the main components, with average proportions of 38% and 32% respectively with small amounts of other substances such as proteins, vitamins and minerals (**Qiu *et al.*, 1999**). Also, the composition of honey varies greatly according to the foraging areas of the bees (**Qiu *et al.*, 1999; Serrano *et al.*, 2004**). The moisture content is recommended not to be more than 20%, and the level of hydroxymethylfurfural (HMF), which is related to the ageing of honey, not to exceed 40 mg/kg, excluding honey produced in tropical areas, for which the highest level of HMF allowed is 80 mg/kg. Due to the high sugar content and pH of about 3.9, microorganisms are not able to grow in ripe honey and the only microorganisms present are spores of certain bacteria and yeasts. Factors contributing to the inhibitory activity of honey are believed to be the high osmotic pressure as a consequence of high sugar content, low water activity, low protein content, low pH value, low redox potential due to the high number of reducing sugars, high viscosity, the glucose oxidase system forming hydrogen peroxide, and other antimicrobial agents such as flavonoids, lysozyme, phenolic acids and terpenes. Honey is used as reserve of food for the hive in the event of shortage of nectar and of climatic insulator, in particular the winter. Honey is absorbed very quickly into the tissues and has traditionally been used to relieve the symptoms of asthma and diabetes but does not mean that honey can be added without caution to a diabetic's diet. Honey is also used for burns and other wounds because of its osmotic cleansing effect and its healing properties and as the wound heals, the percentage of fat is reduced. In South America it is also used in pure form as eye drops to treat cataracts (**Mickael Blanc, 2010**).

I-1-3: Royal jelly

The young bees add secretions from glands on their heads to the ingested bee bread to make bee milk or royal jelly. They put this bee milk in cells that contain young larvae. The larvae of worker bees, drones and the egg-laying female (the queen) eat these products, which make them grow. The production of royal jelly is therefore important to many young bees in the colony. The royal jelly is the most nutrient rich food for the queen and this is partly why the queen becomes much bigger and stronger than the workers. She can live for a few years, and thus much longer than the 4 weeks to 6 months, depending on the season, that the worker bees live. Royal Jelly is a known source of 10-hydroxydecanoic acid, a compound with anti-tumoral and antibacterial potential. The chemical composition of Royal Jelly shows that it is a mixture of vitamins, amino acids and some unidentified compounds amounting to about 2.8% (**Boselli *et al.,* 2003**). Literature shows that, some of the important components of royal jelly are proteins, sugars and lipids (**Lerker *et al.,* 2003**). Royal jelly contains peptides, lipids, sterols, aromatic oils, carbohydrates, enzymes, anthocyanins, carotenoids, flavonoids, ferulic acids, as well as minerals and spore elements from the bee bread like calcium, potassium and iron. Royal jelly shows metabolic, nutritive and energetic properties and can favour the oxygenation of tissues, increase resistance to cold, stimulate the appetite and increase vitality (**Mickael Blanc, 2010**). It can also be used as an antiviral against the herpes or influenza viruses and as an antibacterial agent against *Escherichia coli*. Royal jelly is recommended for stomach, liver and digestion problems, high blood pressure, loss of appetite, weight loss, fatigue, listlessness, insomnia, pregnancy, menopause, old-age problems, convalescence and athletics. Royal jelly can be viewed as a tonic to make you feel stronger, healthier and less tired. It stimulates the formation of healthy tissue and hair growth (**Mickael Blanc, 2010**).

I-1-4: Beeswax

Worker bees secret wax from the ventral face of their abdomens through the eight wax producing glands situated there. The wax drips like a curtain out of the gland's narrow opening and hardens as it comes in contact with the air. It thus becomes transparent, white, ellipse-shaped scales. The bees then hold these scales with their forelegs and chew them into the right form to build a comb. This wax will constitute the structure of support of the hive, cells and covers (**Mickael Blanc, 2010**). Secreting wax is an energy-consuming biochemical process. Thus, while producing and building with wax, the bees eat and digest a lot of honey. Their high metabolism leads to a high

body and surrounding temperature, which is necessary to keep the wax fluid. Beeswax is a greasy substance containing up to about 300 components, two thirds of which are esters formed between fatty acids and high molecular weight alcohol with less than one third being free fatty acids, lactones, saturated hydrocarbons, free alcohols, chrysin, water, certain pigments, vitamin A and unidentified substances and other compounds from propolis, (**Mickael Blanc, 2010**). Beeswax is employed in cosmetology because it is known to clean the epidermis, to nourish and soften dermal layer and to prevent cutaneous ageing. Moreover, it possesses anti-inflammatory and cicatrizing proprieties making it useful in the treatment of burns, of scares, wounds, abscess and stretch marks. Beeswax also possesses antioxidant and antibiotic properties (**Mickael Blanc, 2010**). Beeswax is used in rubbing oils, shampoos, mascaras, pomades for dyes and lotions, anti-wrinkle creams, more especially as the beeswax is the only known product gathering all these qualities, desired in the cosmetic industry (**Mickael Blanc, 2010**).

I-1-5: Bee venom

Female bees, namely the worker bees or the queen, have a stinger covered in barbs on the end of their abdomen that they can extend. The queen usually uses this to lays eggs, but she can also sting with it while worker bees only sting with it. A drop of fluid, the bee venom, hangs on the extended stinger. Bee venom is used for the defense of the hive against intruders and also by the queen bee to get rid of rivals. It contains many peptides and enzymes which are necessary for human beings. It has a negative impression to the general public because the pain immediately associated to it usually with different symptoms and allergies observed in individuals (**Alphandery, 2002**). Indeed, this venom contains a toxin which is at the origin of the anaphylactic shock observed in individuals who are victims of a first bee sting and the venom secretes pheromones which will excite the other bees to sting in their turn. For information, the lethal dose/amount is of 19 stings/kg for adults and the large animals and obviously this amount is lower for children and small animals because they are more sensitive animals. The LD_{50} of pure bee venom is 2.8 mg per kg of body weight. However, it is important to differentiate bee venom from apitoxin which corresponds to the venom of bee that has lost its volatile components. Components of bee venom include, among many other substances, mellitin (40-60%), phospholipase A (10-12%), apamine (2-3%) and histamine (1%). The mellitin, a hemolytic peptide responsible for the pain and shock represents 40 to 50% of the dry weight of venom. It causes a lysis of red blood cells and increases the effect of phospholipase A2 which is an active agent of this venom. Bee venom is used in treating arthritis

and psoriasis or eczema. Venom is implicated in a method of treatment of the sclerosis in plate, called Bee Venom Therapy in the United States over 40000 to 60000 patients are treated each year by this substance (**Mickael Blanc, 2010**).

I-1-6: Pollen

Honey bees collect pollen from the stamens of flowers. The pollen sticks to the bee's hairs while the bee is sucking nectar. Pollen is made up of small spherical elements or ovoides of sizes between 20 and 40 microns contained in the pollen bags of the anthers of a flower. It is useful in fertilizing the female part of the flower and constitutes the male gametes in the plant kingdom. It is noticed that those which are transported by the bees or other insects are endowed with prickles to foster their attachment. There exist many types of pollens of different constitution depending on the plant species, climate, geographic location, period of harvest or and the nature of the soil (**Mickael Blanc, 2010**). Pollen can serve as source of proteins for the hive but also contains tetraterpenes, carotenoids, fatty acids (linoleic, linolenic and arachidonic acids), glycerides, sterols, terpenes, essential oils, hydrocarbons, flavonoids, mineral substances like zinc, copper, iron, magnesium, calcium as well as liposoluble vitamins and enzymes. It can be used in cases of constipation, nervousness, prostatitis, hypertrophy of the prostate, in animal feeds and in cosmetics to increase resistance to infections (**Mickael Blanc, 2010**). According to studies carried out on animals, it is beneficial for reproduction, growth, intestinal problems, constipation and diarrhea. It has antibiotic proprieties in particular on salmonella and can be used as tonic in psychic or physical tiredness. Other works report its action on certain hepatic affections, hypertension and prostate disorders. Pollen, thanks to its components, thus presents interesting potentials in several fields (**Molan, 1999**). Pollen is used for improving fertility, relieving the discomforts of old age, and combating the symptoms of menopause, an enlarged prostate, listlessness and stress, hay fever, asthma, sore throats, colds and is a good remedy for intestinal problems.

I-1-7: Bee bread

Bees make bee bread out of the pollen that they have collected. Pollen loads undergo biochemical processes caused by enzymes added through the bees' saliva and stomach fluids. Thanks to the work of micro-organisms and the influence of moisture and temperature in the beehive (35-36 ºC), the mixture ripens in two weeks into bee bread. Bee bread is a source of proteins, fats, micro-elements and vitamins for the bees. It is the raw material for production of bee milk and royal jelly,

which the young nurse bees make with the help of secretions from glands in their heads. Bee bread has a different composition than pollen. Bee bread contains fewer proteins than the original pollen, but they are easier to absorb. Bee bread also contains the following substances: proteins with essential amino acids, vitamins, nicotinic acid, folic acid and pantothenic acid, pigments, carotenoids, anthocyanins, enzymes, flavonoids and more than 25 different minerals and spore elements such as iron, calcium, magnesium, phosphorus, potassium, copper, zinc and selenium. The combination of various biologically active substances in bee bread makes it effective for the prevention and treatment of various diseases. Both bee bread and pollen have a positive effect on the immune and anti-oxidant systems of healthy people. It can improve the physical performance of athletes by providing extra energy. Bee bread also has antibiotic properties, stimulates tissue growth and recovery and it cleanses the blood, slows down the aging process, helps increase a person's appetite, gives added strength to the elderly and speeds recovery. In apitherapy bee bread is used quite successfully in combination with other methods to treat the elderly and children. The use of bee bread is recommended for anemia, hepatitis, diabetes and gastrointestinal problems such as colitis, constipation and diarrhea (**Mickael Blanc, 2010**).

I-1-8: Brood: (eggs, larvae and pupae)

Bee brood is made up of eggs, larvae and pupae in the comb. The larvae and pupae are especially suited for consumption. Harvesting brood is bad for the colony, so the larvae and pupae of drones are usually used because the colony is less dependent on them. In Africa the larvae and pupae are removed from the comb and used as an ingredient in various dishes. In Indonesia the sealed brood comb of *Apis cerana*, the indigenous honey bee, is sold at markets. In Eastern Europe beekeepers eat drone larvae because of their hormone-like strengthening properties. They are used to treat old-age ailments, enhance convalescence and are recommended in times of digestive problems, insomnia, respiratory problems, nutritional deficiencies, premenstrual syndrome and amenorrhea. Moreover, it stimulates libido and is beneficial to aged people and possesses protective propriety against radiations or certain cancers when associated with honey. The larvae from harvested royal jelly are also processed by drying and then grinding them into powder. Those of false-bumblebee contains water about 65 to 68%, proteins (20%), certain enzymes (amylase, esterase), a high level of globulins, lipids 6%, glucides 1 to 3%, minerals 1%, vitamins (primarily A and D) and 5% of products not yet identified (**Mickael Blanc, 2010**).

I-2: RESIN COLLECTION BY BEES (ORIGIN OF PROPOLIS)

Bees gather propolis from various plant sources (pine, fir, willow, birch, several species of poplar, ash, oak, etc.). Honey bees harvest resins from various plant species and bring them back to the colony where they are then utilized as propolis (propolis is an apicultural term for the resins when used by bees within a hive). Depending on the bees, climate, forest resources and the trapping mechanism, the average production of propolis ranges from 10 g to 300 g per colony per year (**Krell, 1996**). The harvesting of antimicrobial compounds in the form of resins from the environment and their incorporation into the social nest architecture as propolis is an exciting but relatively unexplored colony-level defense against pathogens. Much of the current literature concerning propolis has focused on the chemical constituents and biological activity of propolis and the botanical origins of the resins from which the propolis mixtures are derived (**Banskota *et al.*, 2001; Bankova *et al.*, 2008**). Propolis collection is performed following a specific foraging pattern, foragers extract propolis from buds using their mandibles and their first pair of legs; their mandibular gland secretion (10-hydroxydecenoic acid) makes it possible to chop and soften the material for transport. While there is considerable variation among colonies in resin collection and propolis use, all colonies do appear to use at least some (**Page *et al.*, 1995; Manrique and Soares, 2002**). In tropical climates, honey bees mostly collect resins from *Clusia minor* and *Clusia rosea* flowers and also from alecrim plants (e.g. *Baccharis dracuncufolia*), which are common plants to tropical bee species (**Pereira *et al.*, 2003; Salatino *et al.*, 2005**). Recently a leguminous species (*Dalbergia sp*.) has also been identified as a common source in tropical regions (**Silva *et al.*, 2008**). In temperate climates poplar trees (*Populus sp*.) appear to be the primary source for resins (**Bankova *et al.*, 1992; 2006; Markham *et al.*, 1996; Salatino *et al.*, 2005**). However, it is clear that other trees, like pine, birch, elm, alder, beech and horse-chestnut species, are adequate resin sources for temperate honey bees, particularly when poplar species are unavailable (**Ghisalberti, 1979; Crane, 1990**). Additionally, honey bees in Uganda appear to forage for resins selectively on *Alnus sp.* and can actually defoliate these trees; whether there are other possible sources in the region remains unclear (**Nyeko *et al.*, 2002**). Honey bees will forage for resins from droplets appearing on the bark of the trunks or limbs of trees, from the surfaces of some fruits like *Macaranga tanarius* (**Kumazawa *et al.*, 2008**), or more typically on the vegetative apices (buds and young leaves). The bees must extract the resins from the trichomes and ducts by fragmenting these early leaves using their mandibles (**Nyeko *et al.*, 2002; Teixeira *et al.*, 2005**). Resin-foragers

have shown a preference for young leaves and vegetative buds over more expanded leaves (**Park *et al.*, 2004**). Honey bees have been observed probing the apex of one plant with their antennae then moving to another one, probing it and subsequently collecting resin from it (**Teixeira *et al.*, 2005**). The same study also provided evidence that the resin-foragers preferred female versus male *Baccharis dracuncufolia* as resin sources. The young leaves and buds have a similar chemical composition that changes as the leaves become more expanded (**Park *et al.*, 2004**), which implies that there may be a chemical compound released by the resin source that the foragers are able to detect. Once the bees find the resin source, they then have to collect it. There are four basic steps that a resin forager follows to pack her corbicula: (1) Break off a particle of propolis with the mandibles; (2) work it with the mandibles and take it with the forelegs; (3) transfer it from the forelegs to the middle leg; (4) transfer it from the middle leg to the corbicula on the same side. This sequence is repeated until there is a full resin load on both corbicula. No corbiculate bees can collect resin and pollen during a single foraging trip because of this behavior. After completing the four steps, bees have been observed flying around for a few seconds above the resin source, then landing again to add more to each corbicula. The purpose of these flights is unknown but may be used to assess the weight of the current corbicular load. The process of obtaining a full corbicular load of resin has been noted to take about seven minutes (**Teixeira *et al.*, 2005; Kumazawa *et al.*, 2008**), but can take from 15 minutes to an hour depending on the weather. Once the bee has a full load, she returns to her colony to unload the resin from her corbiculae. The unloading process typically takes approximately 15 minutes, but can extend from one to seven hours or even overnight. Cementing bees, bite off chunks of resin from the corbiculae of resin-foragers and immediately attach the resin to a site along the hive wall, then smoothens the resin, now officially propolis, with her mandibles in a manner that is similar to that of wax construction (**Nakamura and Seeley, 2006**). During the cementing process, the resins do not appear to be chemically modified while there is some evidence that the general chemical profiles of resins collected directly from a forager and in-hive collected propolis can vary slightly from the leaf buds of the plant source (**Peev *et al.*, 2009**). After resin foraging has been initiated by one or several bees, it is possible bees use waggle dances as a colony-level recruitment signal, in a similar way to how pollen and nectar-foragers use dances as communication signals to recruit other foragers to their food sources. It was found that 26% of the 77 observed resin foragers performed dances near cementing sites deep within the hive unlike pollen and nectar dances, which are done near the

hive entrance (**Nakamura and Seeley, 2006**). Waggle dances by resin foragers have also been observed near cementing sites. However, the purpose and subsequent effect of these dances is unknown, and could simply be a vestige of more general foraging behaviors.

I-2-1: Some plants sources of resin used by bees in manufacturing propolis

The plant source is identified by observing the collection activities of bees and comparing the chemical profiles of propolis and plant materials. Other researchers found that honeybees collect plant material by cutting fragments of vegetative tissues, so the anatomical characteristics of plant tissue in the propolis can be used as evidence of propolis origin (**Nagy *et al.*, 1986**).

Table 1: Plants sources of resin from some geographical regions

Plants	**Geographical region**	**Reference**
Populus nigra, P. italic	Bulgaria	**Bankova *et al.*, 1994**
M. schweinfurthii, Macaranga spp	Kenya	**Petrova *et al.*, 2010**
Populus nigra	Albania	**Bankova *et al.*, 1994**
Populus tremula	Bulgaria	**Marcucci, 1995**
Populus suaveolens	Mongolia	**Marcucci, 1995; Bankova *et al.*, 1994**
Populus fremontii, Fraxinus excelsior, Castanea sativa, Larrea tridentate, P. angustifolia, P. deltoides P. trichocarpa, P. balsamifera	USA (Mainland)	**Marcucci, 1995; Crane, 1990**
Plumeria acuminate, P. acutifolia, P. rubra, Schinus terebinthifolius, Psidium gaujava	USA (Hawaiian Islands)	**Marcucci, 1995; Crane, 1990**
Populus euramericana	United Kingdom	**Marcucci, 1995**
Betula, Populus, Pinus, Prunus, and Acacia spp.; Aesculus hypocastane	Hungary	**Marcucci, 1995**
Betula, Alnus spp.	Poland	**Marcucci, 1995**
Delchampia spp	Equatorial regions	**Marcucci, 1995**

Table 1: continuation

Clusia spp.	Equatorial regions	**Bankova *et al.*, 1995**
Xanthorrhoea	Australia	**Ghisalberti, 1979**
Betula verrucosa Ehrh. *Populus tremula*	Russia (birch propolis)	**Popravko, 1978**
Baccharis spp. Baccharis dracunculifolia DC. (Compositae)	Brazil (green-alecrim-propolis)	**Park *et al.*, 2002; Funari *et al.*, 2007**
Clusia minor, Clusia scrobiculata, Clusia nemorosa	Cuba, Venezuela	**Marcucci, 1995; Trusheva *et al.*, 2004; Cuesta-Rubio *et al*, 2002**
Populus alba, Hyptis divaricata, Dalbergia ecastophyllum, Baccharis dracunculifolia, D. odorifera, D. cultrate, D. frutescens	South Brazil (type 3), Argentine, and Uruguay	**Park *et al.*, 2002; Daugsch *et al.*, 2008 ; S. Awale *et al*., 2008**
Hyptis divaricate, Corymbia citriodora, Araucaria angustifolia, Hyptis divaricata	Brazil (type 6 from North-Eastern Brazil)	**Crane, 1990; Park *et al.*, 2002**
Dalbergia ecastaphyllum,	Brazil (type 13 from North-Eastern Brazil)	**Alencar *et al.*, 2007;**
Azadirachta indica, Acacia spp., Mangifera indica	Oman	**Popova *et al.*, 2013**
Acacia spp.	Ethiopia	**Rushdi *et al.*, 2014**
Styrax trees	Thailand	**Athikomkulchai *et al.*, 2013**
Macaranga tanarius	Okinawa Hawaii, and Taiwan	**Kumazawa *et al.*, 2004; Chen *et al.*, 2012 ; Inui *et al.*, 2014**
Mangifera indica	Indonesia, Myanmar	**Trusheva *et al.*, 2011; Li *et al.*, 2009**
Populus spp., Eucalyptus spp., Castanea sativa, Populus euphratica, Pinus brutia L.	Turkey	**Seda Vatansever *et al.*, 2010 ; Popova *et al.*, 2005 ; Kartal *et al.*, 2002**
Populu snigra	Iraq	**Sulaiman *et al.*, 2012**

Table 1: continuation

Baccharis dracunculifolia, Populus nigra, Mangifera indica.	Myanmar	**Li *et al*., 2009**
Poplar spp., Ferula ovina	Iran	**Szliszka *et al*., 2011**
P. trichocarpa, P. tremuloides. Betula Verrucosa, Populus tremula, P. maximoviczi, P. fremontii, P. deltoides, P. balsamifera L., P. suaveolens	Canada	**Christov *et al.*, 2006, Garcia-Viguera *et al.*, 1993,**
Cupressus sempervirens.	Malta	**Popova *et al.*, 2012**
Ceratonia siliqua, Olea europeae,	Morocco	**Mouse *et al.*, 2012**
Bursera simaruba	Mexico	**Crane, 1990**
Ambrosia deltoidea, Encelia farinose	Sonoran Desert	**Wollenweber and Buchmann, 1997**
P. tremula	Italy	**Crane, 1990**

The plants listed above are the sources of the chemical compounds found in propolis that bees gather from available plants in their environment.

I-3: SOME COMPOUNDS ISOLATED FROM PROPOLIS

Typically, propolis is broadly classified into (i) samples from temperate regions mainly originating from poplar tree exudates and rich in phenolics such as flavonoids, aromatic acids and esters (**Bankova *et al.*, 2002**) and (ii) samples from tropical areas, void or containing only traces of poplar constituents but rich in other substances including prenylated derivatives of p-coumaric acids, terpenoids and lignans (**Marcucci and Bankova, 1999**), prenylated benzophenones (**Cuesta Rubio *et al.*, 2002**) and prenylated flavonoids (**Raghukumar *et al.*, 2010**). Propolis is composed of 50% resin and balsams, 30% wax and fatty acid, 10% aromatic and essential oil and 5% of pollen and various other substances (**Burdock, 1998**). Some classes of compounds identified in propolis include: flavonoids, prenylated p-coumaric acids and acetophenones, lignans, phenolic compounds, di- and triterpenes, caffeoylquinic acids, sugars, sugar alcohols, hydrocarbons, and mineral elements (**Bankova *et al.*, 2000**).

I-3-1: Some compounds isolated from propolis from temperate regions

In the temperate zones all over the world, the main source of bee glue is poplar tree, mainly the black poplar *Populus nigra* (**Bankova *et al.*, 2000**). For this reason, propolis from temperate regions contains the typical poplar bud phenolics such as flavonoid aglycones, phenolic acids and their esters (**Bankova *et al.*, 2002**). The quantity of flavonoids is used as a criterion to evaluate the quality of temperate propolis (**Zhang *et al.*, 2014**). Compounds found in propolis from temperate zones are mainly flavones, flavonols, flavanones, flavanonols, chalcones, dihydrochalcones, isoflavones, isodihydroflavones, flavans, isoflavans, neoflavonoids, aurones, pterocarpans, lignans, coumarines, phenolic acids and their derivatives (**Bankova *et al.*, 2000, Zhang *et al.*, 2014, Huang *et al.*, 2014**).

Table 2: Some flavones and flavonols isolated from propolis

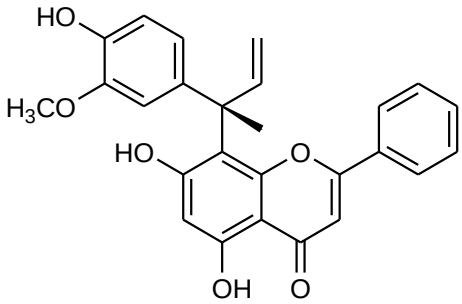
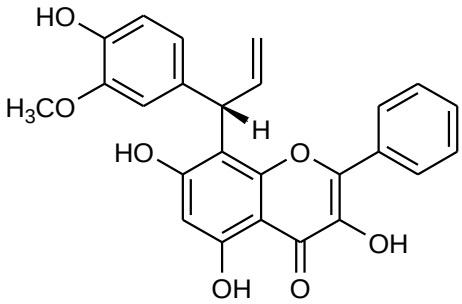
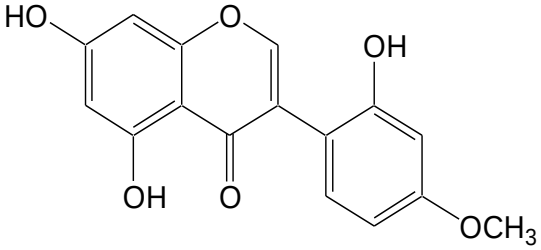
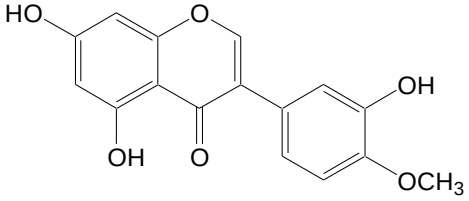
 (7″ R)-8-[1-(4′-hydroxy-3′-methoxyphenyl) prop-2-en-1-yl] chrysin (**Li *et al* ., 2010**) **(1)**	 (7″R)-8-[1-(4′-hydroxy-3′-methoxyphenyl) prop-2-en-1-yl] galangin (**Li *et al* ., 2010**) **(2)**
 2'-hydroxybiochanin A (**Awale *et al.*, 1998**) **(3)**	 calycosin (**Awale *et al.*, 2006**) **(4)**

Table 2: continuation

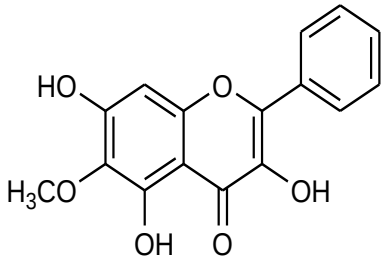
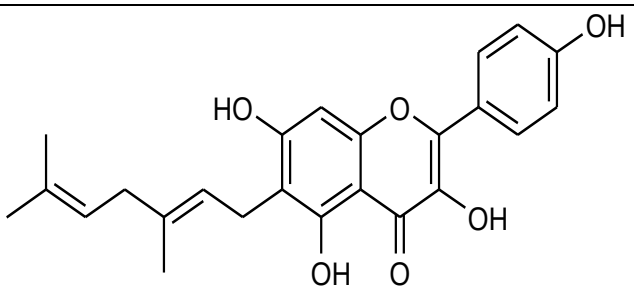
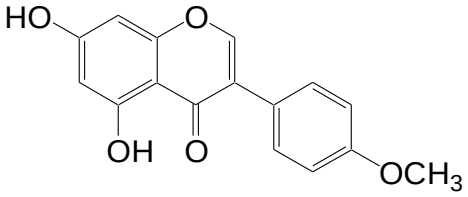
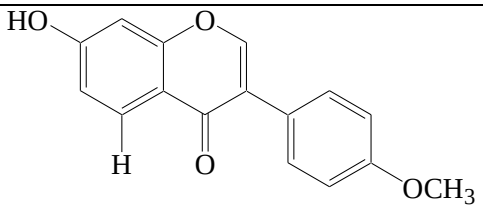
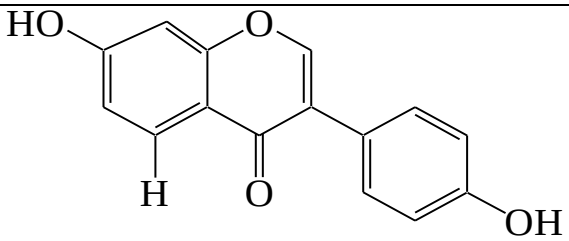
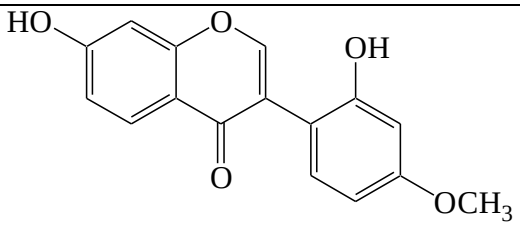
 Alnusin (**Awale *et al*., 2008**) **(5)**	 Macarangin (**Petrova *et al*., 2010**) **(6)**
 Biochanin A (**Piccinelli *et al.,* 2005**) **(7)**	 Formononetin (**Piccinelli *et al.,* 2005**) **(8)**
 daidzein (**Awale *et al.,* 2006**) **(9)**	 Xenognosin B (**Awale *et al.,* 2006**) **(10)**

Table 3: Some flavanones and flavanonols isolated from propolis

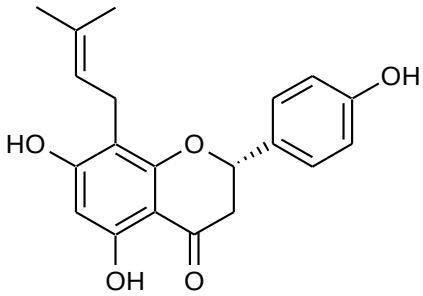
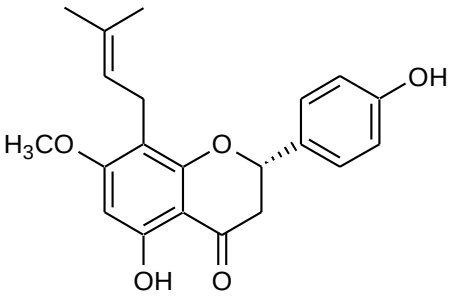
 8-prenylnaringenin (**Popova *et al*., 2013**) **(11)**	 7-O-methyl-8-prenylnaringenin (**Popova *et al*., 2013**) **(12)**

Table 3 continuation

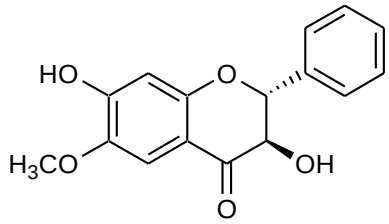
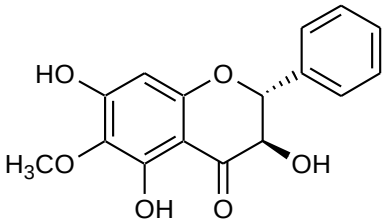
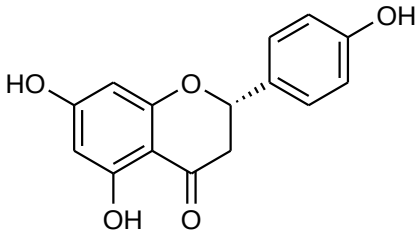
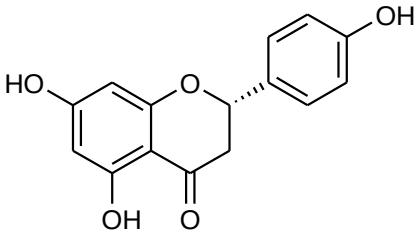
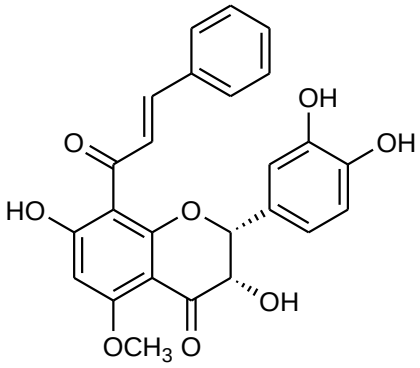
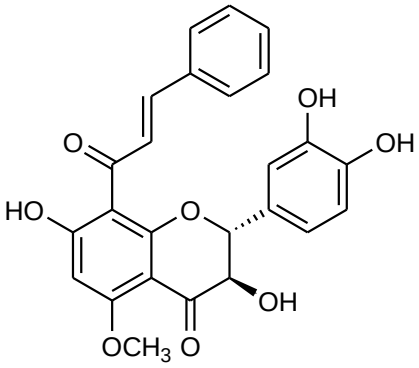
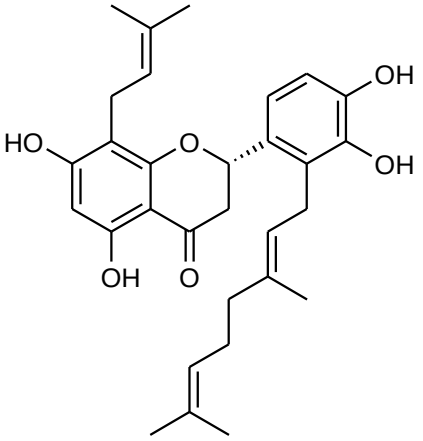
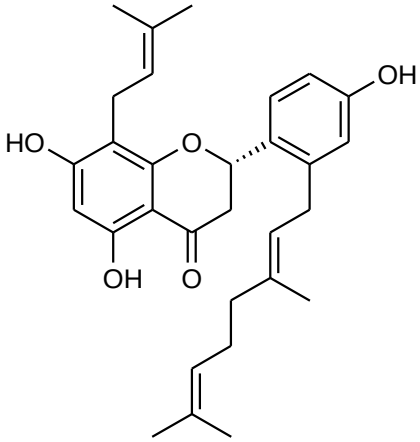
 (2R,3R)-3,7-dihydroxy-6-methoxyflavonol (**Awale *et al*., 2008**) **(13)**	 Alnustinol (**Awale *et al*., 2008**) **(14)**
 Liquiritigenin (**Peccinelli *et al*., 2011**) **(15)**	 Naringenin (**Peccinelli *et al*., 2011**) **(16)**
 8-((E)-phenylpro-2-en-1-one)-(2R,3S)-5-methoxycatechin (**Boisard *et al*., 2015**) **(17)**	 8-((E)-phenylpro-2-en-1-one)-(2R, 3R)-5-methoxycatechin (**Boisard *et al*., 2015**) **(18)**
 Solophenol A (**Inui *et al*., 2012**) **(19)**	 Sophoraflavanone A (**Inui *et al*.,2012**) **(20)**

Table 3 continuation

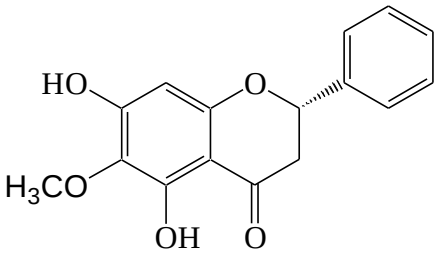
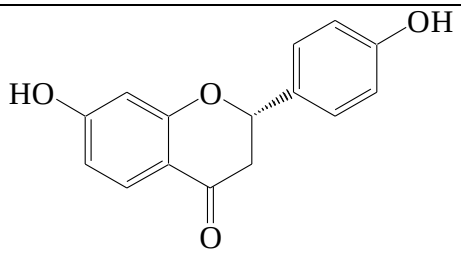
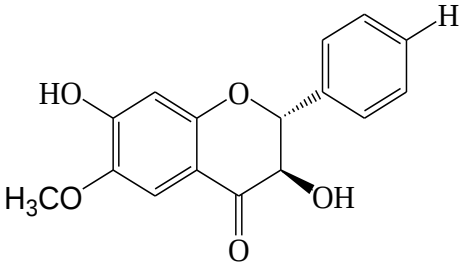
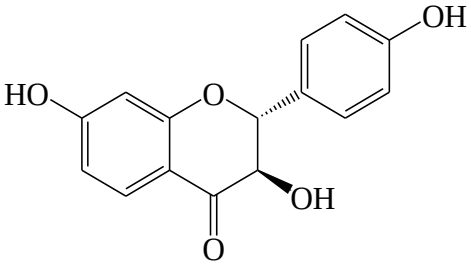
 (2S)-dihydrooroxylin A (**Awale *et al.*, 2008**) **(21)**	 (2S)-liquiritigenin (**Piccinelli *et al.*, 2005**) **(22)**
 (2R,3R)-3,7-dihydroxy-6-methoxyflavanone (**Awale *et al.*, 2006**) **(23)**	 garbanzol (**Park *et al.*, 2004**) **(24)**

Table 4: Some chalcones and aurones isolated from propolis

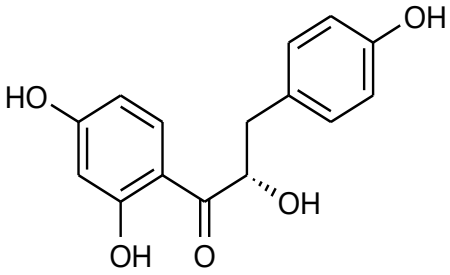
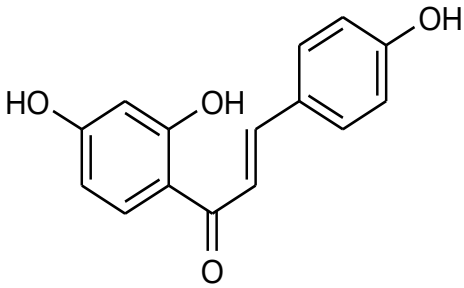
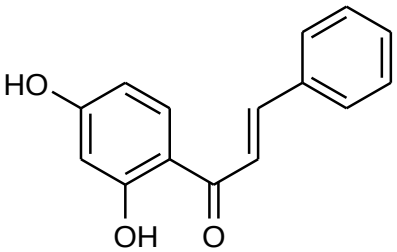
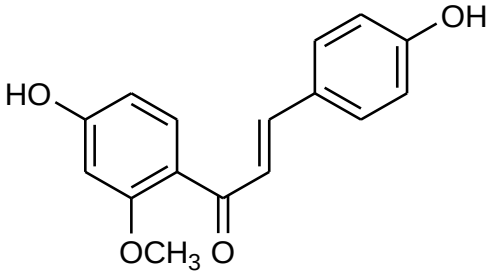
 (αS)-α2′,4, 4′-tetrahydroxydihydrochalcone (**Awale *et al.*, 2008**) **(25)**	 Isoliquiritigenin (**Piccinelli *et al.*, 2011**) **(26)**
 2′,4′-dihydroxychalcone (**Awale *et al.*, 2008**) **(27)**	 Isoquitigenin (**Awale *et al.*, 2008**) **(28)**

Table 4 continuation

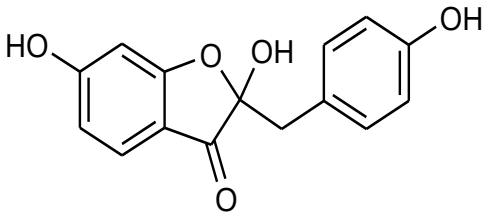
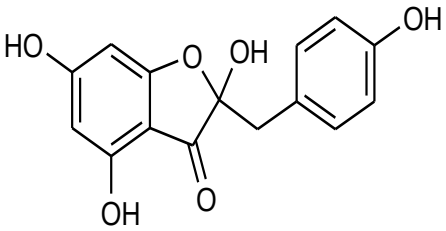
 2,6-dihydroxy-2-[(4'-hydroxyphenyl) methyl]-3-benzofuranone (**Awale *et al*., 2008**) **(30)**	 Maesopin (**Awale *et al*., 2008**) **(29)**

Table 5: Some pterocarpans isolated from propolis

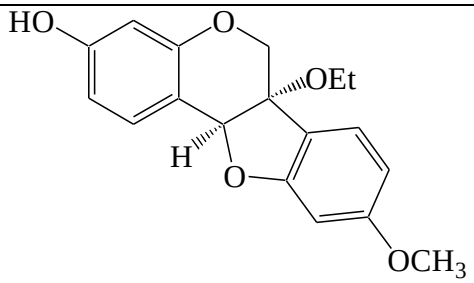
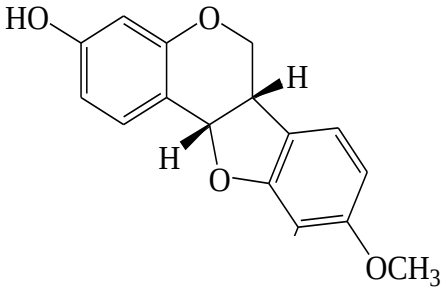
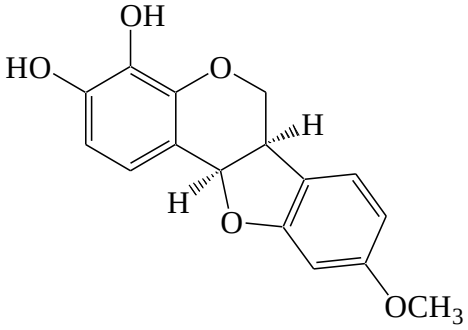
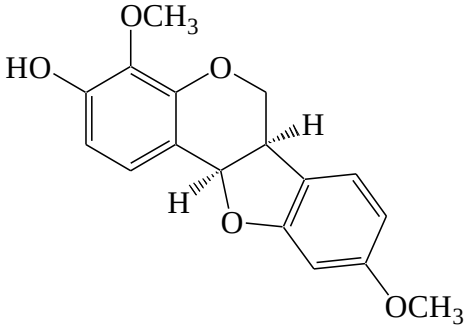
 (6aS,11aS)-6a-ethoxymedicarpan (**Awale *et al.*, 2008**) **(31)**	 (6aS,11aS)-medicarpan (**Piccinelli *et al.*, 2005**) **(32)**
 (6aR,11aR)-3,4-dihydroxy-9-methoxypterocarpan (**Piccinelli *et al.*, 2005**) **(33)**	 (6aR,11aR)-4-methoxymedicarpan (**Piccinelli *et al.*, 2005**) **(34)**

Table 5 continuation

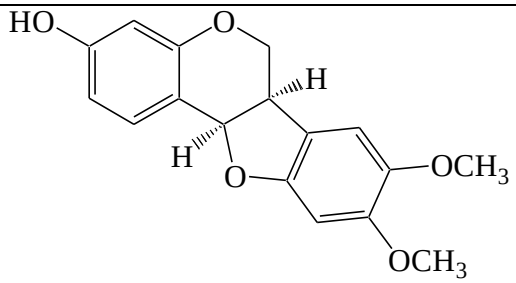
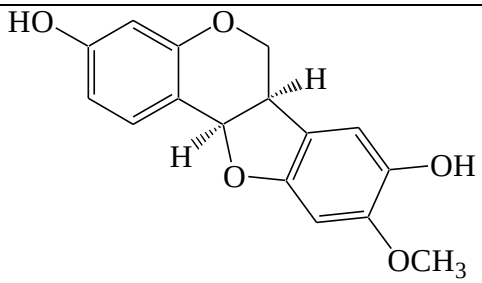
 (6aR,11aR)-3-hydroxy-8,9-dimethoxypterocarpan (**Awale *et al.,* 2008**) **(35)**	 (6aR,11aR)-3,8-dihydroxy-9-methoxypterocarpan (**Piccinelli *et al.,* 2005**) **(36)**

Table 6: Some lignans isolated from propolis

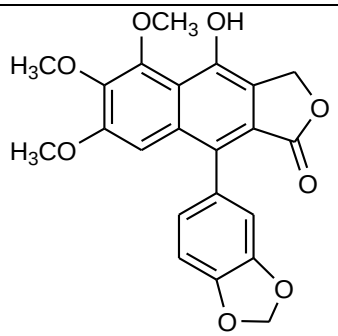
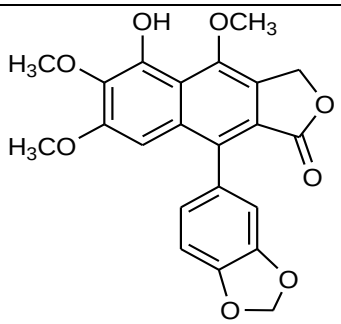
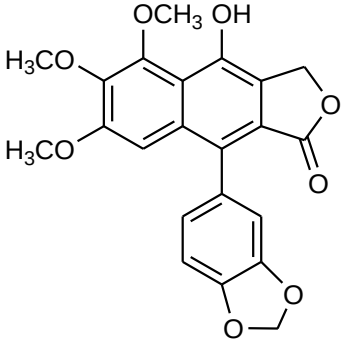
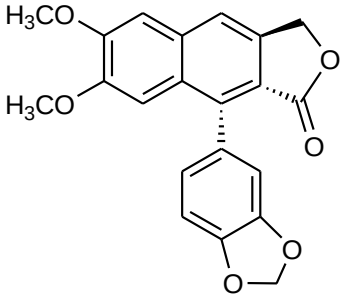
 6-methoxydiphyllin (**Petrova *et al.,* 2010**) **(37)**	 6-hydroxyjustidin A (**Petrova *et al.,*2010**) **(38)**
 6-methoxydiphyllin (**Petrova *et al.,* 2010**) **(39)**	 Tetrahydrojustidin B (**Petrova *et al.,* 2010**) **(40)**

Table 6 continuation

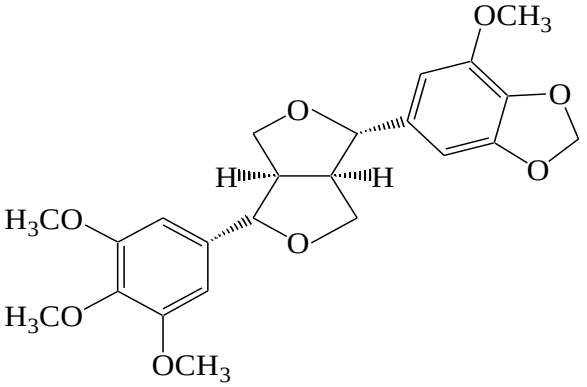
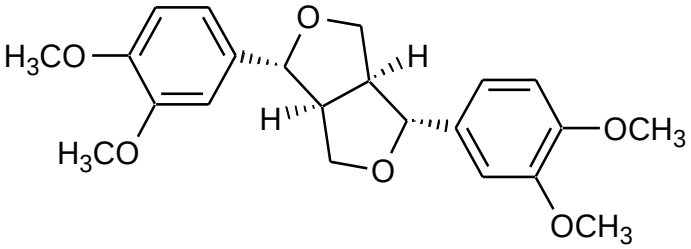
 Sesartenin (**Christov *et al*., 1999**) **(41)**	 (+)-pinoresinol dimethyl ether (**Awale *et al*., 2008**) **(42)**

Table 7: Some phenolic acids and their esters isolated from propolis

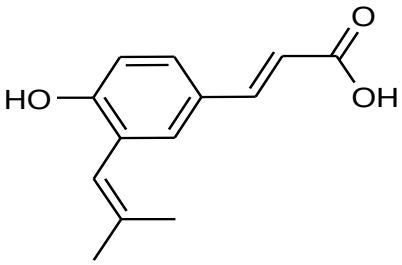
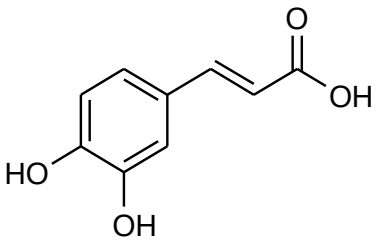
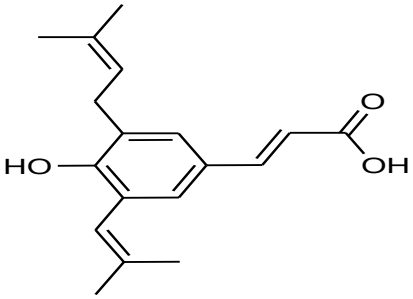
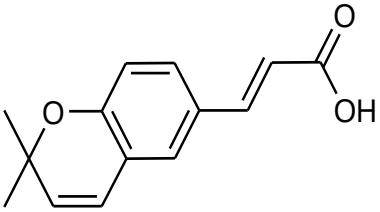
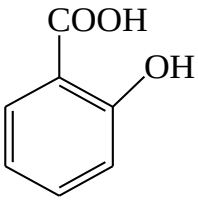
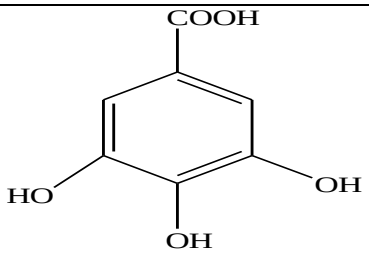
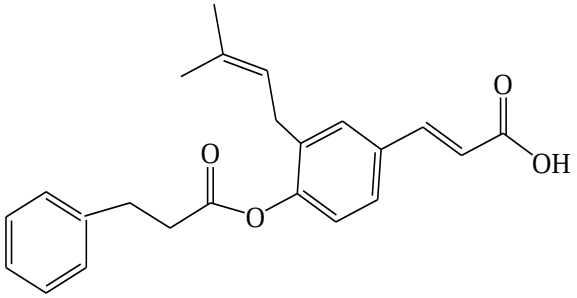
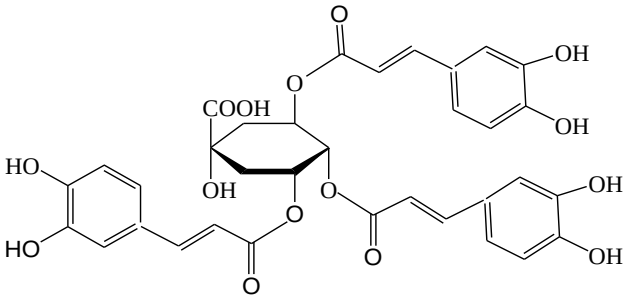
 3-prenyl-4-hydroxylcinnamic acid (**Marcucci *et al*., 2001**) **(43)**	 Caffeic acid (**Banskota *et al*., 2002**) **(44)**
 3,5-diprenyl-4-hydroxylcinnamic acid (**Marcucci *et al*., 2001**) **(45)**	 2 ,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopyran (**Marcucci *et al*., 2001**) **(46)**
 Salicylic acid (**Marcucci, 1995**) **(47)**	 Gallic acid (**Marcucci, 1995**) **(48)**

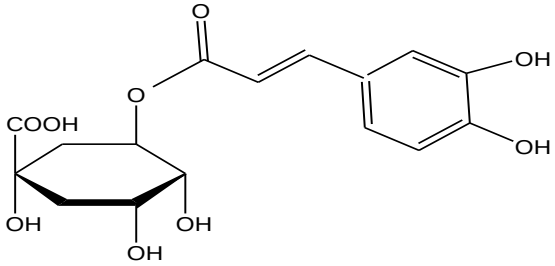
Table 7 continuation



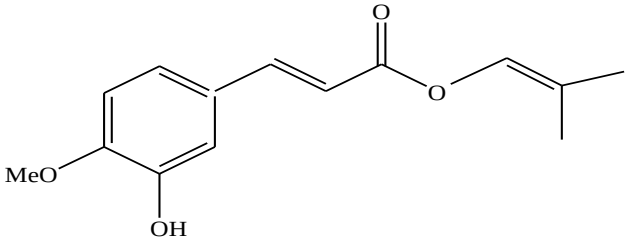
Baccharin (**Simoes-Ambrasio *et al.*, 2010**) **(49)**



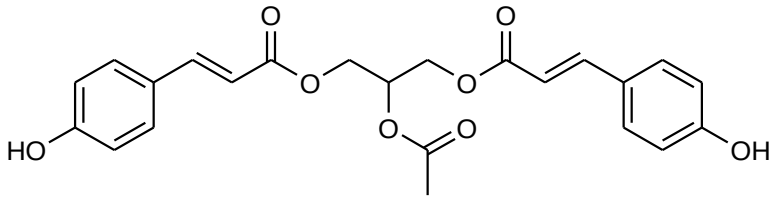
3,4,5-Tricafeoylquinic acid (**Urushisaki *et al.*, 2011**) **(50)**



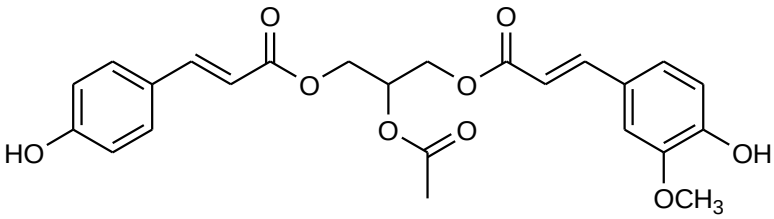
Chlorogenic acid (**Urushisaki *et al.*, 2011**) **(51)**



Prenyl ferulate (**Marcucci, 1995**) **(52)**



2-acetyl-1,3-dicoumaroylglycerol (**Banskota *et al.*, 2002**) **(53)**



2-acetyl-1-coumaroyl-3-feruloylglycerol (**Banskota *et al.*, 2002**) **(54)**

I-3-2: Some compounds isolated from propolis from tropical and subtropical regions

The bee glue from different tropical regions is chemically diverse and devoid or containing only traces of poplar constituents (**Marcucci and Bankova, 1999**). They are rich mainly in terpenes and their derivatives but might equally contain other constituents including xanthones, steroids, prenylated derivatives of p-coumaric acids lignans, different phenolics, flavonoids and benzophenones (**Marcucci and Bankova, 1999; Bankova, 2009; Sanpa *et al.*, 2015**). However,

data about the chemical composition and biological activity of propolis from the subtropical areas are limited.

Table 8: Some terpenoids and steroids isolated from propolis

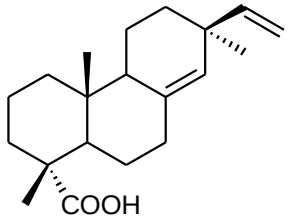
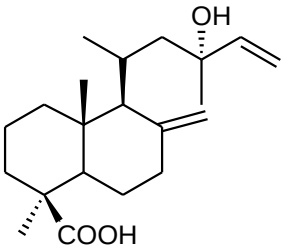
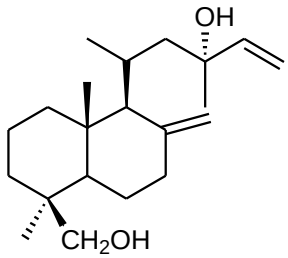
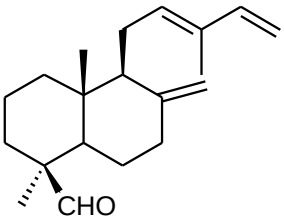
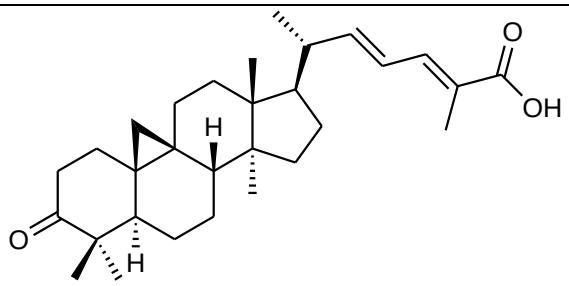
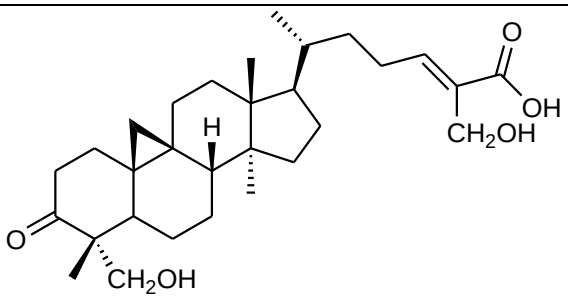
 Pimaric acid (**Popova *et al.*, 2009**) **(55)**	 13-epi-isocupressic acid (**Popova *et al.*, 2009**) **(56)**
 13-epi-torulosole (**Popova *et al.*, 2009**) **(57)**	 Commurale (**Popova *et al.*, 2009**) **(58)**
 (22E,24E)-3-oxocycloart-22,24-dien-26-oic acid (**Li *et al.*, 2009**) **(59)**	 (24E)-3-oxo-27,28-dihydroxycycloart-24-ene-26-oic acid (**Popova *et al.*, 2009**) **(60)**

Table 8 continuation

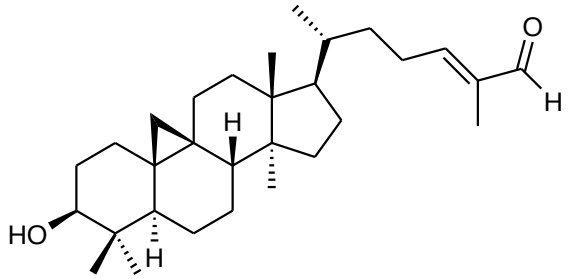
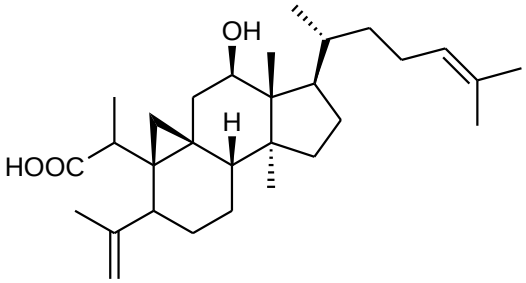
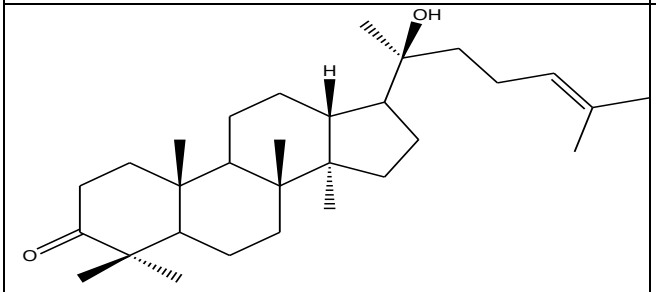
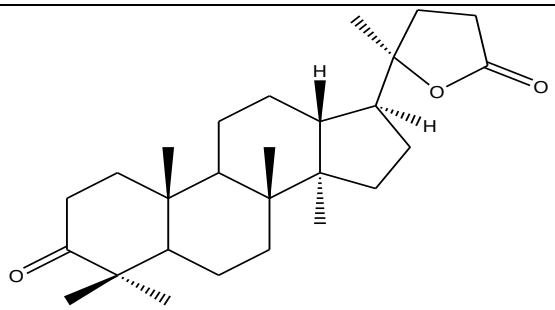
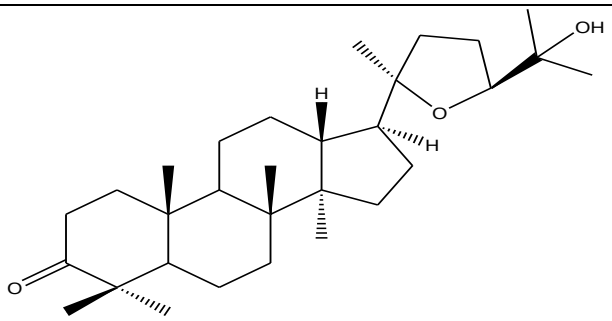
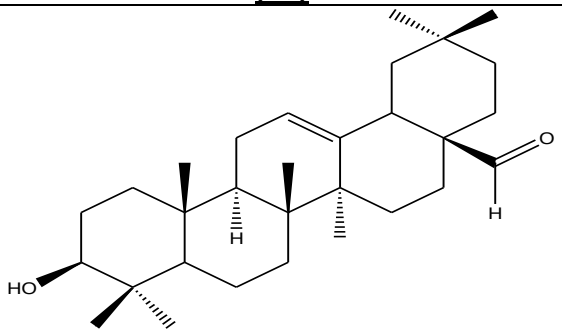
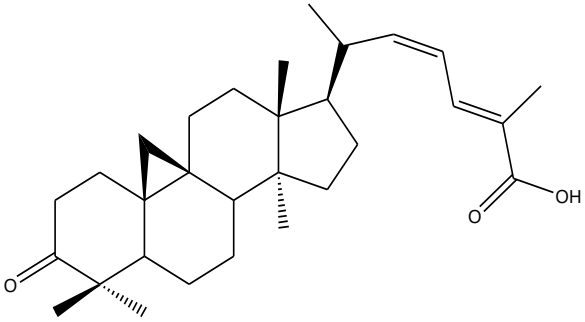
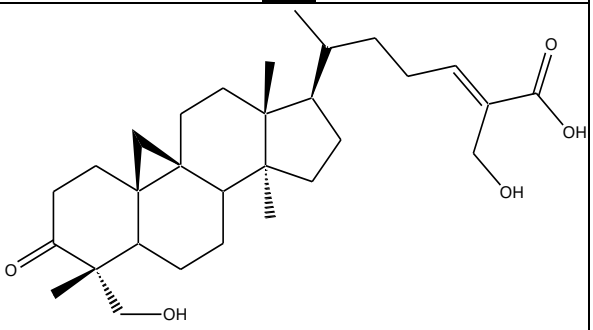
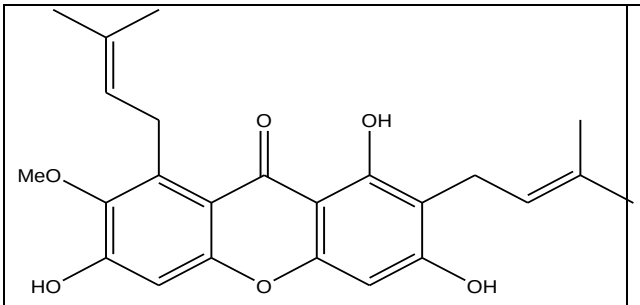
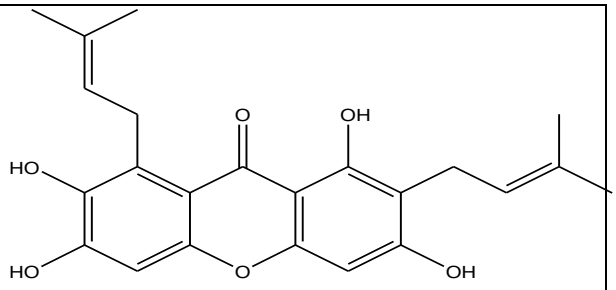
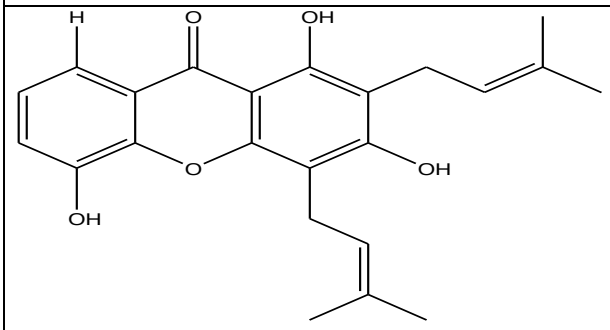
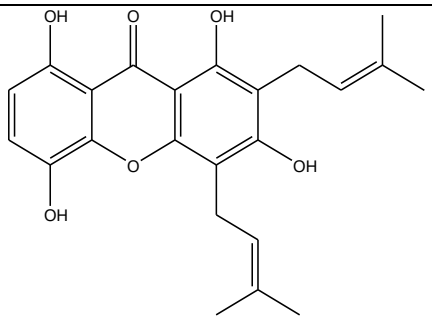
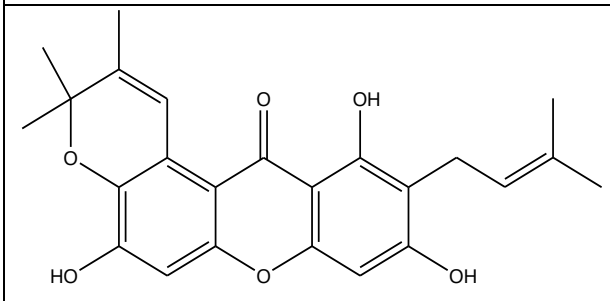
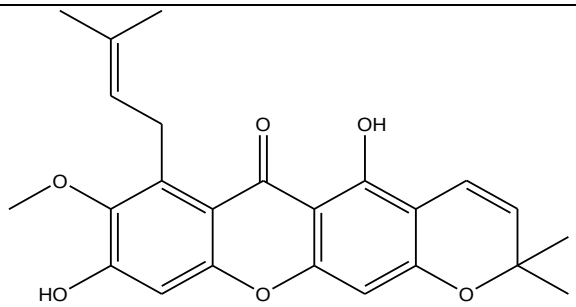
 (24E)-3β-hydroxycycloart-24-en-26-al (**Li *et al*., 2009**) **(61)**	 3,4-secocycloart-12-hydroxy-4(28)-24-dien-3-oic acid (**Popova *et al.,* 2009**) **(62)**
 Dipterocarpol (**Sanpa *et al.,* 2015**) **(63)**	 Cabralealactone (**Sanpa *et al.,* 2015**) **(64)**
 Ocotillone I (**Sanpa *et al.,* 2015**) **(65)**	 Oleanolic aldehyde (**Sanpa *et al.,* 2015**) **(66)**
 (22*Z*,24*E*)-3-oxocycloart-22,24-dien-26-oic acid (**Li *et al*., 2009**) **(67)**	 (24*E*)-3-oxo-27,28-dihydroxycycloart-24-en-26-oic acid (**Li *et al*., 2009**) **(68)**

Table 9: Some xanthones isolated from propolis (Sanpa *et al.*, 2015)

 α-mangostin **(69)**	 γ-mangostin **(70)**
 8-deoxygartanin **(71)**	 Gartanin **(72)**
 Garcinone B **(73)**	 Mangostanin **(74)**

I-3-3: Some compounds isolated from propolis from Cameroon

There are numerous reports in the literature on the isolation and structural elucidation of phytochemicals from propolis collected in Europe, South America, Asia and the Pacific region. Little is known about the exact chemical constituents of African propolis in general and Cameroonian propolis in particular. Cameroonian propolis is an important traditional medicine which has antibacterial and antiradical activity and some compounds mainly triterpenes have been isolated from it (**Mbawala *et al.*, 2010; Talla *et al.*, 2013; Sakava *et al.*, 2014; Almutairi *et al.*, 2014**).

Table 10: Some compounds isolated from Cameroonian propolis

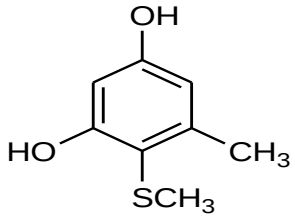
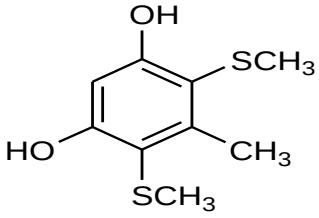
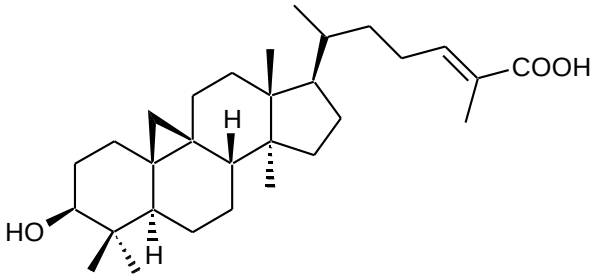
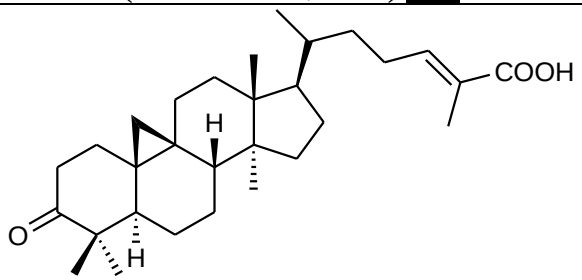
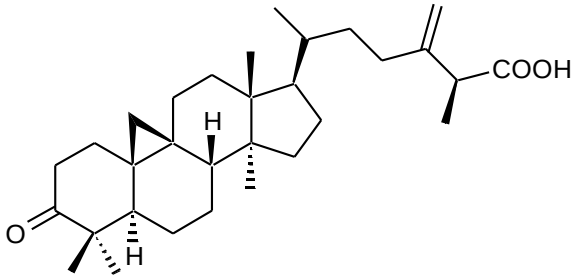
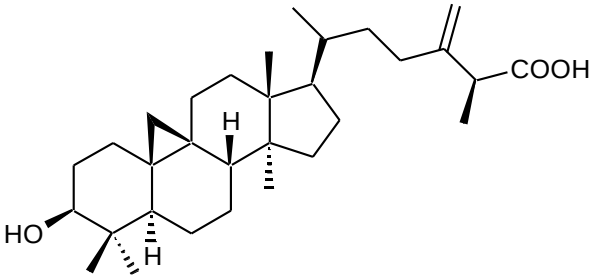
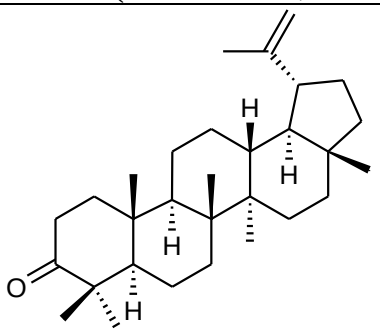
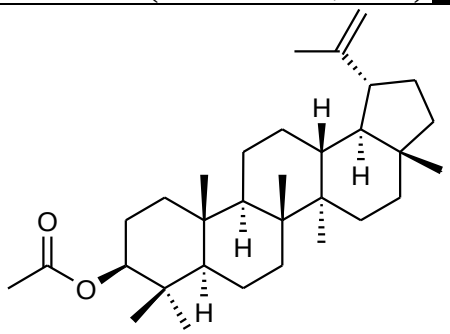
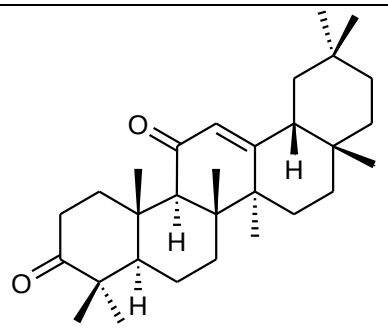
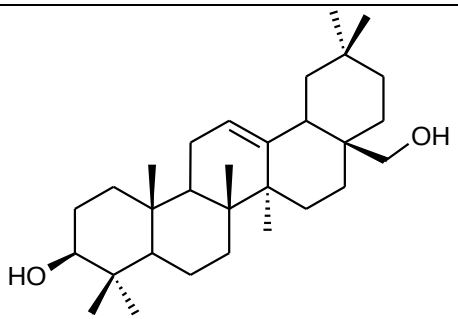
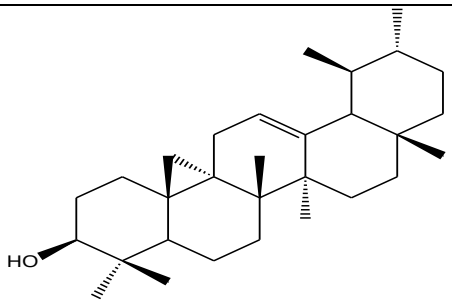
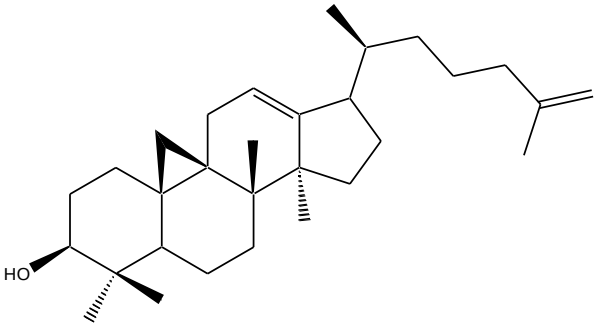
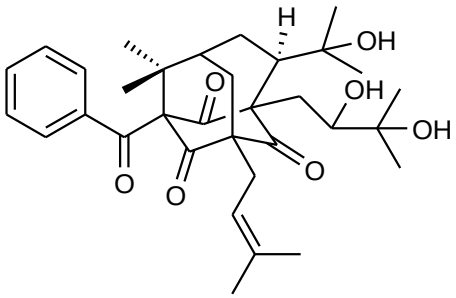
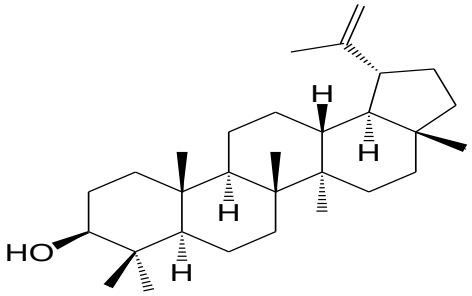
 6-methylmercapto-orcinol (**Kardar *et al*., 2014**) **(75)**	 4,6-dimethymercapto-orcinol (**Kardar *et al*., 2014**) **(76)**
 Isomangiferolic acid (**Kardar *et al*., 2014**) **(77)**	 Mangiferonic acid (**Kardar *et al*., 2014**) **(78)**
 Ambonic acid (**Kardar *et al*., 2014**) **(79)**	 Ambolic acid (**Kardar *et al*., 2014**) **(80)**
 Lup-20(29)-en-3-one (**Talla *et al*., 2013**) **(81)**	 Lupeol acetate (**Sakava *et al*., 2014**) **(82)**

Table 10: continuation

 18-iso-olean-12-en-3,11-dione (**Talla *et al*., 2013**) **(83)**	 Olean-12-en-3β,28-diol (**Sakava *et al*., 2014**) **(84)**
 25-cyclopropyl-3β-hydroxyurs-12-ene (**Sakava *et al*., 2014**) **(85)**	 3β-hydroxy-cycloart-12,25(26)-diene (**Sakava *et al*., 2014**) **(86)**
 Phlogucinonone (**Almutairi *et al*., 2014**) **(87)**	 3β-hydroxylup-20(29)-ene (**Talla *et al*., 2013**) **(88)**

It is clear that these compounds are responsible for the numerous biological activities of propolis.

I-4: SOME BIOLOGICAL PROPERTIES OF PROPOLIS

Propolis is known to have a wide range of biological activities and some of these activities have made it to find applications in medicine, cosmetics and food technology.

I-4-1: Propolis antimicrobial activity

Propolis antimicrobial activities are well documented against different bacteria (**Sforcin *et al.*, 2000**), yeasts (**Sforcin *et al.*, 2001**), viruses (**Gekker *et al.*, 2005**) and parasites (**Freitas *et al.*, 2006**). In vitro, propolis may act directly on microorganisms, and in vivo it may stimulate the immune system, activating the mechanisms involved in the microorganisms killing. *Paenibacillus larvae,* the agent behind American foulbrood, a key larval pathogen of the honey bee *Apis mellifera,* has become increasingly resistant to conventional antibiotics, and propolis extracts from various states of Brazil significantly inhibited this microorganism (**Bastos *et al.*, 2008**). Propolis may also show synergistic effects with antimicrobial drugs, and its association to commercially disposable drugs is a field of interest to the development of new products by the pharmaceutical industry. It has been reported that propolis diminishes the resistance of the bacteria wall to antibiotics such as amoxicillin, ampicillin and cefalexin and had synergistic effects with antibiotics acting on the ribosome e.g. chloramphenicol, tetracycline and neomycin (**Orsi *et al.*, 2006**). Nevertheless, propolis does not seem to interact with the antibiotics acting on the DNA e.g. ciprofloxacin, norfloxacin, folic acid and cotrimoxazole (**Orsi *et al.*, 2006**). These data enable us to compare the action of propolis with antimicrobial drugs. A review dealing with the effects of propolis on *Streptococcus mutans* group was published suggesting the potential of propolis or its compounds as cariostatic agents and for the development of biotechnological products to control caries and other infectious diseases (**Liberio *et al.*, 2009**). The clinical efficacy of a new Brazilian propolis gel formulation in patients diagnosed with denture stomatitis was performed and results showed that this gel was efficient and could be an alternative topical choice for the treatment of denture stomatitis (**Santos *et al.*, 2008**).

I-4-1-1: Antibacterial Activity

Many researchers had investigated the antibacterial activity of propolis and its extracts against gram-positive and gram-negative strains and they found that propolis had antibacterial activity against a wide range of Gram-positive rods but had a limited activity against gram-negative bacilli. Ugur and Arslan investigated the antibacterial and antifungal activities of acetone and dimethyl

sulfoxide (DMSO) extracts of 45 different propolis samples from the Mugla province of Turkey. They found that the antimicrobial activity of propolis varied depending on propolis sample, dosage of propolis, and the extraction solvents for all tested propolis samples. Antibacterial activity of all propolis samples increased with increasing dosage without reaching a plateau at the highest dosage tested. Except for *Brucella melitensis,* the DMSO extracts of all propolis samples were more active than the acetone extracts of the same samples. For *B. melitensis*, the acetone extracts of all propolis samples showed greater activity. The most sensitive microorganism to propolis was *Shigella sonnei* in the Gram-negative group and *Streptococcus mutans* in the Gram-positive. Standard antibiotics were used and the results revealed that propolis samples from the Mugla province of Turkey has a similar or greater inhibitory effect on *S. mutans, Salmonella typhi, Pseudomonas aeruginosa*, and *S. sonnei* (**Ugur and Arslan, 2004**). Ethanolic extracts from samples of propolis were collected from 18 regions of the Russia at 125-500 ug/mL inhibited the growth of *B. cereus* and *S. aureus* (**Shub *et al.*, 1978**). One hundred and six strains of *S. aureus* were tested, all of them were susceptible to 0.5-1.0 mg propolis/mL. Strains resistant to benzyl/penicillin, tetracycline, and erythromycin were sensitive to propolis. Propolis had a synergistic effect when combined with any of the three antibiotics used against the antibiotic resistant strains (**Shub *et al.*, 1981**). Inhibition of growth of five mycobacterium species was proportional to the concentration of flavonoids in the propolis. The strain *Mycobacterium sp.* was the most sensitive to flavonoids and was therefore useful in comparative tests. The lowest concentration of flavonoids at which inhibition was observed was 0.00996 mg/mL (**Jozwik and Trytek, 1985**). Three antibacterial compounds isolated from Brazilian propolis and identified as 3,5 diprenyl-4- hydroxycinnamic acid, 3-prenyl - 4- dihydrocinnamoloxycinnamic acid and 2,2- dimethyl -6- carboxyethenyl-2H-1-benzopyran had their antibacterial activities investigated against *Bacillus cereus, Enterobacter erogenous* and *Arthroderma benhamiae* and it was found that the first compound showed the highest activity and is likely to be one of the major antibacterial compounds in Brazilian propolis (**Aga *et al.*, 1994**). Takasi and co-workers stated that propolis inhibits bacterial growth by preventing cell division, thus resulting in the formation of pseudo-multicellular *streptococci*. In addition, propolis disorganized the cytoplasm, the cytoplasmic membrane and the cell wall, caused a partial bacteriolysis and inhibited protein synthesis. It was evidenced that the mechanism of action of propolis on bacterial cell is complex and a simple analogy cannot be made to the mode of action

of any classic antibiotics. This conclusion came from the micro-calorimetric and electron microscopic studies (**Takasi *et al.*, 1994**).

Table 11: Some antibacterial compounds isolated from propolis

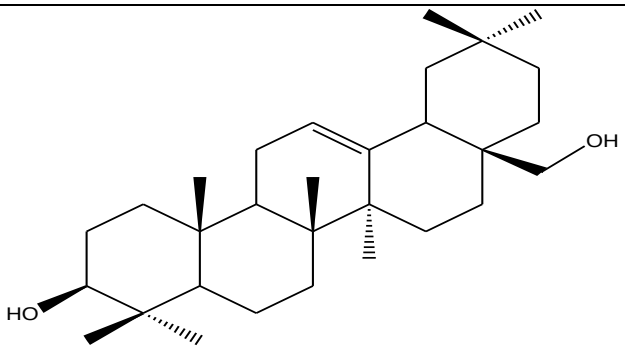
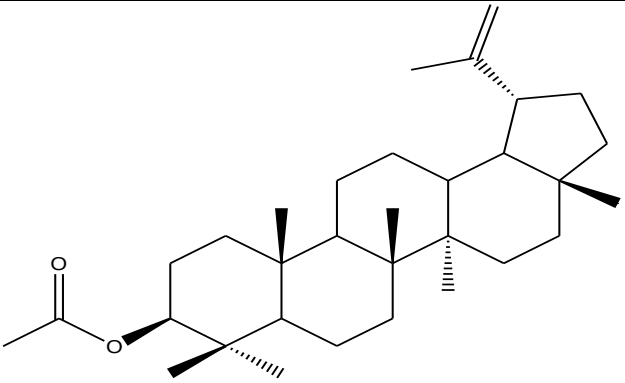
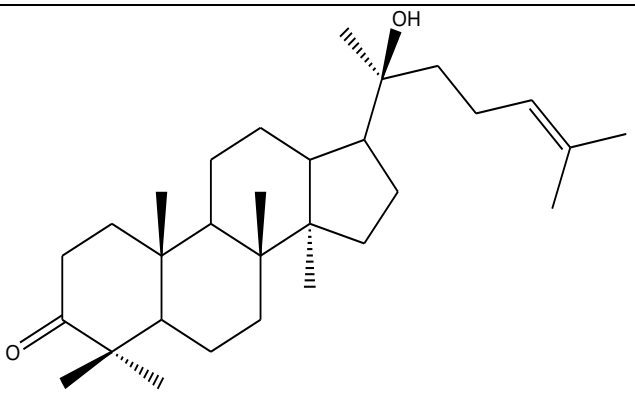
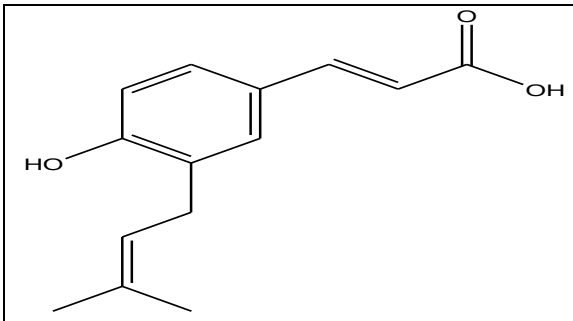
Compound	**Region**	**Reference**
 Olean-12-en-3**β**,28-diol **(84)**	Cameroon	**Sakava *et al.*, 2014**
 Lupeol acetate **(82)**	Cameroon	**Sakava *et al.*, 2014**
 Dipterocarpol **(89)**	Thailand	**Sanpa *et al.*, 2015**

Table 11 continuation

 3-prenyl-4-hydroxycinnamic acid **(90)**	Brazil	**Marcucci *et al.*, 2001**

I-4-1-2: Antiviral Activity

In vitro activity of 3-methyl-but-2-enyl caffeate isolated from poplar buds a chief propolis source plant in temperate zone against Herpes simplex virus type 1 was investigated and it was found that this compound, as a minor constituent of propolis, reduce the virus titer and viral DNA synthesis effectively (**Amoros *et al.*, 1994**). It was found that isopentyl ferulated isolated from propolis inhibited significantly the infectious activity of influenza virus in vitro (**Serkedjieva *et al.*, 1997**). Administration of aqueous extract of propolis was accompanied by a decrease in mortality and increase in mean survival length in mice infection with influenza virus (**Ecsanu *et al.*, 1981**). A triterpenoid named melliferone, three known triterpenoids, moronic acid, anwuweizonic acid, and betulonic acid, and four known aromatic compounds were isolated from Brazilian propolis and tested for anti-HIV activity in H9 lymphocytes. Moronic acid showed significant anti-HIV activity (**Ito *et al.*, 2001**). The antiviral activity of propolis was studied by a limited number of authors but this study proved to be interesting. Amoros and others studied the *in vitro* antiviral activity of propolis against a representative range of viruses with standard Adenovirus II, Herpes simplex standard virus I and II, virus of the stomatitis vesiculaire and standard Poliovirus II and found that propolis exerts a virucide effect on the wrapped viruses Herpes standard simplex virus I and II, Virus of the stomatitis vesiculaire and this effect is net with the high concentrations 250 and 500 µg / mL. The Poliovirus is most strongly inhibited but the activity of propolis on the three herpetic stocks is also interesting, inhibition reaching 99.9% with the concentration of 30 µg / mL. Adenovirus and the Virus of the stomatitis vesiculaire are less sensitive (**Amoros *et al.*, 1992**). In a Propolis review: chemical composition, biological properties and therapeutic activity, some results on the antiviral activity of propolis: propolis affects the reproduction of Influenza Virus A and B, Vaccinia virus and Avulovirus were enumerated (**Marcucci, 1995**). The antiviral activity

of the aqueous extract of propolis against Influenza virus is attributed to the cafeoylquinic acids which it contains. Caffeic acid has an antiviral activity on the virus whereas quinic acid and the other compounds such as p-coumaric acid, Artepillin-C, baccharine, drupanine, kaempferide which do not have the cafeoylquinic acid structure do not exhibit this property (**Urushisaki *et al.*, 2011**).

Table 12: Some antiviral compounds isolated from propolis

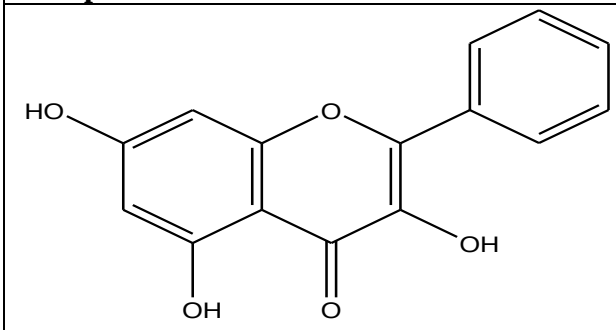
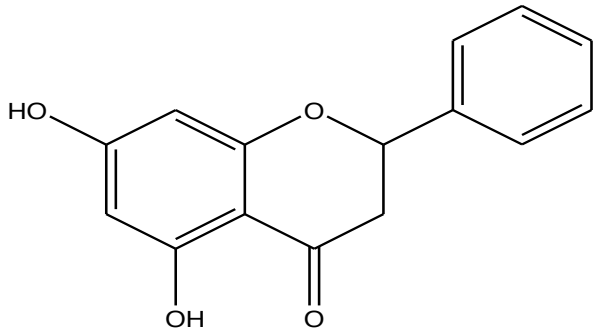
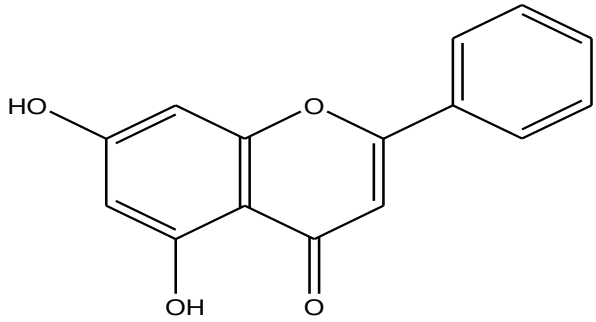
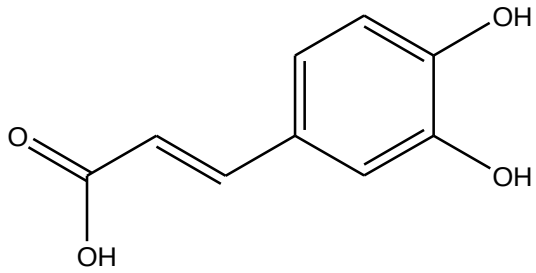
Compound	Region	Reference
 Galangin **(91)**	Brazil	**Schnitzler *et al.*, 2009**
 Pinocembrin **(92)**	Brazil	**Schnitzler *et al.*, 2009**
 Chrysin **(93)**	Brazil	**Schnitzler *et al.*, 2009**

Table 12 continuation

 Caffeic acid **(44)**	Brazil	**Schnitzler *et al.*, 2009**

I-4-1-3: Antifungal Activity

Ota and co-workers studied the antifungal activity of propolis in sensitivity tests on 80 strains of Candida yeasts: 20 strains of *Candida albicans*, 20 strains of *Candida tropicalis*, 20 strains of *Candida krusei* and 15 strains of *Candida guilliermondii*. The yeasts showed a clear antifungal activity with the following order of sensitivity: *C. albicans* > *C. tropicalis* > *C. krusei* > *C. guilliermondii* (**Ota *et al.*, 2001)**. The ethanolic extract of propolis inhibited 60 strains of yeasts, 38 strains of fungi and *Aspergillus parasiticus* strain (**Ozcan, 2004**). Paracoccidioidomycosis is the most important systemic mycosis in Latin America. Its etiological agent, *Paracoccidoides brasiliensis*, affects individuals living in endemic areas through inhalation of airborne conidia or mycelial fragments. The disease may affect different organs and systems, with multiple clinical features, with cell mediated immunity playing a significant role in host defense. Peritoneal macrophages from mice were stimulated with Brazilian and Bulgarian propolis and subsequently challenged with *P. brasiliensis.* Data suggest an increase in the fungicidal activity of macrophages by propolis stimulation, independently from its geographic origin (**Murad *et al.*, 2002**).

Table 13: Some antifungal compounds isolated from propolis

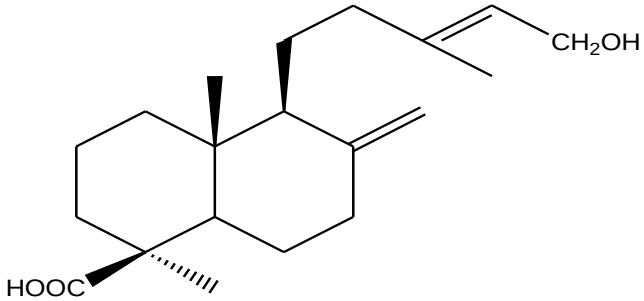
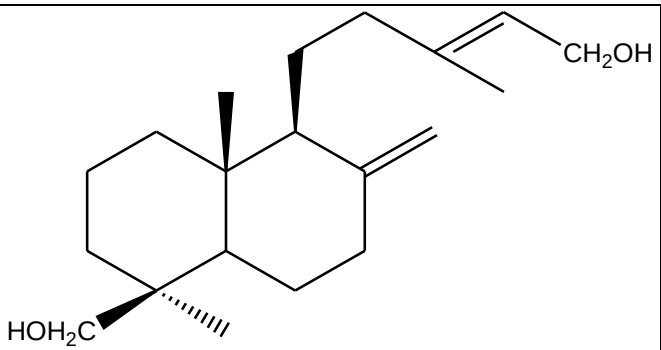
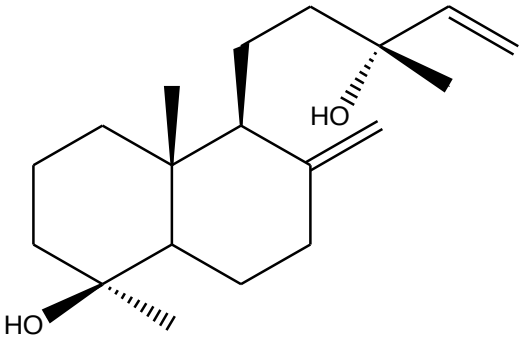
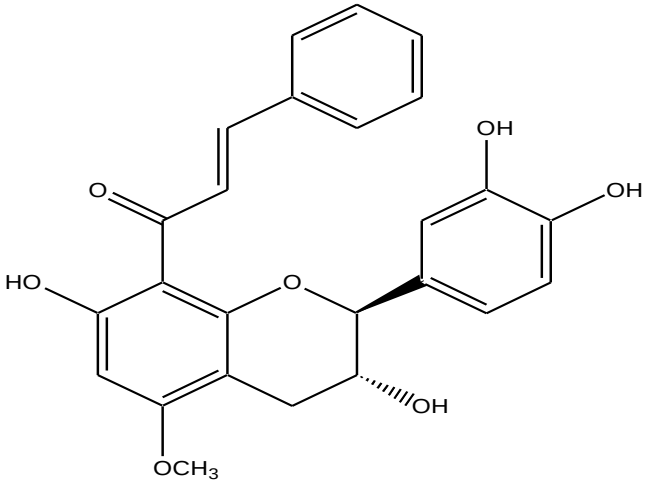
Compound	**Region**	**Reference**
 Isocupressic acid **(94)**	Columbia	**Meneses *et al.*, 2009**

Table 13 continuation

 (+)-agathadiol **(95)**	Columbia	**Meneses *et al.*, 2009**
 Epi-13-torulosol **(96)**	Columbia	**Meneses *et al.*, 2009**
 8-[(E)-phenylprop-2-en-1-one]-5-methoxycatechin **(18)**	France	**Boisard *et al.*, 2015**

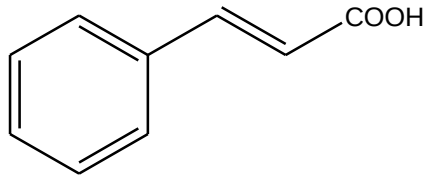
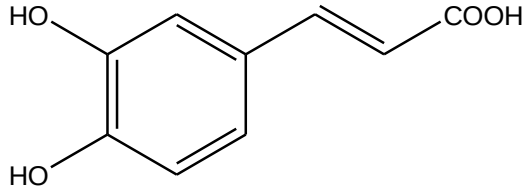
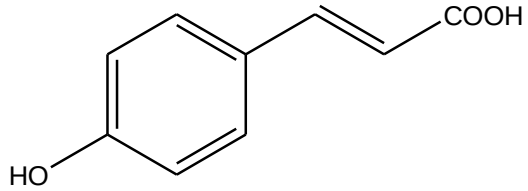
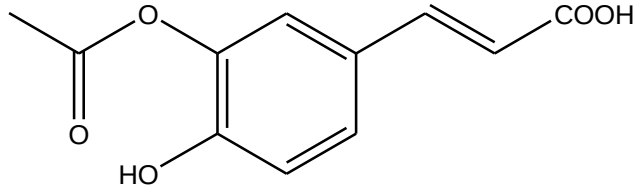
I-4-2: Propolis anti-ulcer activity

Gastroduodenal ulcer may be the result of the imbalance between aggressive and protective factors in the stomach, such as acid–pepsin secretion, mucosal barrier, mucus secretion, cellular regeneration and epidermal growth factors (**Lima *et al.*, 2006**). The treatment of peptic ulcer is often based on the inhibition of gastric acid secretion by histamine H2-antagonists, proton pump

inhibitors, and antimuscarinics. Acid-independent therapy including sucralfate and bismuth cholinergics is used as well (**Bighetti *et al.*, 2005**). Omeprazole, indomethacin and cimetidine have been commonly used as a positive control to induce gastric ulcer. Barros and co-workers reported the gastric protective effects of propolis (50, 250 and 500 mg/kg) and described the antiulcerogenic properties of the main phenolic acids from Brazilian propolis (50 and 250 mg/kg), in different models: non-steroidal-anti-inflammatory drug-induced ulcer, ethanol-induced ulcer, and stress-induced ulcer, evidencing that caffeic, ferulic, p-coumaric and cinnamic acids displayed antiulcer activity (**Barros *et al.*, 2008**). Massignani and others investigated the effects of the essential oil (50, 250 and 500 mg/kg) obtained from *Baccharis dracunculifolia*, the most important botanical source of Brazilian green propolis, on gastric ulcers, suggesting that it could probably be a good therapeutic agent for the development of new phytotherapeutic medicine for the treatment of gastric ulcer (**Massignani *et al.*, 2009**). Honey and propolis as management of chronic skin ulcers were found effective (**Tossoun *et al.*, 1997**). The inhibitory effect of Bulgarian propolis on *Helicobacter pylori* growth in vitro was investigated by Boyanova and co-workers. Activity of 30 % ethanolic extract of propolis against 38 clinical isolates of *H. pylori* was evaluated by using the agar-well diffusion method. Ethanol was used as a control. In addition, the effect of propolis on the growth of 26 *H. pylori* and 18 *Campylobacter* strains was tested by the disc diffusion method. Mean diameters of *H. pylori* growth inhibition by the agar-well diffusion method, using 30, 60 or 90 μL ethanol extract of propolis or 30 μL ethanol per well, were 17.8, 21.2, 28.2 and 8.5 mm, respectively. Ethanol extract of propolis was significantly more active than ethanol against *H. pylori* (**Boyanova *et al.*, 2003**). In addition to these results, some authors had also concluded that the Bulgarian propolis possesses considerable antibacterial activity against *H. pylori*, and can inhibit the growth of *Campylobacter jejuni* and *Campylobacter coli* (**Kimoto *et al.*, 1998**). The preventive and curative effects of Indian propolis for ulcers were evaluated using models of acute gastric lesions induced by ethanol and indomethacin in rats. Moreover, the effects of ethanol extract of propolis on gastric content volume, total acidity and pH, using the pylorus ligated model were also evaluated. Animals pretreated with propolis extract showed a significant reduction in lesion index in both ethanol and indomethacin induced ulcer models in a dose dependent manner when compared to the control group. Similarly, post-treatment with propolis (300 mg/kg body weight) for a period of 15 days revealed a statistically significant improvement in the ulcer healing process. In the pylorus ligated model, it was observed that the Indian propolis extract displayed an

antisecretory activity, which led to a significant reduction in the gastric juice volume, total acidity and pH (**Iyyam Pillai *et al.*, 2010**).

Table 14: Some anti-ulcer compounds isolated from propolis

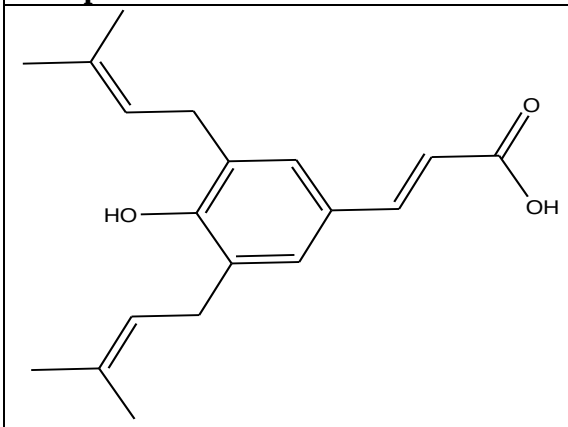
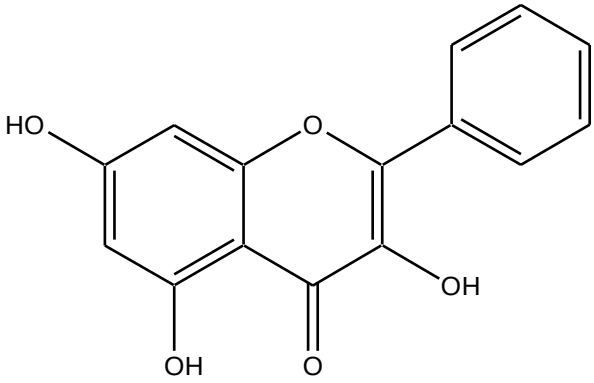
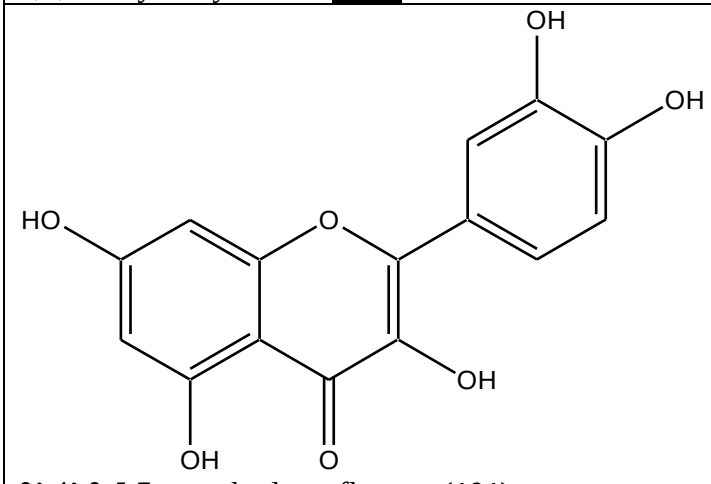
Compound	Region	Reference
 Cinnamic acid **(97)**	Brazil and Belgium	**Barros *et al.*, 2008**
 Caffeic acid **(44)**	Brazil and Belgium	**Barros *et al.*, 2008**
 p-coumaric acid **(98)**	Brazil and Belgium	**Barros *et al.*, 2008**
 Ferulic acid **(99)**	Brazil and Belgium	**Barros *et al.*, 2008**

I-4-3: Propolis anti-inflammatory Activity

The effects of ethanol extract of propolis on chronic inflammation were evaluated using rat adjuvant arthritis. In the chronic inflammatory animal model, the arthritis index was suppressed by ethanol extract of propolis treatments (50 mg/ kg/day and 100 mg/kg/day). Moreover, physical weakness, induced by the chronic disease state, was dose-dependently improved in the ethanol extract of propolis treated groups. Its analgesic effect was assessed using the tail-flick test and was comparable to prednisolone (2.5 mg/kg/day) and acetyl salicylic acid (100 mg/kg/day). In carrageenan rat hind paw edema, which was conducted to test the effects of subfractions of ethanol extract of propolis, the petroleum ether sub-fraction (100 mg/kg) showed an inhibitory effect on

the paw edema whereas ethanol extract of propolis (200 mg/kg) showed a significant anti-inflammatory effect at 3 and 4 hrs after carrageenan injection. From these results, they concluded that the ethanolic extract of propolis had profound anti-inflammatory effects on both chronic and acute inflammations (**Park and Kahng, 1999**). Caffeic acid phenethyl ester, derived from propolis, has been shown to reveal anti-inflammatory properties. Since T-cells play a key role in the onset of several inflammatory diseases, Marquez and co-workers have evaluated the immunosuppressive activity of caffeic acid phenyl ester in human T-cells, discovering that this phenolic compound is a potent inhibitor of early and late events in T-cell receptor-mediated T-cell activation. Moreover, they found that caffeic acid phenyl ester specifically inhibited both interleukin (IL)- 2 gene transcription and IL-2 synthesis in stimulated T-cells (**Marquez *et al.*, 2004**). To further characterize the inhibitory mechanisms of caffeic acid phenethyl ester at the transcriptional level, they examined the DNA binding and transcriptional activities of nuclear factor (NF)-κB, nuclear factor of activated cells (NFAT), and activator protein-1(AP-1) transcription factors in Jurkat cells. They found that caffeic acid phenethyl ester inhibited NF-κB-dependent transcriptional activity without affecting the degradation of the cytoplasmic NF-κB inhibitory protein, IBκB. However, both NF- B binding to DNA and transcriptional activity of a Gal4-p65 hybrid protein were clearly prevented in caffeic acid phenethyl ester -treated Jurkat cells. Moreover, caffeic acid phenethyl ester inhibited both the DNA-binding and transcriptional activity of NFAT, a result that correlated with its ability to inhibit phorbol 12-myristate 13-acetate plus ionomycin-induced NFAT1 dephosphorylation. They stated that, these findings provide new insights into the molecular mechanisms involved in the immuno-modulatory and anti-inflammatory activities of this natural compound (**Marquez *et al.*, 2004**). Propolis has inhibitory effects on myeloperoxidase activity, NADPH-oxidase, ornithine decarboxylase, tyrosineprotein-kinase, and hyaluronydase from guinea pig mast cells.

Table 15: Some anti-inflammatory compounds isolated from propolis

Compound	Region	Reference
 3,5-diprenyl-4-hydroxycinnamic acid **(45)**	Brazil	**Paulino *et al.*, 2008**
 3,5,7-trihydroxyflavone **(100)**	South Africa	**Toit *et al.*, 2009**
 3',4',3,5,7-pentahydroxyflavone **(101)**	South Africa	**Toit *et al.*, 2009**

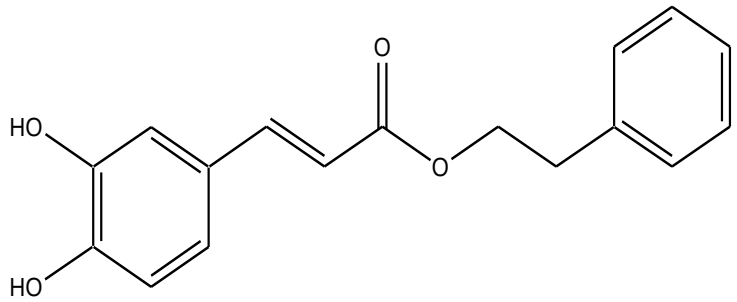
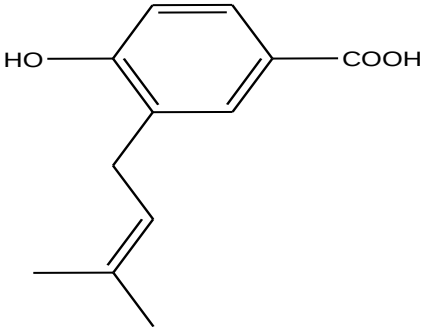
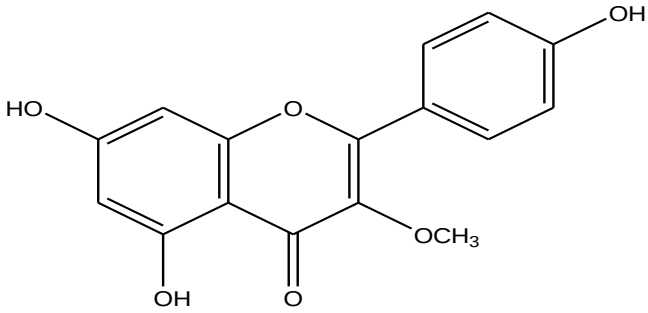
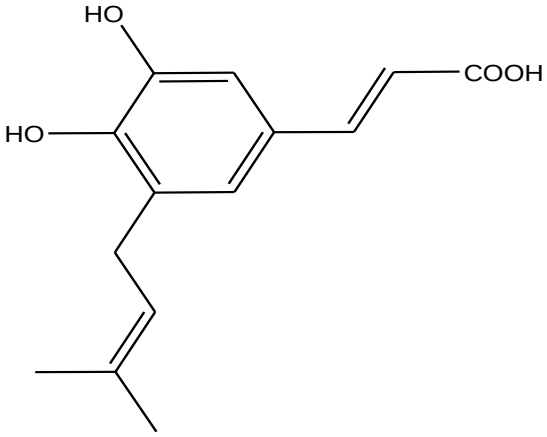
I-4-4: Propolis antioxidant activity

Of the antioxidant evaluation methods used, the DPPH assay, based on the reaction of the stable free-radical (2,2-diphenyl-1-picrylhydrazyl) with components of the sample, evaluating a change

in the color of the solution, is the most frequently used. A comparison of the antioxidant activity of propolis samples from various countries, including Brazil, was carried out using this method (**Vague Rodrigues, 2012**). The phenolic contents correlated well with the results of both assays. Another method that can evaluate the antioxidant effect of propolis is the chemoluminescenscence assay, which was applied to samples of green propolis collected monthly for one year. The antioxidant activity was found to vary with the different seasons (**Silva-Carvalho *et al.*, 2015**). The capacity of a compound to inhibit lipid peroxidation is another way to evaluate the antioxidant activity. Green propolis samples, as well as isolated compounds, were tested and 3-prenyl-4-hydroxycinnamic acid was found to have the highest antioxidant activity by this method and the antioxidant activity deserves special interest because propolis could be topically applied successfully to prevent and treat skin damaged (**Awale *et al.*, 2005; Vagues Rodrigues, 2012**). Phenolic compounds found in high concentrations in Brazilian green propolis, including Artepillin C, have a wide range of biological properties including the ability to act as an anti-oxidizing free radicals and nitric oxide radicals (**Mani *et al.*, 2006**). Although studies of propolis ethanol extracts are very common, it is reported that the aqueous extract has good antioxidant activity, associated with high content of phenolic compounds (**Russo *et al.*, 2002**). The antioxidant activity of propolis and its main phenolic compounds, caffeic acid, p-coumaric acid, ferulic acid, and caffeic acid phenethyl ester, were investigated in yeast *Saccharomyces cerevisiae.* Yeast cells showed decreased intracellular oxidation, with no significant differences seen for the individual phenolic compounds. It is well known that endogenous stimuli, like cellular metabolism, and exogenous agents like UV, toxins, and drugs, among others, generate reactive oxygen species. Carbohydrates, proteins, lipids, and nucleic acids, among other biomolecules, when exposed to the reactive species, suffer oxidative modifications that modify the cell and lead to its death (**Silva-Carvalho *et al.*, 2015**). Oxidative stress is responsible for the occurrence of a wide variety of human diseases, such as neurodegenerative or cardiovascular diseases, cancer, diabetes, and atherosclerosis (**Silva-Carvalho *et al.*, 2015**). In the last years, several studies have been performed to evaluate the antioxidant capacity of natural products. Propolis extracts, composed of different polyphenols, have been reported to possess a potent antioxidant activity. Additionally, the chemical varieties in different propolis samples from different regions have an influence on the antioxidant activity. It was recently shown that a sample of Italian and Russian propolis ethanol extract, which have a similar polyphenolic composition, have a similar antioxidant activity, while Brazilian propolis

ethanol extract, which have low polyphenolic composition, have a weak antioxidant activity (**Fabris *et al.,* 2013**). Phenolic acids and flavonoids are characterized by a powerful antioxidant activity, which is closely related to the chemical structure of the compounds. Briefly, the antioxidant activity is exerted by inhibiting the activity of some enzymes which inhibit the production of reactive oxygen species by scavenging, interrupting the reactions that lead to the lipid peroxidation; by chelating metal ions, mainly iron and copper, that are involved in the process of free radical creation; or by potentiating the action of other antioxidants (**Kurek-Gorecka *et al.,* 2014**). Portuguese propolis, an important source of total phenols, flavones, and flavonols, could be beneficial for human health due to its antioxidant properties. Portuguese propolis also protects human erythrocytes from free radicals damaging by decreasing lipid peroxidation (**Valente *et al.,* 2011**). These studies suggest that Portuguese propolis is a powerful antioxidant agent that can be used against oxidative stress, thus maintaining the structural and functional integrity of the cells. Ethyl acetate extract of Kangaroo Island propolis, which is rich in stilbenes, showed a stronger scavenging activity (**Cuesta-Rubio *et al.,* 2002**) while ethyl acetate of propolis collected in Anhui, China, has strong scavenging activity and ferric reducing activity, those activities being influenced by caffeic acid, phenyl caffeate, cinnamyl caffeate, and benzyl caffeate (**Silva-Carvalho *et al.,* 2015**). Another study showed that samples of methanolic extracts of Algerian propolis that contains high amounts of caffeic acid esters and flavanones, kaempferol, and galangin possess strong scavenging activity and ferric reducing activity (**Pinicelli *et al.,* 2013**). Uruguay propolis with high polyphenolic composition inhibits low density lipoprotein peroxidation and protein nitration in vitro. Moreover, it induces the expression of nitric oxide synthase and inhibits NADPH oxidase in bovine aortic endothelial cells and in another study, the topical administration of Romanian propolis ethanol extract in mouse significantly attenuated the malondialdehyde (MDA) formation and restored glutathione peroxidase activity (**Silva-Carvalho *et al.,* 2015**). Turkish propolis ethanol extract has antioxidant properties in the liver tissue of nitric oxide synthase inhibited rats. In fact, nitric oxide synthase inhibition caused an increase in MDA levels, effect that was significantly decreased when the rats were treated with propolis ethanol extract (**Talas *et al.,* 2015**). Antioxidant activity is one of the most studied and important activities of propolis, though there are no studies with data on the safe dose to be used in humans.

Table 16: Some antioxidant compounds isolated from propolis

Compound	Region	Reference
 Caffeic acid phenylethyl ester **(102)**	China	**Gong *et al.*, 2012**
 4-Hydroxy-3-prenylbenzoic Acid **(103)**	Brazil	**Katsuhiro *et al.*, 1999**
 3-Methylkaempferol **(104)**	Brazil	**Katsuhiro *et al.*, 1999**
 3,4-dihydroxy-5-prenylcinnarnic acid **(105)**	Brazil	**Katsuhiro *et al.*, 1999**

I-4-5: Propolis toxicity

It must be emphasized that propolis has the advantage of being a natural product, with a higher molecular diversity. It has many therapeutic substances compatible with the metabolism of mammals in general, which reduces the possibility of causing adverse reactions to oral tissue as compared to industrial products tested (**Simoes *et al.*, 2008**). The aqueous and alcoholic extracts of propolis do not cause irritation to the tissues and are considered relatively non-toxic (**Burdock, 1998**). Propolis is relatively non-toxic and studies have exhibited a no-effect level in a mice study of 1400 mg/kg weight/day leading the authors to propose that a safe dose in humans would be 1.4 mg/kg weight/day, or approximately 70 mg/day (**Miguel *et al.*, 2010**). On other hand the high effectiveness of mouthwash containing propolis was demonstrated in control of dental plaque and gingivitis in humans and not observed no toxic or side effects in the administration of the rinse during 90 days. Propolis is considered safe in small doses. Therefore, adverse effects are common at doses above 15 g/day (**Pereira *et al.*, 2011**). The most commonly experienced adverse effects are allergic reactions, as well as irritation of the skin or mucous membranes but caution should be used in the treatment of individuals with asthma and eczema and nettle rash (**Vague Rodrigue, 2012**).

Table 17: Some compounds with evaluated toxicity isolated from propolis

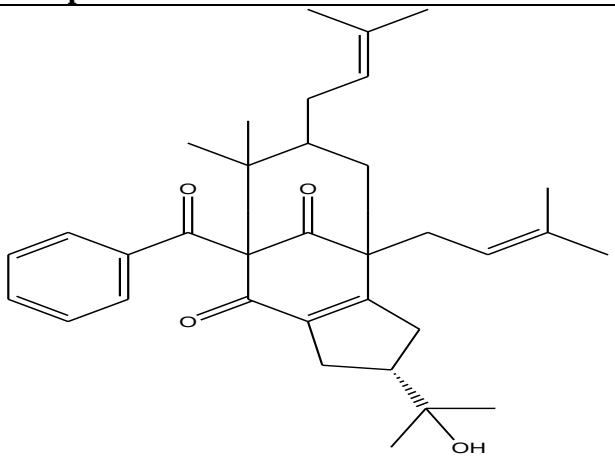
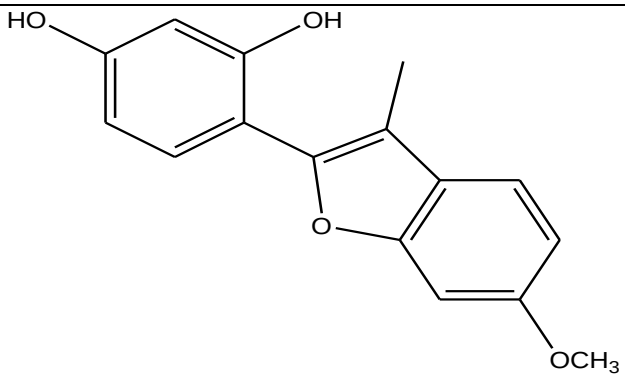
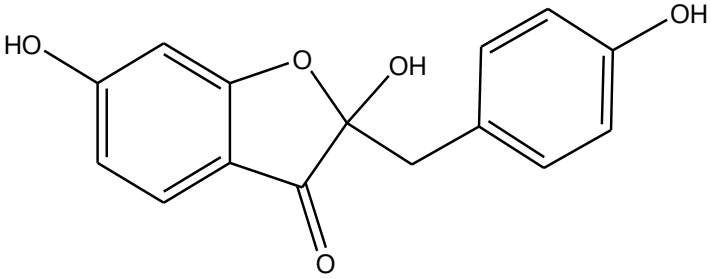
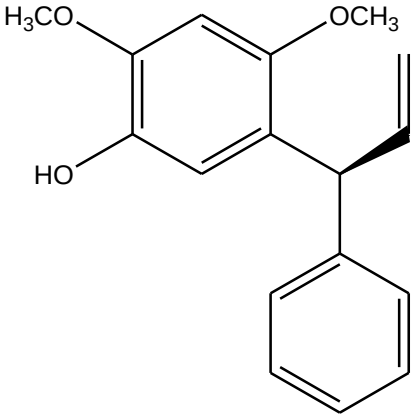
Compound	**Region**	**Reference**
 Hyperibone A **(106)**	Brazil	**Castro *et al.*, 2009**

Table 17 continuation

 2-(2,4-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran **(107)**	Brazil	**Awale *et al.*, 2008**
 2,6-dihydroxy-2-[(4-hydroxyphenyl) methyl]-3-benzofuranone **(30)**	Brazil	**Awale *et al.*, 2008**
 (7S)-dalbergiphenol **(108)**	Brazil	**Feng *et al.*, 2008**

Based on reported data on propolis from tropics and subtropical zones, most compounds isolated from Cameroonian propolis will be triterpenoids. Therefore, it is necessary to make an overview of triterpenoids, their classes as well as some of their structural features and spectral characterisation.

I-5: AN OVERVIEW OF TRITERPENES

Tropical and subtropical propolis samples such as Cameroonian propolis are rich mainly in triterpenes and triterpene derivatives (**Marcucci and Bankova, 1999; Kardar *et al.*, 2014)**. Phytochemical studies of Cameroonian and some African propolis samples led to the isolation of or identification of a significant number of triterpenes (**Talla *et al.*, 2013; Sakava *et al.*, 2014; Kardar *et al.*, 2014; Almutairi *et al.*, 2014; Papachroni *et al.*, 2015**). The source of these triterpenoids in propolis is terrestrial vegetation (**Hernandez-Vazquez *et al.*, 2010**).

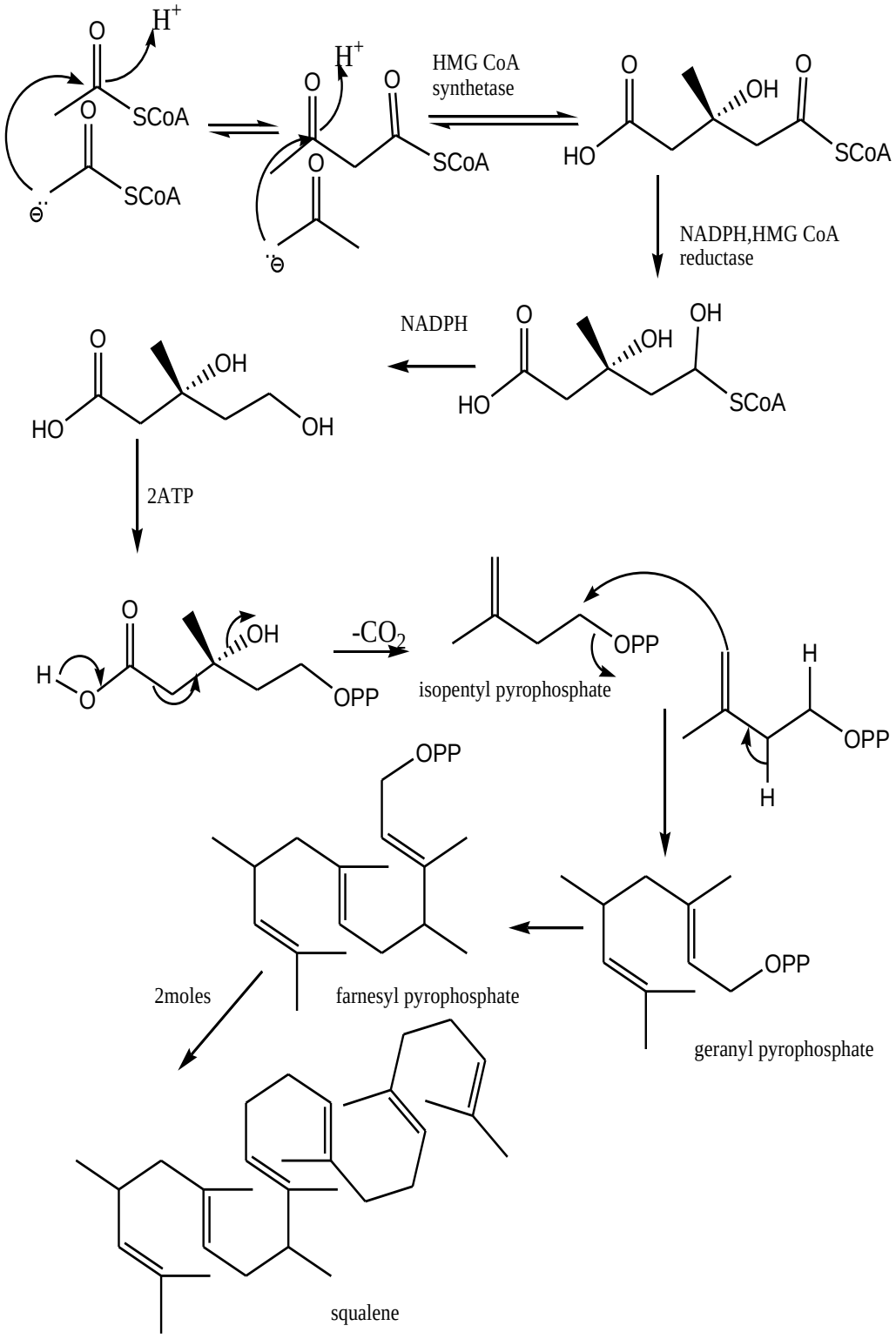
Terpenoids or isoprenoids constitute one of the richest classes of natural products with a great diversity in structure even though all are constructed from the same 5-carbon compound called isoprene. More than 30,000 terpenoids have been isolated from plants, both marine and terrestrial with new compounds being discovered yearly. Terpenoids include terpenes and steroids which is gradually gaining the status of a class of its own accord. The different terpenoids could be acyclic, mono-, di-/bi-, tetra- or penta-cyclic structures which frequently carries a functional group (**Adenot, 2000**). Terpenoids are common in living organisms where they could be found in the free form, as esters or glycosides. Mono- and sesqui-terpenes are common in essential oils of plants, di-, sesqui-, and triterpenes are found in balsam, resins and higher plants. The majority of triterpenoids are 6-6-6-5 tetracycles, 6-6-6-6-5 pentacycles, or 6-6-6-6-6 pentacycles types.

I-5-1: Biosynthesis of triterpenes

In a living cell, the biosynthesis of pentacyclic triterpenes takes place in two steps: the synthesis of squalene and then its cyclization.

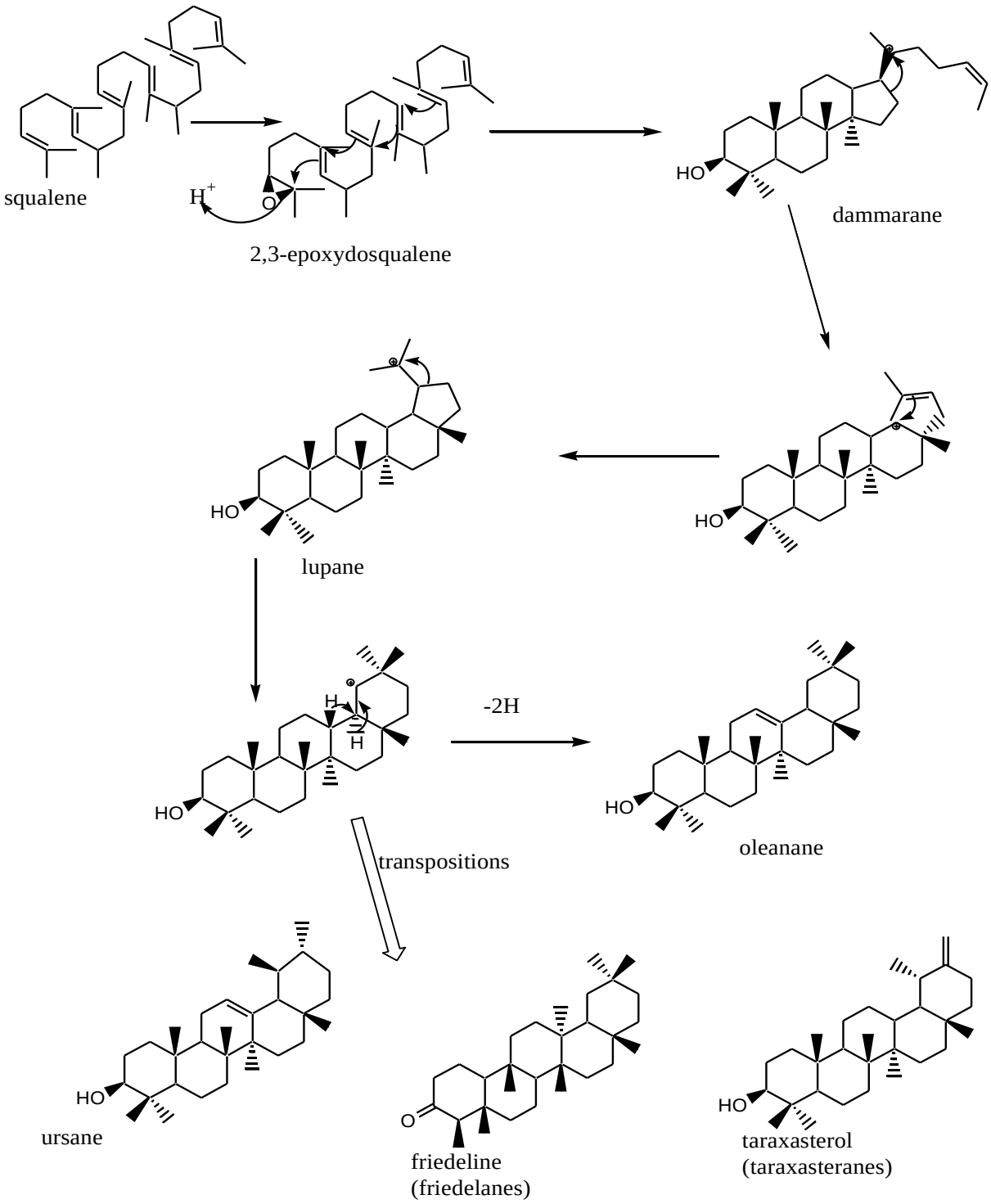
I-5-1-1: Biosynthesis of squalene

The biosynthesis of squalene is generally done via the mevalonic pathway starting with acetic acid and more precisely its active equivalent, acetyl coenzyme A (acetyl-SCoA). This leads to mevalonic acid through successive aldol condensation reactions between two molecules of the same type or (R)-3-hydroxy-3-methylglutaryl SCoA (HMG - SCoA) which is reduced by Nicotinamide Adenine Dinucleotide Phosphate (NADP). This acid undergoes a double phosphorylation in the presence of Adenosine Triphosphate (ATP) to form the mevalonic-5-pyrophosphate acid which in its turn undergoes decarboxylation to give the precursor in five carbon atoms, isopentenyl pyrophosphate (IPP). Pyrophosphates of geranyl and farnesyl undergo a dimerization to give the squalene (**Bruneton, 1999**).



Scheme 1: Reaction scheme for the synthesis of squalene

I-5-1-2: Cyclisation of squalene



Scheme 2: Reaction scheme for the cyclisation of squalene (Bruneton, 1999)

I-5-2: Various structural groups of pentacyclic triterpenes

The pentacyclic triterpenes are classified in several structural groups and are made up of rings generally noted A, B, C, D, and E. Certain groups consist of five rings with six carbon atoms in each of the rings, others have rings with five or seven carbon atoms in their skeleton (**Mahato and Kundu, 1994**). Glycosylation can take place on each one of these structural groups. When this occurs, the natural products formed are called saponins. Triterpene skeleton always have at the beginning a hydroxyl group fixed on C-3 carbon. This hydroxyl group can serve as the point of linkage between triterpenic and osidic skeletons (**Mahato and Kundu, 1994**).

Table 18: Some structural groups of pentacyclic triterpenoids

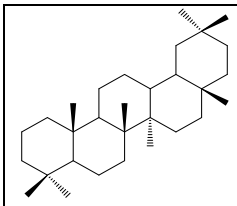
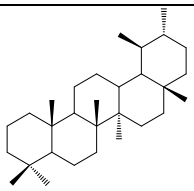
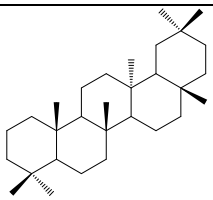
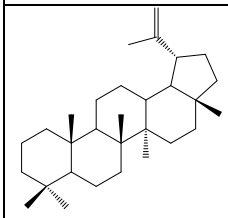
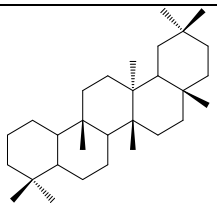
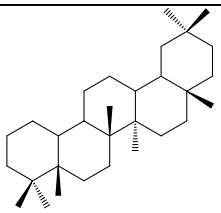
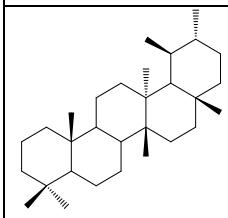
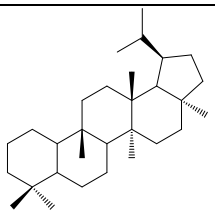
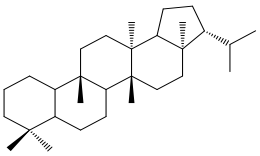
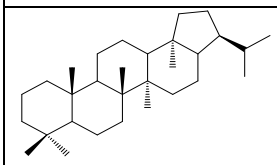
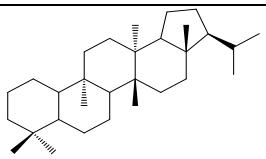
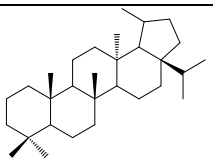
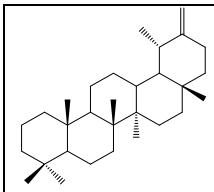
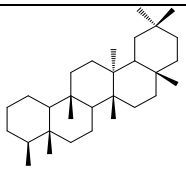
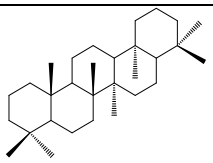
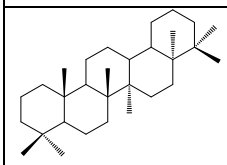
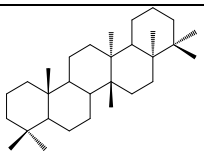
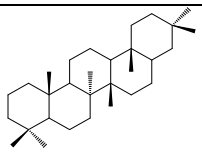
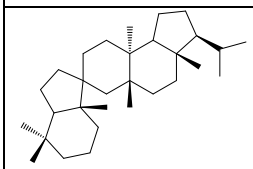
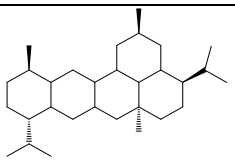
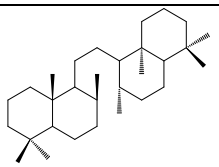
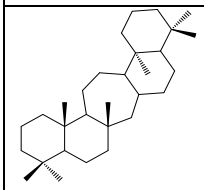
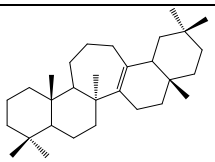
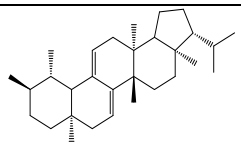
 Oleanane **(109)**	 Ursane **(110)**	 Taraxarane **(111)**
 Lupane **(112)**	 Glutinane **(113)**	 Mimusopane **(114)**
 Bauerane **(115)**	 Hancokinane **(116)**	 Fernane **(117)**
 Hopane **(118)**	 Sorghumane **(119)**	 Friedomadeirane **(120)**

Table 18: continuation

 Taraxastane **(121)**	 Friedelane **(122)**	 Gammacerane **(123)**
 Kairatane **(124)**	 Swertane **(125)**	 Stictane **(126)**
 Spirosupinane **(127)**	 Bicadinane **(128)**	 Onocerane **(129)**
 Serratane **(130)**	 Dubosane **(131)**	 Canarane **(132)**

I-5-3: Elucidation of the structures of triterpenoids

NMR methods have indisputably become the single most important spectroscopic techniques for the identification and structure elucidation of most organic compounds including triterpenoids. Several 1D and 2D NMR experiments are now commonly used for the characterisation of pentacyclic triterpenes. These methods include ^{1}H- and ^{13}C-NMR, APT, DEPT, COSY, HMQC, HMBC and TOCSY (**Dias *et al.*, 2011**). Notwithstanding, other spectroscopic techniques such as IR, UV, and especially mass spectrometry are still frequently useful.

Triterpenoids are easily identified on the ^{1}H NMR by the appearance of between four and eight very intense peaks in the 0.50 to 2.00 ppm region, each integrating for three protons. These readily discernable peaks are the angular methyls on the triterpenoid structure. Most triterpenoids are hydroxylated at position 3 of the triterpenoids structure, hence the oxymethine proton appear between 3.00 and 4.00 ppm. But if the proton attached to the oxygen is substituted by an ester or ether bond, then the oxymethine proton will appear further downfield after 4.00 ppm. Protons

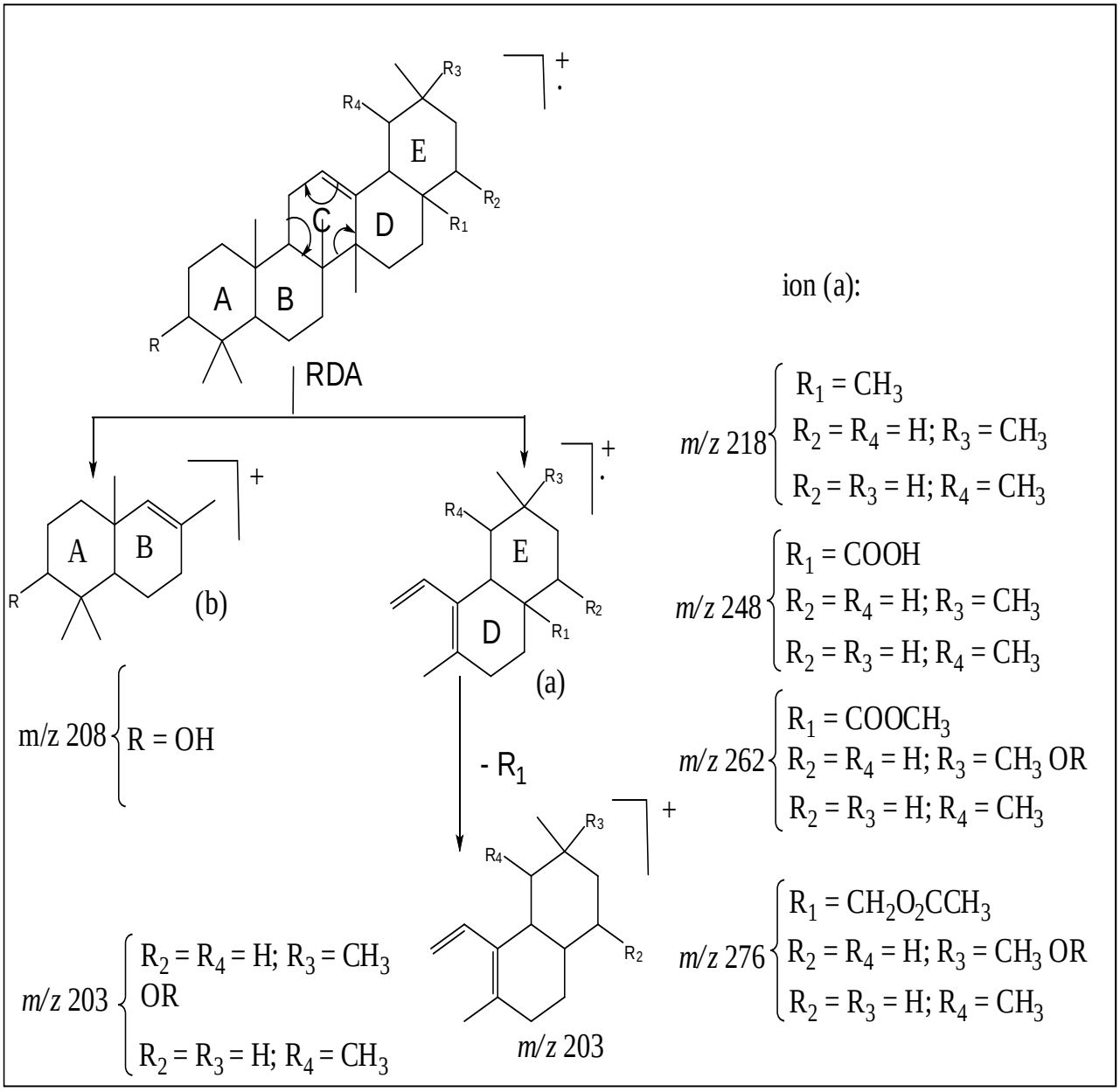
attached to any other oxygenated carbon appear downfield after 3.00 ppm; meanwhile protons attached to unsaturated carbons appear further up-field after 5.00 ppm. In the ^{1}H NMR spectrum of oleanane-type triterpenoids, H-18 appears around δ_H 2.20 as a doublet of doublet whereas it appears as a doublet around δ_H 2.50 in ursane-type triterpenoids. When the methyl C-17 is oxidized to carboxylic acid, the proton H-18 undergoes an attractor effect of the acid which then moves it downfield to about δ_H 2.84 for oleanane-type molecules and δ_H 2.40 for ursane-type. The proton NMR spectrum must be corroborated by other NMR experiments.

The ^{13}C NMR experiment is the finger print of any molecule; it is different even for two very close molecules. Triterpenoids show 30 signals on their broad band proton decoupled ^{13}C NMR spectrum, except in cases where more than one carbon atoms possess same magnetic environment and hence the same chemical shift value, reducing the number of signals or when other molecules add up to triterpenoids, increasing the number of signals. Some triterpenoids are said to be "Oleanane-type" or "Ursane-type" and "Lupane-type". These model compounds are quickly distinguished on their ^{13}C NMR spectrum by the appearance of some unique signals pertaining to the olefinic carbons at C-12 and C-13: Olean-12-enes have signals approximately at δ_C 122 and 145 ppm respectively; Urs-12-enes have signals at δ_C 124 and δ_C 139 ppm; and lup-20(29)-enes have signals at δ_C 109 and 150 ppm for C-20 and C-29 respectively. Evidently these resonances are affected by the introduction of substituents (Mahato and Kundu, 1994). The ^{13}C DEPT experiments, especially DEPT 135 help in the differentiation of CH, CH_2 and CH_3 in the molecule.

In addition to the above, 2D NMR experiments; HMQC, HMBC, NOESY and TOSY experiments are important tools for the unambiguous elucidation of the structures of unknown molecules. The HMQC or HSQC permits attribution of protons to particular carbon-atoms, the HMBC allows for connectivity between protons and the carbon atoms adjacent to the one bearing the proton usually up to 2 or 3 bonds apart (^{2}J, ^{3}J), but sometimes ^{4}J occurs. The HMBC is very important in establishing a link between parts of the same molecule. The proton on C-18 is very useful in that it connects easily to other carbons of the molecule on HMBC, thus indicating the position of olefinic carbons and usually the carbonyl function at C-28. NOESY helps in the attribution of the relative stereochemistry around stereogenic centres. TOCSY is very useful in the elucidation of the structure of saponins.

Mass spectroscopy is used to establish the molecular weight of the compound under analysis. Soft ionisation techniques such as ESI, FAB, MALDI, direct ionization and chemical ionization are

commonly used to establish the mass of steroids, triterpenoids and their saponins from pseudo-molecular ion peaks on the spectrum (**Yang *et al* 2007; Bonfill *et al.*, 2005**). EI is also frequently used to get the molecular mass directly from the molecular ion peak and information concerning the structure of a molecule is provided by fragmentation patterns observed on the EI-MS. The most prominent fragmentation pattern shown by pentacyclic triterpenoids is that due to a RDA reaction common in tritepenoids with a double bond. This usually leads to a base peak at m/z 218 and a prominent peak at m/z 203 on simple unsubstituted triterpenoids like α and β-amyrin. It is thus possible to get more information about the structure of a substituted triterpenoid by making deductions from the distinctive peaks, oleanolic and ursolic acid show base peak at m/z 203 and other prominent peaks at m/z 203 + COOH and 218 +COOH (**Ogunkoya, 1981**).



Scheme 3: Retro-Diels-Alder fragmentation pattern for Oleanane and Ursane type triterpenoids (Ogunkoya, 1981)

By simple inspection of the mass spectrum of an unknown triterpenoid, it is possible to decide whether the compound belongs to the oleanane and ursane-type (Δ^{12}) or otherwise (Δ^{14}, Δ^{18}). The RDA reaction usually occurs in the ring which bears a double bond. For the Δ^{12} triterpenoids it occurs in ring C, leading to base peak at 218 or 203, and diagnostic peaks at m/z between 200 and 300 as shown in the chart above. Δ^{14} triterpenoids like taraxerol undertake a ring D RDA reaction in which rings A, B, and C are retained, leading to base peak at m/z 302, 300 for taraxerol and

taraxerone respectively with prominent peaks at 204 (**Djerassi, 1964**). However, oleanane and ursane-type triterpenoids cannot be distinguished by mass spectrometry, since they show identical fragmentation process- the RDA reaction involving the Δ^{12} double bond.

Summarily, literature study is indispensible for the elucidation of the structures of triterpenoids. It is relatively easier nowadays to identify an unknown triterpenoid molecule from its spectroscopic and physical data than it was two decades now. This is possible due to the presence of tables that compile some outstanding features of triterpenoids, permitting quick comparison with new NMR data (**Mahato and Kundu, 1994**). In this compilation, pentacyclic triterpenoids are further classified into different classes based on their carbon-skeleton. Oleanane type and Ursane type triterpenoids, based on the way methyl groups are attached on position 19 and 20 of the pentacyclic skeleton. Oleanane when two methyls are attached to position 20 and Ursane when one methyl is attached to each of the positions. The above arrangement has a remarkable impact on the ^{1}H NMR spectrum, appearance of two doublet methyl doublets on the spectrum of an ursane type molecule whose protons at 19 and 20 have not been oxidised (**Mahato and Kundo, 1994**).

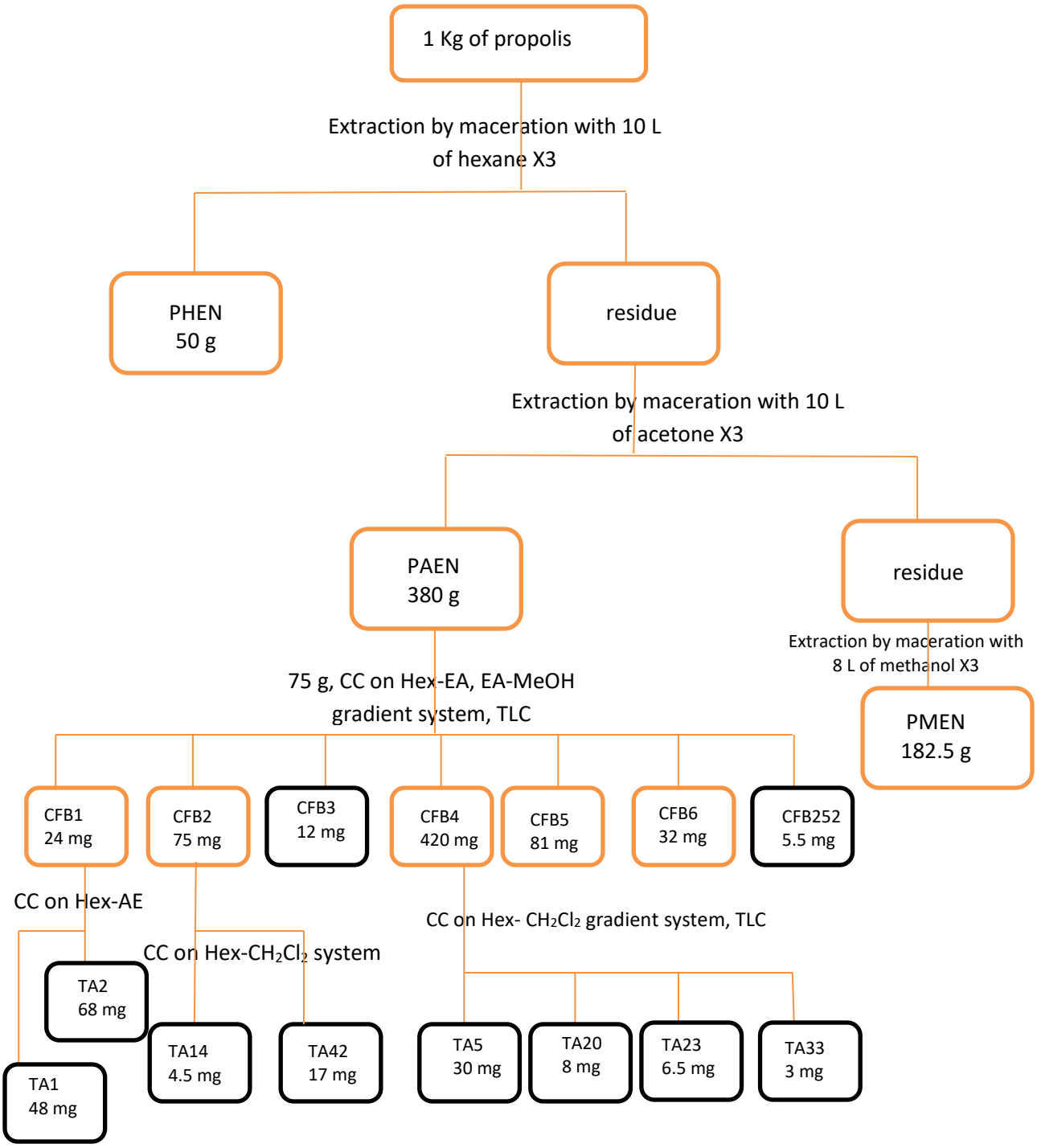
This chapter has given a general knowledge of propolis and has been focused on the major chemical composition as well as some biological activities of propolis previously studied. A general overview of the class of compounds, the triterpenes, was also examined based on works of other authors. The goal of this literature review was to provide background knowledge of what our research work is based on and to also have an idea of expected outcomes. It is also essential to know what other researchers have done so far.

CHAPTER II: RESULTS AND DISCUSSION

Extracts prepared from samples of propolis from Nkambe, Foumban and Ndian and were subjected GC-MS and CC. CC led to isolation of pure compounds out of which the structures of some were established by interpretation of their spectral data and reported herein. Some of these extracts and isolated compounds where screened for anti-ulcer, antimicrobial, antiradical and anti-inflammatory activities. Other extracts of propolis samples from Djerem, Mbere, Mbouda, Boyo and Mokolo were screened for antimicrobial and antioxidant activities and their chemical profiles established by GC-MS. Lupeol and β-amyrine were further subjected to chemical transformations to give some ester and ketone derivatives.

II-1: PREPARATION OF EXTRACTS AND ISOLATION OF SECONDARY METABOLITES FROM THE PROPOLIS OF NKAMBE

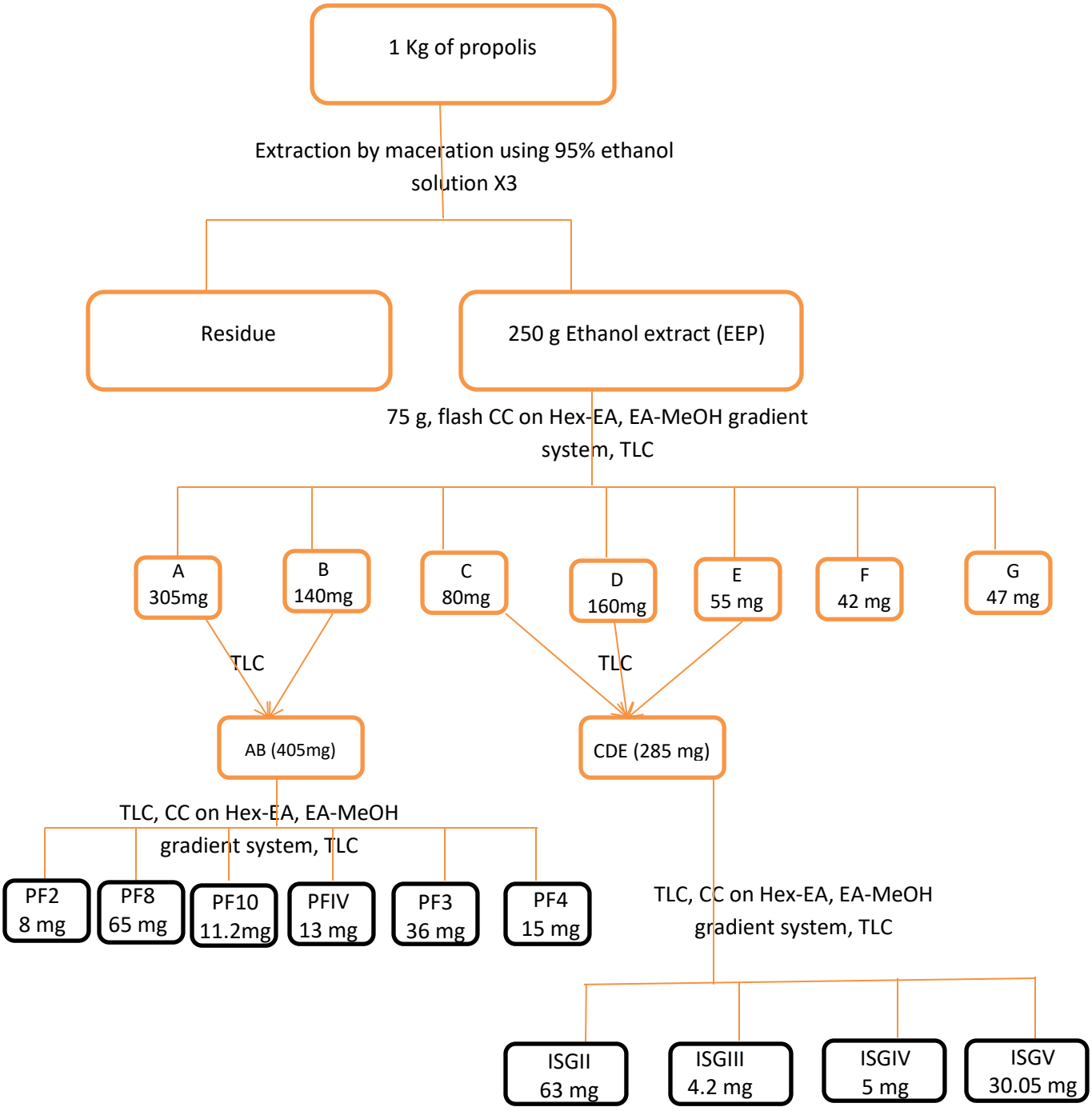
The propolis was harvested from bee hives of an apiary located within the same area in Njap village, Nkambe town, North-West region of Cameroon during the months of February to March 2013. 1 kg of raw propolis sample was dried and cooled (20 ^{o}C) and ground in a mortar using a pestle to obtain a powder. The propolis powder was extracted successively by maceration with 10 volumes (10 litres) of hexane, acetone and methanol in a tightly closed glass jar kept in a dark cupboard at ambient temperature for 48 hours with intermittent stirring. The supernatant was carefully decanted and filtered through a Whatman No. 1 filter paper. The final filtrates were evaporated to near dryness on a rotary evaporator under reduced pressure to remove the solvent and the extract was collected in a clean vial. The maceration, filtration and evaporation processes were repeated three times for each solvent after which the residual powder was dried before introduction of a new solvent. This yielded the hexane extract (PHEN), the acetone extract (PAEN) and the methanol extract of propolis (PMEN).75 g of the acetone extract (PAEN) were adsorbed on silica gel and subjected to column chromatography with silica gel on a gradient of Hexane-EtOAc (0-100%) then EtOAc-MeOH (0-40%) with increasing polarity to yield 352 fractions. Based on their TLC profiles, some of the fractions were regrouped into sub-fractions and subjected to further repeated silica gel chromatographic column eluting with hexane (Hex), hexane-ethyl acetate (EA) or hexane-CH_2Cl_2 gradient conditions as shown in scheme 4.



Scheme 4: Extraction and isolation procedure of Nkambe propolis

II-2: PREPARATION OF EXTRACTS AND ISOLATION OF SECONDARY METABOLITES FROM THE PROPOLIS OF FOUMBAN

1 kg of propolis sample was collected from the peripheries of Foumban town, Noun division from West Region of Cameroon during the months of October and November 2013. This sample was dried in a shed after which it was powdered using a mortar and pestle. The powder was extracted successively with hexane, ethyl acetate and 95% ethanol solution. Each extraction was done trice and the solvent was removed using a Rotavapor before introduction of a new solvent. This gave the hexane extract of Foumban propolis (PHEF, 362 g), ethyl acetate extract of Foumban propolis (PEAEF, 150 g) and the ethanol water residue was lyophilized and dissolved in methanol as the methanol extract of Foumban propolis (PMEF, 130 g). Another 1 kg of this sample was extracted uniquely with 95% ethanol solution and concentrated *in vacuo* to afford a dark red ethanol extract. 75 g of this ethanol extract of propolis from Foumban was subjected to flash column chromatography using silica gel using hexane-ethyl acetate (0-100%) then ethyl acetate-MeOH (0-60%) gradient system to afford 110 fractions which were later grouped into 7 pooled fractions (A-G) based on their TLC profiles. The fractions A and B were regrouped while C, D and E too were combined and each of the combinations adsorbed on silica gel and subjected to column chromatography with silica gel on a gradient of Hexane-EtOAc (0-100%) then EtOAc-MeOH (0-100%) separately.



Scheme 5: Extraction and isolation procedure from Foumban propolis

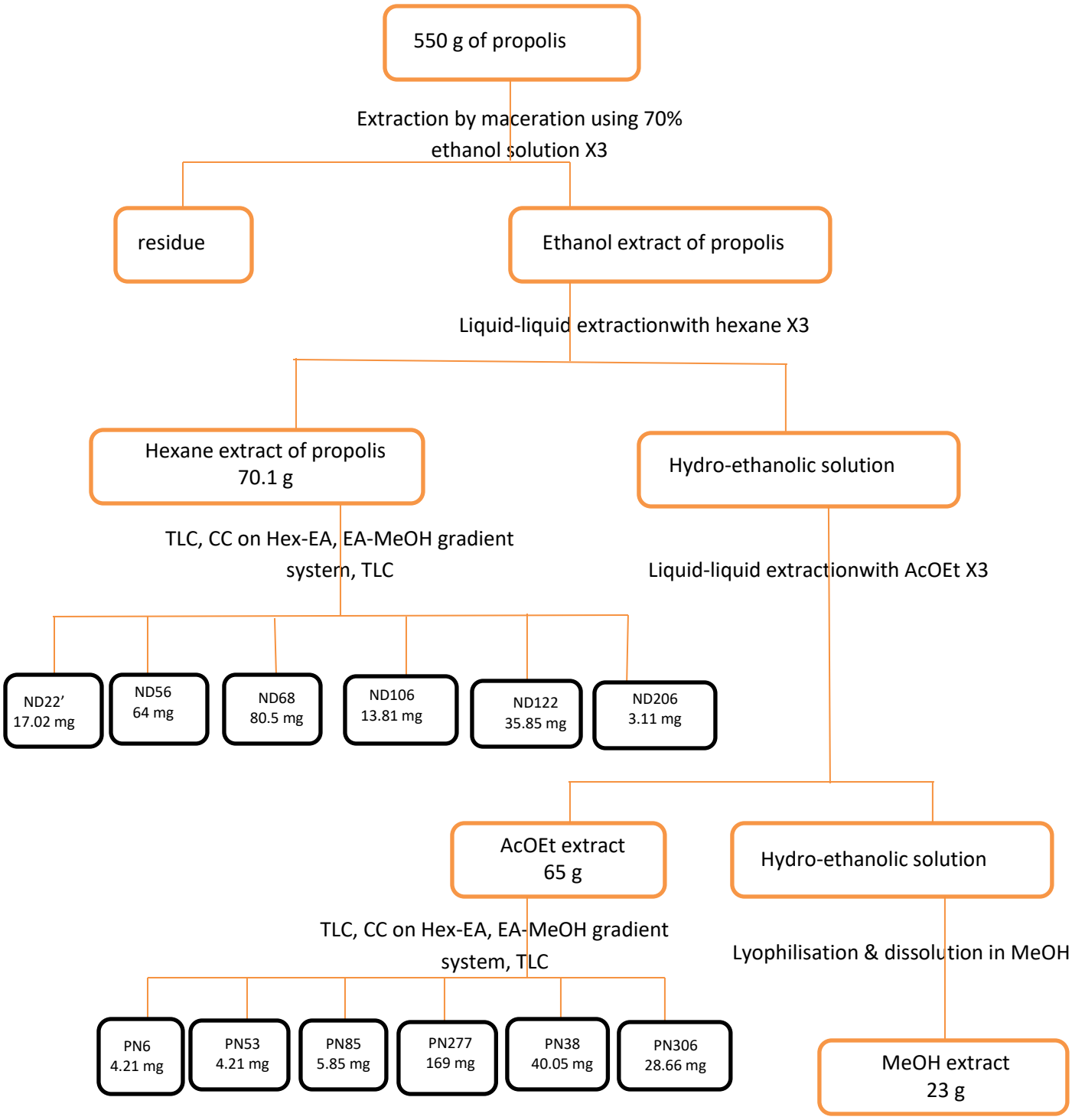
II-3: PREPARATION OF EXTRACTS AND ISOLATION OF SECONDARY METABOLITES FROM THE PROPOLIS OF NDIAN

550 g of propolis from Ndian, South-West region of Cameroon which was collected in February 2015 was chilled and ground. The powder was then extracted with a 10 folds volume of 70% ethanol at room temperature for 48 hrs. This process was repeated three times to yield a hydro-alcoholic solution of the propolis which was concentrated to near dryness to give the ethanol extract. The ethanol extract was extracted successively by liquid-liquid extraction with hexane (3 times) and ethyl acetate (3 times) to obtain a hexane extract of Ndian propolis (70.1 g) and ethyl acetate extract of Ndian propolis (65 g) respectively. The hydro-alcoholic residue was lyophilized and the solid obtained (9.3 g) was dissolved in MeOH and considered as a methanol residue or extract.

35 g of hexane extract of propolis from Ndian were subjected to column chromatographic separation with silica gel as absorbent using hexane-ethyl acetate (0-100%) and ethyl acetate-MeOH (0-20%) to give 302 fractions which were regrouped into major fractions (A-Z and A1-A6) based on their TLC profiles.

35 g of ethyl acetate extract of propolis from Ndian were subjected to column chromatographic separation with silica gel as absorbent using hexane-ethyl acetate (0-100%) and ethyl acetate-MeOH (0-100%) to give 578 fractions. These fractions were regrouped into major fractions (A'-Z' and A1-G1) based on their TLC profiles.

Some of the fractions were pure and crystallized to give some compounds as shown in scheme 6.



Scheme 6: Extraction and isolation procedure from Ndian propolis

The above chromatographic separation and purification processes led to the isolation of the following compounds: 10 compounds from the propolis of Nkambe, 10 compounds from the propolis of Foumban and 12 compounds from the propolis of Ndian as distributed in the table below.The structures of these compounds were elucidated based on some data of their physical properties like melting points, MS data, routine NMR spectroscopic techniques (1D: ^{1}H, ^{13}C, and 2D: COSY, HSQC, HMBC and NOESY) and by comparison with information reported in literature.

Table 19: The compounds isolated from the propolis of Nkambe, Foumban and Ndian with their masses

Nkambe propolis			**Foumban propolis**			**Ndian propolis**		
compound	**Mass/ mg**	**remark**	**compound**	**Mass/ mg**	**remark**	**compound**	**Mass/ mg**	**remark**
TA1	48.00	known	**PF2**	8.00	known	**ND22'**	17.02	known
TA2	68.00	known	**PF8**	65.00	known	**ND56**	64.00	*NA*
TA14	4.50	known	**PF10**	11.20	known	**ND68**	80.50	=ISGII
CFB3	12.00	known	**PF3**	36.0	=ND22 ,	**ND106**	13.81	*NA*
TA42	17.00	known	**PF4**	15.00	known	**ND122**	35.85	=ANT252
TA5	30.00	known	**PFIV**	13.50	*NA*	**ND206**	3.11	*NA*
TA20	8.00	known	**ISGII**	63.00	known	**PN6**	4.21	known
TA23	6.50	known	**ISGIII**	4.22	*NA*	**PN53**	104.10	known
TA33	3.00	=TA1	**ISGIV**	5.00	*NA*	**PN85**	5.85	=ISGV
CFB252	5.50	known	**ISGV**	30.05	new	**PN277**	169.00	=ANT252
						PN38	43.05	new
						PN306	28.66	new

NA = not analyzed (total = 6 compounds)

The spectral analyses of 6 compounds are awaited while 7 compounds were identical to each other based on the comparison of their spectral data. Hence the number of compounds whose structures were identified or elucidated was reduced to 20. The structures of the compounds were established and described as follows.

II-4: CHARACTERISATION OF THE COMPOUNDS ISOLATED

The structure of the compounds isolated were characterised using routine spectroscopic techniques such MS, IR and NMR (^{1}H, ^{13}C, COSY, HSQC, HMBC and NOESY) in addition to characteristic chemical tests and comparaison of their physical (melting points) and spectroscopic data with those reported in literature.

II-4-1: Characterisation of triterpenoids

II-4-1-1: Structural elucidation of SB38

The compound PN38 crystallized in the form of white crystals in hexane-ethyl acetate (55/45, v/v) gradient system. It melted between 167-170°C and gave a blue-green colouration with the Liebermann-Burchard reagent, suggesting that it is a sterol.

The ESI-TOF MS spectrum of PN38 (positive mode) showed a pseudo-molecular ion peak of $[M+K]^+$ at m/z = 537.3 and another diagnostic peak $2[M+K]^+$ at m/z 1074.6 from which the molecular mass of the compound PN38 was deduced as 498.3 g/mol for $C_{32}H_{50}O_4$ with 8 double bond equivalences.

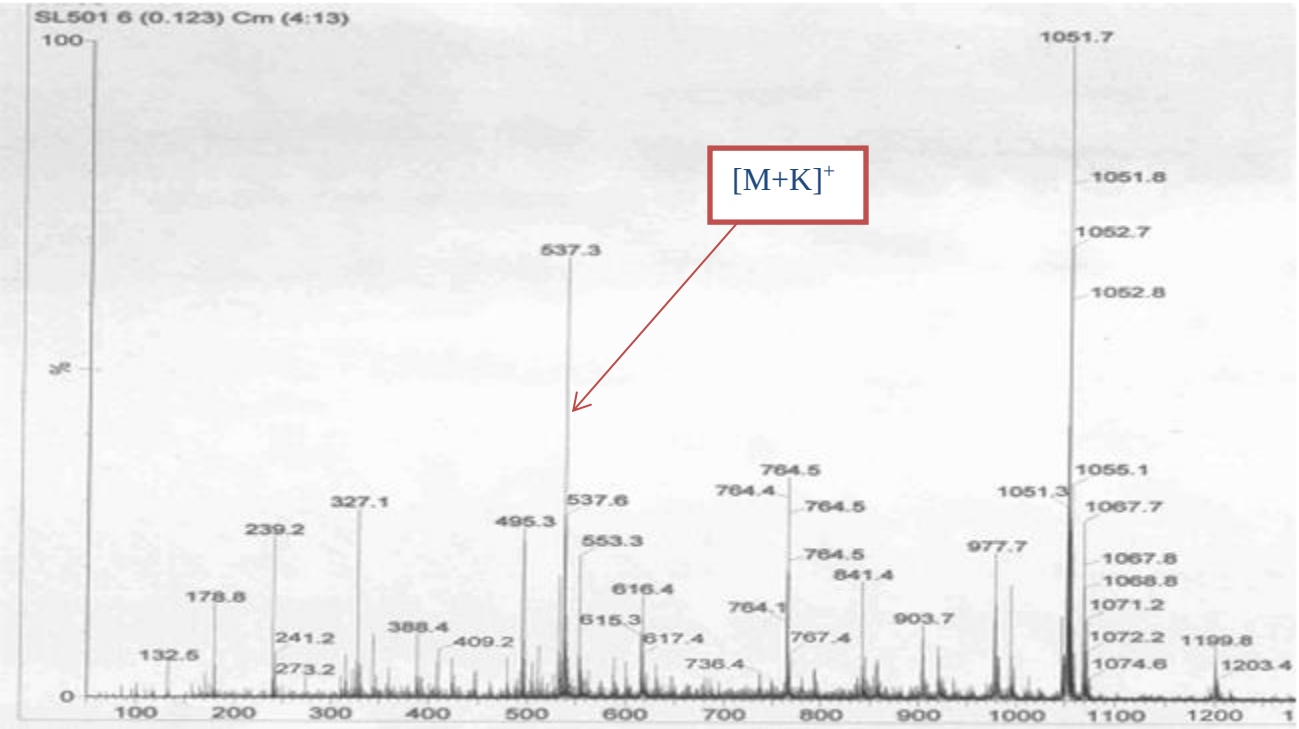


Figure 2: ESI-TOF MS spectrum of PN38

The ^{1}H NMR (d6-acetone, 500 MHz) spectrum of PN38 showed signals of two separated allylic oxymethines at δ_H 4.05 ppm and 4.25 ppm together with two distinct olefinic protons at δ_H 5.05 ppm and δ_H 5.35 ppm thereby suggesting that PN38 is a compound with a spinasteryl skeleton in which the two double bonds have a hydroxyl substituent in an α-position to the double bond. This is similar to a system of substituted 8,22-dihydroxyspinast-5,23-dienyl skeleton described for 16-β (β-D–glucopyranosyl 3,8,22-trihydroxy-cholest-5,14,16,23-tetraiene 1β-yl, 6-O-(3,4,5-trimethoxybenzonyl) β-D glucopyranoside (**Ebere and Offiong, 2010**). Signals of 8 angular methyls were present: five singlets at δ_H 0.70 ppm (3H, H-32), 0.82 ppm (3H, H-31), 0.85 ppm (3H, H-30), 0.98 ppm (3H, H-18) and 1.05 ppm (3H, H-19); three doublets at δ_H 0.67 ppm (3H, H-27), δ_H 0.68 ppm (3H, H-26) and δ_H 0.87 ppm (3H, H-21). The absence of a triplet of three protons for H-29 and the multiplet of two protons for H-28 in the zone δ_H 0.83-1.05 ppm suggests that the position 29 is substituted. This is substantiated by the presence a carbonyl proton at δ_H 10.10 ppm (1H, H-29, s) and a pair of doublets at 2.60 ppm (1H, H-28a) and 2.90 ppm (1H, H-28b) enabling us to place a carbonyl function at position 29.

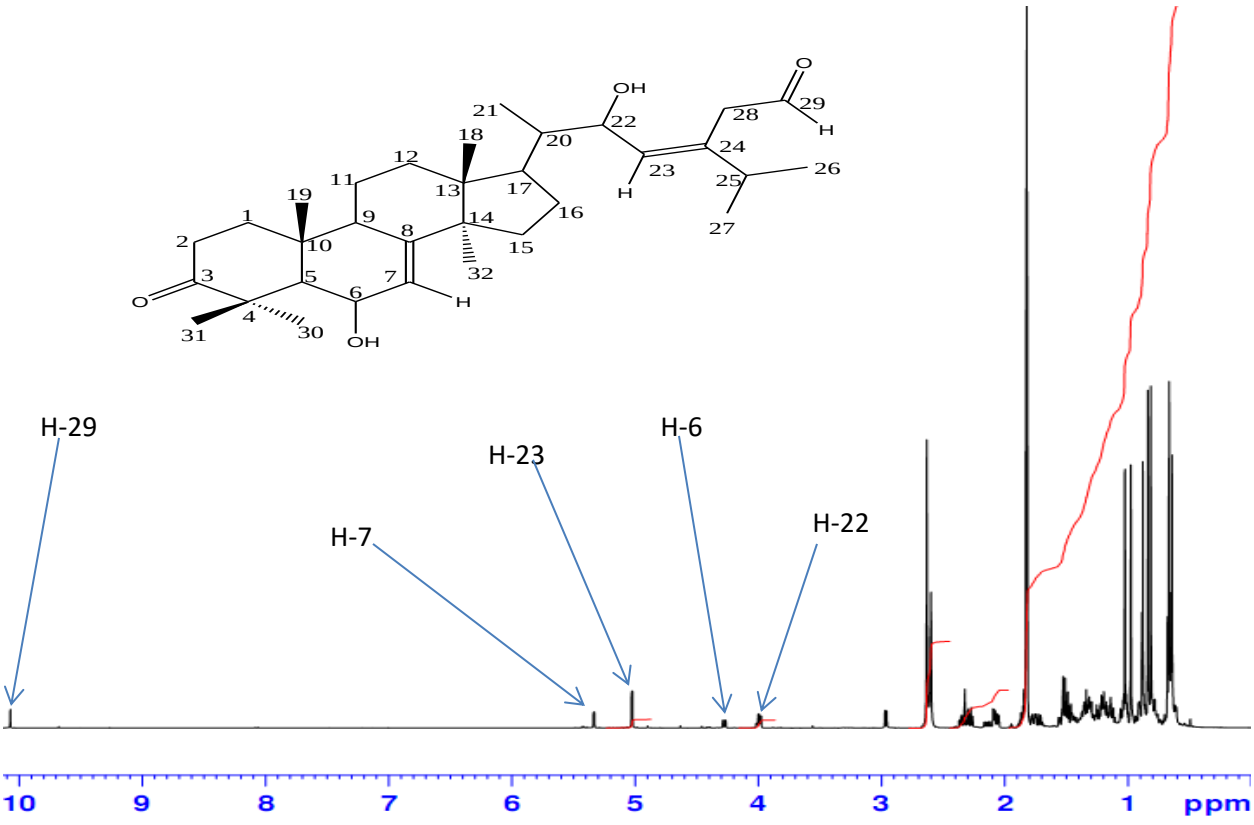


Figure 3: ^{1}H NMR (d6-acetone, 500 MHz) spectrum of PN38

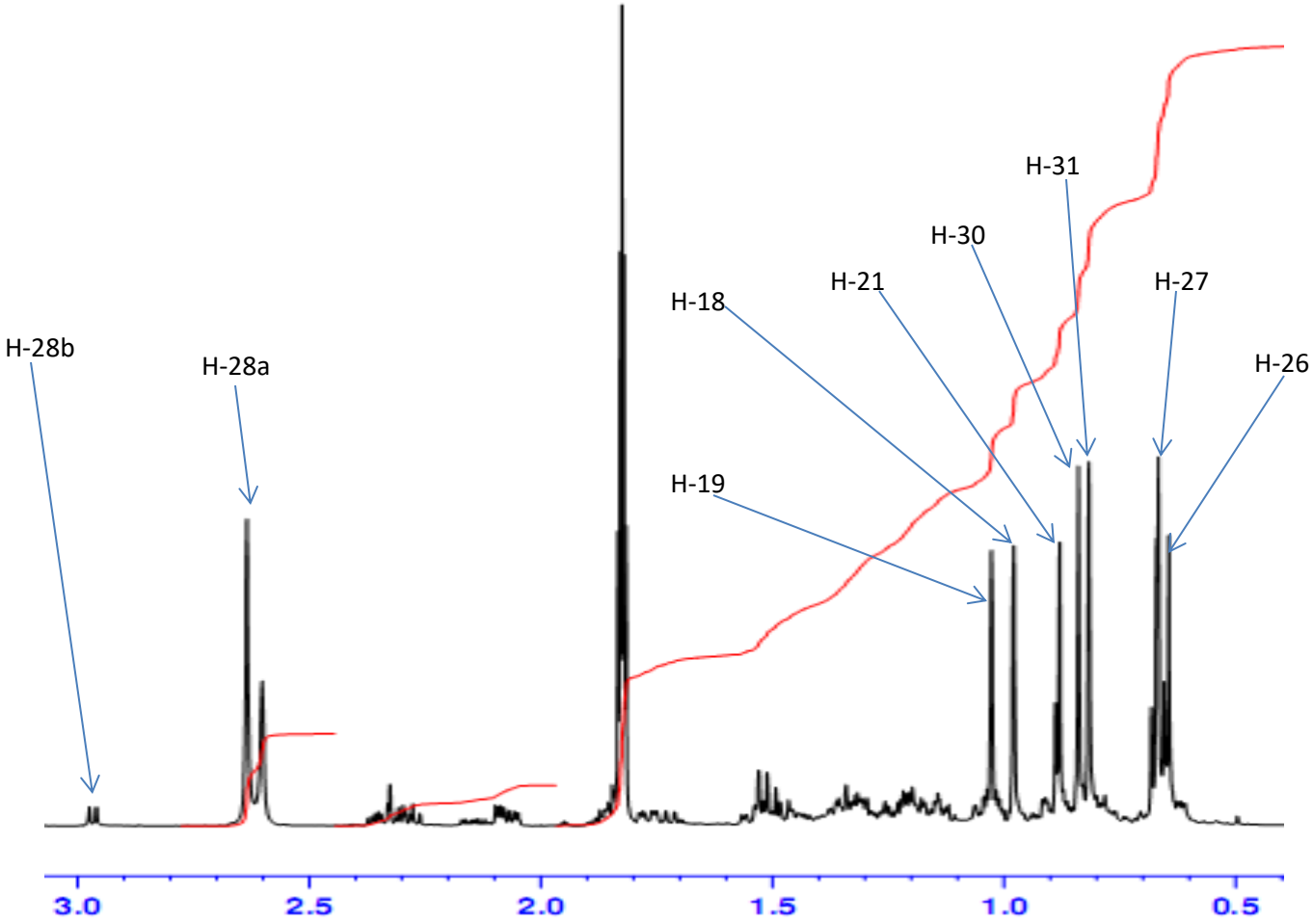


Figure 4: The ^{1}H NMR (d6-acetone, 500 MHz) spectrum of PN38 (0.5-3.0 ppm)

^{13}CNMR Spectrum (d6-acetone, 150 MHz) of the compound PN38 shows characteristic olefinic signals of olefinic carbon atoms at δ_C 123.1 ppm and δ_C 147.0 ppm for Δ^7 double bond and δ_C 128.1 ppm and δ_C 150.2 ppm for Δ^{23} of a spinastan-7,23(24)-dienyl skeleton and the observed downfield shifts is explained by the presence of hydroxyl groups in α-positions to the respective double bonds. Two carbonyl carbon signals present at δ_C 216.5 ppm and δ_C 216.2 ppm are attributable to the 3-oxo and 29-al carbonyl functions confirmed by the presence of protons at δ_H 2.10 ppm (1H, H-2a) and δ_H 2.25 ppm (1H, H-2b) and 2.60 ppm (1H, H-28a) and 2.90 ppm (1H, H-28b) all at α-positions to the two carbonyls. Furthermore, two allylic oxymethine carbon atoms appear at δ_C 81.6 ppm and δ_C 67.9 ppm corresponding to the carbon atoms C-6 and C-22 respectively.

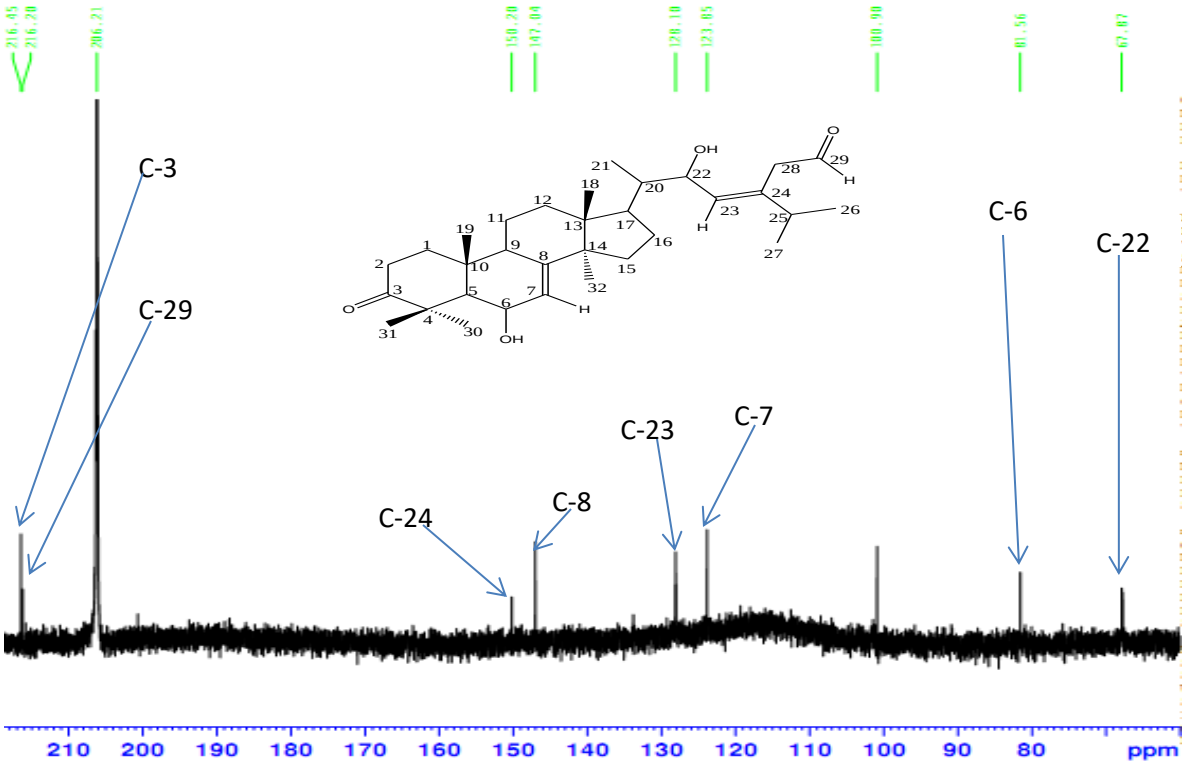


Figure 5: ^{13}C NMR Spectrum (d-acetone, 150 MHz) of PN38 (60-220 ppm)

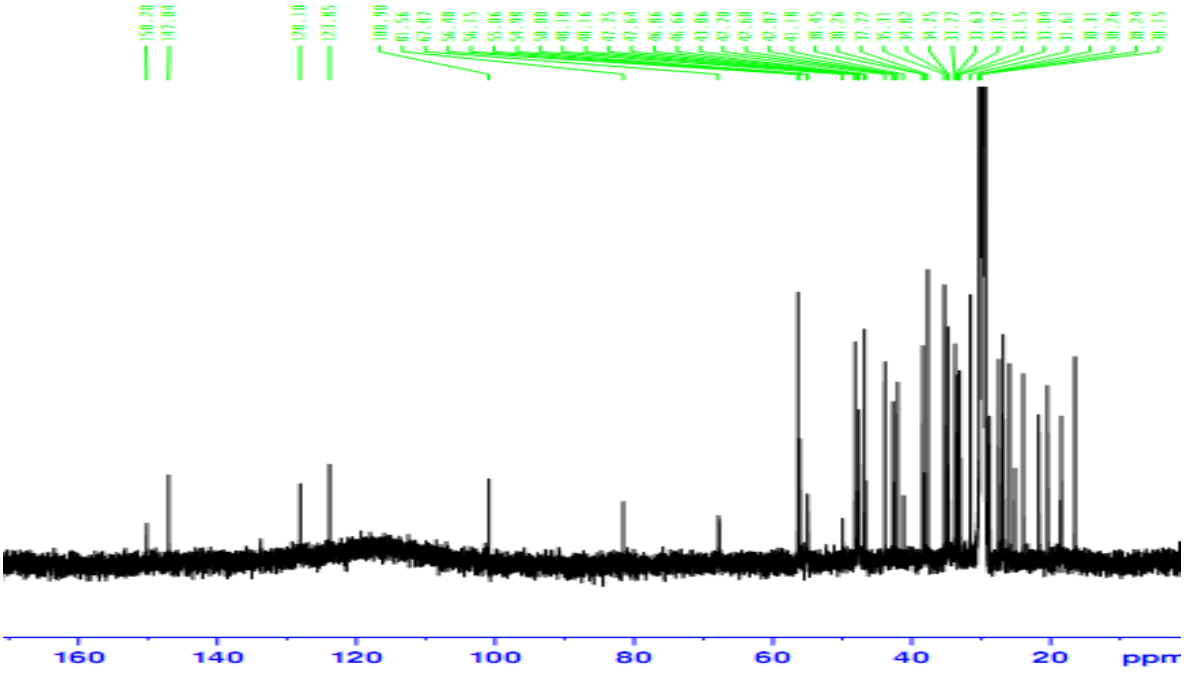


Figure 6: ^{13}C NMR Spectrum (d6-acetone, 150 MHz) of SB38 (0-170 ppm)

The HSQC ($^{1}J_{C-H}$) spectrum of PN38 showed some correlation spots between the following pairs of protons and carbon atoms: δ_H = 0.98 ppm (H-18) and δC = 16.5 ppm (C-18), δ_H = 0.87 ppm (H-21) and δC = 18.6 ppm (C-21), δ_H = 0.67 ppm (H-27) and δC = 20.1 ppm (C-27), δ_H = 4.05 ppm (H-22) and δC = 67.9 ppm (C-22) amongst others.

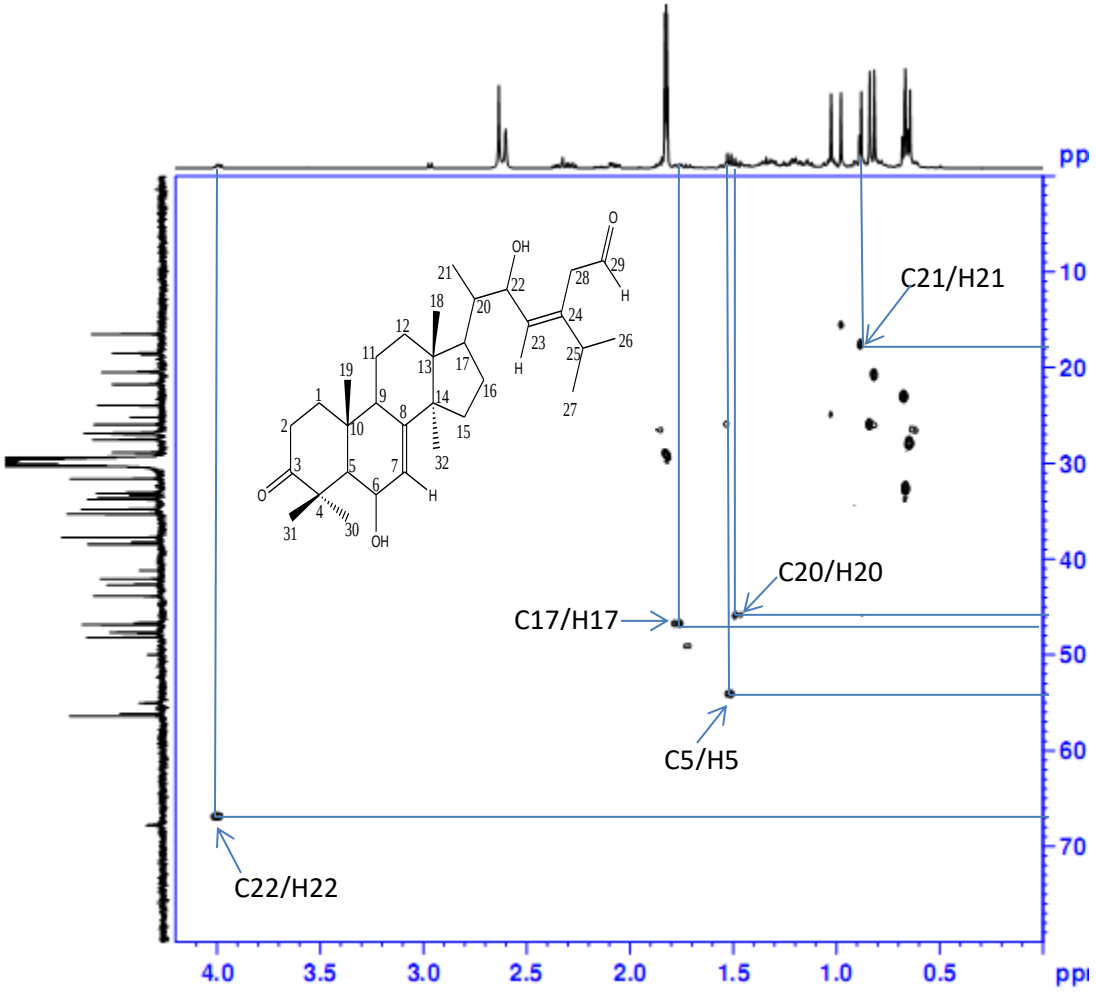


Figure 7: HSQC ($^{1}J_{C-H}$) spectrum of SB38

The HMBC spectrum of PN38 shows long distance proton/carbon correlation, a $^{4}J_{H-C}$ coupling between the proton at δ_H= 4.05 ppm (H-22) and the carbon atom at δ_C = 41.2 ppm (C-28).

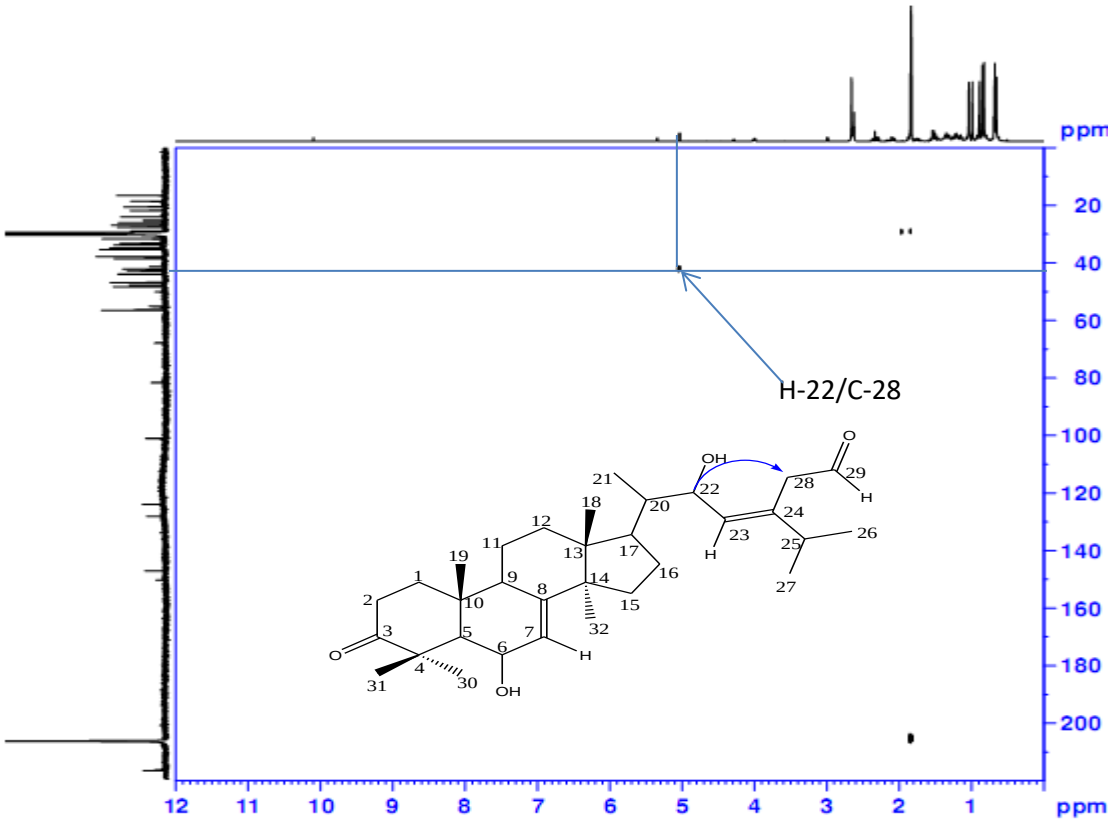


Figure 8: HMBC spectrum of PN38

^{1}H-^{1}H COSY spectrum of PN38 showed cross peaks between some adjacent protons as follows: H-23 and H-22 which further spin couples with the proton H-20 and through a long-range coupling with H-28b thanks to the orientation of the molecule in space. The two doublets H-28a and H-28b spin couple with each other. Also, the olefinic proton H-7 showed correlation peak with the oxymethine H-6 while this H-6 spin couples with H-5. Other important correlations visible on the ^{1}H-^{1}H COSY spectrum of PN38 include correlations between H-1/H-2, H-21/H-17, H-25/H-27 etc. From biosynthetic pathway, the most probable configuration of the carbon atom of 22-hydroxyl sterol is S, that is a 22α-OH group orientation. This biosynthetically defined stereochemistry has been extensively described and experimentally proven (**Hiromu *et al.*, 1968**). In addition, C-6 β-OH orientation for C-6 hydroxylation resulting from biosynthetic pathway for C-6 hydroxyl triterpenes and steroids has been confirmed as sole possible stereo-isomer, while confirming the α-orientation of C-22 hydroxyl using 22-hydroxycholesterol-25-al (**Sumit Ghosh, 2017**). These resulting biosynthesis configurations for C-6 hydroxyl and C-22 hydroxyl groups of

sterols and other hydroxyl patterns have been explicitly and experimentally proven and have been also used in structural configuration assignments of steroidal alkaloids (**Megan *et al.*, 2015**).

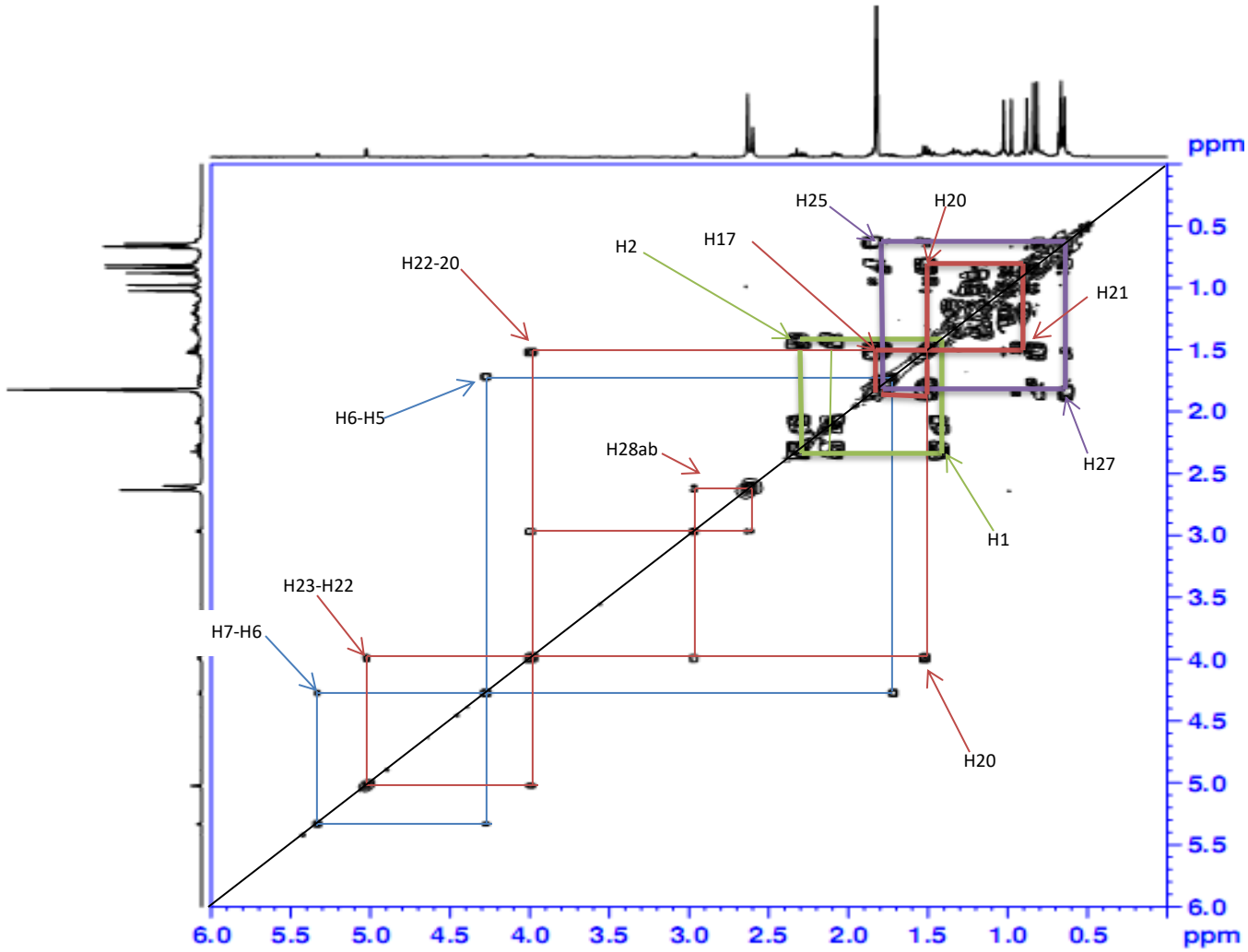
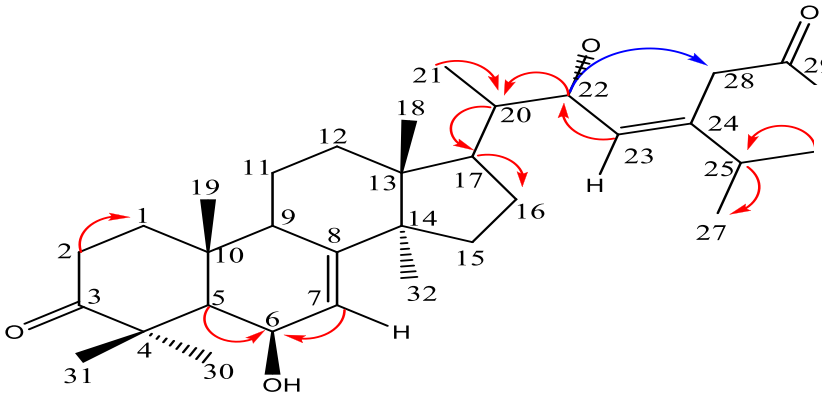


Figure 9: ^{1}H-^{1}H COSY spectrum of PN38

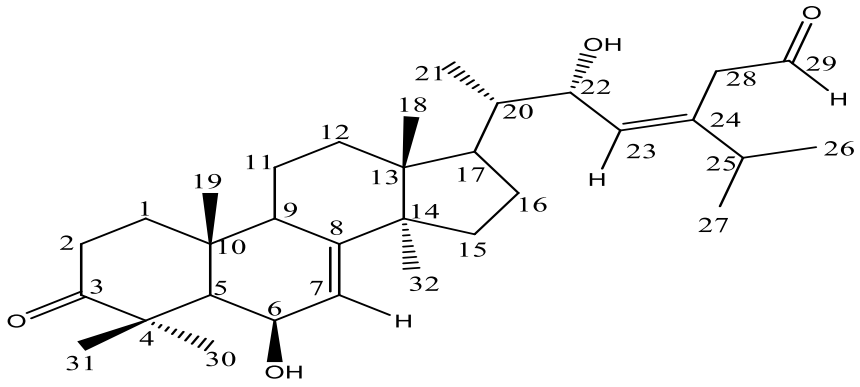


Major correlations represented on the COSY spectra above are shown in the molecule besides.

Key COSY= red arrows

HMBC and COSY= blue arrow

The foregoing spectral information and by comparison with some related data reported in literature for some closely related molecules enable us to attribute the structure below to PN38 which is that of 3-oxo-6,22-dihydroxy-4,4,14-trimethylspinast-7,23(24)-dien-29-al which is a new derivative.



3-oxo-6β,22α-dihydroxy-4,4,14-trimethylspinast-7,23-dien-29-al **(133)**

Table 20: NMR data of PN38 (3-oxo-6β,22α-dihydroxy-4,4,14-trimethylspinast-7,23-dien-29-al)

PN38 (3-oxo-6β,22α-dihydroxy-4,4,14-trimethylspinast-7,23-dien-29-al)			PN38 (3-oxo-6β,22α-dihydroxy-4,4,14-trimethylspinast-7,23-dien-29-al)		
Position	**^{13}C**	**^{1}H**	**Position**	**^{13}C**	**^{1}H**
1	38.5	1.40;1.6, m, 2H	**17**	46.8	1.70, m, 1H
2	34.7	2.25;2.10, m, 2H	**18**	16.5	0.98, s, 3H
3	216.5	/	**19**	21.8	1.05, s, 3H
4	41.2	/	**20**	43.8	1.45, m, 1H
5	56.4	1.50, d, 1H	**21**	18.6	0.87, d, 3H
6	81.6	4.25, dd, 1H, J = 5.2 Hz	**22**	67.9	4.25, dd, 1H, J = 9 Hz
7	123.1	5.35, d, 1H	**23**	128.1	5.05, d, 1H
8	147.0	/	**24**	150.2	/
9	48.2	1.93, t, 2H	**25**	33.8	1.65, m, 1H
10	31.6	/	**26**	25.9	0.68, d, 3H
11	26.9	1.41, m, 2H	**27**	20.1	0.67, d, 3H
12	33.2	1.35, m, 2H	**28**	41.2	2.60; 2.90, d, 2H
13	46.6	/	**29**	216.2	10.10, s, 1H
14	55.1	/	**30**	25.2	0.85, s, 3H
15	34.8	1.25, dd, 2H	**31**	23.9	0.82, s, 3H
16	26.8	1.25;1.50, m, 2H	**32**	20.5	0.70, s, 3H

II-4-1-2: Structural identification of TA1

The compound TA1 crystallized as white crystals in the eluent mixture hexane-CH_2Cl_2 (85:15, v/v). Its melting point was between 170-172°C and gave a positive test with the Liebermann-Burchard reagent indicating that the compound TA1 is a triterpene.

The ITMS-ESI Full MS mass spectrumof TA1 showed a pseudo-molecular ion peak $[M+Na]^+$ at m/z = 447.8 calculated for 424.8 g/mol corresponding to the molecular formula $C_{30}H_{48}O$ with a double bond equivalent of 7. Another diagnostic ion [2M+Na] is present at m/z = 872.5.

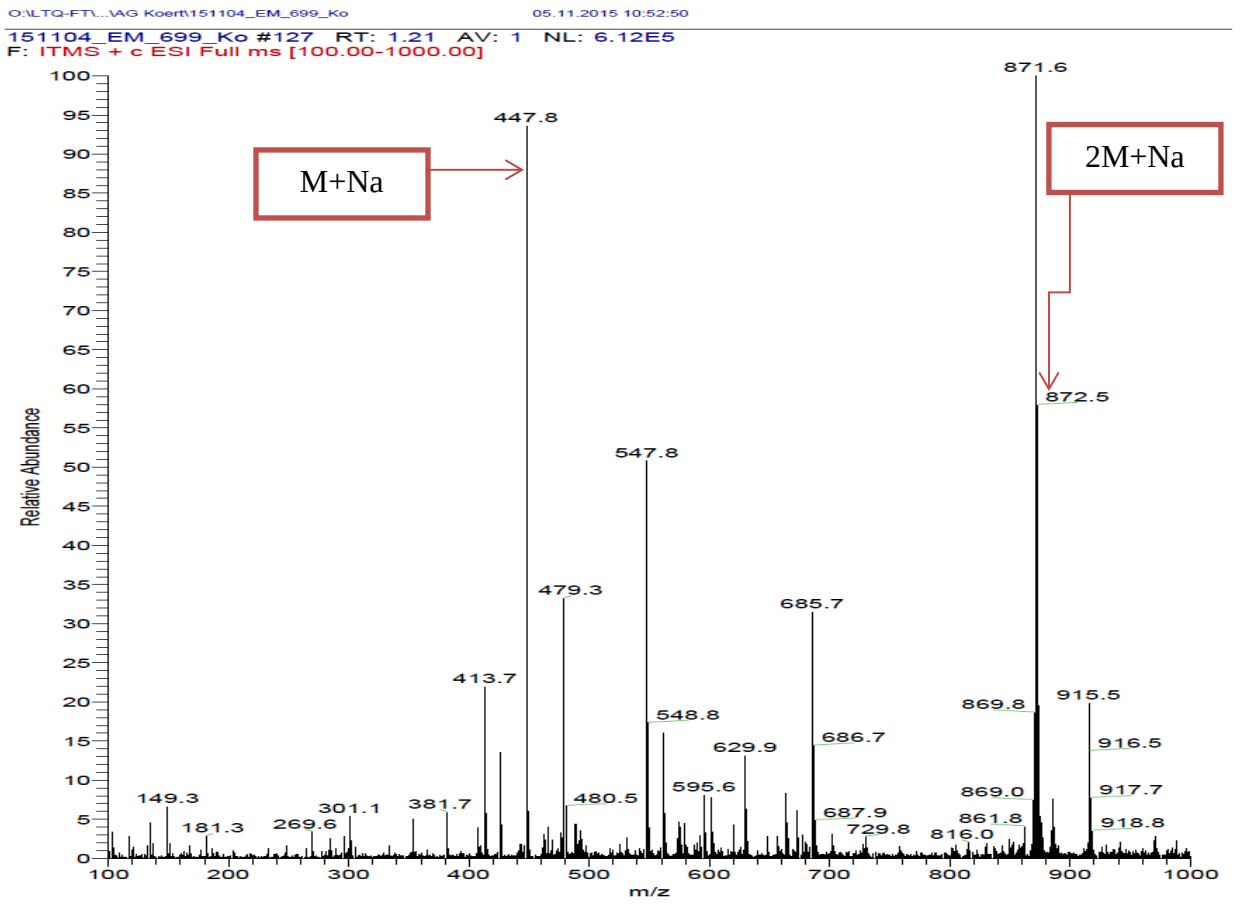


Figure 10: ITMS-ESI Full MS Mass spectrum of TA1

The ^1H NMR (300 MHz, $CDCl_3$) spectrum of TA1 showed four signals of three protons at δ_H 0.77 ppm (s, 3H, H-28), 0.90 ppm (s, 3H, H-25), 0.93 ppm (s, 3H, H-27), 1.00 ppm (s, 3H, H-24) attributable to the four methyls bonded to quaternary carbon atoms. Other singlets appeared at δ_H 1.04 ppm (s, 3H, H-23) and 1.22 ppm (s, 3H, H-26) corresponding to the methyls on position 23

and position 26 respectively. An olefinic methyl appeared at δ_H 1.66 ppm (s, 3H, H-30). Two multiplet were observed between δ_H 1.84-1.97 ppm (m, H-21) and 2.24-2.52 ppm (m, H-19) corresponding to the methylene protons at positions 21 and 19 respectively. Two olefinic protons appeared as singlets of one proton each at δ_H 4.55 ppm (s, 1H, H-29) and 4.80 ppm (s, 1H, H-29) all bonded on the same and terminal carbon atom of the double bond.

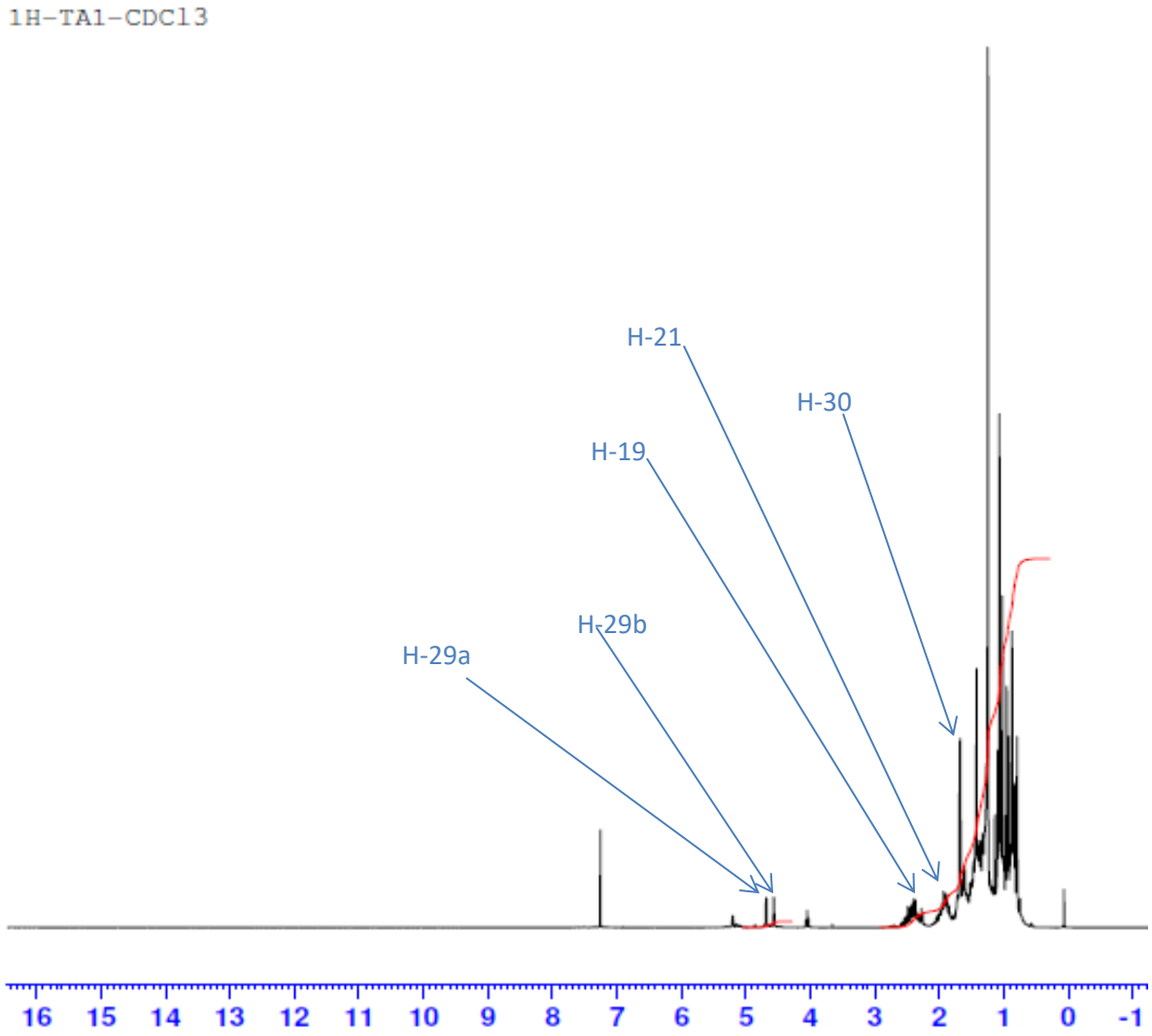


Figure 11: ^{1}H NMR (300 MHz, $CDCl_3$) spectrum of TA1

The ^{13}C NMR spectrum ($CDCl_3$, 150 MHz) of TA1 showed thirty peaks thus confirming a triterpene skeleton. The spectra showed the following peaks;

Seven methyls with *δC* 14.1 (C-27), 21.5 (C-24), 14.4 (C-26), 15.7 (C-25), 15.9 (C-28), 19.7 (C-30) and 26.9 (C-23). Eleven methylenes at *δC* 39.6 ppm (C-1), 28.6 ppm (C-2), 18.0 ppm (C-6), 33.6 ppm (C-7), 26.6 ppm (C- 11), 26.3 ppm (C-12), 27.4 ppm (C-15), 35.5 ppm (C-16), 29.8 ppm

(C-21), 39.9 ppm (C-22) and 109.4 ppm (C- 29). Five methines at 54.9 ppm (C-5), 49.8 ppm (C-9), 39.3 ppm (C-13), 48.3 ppm (C-18), 47.9 ppm (C-19). Six quaternary carbon atoms at *δC* 41.0 ppm (C-10), 47.3 ppm (C-4), 42.9 ppm (C-17), 150.8 ppm (C-20), 43.8 (C-8), 42.9 (C-14). Also, the peaks at *δC* 109.8 ppm and *δC* 150.8 ppm confirms the presence of olefinic carbon atoms of lup-20(29)-ene (**Mahato and Kundu, 1994**).

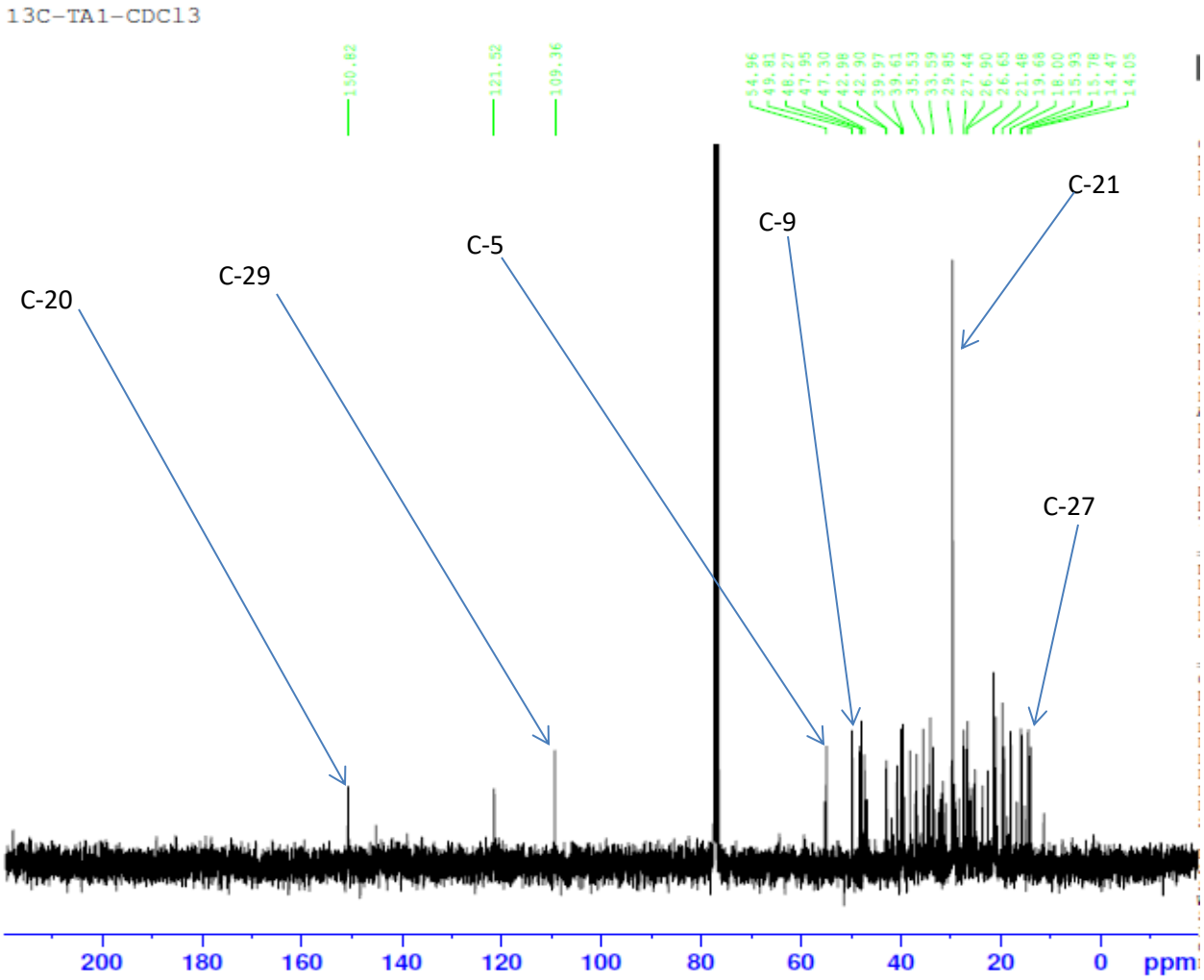
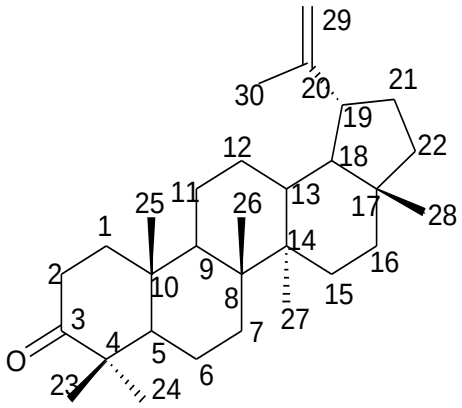


Figure 12: ^{13}C NMR (150 MHz, $CDCl_3$) spectrum of TA1

From the forgoing spectral data and by comparison with similar data reported in literature (**Mahato and Kundu, 1994**) TA1 is unambiguously attributed the structure of a pentacyclic triterpenoid lup-20(29)-en-3-one previously isolated from *diospyros rubra* (**Prachayasittikul *et al.*, 2010**) whose structure is given below.



Lup-20(29)-en-3-one **(81)**

Table 21: NMR data of TA1 compared with that reported in literature

TA1			Lupenone (Prachayasittikul *et al.*, 2010)	TA1			Lupenone (Prachayasittikul *et al.*, 2010)
Position	**^{13}C**	**^{1}H**	**^{13}C**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**
1	39.6		39.6	**17**	42.9		42.9
2	28.6		29.1	**18**	48.3		48.2
3	-		218.2	**19**	47.9	2.4 (m, 1H)	47.9
4	47.3		47.3	**20**	150.8		150.8
5	54.9		54.9	**21**	29.8	1.84 (m, 2H)	29.6
6	18.0		19.2	**22**	39.9		39.4
7	33.6		33.5	**23**	26.9	1.04 (s, 3H)	26.6
8	43.8		42.7	**24**	21.5	1.00 (s, 3H)	21.0
9	49.8		49.7	**25**	15.9	0.90 (s, 3H)	15.9
10	41.0		39.8	**26**	14.4	1.22 (s, 3H)	14.7
11	26.6		26.4	**27**	14.1	0.93 (s, 3H)	14.4
12	26.3		26.1	**28**	17.9	0.77 (s, 3H)	17.9
13	39.3		38.9	**29**	109.4	4.55 & 4.85 (s, 1Ha & 1Hb)	109.3
14	42.9		42.8	**30**	19.7	1.66 (s, 3H)	19.6
15	27.4		27.4				
16	35.5		35.5	-			

II-4-1-3: Structural identification of TA2

The mixture TA2 crystallized from the mixture hexane-CH_2Cl_2 (80/20, v/v) in the form of dirty-white crystals. It was found to be soluble in methanol and had a melting point of 208ºC -214 ºC. Two quasi-molecular ion peaks were observed on the ITMS-ESI Full MS mass spectrum of TA2 suggesting that TA2 is a mixture of two compounds that is an ion [M+H] at m/z 413.5 calculated for $C_{29}H_{48}O$, 412 g/mol with double bond equivalent of 6 and another ion [2M+Na] at 875.4 corresponding to $C_{30}H_{50}O$, 426 g/mol equally with double bond equivalent of 6. From the following masses, TA2 is evidently a mixture of a triterpene and a sterol.

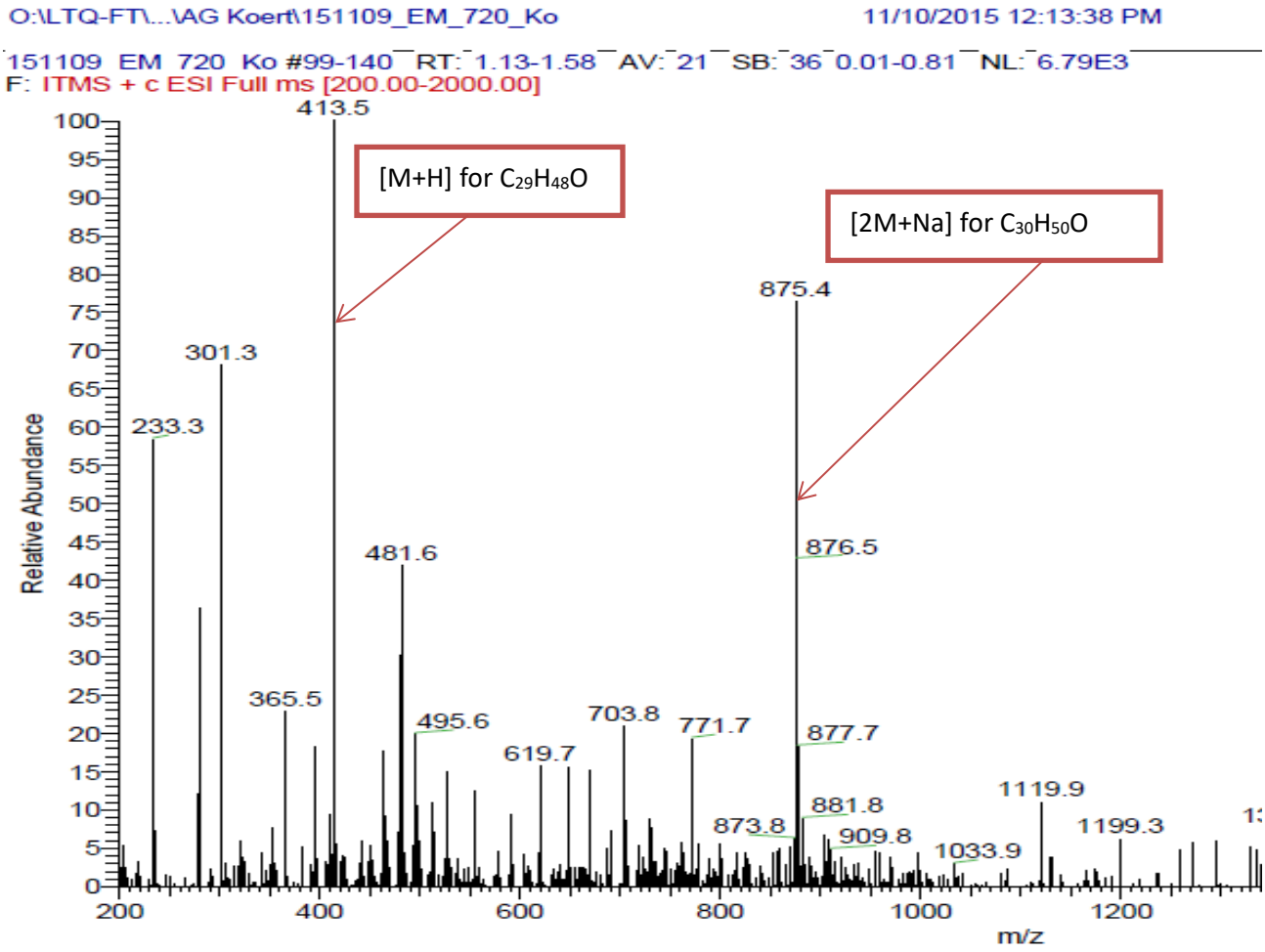


Figure 13: ITMS-ESI Full MS mass spectrum of TA2

The IR spectrum of TA2 indicated the presence of some functional groups including the following: broad band at 3300 cm^{-1} (O-H stretching), 2940 cm^{-1}(C_{sp2}-H stretching), 2840 and 2870 cm^{-1} (C_{sp3}-H stretching), 1453 and 1380 cm^{-1} (CH_3 and CH_2 bending) and finally 1043 cm^{-1} (C –O stretching)

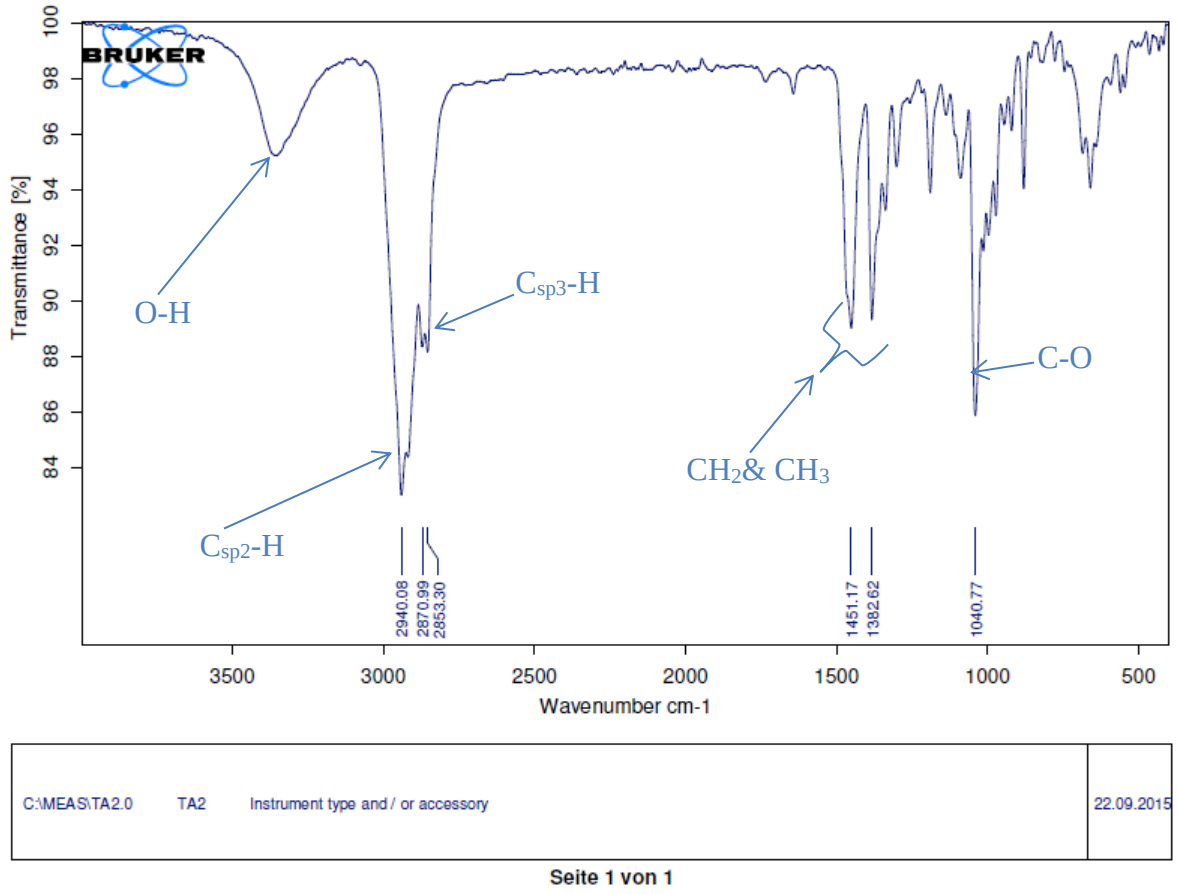


Figure 14: IR spectrum of TA2

On the ^1H NMR spectrum of TA2 ($CDCl_3$, 400 MHz) the following characteristic peaks are observed: between δ_H 0.70 ppm and 1.05 ppm six singlets ascribable to the methyl protons of the sp3 carbon atoms. Another singlet at δ_H 1.68 ppm reveals vinyl methyl protons carried by C-30 of triterpenes of the lupene type pentacyclic triterpenes. A multiplet at δ_H 3.25 ppm ascribable to the methine protons of pentacyclic triterpenes H-3, geminal with and at α-position to the β-hydroxyl group at position 3. Two singlets at δ_H 4.60 ppm and 4.75 ppm for H-29a and H-29b respectively, which are those of terminal methylene on C-29 position of the lup-20(29)-enes. A multiplet signal at δ_H 2.38 ascribable to 19β–H (m,1H, J = 10.9, 5.5 Hz) is characteristic of lupane type triterpenoids. These data agree with the ^1H NMR spectrum of lupeol as reported elsewhere (**Prachayasittikul *et al.*, 2010**). In addition, the ^1H NMR spectra of TA2 showed the presence of

additional methyl singlets, doublets and triplet between δ_H 0.71 ppm, and 1.03 ppm, olefinic protons at δ_H 5.14 ppm (m, 1H, H-20), 5.20 ppm (m, 1H, H-21) and 5.31 ppm (t, 1H, H-5) suggesting the presence of three olefinic protons corresponding to that of a tri-substituted C-5(6) and a di-substituted C-22(23) olefinic bonds of stigmasterol respectively.

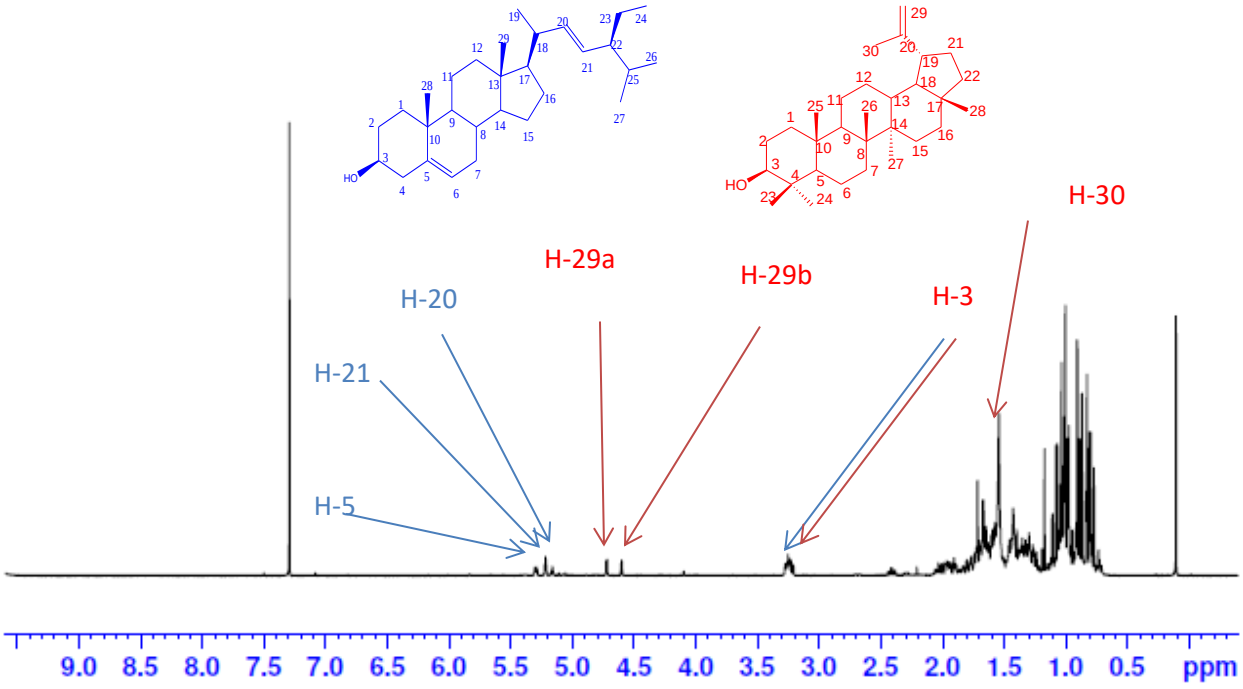


Figure 15: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of TA2

Key HSQC ($^1J_{C\text{-}H}$) correlations were observed between the proton H-21 δ_H 5.20 ppm and a carbon atom at 122.0 ppm (C-21), H-20 at δ_H 5.14 ppm and a carbon atom at 123.0 ppm (C-20), H-6 at δ_H 5.14 ppm and a carbon atom at 121.0 ppm (C-6) for stigmasterol and between the proton H-5 δ_H 0.66 ppm and a carbon atom at 56.0 ppm (C-5), the proton H-19 at δ_H 2.38 ppm and a carbon atom at 49.0 ppm (C-19) for lupeol and finally the proton H-3 at δ_H 3.25 ppm and a carbon atom at 79.0 ppm (C-3) common for lupeol.

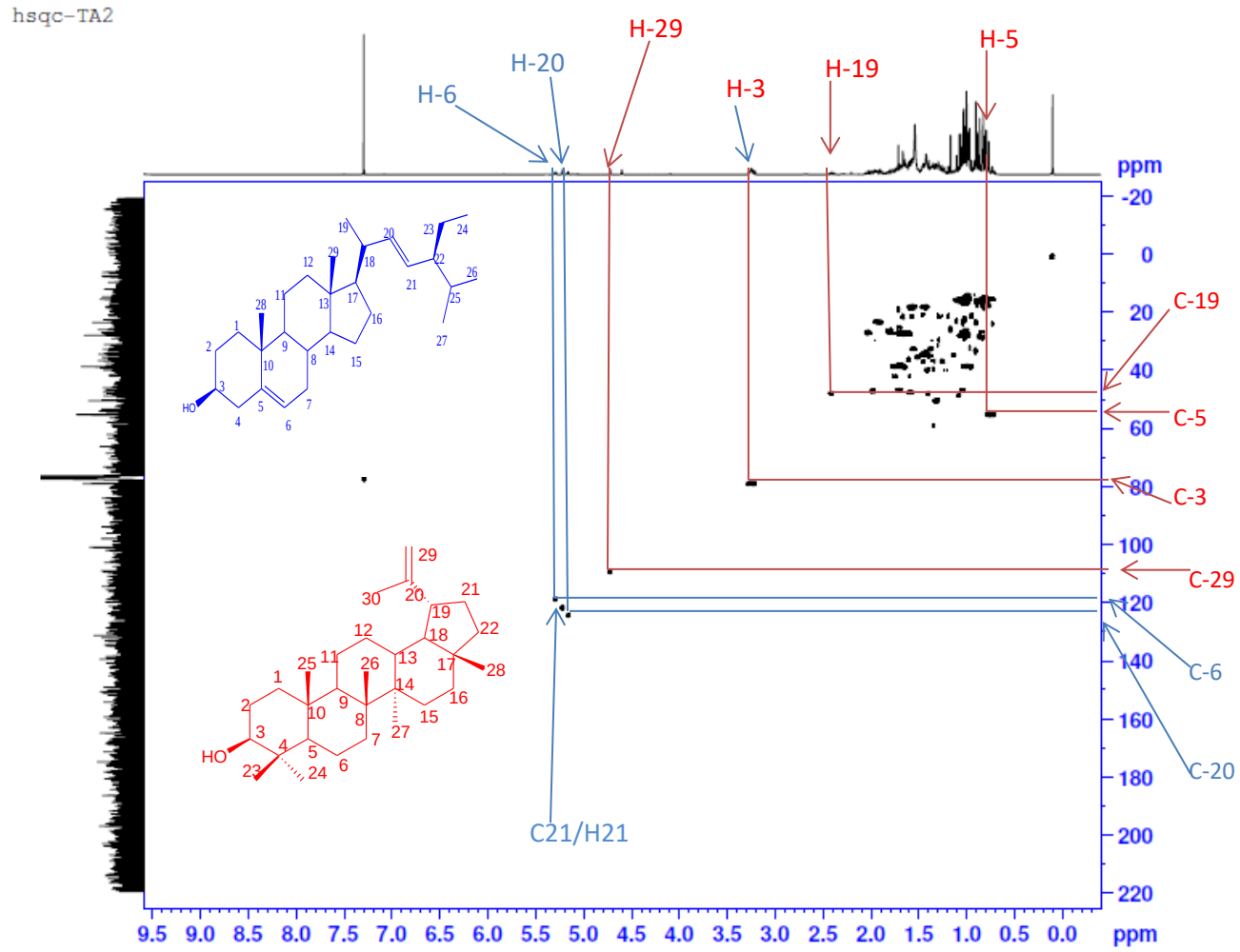


Figure 16: HSQC ($CDCl_3$) spectrum TA2

In the HMBC spectrum, the methine proton signal at δ_H 3.21 (H-3) showed cross peaks with a methyl carbon signal (δ_C 28.5, C-23) by J_3 correlation and a methyne carbon signal (δC 18.5, C-5) by J_3 correlation. The vinyl methyl signal at δ_H 1.68 (H-30) showed cross peaks with two carbons, a quaternary carbon and a methylene carbon signal at δC 150.9 (C-20) and δC 110.2 (C-29) respectively. The pair of broad singlets of olefinic proton at δ_H 4.55 ppm and 4.68 ppm showed cross peaks with a methylene carbon signal δC 48.8 ppm (C-19). The protons H-5 and H-3 also showed correlation with C-3.

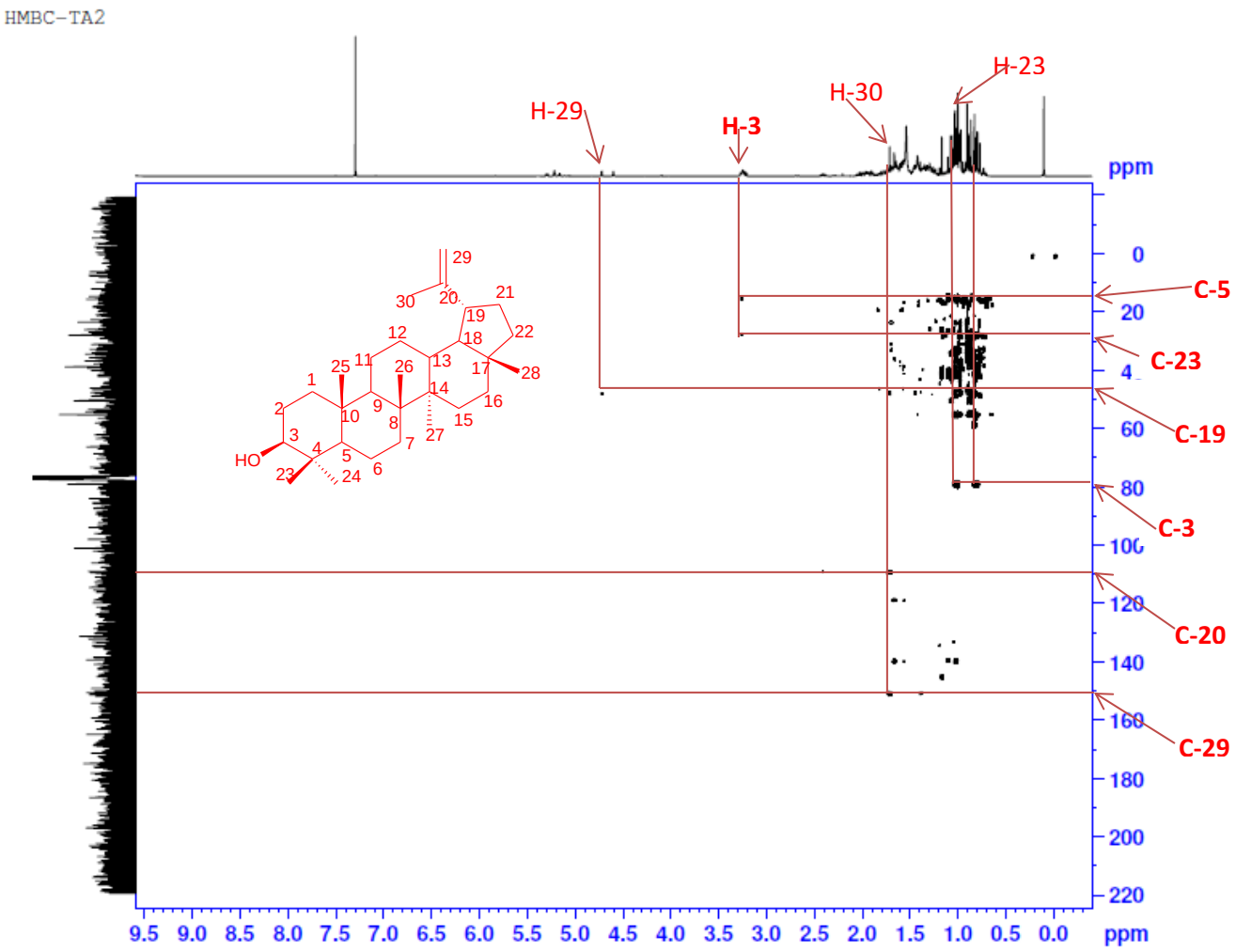


Figure 17: HMBC ($CDCl_3$) spectrum of TA2

The COSY spectrum of TA2 exhibited some cross peaks such as between δ_H 2.38 ppm, H-19 and one Sp3 methylene proton signal δ_H 1.40 ppm, H-21 and between oxygenated methine proton signal δ_H 3.25 ppm, H-3 and Sp3 methylene signal δH 1.60 ppm, H-2. Also, the two olefinic protons δH 4.60 ppm, H-29a and 4.75 ppm H-29b showed cross peaks with each other. Additional cross peaks were observed between the olefinic proton H-21 of stigmasterol and allylic Sp3 methylene proton signal at δ_H 1.80 ppm, H-22.

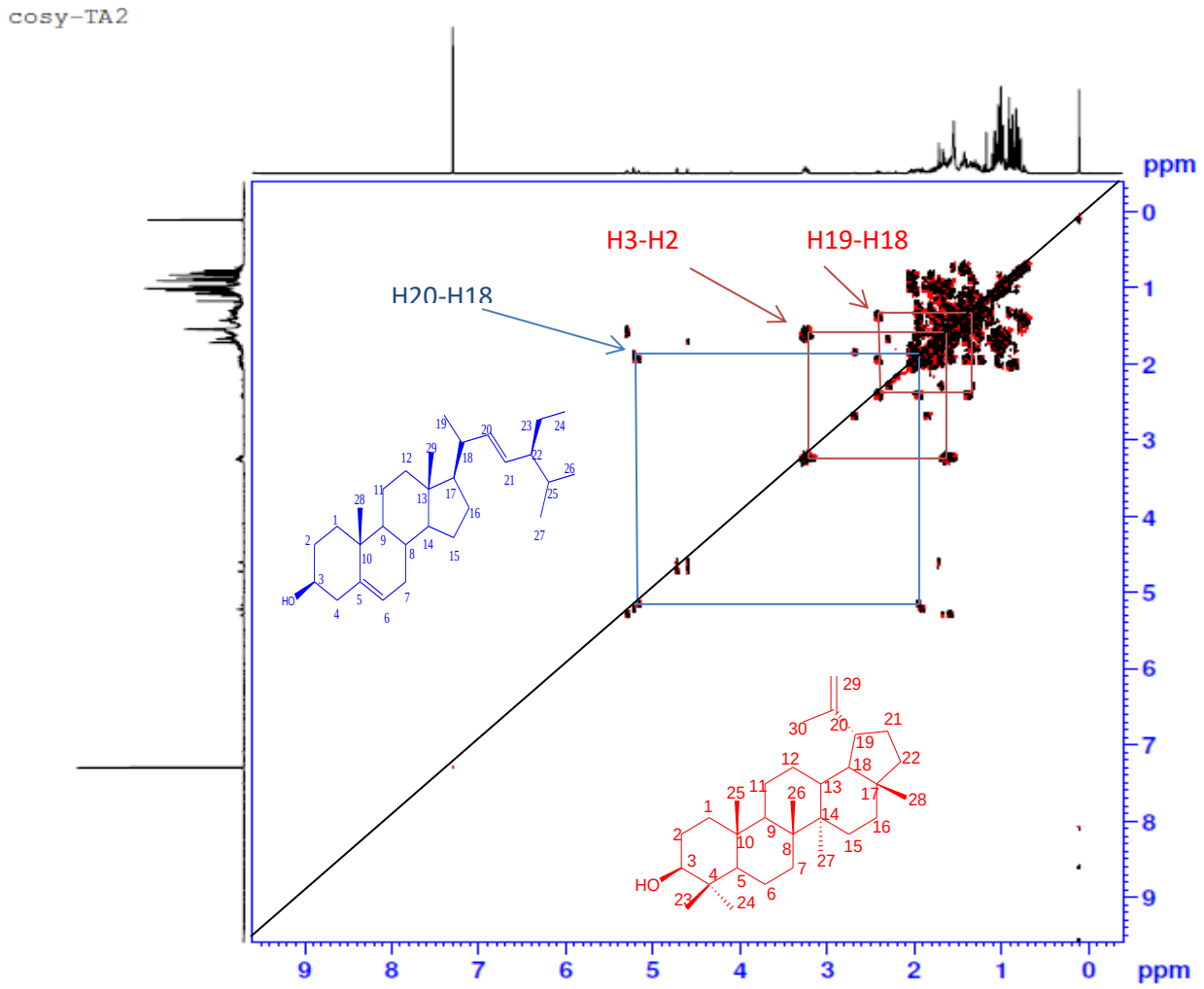
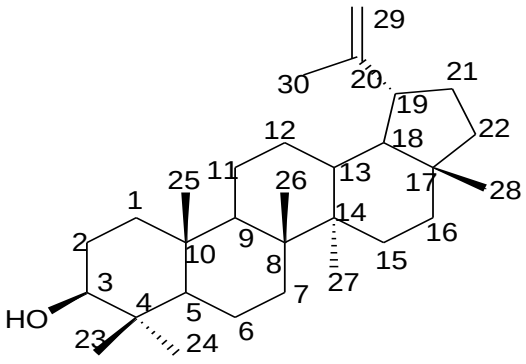
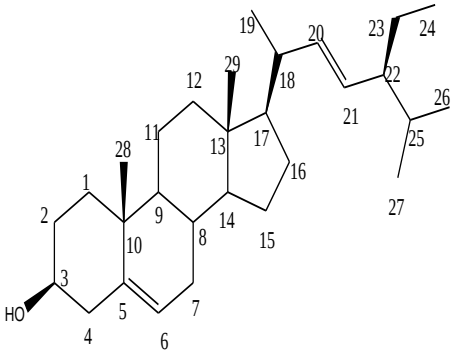


Figure 18: ^{1}H-^{1}H COSY spectrum ($CDCl_3$) of TA2

The set of these spectral data and information found in literature led us to identify TA2 as a mixture of lupeol and stigmasterol both compounds previously isolated from *Diospyros rubra* (**Prachayasittikul et al., 2009**)



Lupeol **(88)**



Stigmasterol **(134)**

II-4-1-4: Structural identification of CFB252

Compound CFB252 crystallized in the form of pale orange crystals in an eluent system of HEX/AcOEt (20/80, v/v). It melted between 186-188°C and gave a positive test with Liebermann-Burchard reagent. The ESI-TOF MS spectrum of CFB252 showed a pseudo-molecular ion peak of $[M+Na]^+$ at m/z = 493.3 and another one [M+2Na-H] at m/z = 515.3 corresponding to a mass of 470 g/mol for which the empirical formula is $C_{30}H_{46}O_4$. Some diagnostic ion fragments were observed as follows: $[M-C_8H_{11}O_3]$ at m/z = 315.2 and $[M-C_8H_{11}O_3-C_9H_{13}O]$ at m/z = 178.8 which are typical for cycloartane type triterpenoids. From the molecular formula, the double bond equivalent of the compound was calculated to be 8.

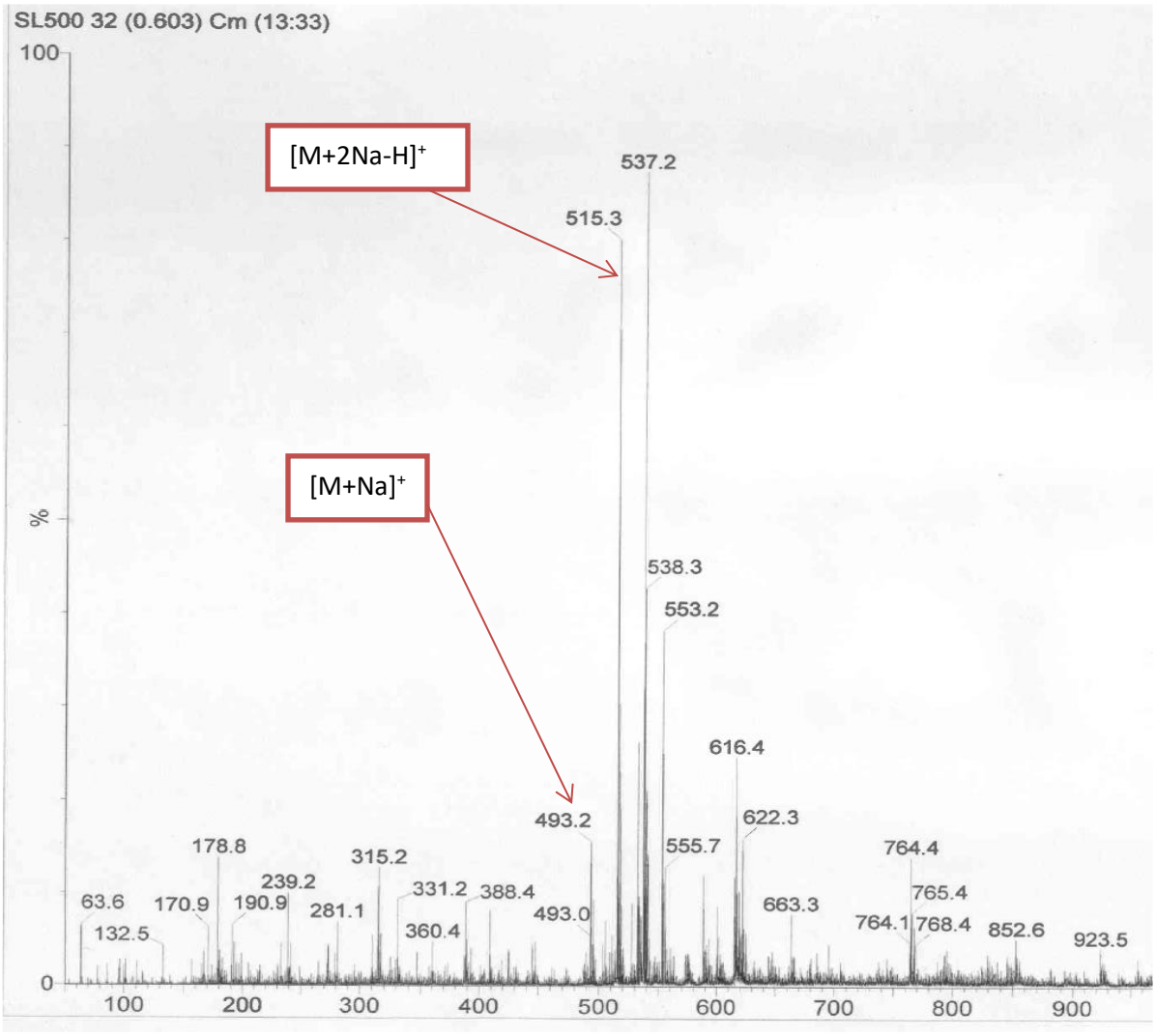


Figure 19: ESI-TOF MS spectrum of CFB252

^{1}H NMR Spectrum CFB252 ($CDCl_3$, 600.3 MHz) showed the following data: two doublets at δ_H = 0.60 and 0.77 ppm (d, 2H, J = 4.2 Hz) characteristic of cyclopropane methylene protons Hβ-19 and Hα-19. Singlets of 4 methyl groups:2 methyl singlets at δ_H 1.05 ppm (s, 3H) and 1.08 ppm (s, 3H) in position for the tertiary methyl protons H-28 and H-29 and 2 other methyl singlets at δ_H 0.91 (s, 3H) and 0.99 ppm (s, 3H) for the methyl protons H-30 and H-18 respectively. A tertiary methyl doublet at δ_H 0.93 ppm (d, 3H, J=6.4 Hz) attributable to the protons H-21. A strong singlet of a deshielded hydroxylated allylic methyl at δ_H 4.37 ppm (s, 2H) corresponding to the protons of the hydroxyl methyl group (-CH_2OH) H-27. An olefinic proton appearing as a triplet at δ_H 7.2 ppm (t, 1H, J=7.8 Hz) this corresponding to the proton H-24 whose coupling constant value indicates a cis vicinal coupling. Two protons of carbon 1 (H-1), one appears as a triple of double doublet (tdd) at δ_H = 1.85 ppm and the other as a multiplet at δ_H 1.56 ppm. Two other protons (H-2) of which one appears as doublet of double doublet (ddd) at δ_H = 2.29 ppm with coupling constants J=13.9, 4.2 and 2.4 Hz respectively and the other proton appears as triple doublet (td) at δ_H = 2.75ppm and J=13.9 and 6.4 Hz.

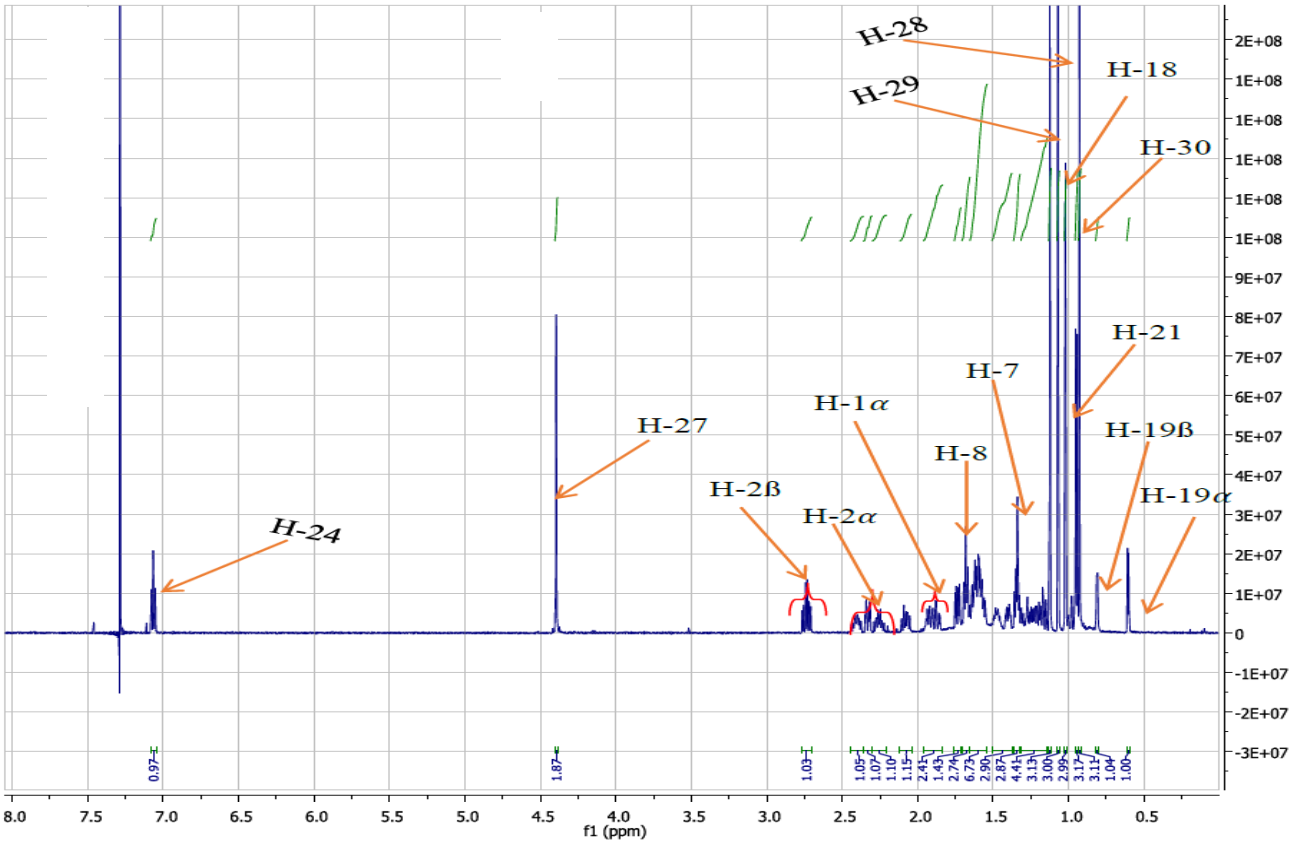


Figure 20: ^{1}H NMR spectrum ($CDCl_3$, 600.3 MHz) of CFB252

^{13}C NMR Spectrum ($CDCl_3$, 125 MHz) of the compound CFB252 shows 5 signals at 18.2 ppm, 22.2 ppm, 20.8 ppm, 19.3 ppm and 18.1 ppm corresponding to five angular methyls C-18, C-28, C-29, C-30 and C-21 respectively of a cycloartane triterpene skeleton. The signal at 29.7 is characteristic of carbon C-19 of the cyclopropane of the cycloartanes. Two signals appear at 149.2 ppm and 130.0 ppm ascribable respectively to olefin carbon atoms C-24 and C-25.

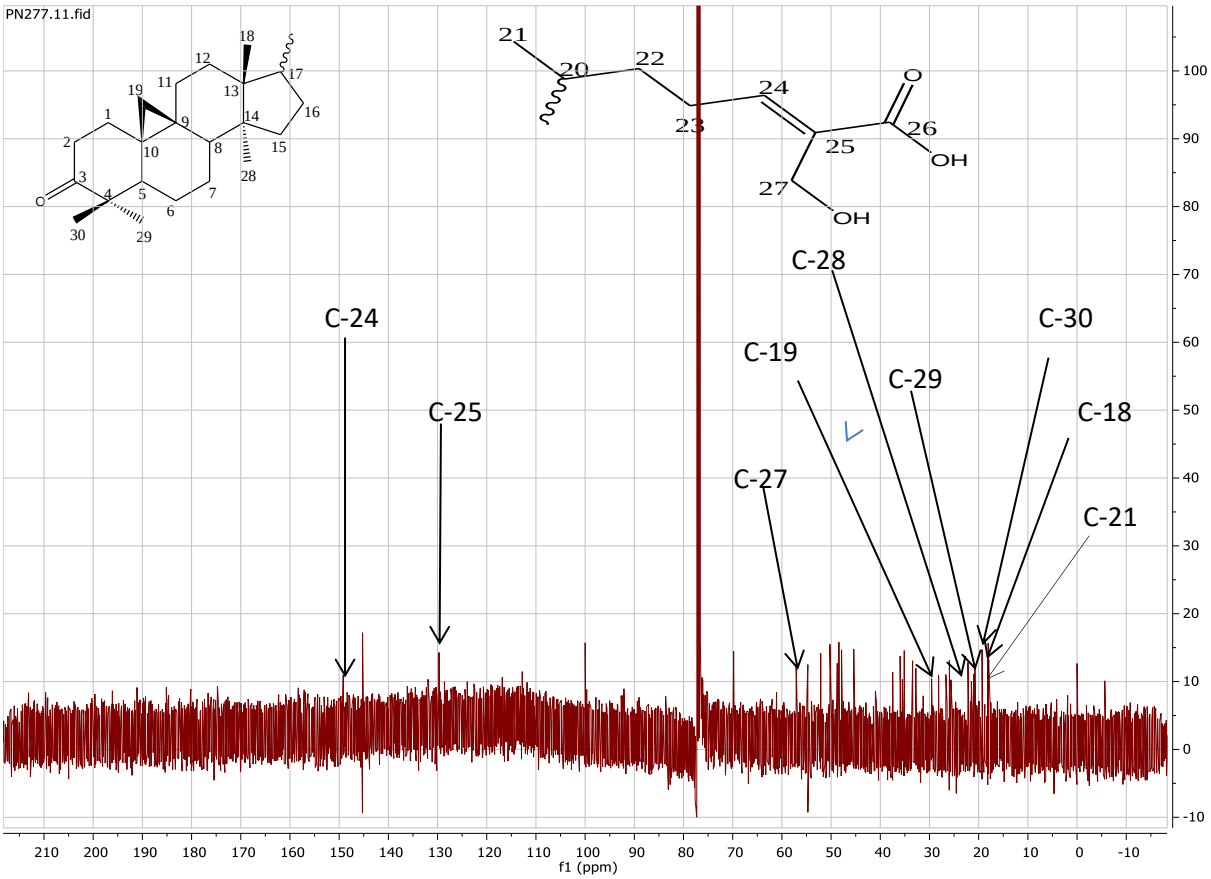


Figure 21: ^{13}C NMR Spectrum ($CDCl_3$, 125 MHz) of CFB252

The HSQC ($^1J_{C-H}$) spectra of CFB252 showed correlation spots between the following pairs of protons and carbon atoms: δ_H =2.75 ppm (H-2), δC =38.2 ppm (C-2), two protons at δ_H=0.77 and 0.60 ppm (H-19) and carbon at δC = 29.9 ppm (C-19), δ_H=2.10 ppm (H-23) and δC =25.7 ppm (C-23), δ_H=4.37 ppm (H-27) and δC =57.1 ppm (C-27) and δ_H=1.61 ppm (H-17) and δC =52.2 ppm (C-17).

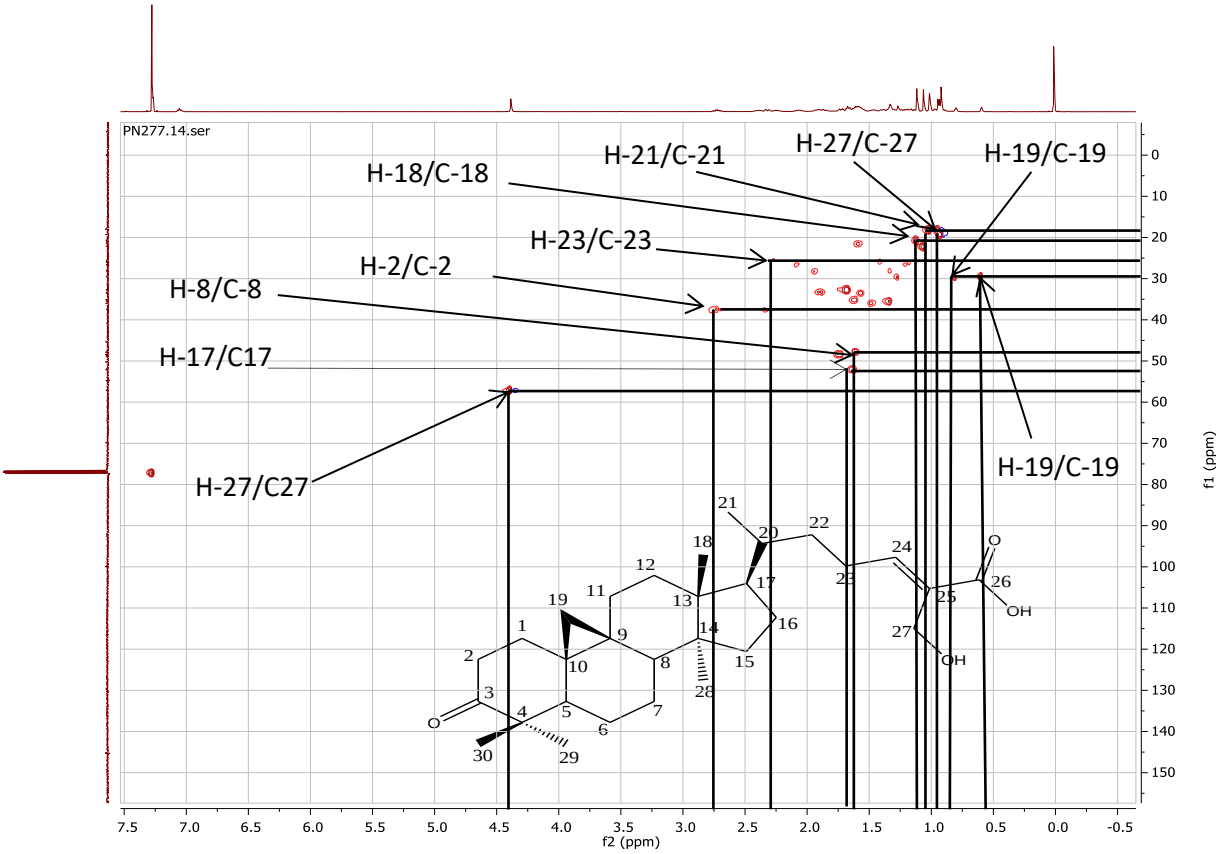


Figure 22: HSQC Spectrum of CFB252

HMBC spectrum of CFB252 shows long distance proton/carbon correlation (^{n}J coupling where n>1) as follows: the proton at δ_H = 1.05 ppm (H-28) and carbon at δ_C = 2 0.8 ppm (C-29), the proton at δ_H = 1.08 ppm (H-29) and carbon with δC =21.5 ppm (C-4), the proton at δ_H = 1.01 ppm (H-18) and carbon at δ_C = 32.7 ppm (C-12), the proton at δ_H = 0.93 ppm (H-21) and carbon at δC = 35.9 ppm (C-20) and the proton at δ_H = 4.37 ppm (H-27) and carbon at δ_C = 171.2 ppm (C-26).

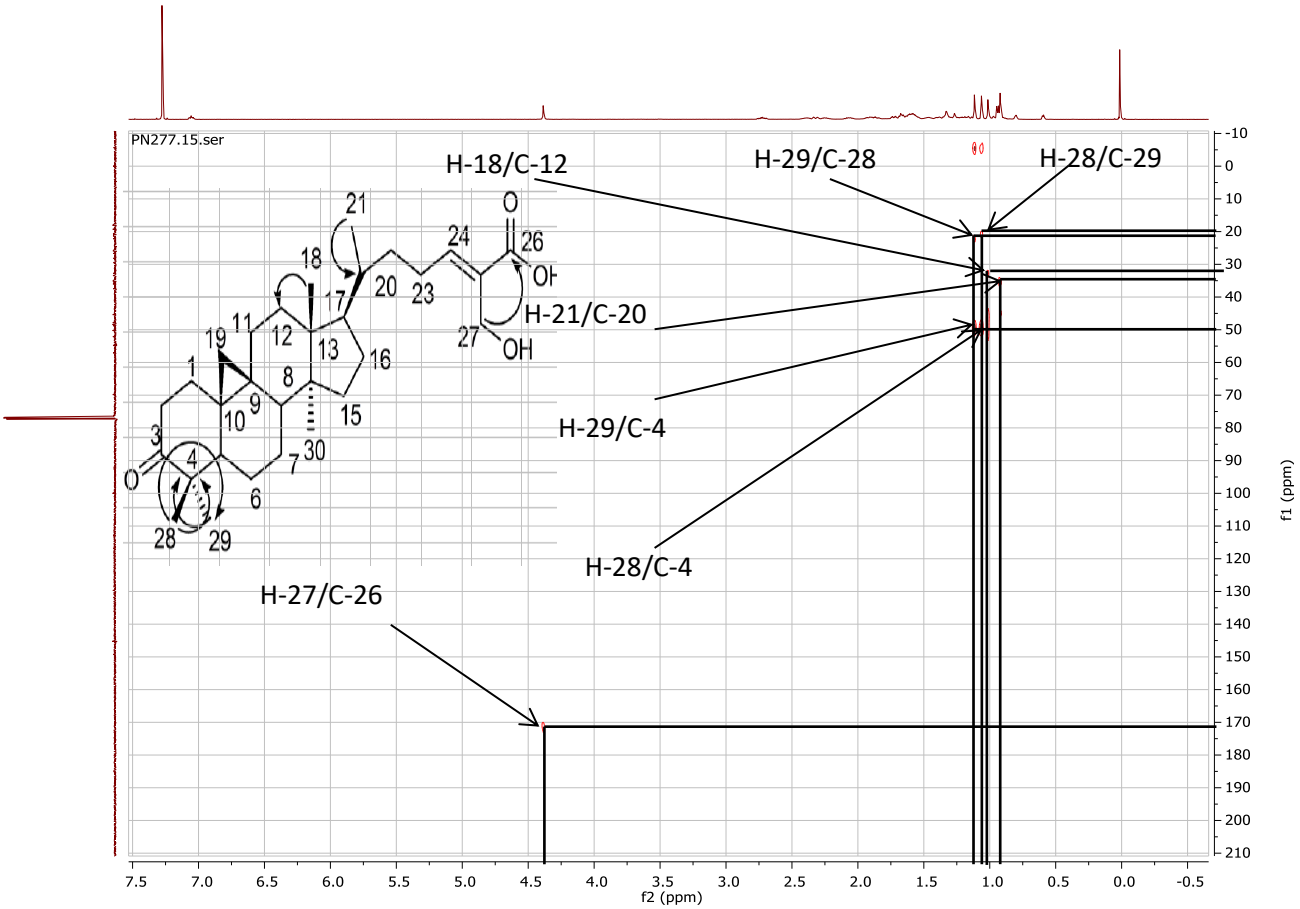


Figure 23: HMBC Spectrum of CFB252

^{1}H-^{1}H COSY spectrum of CFB252 showed correlation peaks between some adjacent protons for example, δ_H=2.10 ppm (H-23) and the proton at δ_H =7.20 ppm (H-24) and the proton at δ_H=2.15 ppm (H-5) and that at δ_H =1.56 ppm (H-6). Also, the pairs of germinal protons Hα -2/ H_β-2 and Hα -19/ H_β-19 showed ^{1}H-^{1}H COSY correlation peaks.

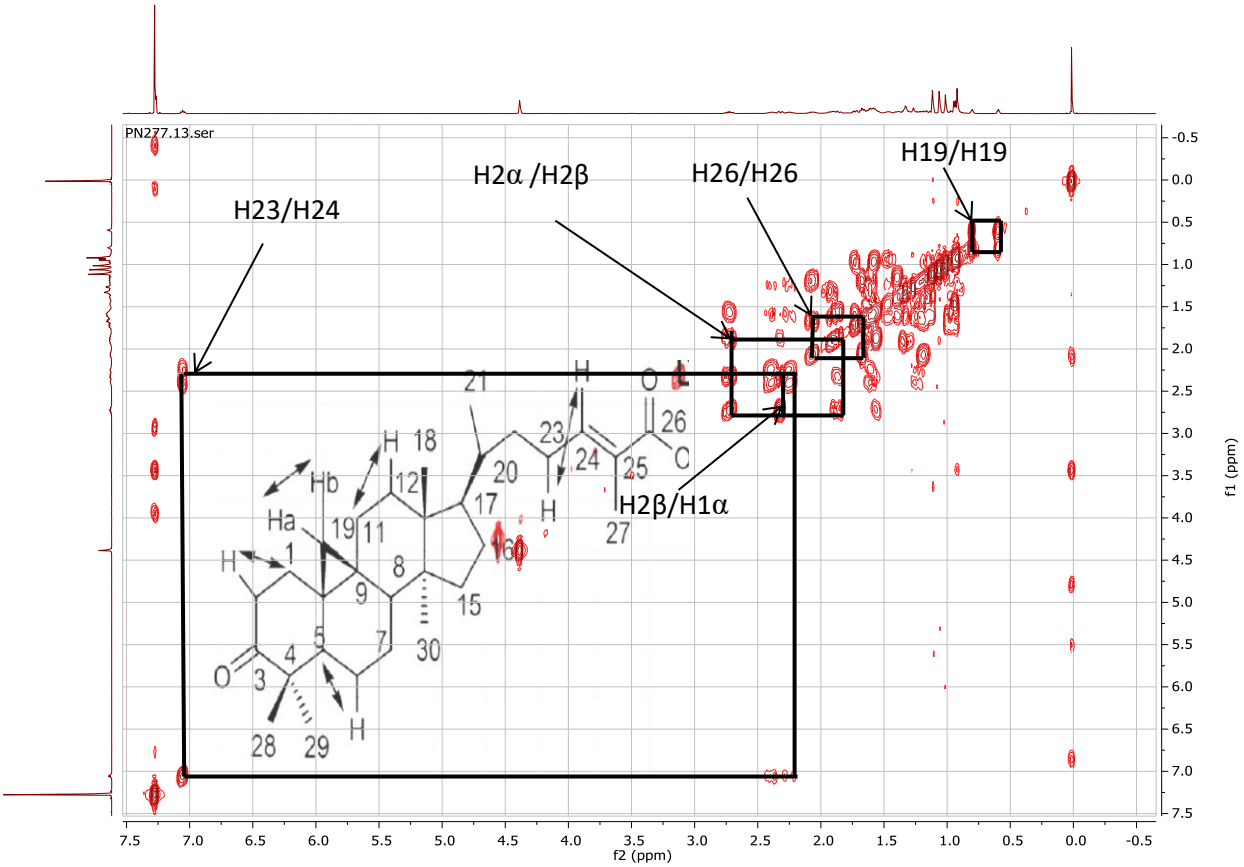
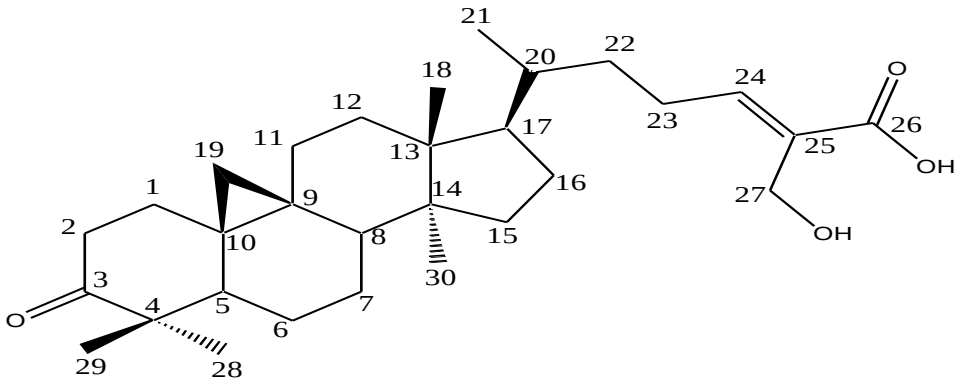


Figure 24: ^{1}H-^{1}H COSY spectrum of CFB252

The collection of this structural information compared with the data reported by Li *et al.*, (2009) enabled us to identify compound CFB252 as 3-oxo-27-hydroxycycloartan-24-en-26-oic acid with trivial name 27-hydroxymangiferonic acid previously isolated from Myanmar propolis (**Li *et al.*, 2009**) whose structure is represented below.



3-oxo-27-hydroxycycloartan-24-en-26-oic acid **(135)**

Table 22: NMR data of CFB252 (27-hydroxymangiferonic acid) compared with literature data

CFB252			3-oxo-27-hydroxycycloartan-24-en-26-oic acid (Li et al., 2009)	CFB252			3-oxo-27-hydroxycycloartan-24-en-26-oic acid (Li *et al*., 2009)
Position	**^{13}C**	**^{1}H**	**^{1}H**	**Position**	**^{13}C**	**^{1}H**	**^{1}H**
1	33.6	1.85 &1.56	1.87 &1.56	**17**	52.2	1.61	1.61
2	38.2	2.29 &2.75	2.29 &2.72	**18**	18.2	0.99	0.99
3	218	/	/	**19**	29.7	0.60 &0.77	0.61 &0.82
4	55.0	/	/	**20**	36.0	1.45	1.45
5	42.8	2.15	2.15	**21**	18.1	0.93	0.93
6	21.2	1.56 &0.97	1.56 &0.97	**22**	35.2	1.60	1.60
7	28.2	1.90 &1.35	1.91 &1.37	**23**	25.7	2.10	2.19
8	47.8	1.61	1.61	**24**	130.0	7.02	7.02
9	21.0	/	/	**25**	149.2	/	/
10	25.7	/	/	**26**	171.2	/	/
11	26.7	2.25 &1.20	2.25 &1.21	**27**	57	4.37	4.37
12	32.8	1.65	1.67	**28**	22.2	1.05	/
13	45.4	/	/	**29**	20.8	1.08	1.08
14	48.8	/	/	**30**	19.3	0.91	0.91
15	35.5	1.29	1.29				
16	25.5	1.35	1.39	**-**			

II-4-1-5: Structural identification of ISGII

Compound ISGII crystallized in the form of white solid in an eluent system of hexane-ethyl acetate (40/60, v/v). It melted between 188-192°C and gave a positive test with Liebermann-Burchard reagent. The ESI-TOF MS spectrum of ISGII showed a pseudo-molecular ion peak of $[M+Na]^+$ at m/z = 477.3 and another one [2M+Na] at m/z = 931.6 corresponding to a mass of 454 g/mol for which the empirical formula is $C_{30}H_{46}O_3$. Some diagnostic ion fragment at m/z = 178.8 is typical for cycloartane type triterpenoids. From the molecular formula, the double bond equivalent of the compound was calculated to be 8.

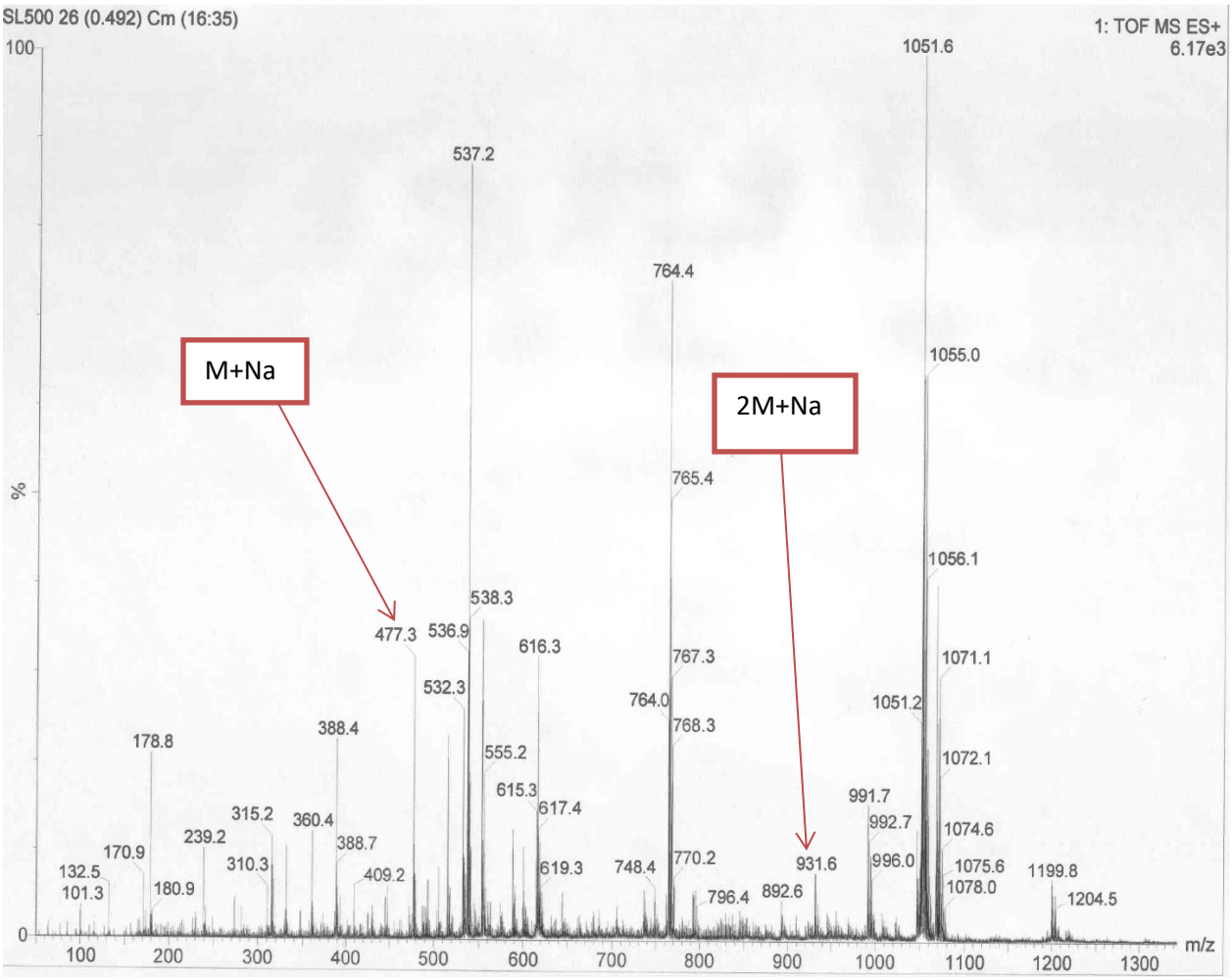


Figure 25: ESI-TOF MS spectrum of ISGII

^1H NMR Spectrum ISGII ($CDCl_3$, 600.3 MHz) showed a characteristic pair of cyclopropane methylene protons at δ_H = 0.58 and 0.78 ppm (1H, J = 4.2 Hz) for the protons H_β-19 and $H\alpha$-19

respectively. Three singlet signals for the 4 tertiary methyl groups appeared at δ_H 1.10 ppm (s, 3H) for H-29, 1.08 ppm (s, 3H) for H-18 and finally 2 singlets at δ_H 1.04 ppm (s, 3H each) for H-28 and H-30. These proton types suggested that ISGII is a cycloartane type triterpenoid. A tertiary methyl doublet at δ_H 0.91 ppm (d, 3H, J=6.4Hz) attributable to the protons H-21. A strong singlet of a lower field shift resonance of a vinyl methyl at δ_H 1.85 ppm (s, 3H) corresponding to the protons H-27. A vinyl proton appearing as a triplet at δ_H 6.90 ppm (t, 1H, J=7.2 Hz) corresponding to the proton H-24 whose coupling constant value indicates a Cis-vicinal coupling. Two protons (H-1) each appears as a multiplet at δ_H = 1.85 ppm and δ_H 1.56 ppm. Two other protons (H-2) with signals as multiplet at δ_H = 2.31 ppm and δ_H = 2.71 ppm.

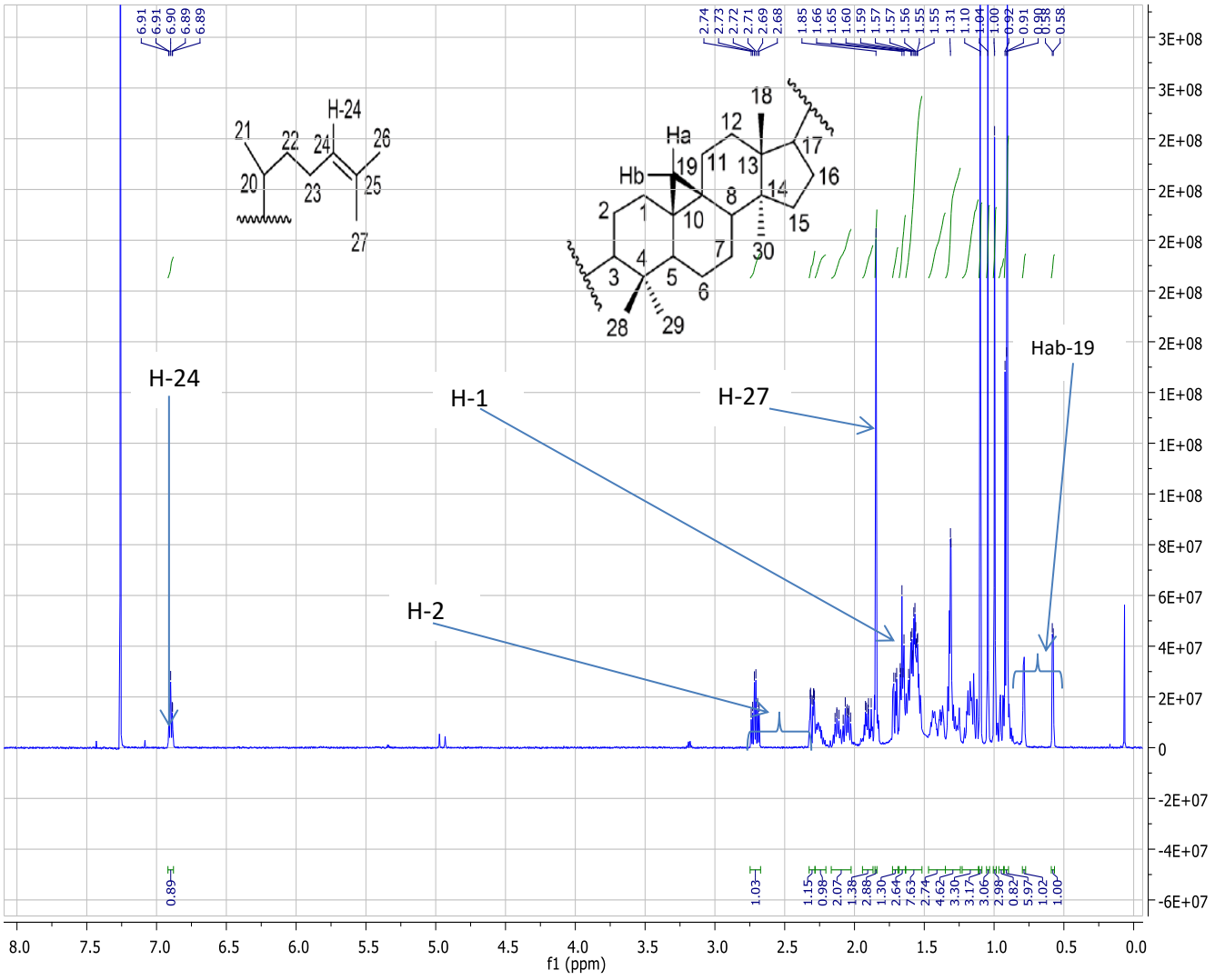


Figure 26: ^{1}H NMR spectra ($CDCl_3$, 600.13 MHz) of ISGII

^{13}CNMR Spectrum ($CDCl_3$, 150 MHz) of the compound ISGII shows a signal at 29.7 ppm which is characteristic of carbon C-19 of the cyclopropane of a cycloartane. Two signals appear at 128.1 ppm and 143.5 ppm ascribable respectively to olefin carbon atoms C-25 and C-24. A carbonyl carbon of an αβ-unsaturated acid (C-26) appears at δ_C 169.1 ppm. The tertiary methyl C-27 appears at about 12.4 ppm.

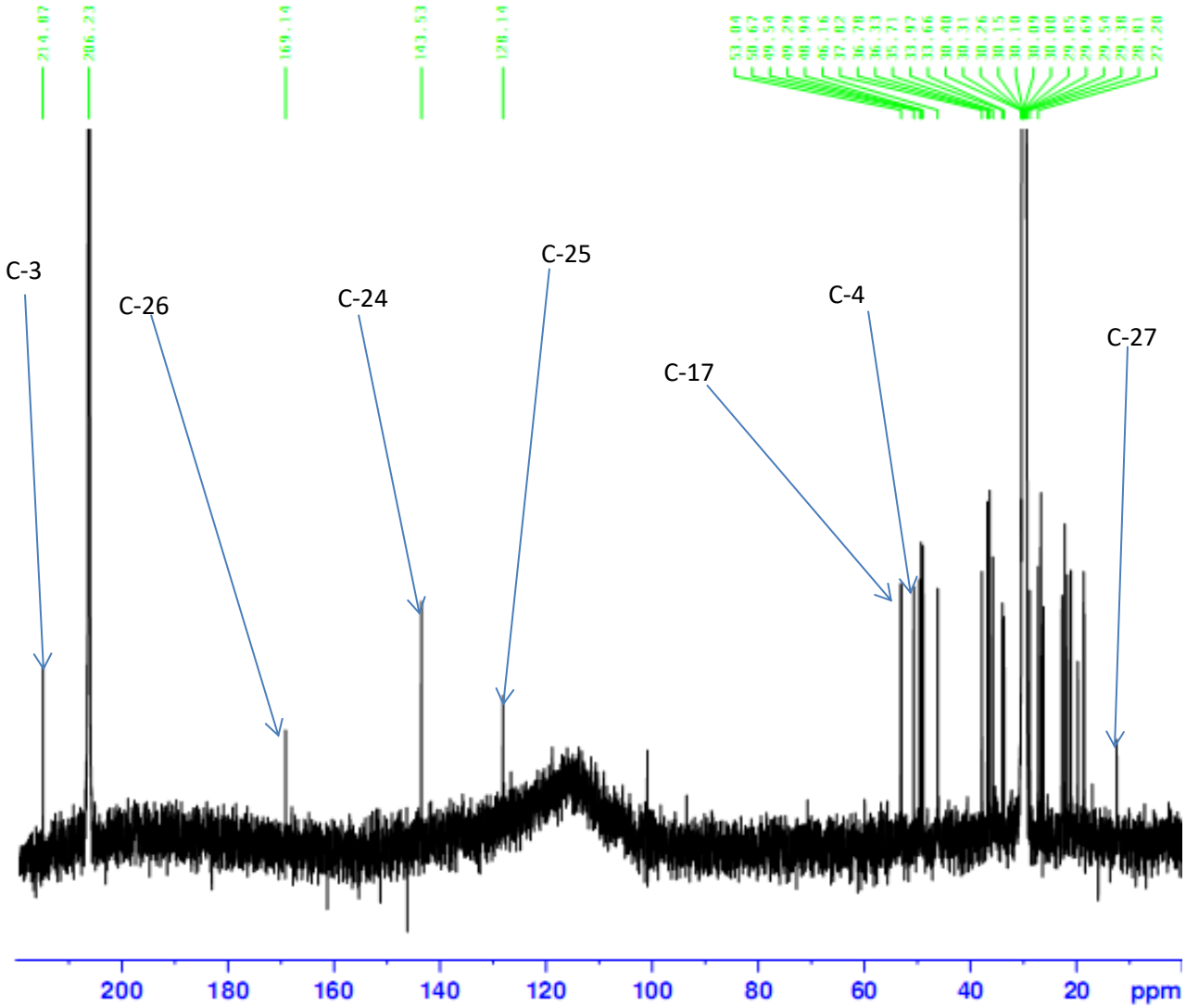


Figure 27: ^{13}C NMR Spectrum (d6-acetone, 125 MHz) of ISGII

The spectral data above was further substantiated by 2D-NMR spectral data notably HSQC, HMBC as shown below.

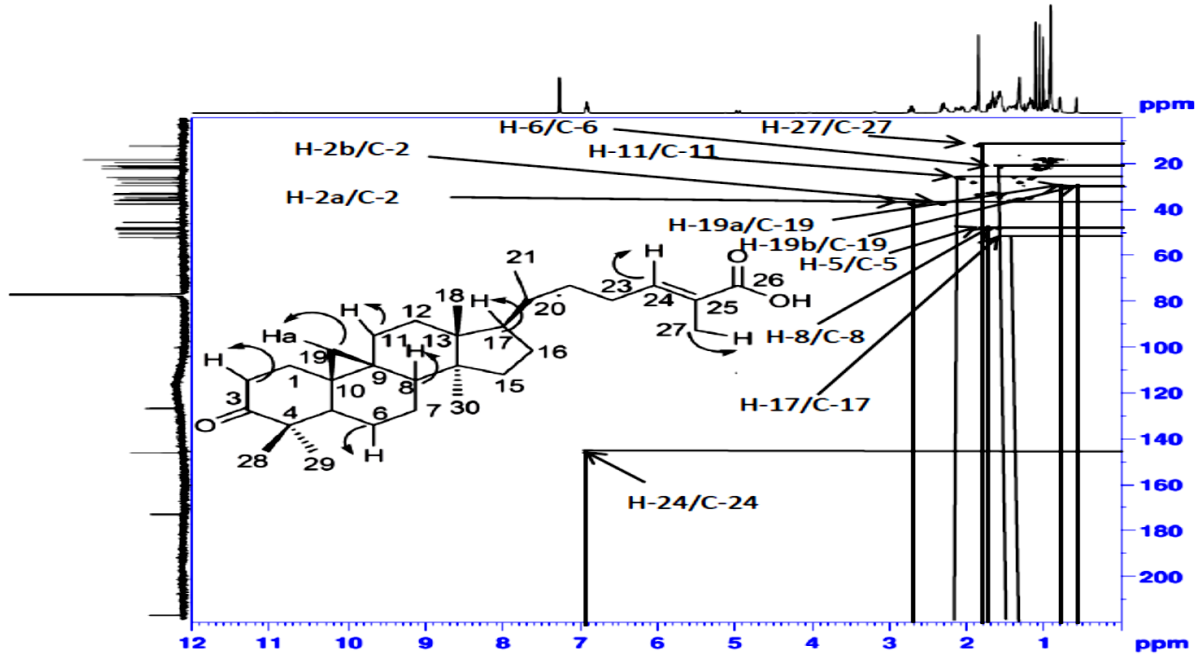


Figure 28: HSQC spectrum of ISGII

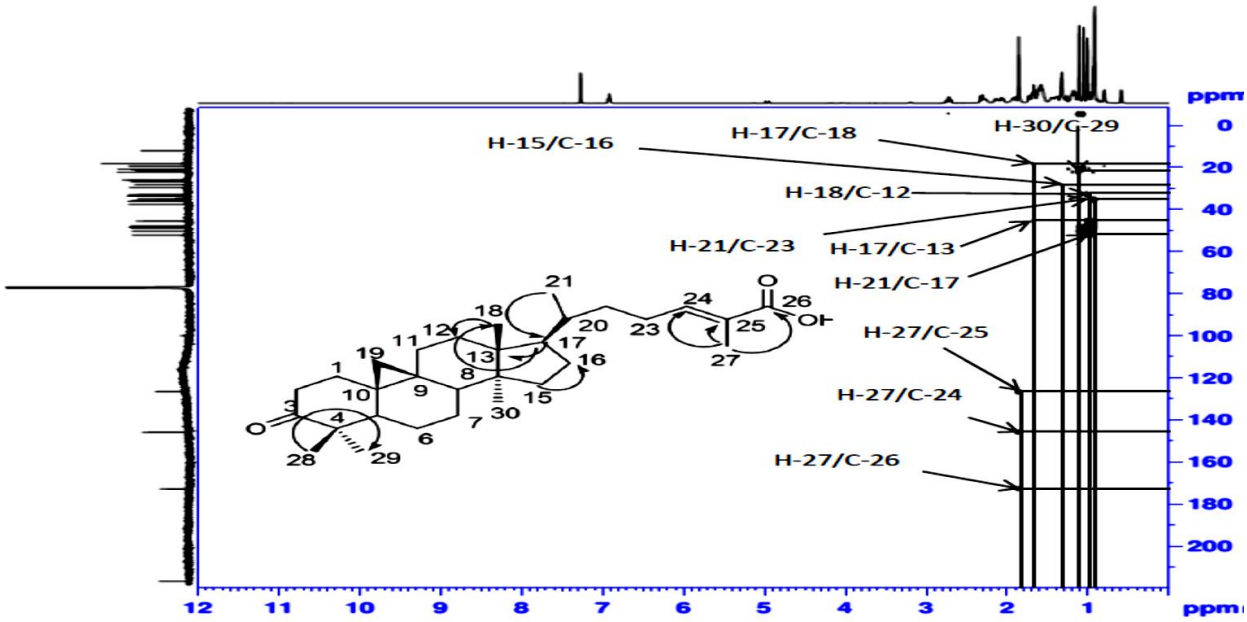
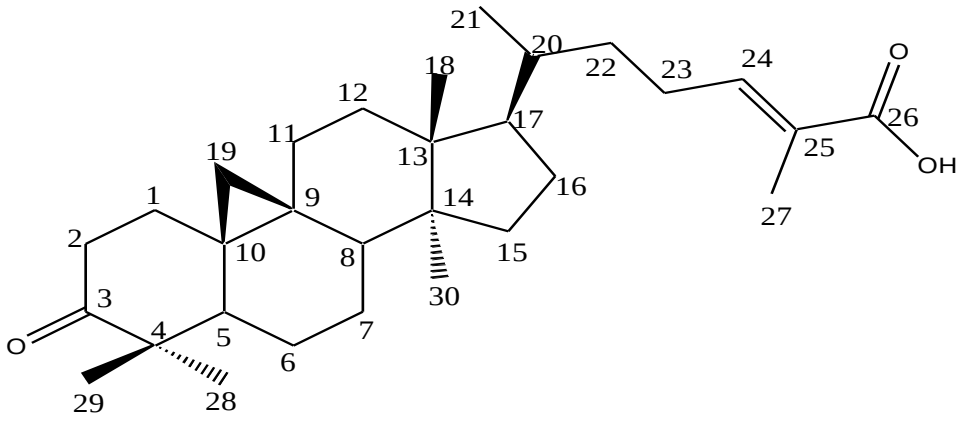


Figure 29: HMBC spectrum of ISGII

The 2D spectral information on bond connectivities lead to the identification of ISGII as mangiferonic acid which has been isolated previously from *mangifera indica* (**Carolina *et al.*, 2011**) and its structure is given below.



Mangiferonic acid **(78)**

Table 23: NMR data of mangiferonic acid compared with literature data

ISGII			Mangiferonic acid (Carolina *et al.*, 2011)	ISGII			Mangiferonic acid (Carolina *et al.*, 2011)
Position	**^{13}C**	**^{1}H**	**^{13}C**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**
1	33.4	1.85, 1.56 (m, 2H)	33.4	**17**	52.2	1.61, m	52.2
2	37.6	2.31, 2.71 (m, 2H)	37.5	**18**	18.1	1.08, m	18.1
3	214.8	-	216.6	**19**	29.6	0.58, 0.78 (2H, d)	29.6
4	50.3	-	50.3	**20**	37.5	-	36.0
5	48.4	1.69, m	48.4	**21**	21.1	0.91, d	18.1
6	21.5	1.53, m	21.5	**22**	25.9	-	29.6
7	25.7	1.34, m	25.8	**23**	34.8	-	34.8
8	47.9	1.5, d	47.8	**24**	143.5	6.9, t, J=7.2 Hz	145.8
9	21.1	-	21.1	**25**	128.1	-	126.6
10	25.9	-	25.9	**26**	169.1	-	173.0
11	26.7	-	26.7	**27**	12.4	1.85, s	11.9
12	32.8	1.64, m	32.8	**28**	19.3	1.04, s	22.2
13	45.4	-	45.4	**29**	20.8	1.10, s	20.8
14	48.8	-	48.7	**30**	22.2	1.04, s	19.3
15	35.5	1.35m	35.6				
16	28.2	-	28.2	**-**			

II-4-1-6: Structural identification of PF2

The compound PF2 crystallized in the form of a white solid in the eluent system Hexane-ethyl acetate (90/10, v/v) with a melting point of 254°C – 257°C. PF2 gave a reddish-violet coloration when treated with the Liebermann-Burchard reagent indicating that this compound is a triterpene. ^{1}H NMR Spectrum PF2 (Pyr, 500 MHz) showed a characteristic doublet of doublets at δ_H 3.13 ppm (dd, 1H, J =12 Hz and 6 Hz), which is characteristic of an α-oriented hydrogen at C-3 (H-3α) of a 3β-hydroxy triterpene. A pair of singlet for geminal protons (Hα-29 and Hβ-29) at δ_H 4.68 ppm (s, 1H) and δ_H 4.55 ppm (s, 1H) alongside the methyl group at δ 1.69 ppm (s, 3H,) attributable to the vinyl methyl protons (H-30) thereby suggesting that PF2 is a lupeol-type triterpene derivative. Another singlet at δ_H3.31 ppm (s, 1H) rather than a seventh methyl singlet around δ_H 0.80 ppm, confirms the presence of a second hydroxyl group at C-28. Other tertiary methyl protons appear as follows: δ_H 0.97 ppm (s, 3H, H-27), δ_H 0.95 ppm (s, 3H, H-26), δ_H 0.85 ppm (s, 3H, H-23), δ_H 0.80 ppm (s, 3H, H-25) and δ_H 0.75 ppm (s, 3H, H-24). This is in agreement with the ^{1}H NMR data reported for betulin previously isolated from *Diospyros rubra* (**Prachayasittikul *et al.*, 2010**).

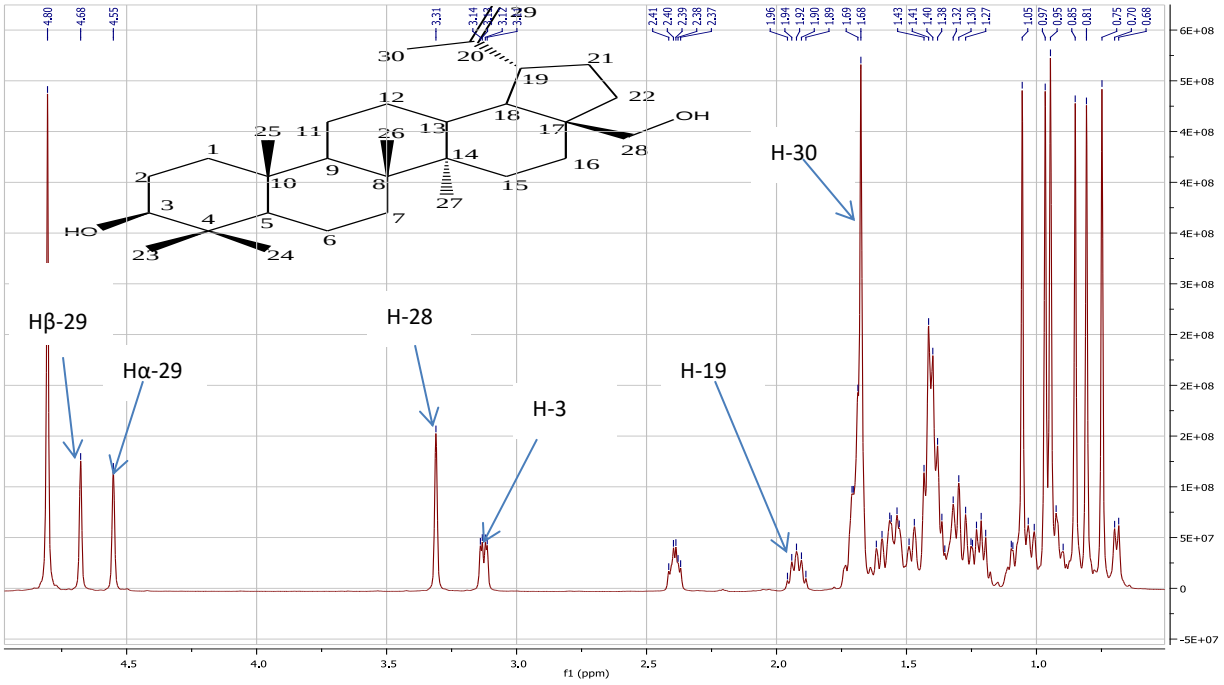


Figure 30 A: ^{1}H NMR Spectrum PF2 (Pyridine, 500 MHz)

The EI-MS spectrum of PF2 showed a molecular ion peak of M+ at m/z= 442.3 from which the molecular formula $C_{30}H_{50}O_2$ is confirmed.

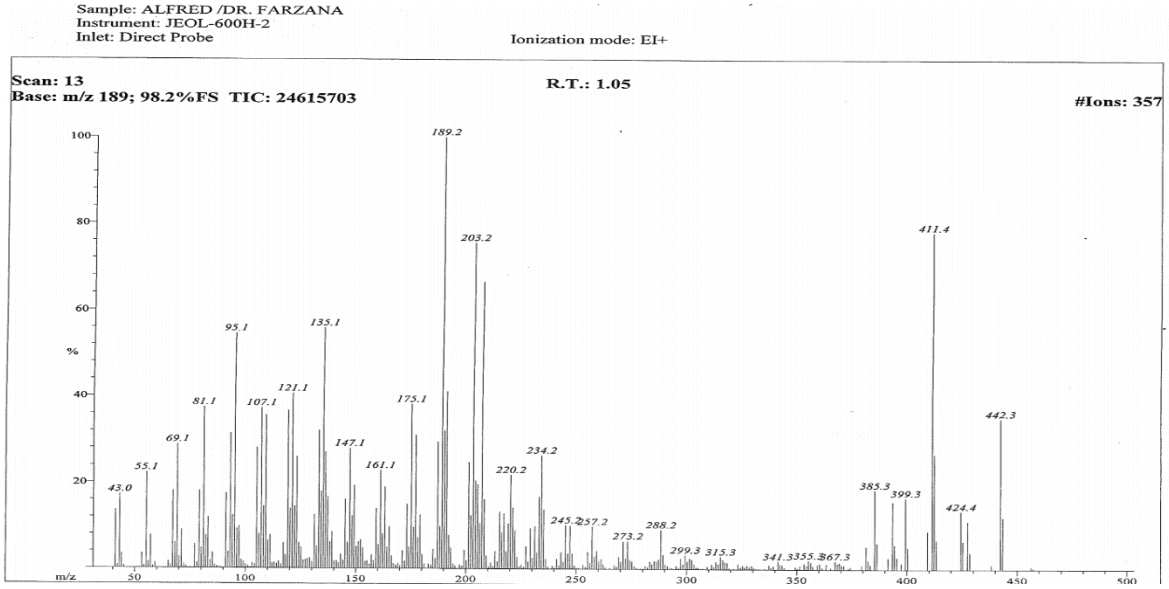
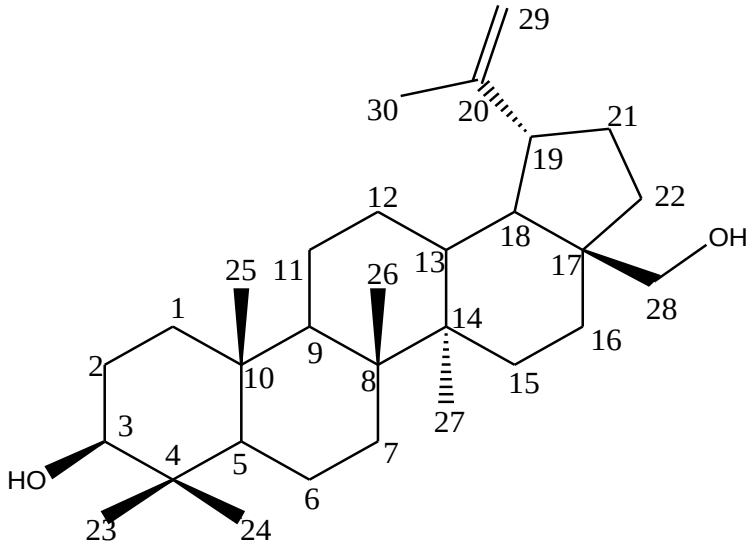


Figure 30 B: EI-MS Spectrum PF2

The ^{13}C NMR Spectrum of PF2 ($CDCl_3$, 125 MHz) showed chemical shifts of a characteristic pair of sp2 carbons at δ_C 151.8 ppm and δ_C 110.2 ppm corresponding to C-20 and C-29 comprising the exocyclic double bond of a lupane type triterpene respectively (Mahato and Kundu, 1994). Chemical shifts of the oxygenated carbon atoms C-3 and C-28 were observed at δ_C 79.6 ppm and δ_C 78.8 ppm respectively. Other characteristic methines appear at δ_C 56.7 ppm (C-5), δ_C 51.7 ppm (C-9), δ_C 49.2 ppm (C-19) and δ_C 49.1 ppm (C-18). The quaternary C-17 and C-4 appear at δ_C 44.1 ppm and six angular methyls between δ_C 15.3 ppm for C-27 and δ_C 28.7 ppm for C-23. In all, the spectra revealed a compound with thirty carbon atoms (which is equivalent to the total number of carbon atoms in triterpenoid) of which six are methyl groups corresponding to a lupene-type triterpenoid nucleus with two hydroxyl groups at C-3 and C-28. Consequently, the compound is identified to be the known compound lup-20(29)-en-3,28-diol, more commonly known as betulin which was isolated from *Diospyros rubra* (**Prachayasittikul *et al.*, 2009**) whose structure is given below.



Lup-20(29)-en-3,28-diol **(136)**

Table 24: NMR data of betulin compared with literature data

PF2			Betulin (Prachayasittikul *et al.*, 2010)	PF2			Betulin (Prachayasittikul *et al.*, 2010)
Position	**^{13}C**	**^{1}H**	**^{13}C**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**
1	39.1		38.6	**17**	44.1		47.7
2	28.3		27.3	**18**	49.1		47.7
3	79.6	3.13, dd	78.9	**19**	49.2	2.35, m	48.7
4	44.1		39.3	**20**	151.8		150.4
5	56.7	0.65, t	55.2	**21**	30.6		29.7
6	18.8		18.2	**22**	37.4		33.9
7	34.9		34.2	**23**	28.7	0.85, s	27.9
8	41.2		40.9	**24**	16.2	0.75, s	15.3
9	51.7		50.3	**25**	16.8	0.80, s	16.0
10	37.9		37.3	**26**	16.4	0.95, s	15.9
11	21.3		20.8	**27**	15.3	0.97, s	14.7
12	26.2		26.1	**28**	78.8	3.31, s	60.5
13	38.7		37.1	**29**	110.2	4.68 &4.55, s	109.6
14	42.9		42.7	**30**	19.3	1.69, s	19.0
15	31.3		31.3				
16	32.6		29.1	**-**			

II-4-1-7: Structural identificationof PF8

The compound PF8 was obtained in the form of white flakes from the eluent mixture hexane-ethyl acetate (75/25, v/v). It melted between 134-136°C. The ESI-TOF MS spectrum of PF8 showed a pseudo-molecular ion peak at [2M+Na] at m/z = 935.6. A diagnostic peak resulting from the loss of the side chain that is [M-$C_8H_{13}O_2$] appears at m/z=315.2. This information led to the deduction of the molecular formula $C_{30}H_{48}O_3$ with a calculated double bond equivalence of 7.

The ^{1}H NMR Spectrum of PF8 ($CDCl_3$, 500 MHz) showed characteristic signals of a lanostane type triterpenoid protons as follows: five tertiary methyl protons at δ_H 0.99 ppm (s, 3H, H-18), δ_H 1.00 ppm (s, 3H, H-19), δ_H 0.75 ppm (s, 3H, H-29), δ_H 0.90 ppm (s, 3H, H-28), δ_H 0.80 ppm (s, 3H, H-30) in addition to two sets of vinyl methyls at δ_H 1.60 ppm (s, 3H, H-26) and δ_H 1.70 ppm (s, 3H, H-27). Two signals of olefinic methine protons appeared at δ_H 5.30 ppm (dd, 1H, H-11) and δ_H 5.20 ppm (t, 1H, H-24). Other characteristic methines were observed at δ_H 1.55 ppm (m, 1H, H-17) and δ_H 2.30 ppm (m, 1H, H-20) indicating the presence of a carboxylic acid group in position 21. The presence of allylic methylenes at δ_H 1.90 ppm (m, 2H, H-23) and δ_H 2.10 ppm (m, 2H, H-12) together with the two olefinic protons mentioned above confirms the presence of two double bonds at positions 9 and 24. Finally a hydroxylated methine signal appears at δ_H 3.50 ppm indicating the presence of a hydroxyl group on carbon 3.

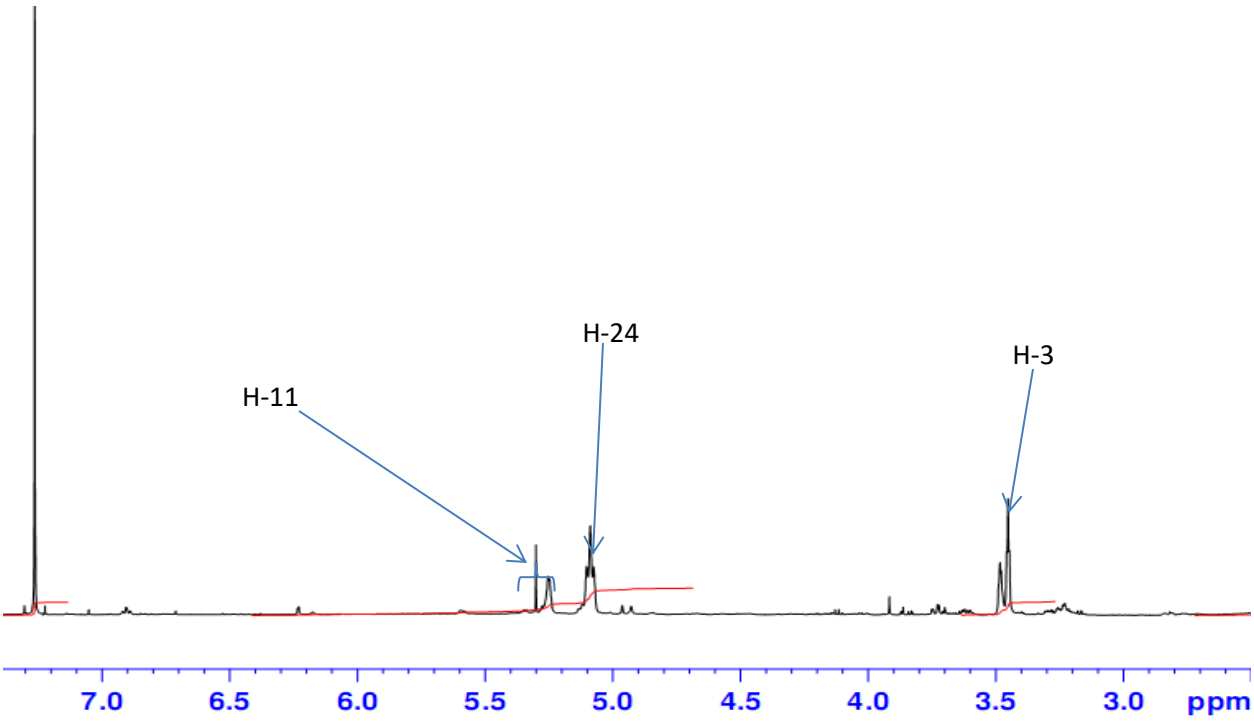


Figure 31: ^{1}H NMR Spectrum PF8 ($CDCl_3$, 500 MHz) enlarged from 2.5 – 7.5 ppm

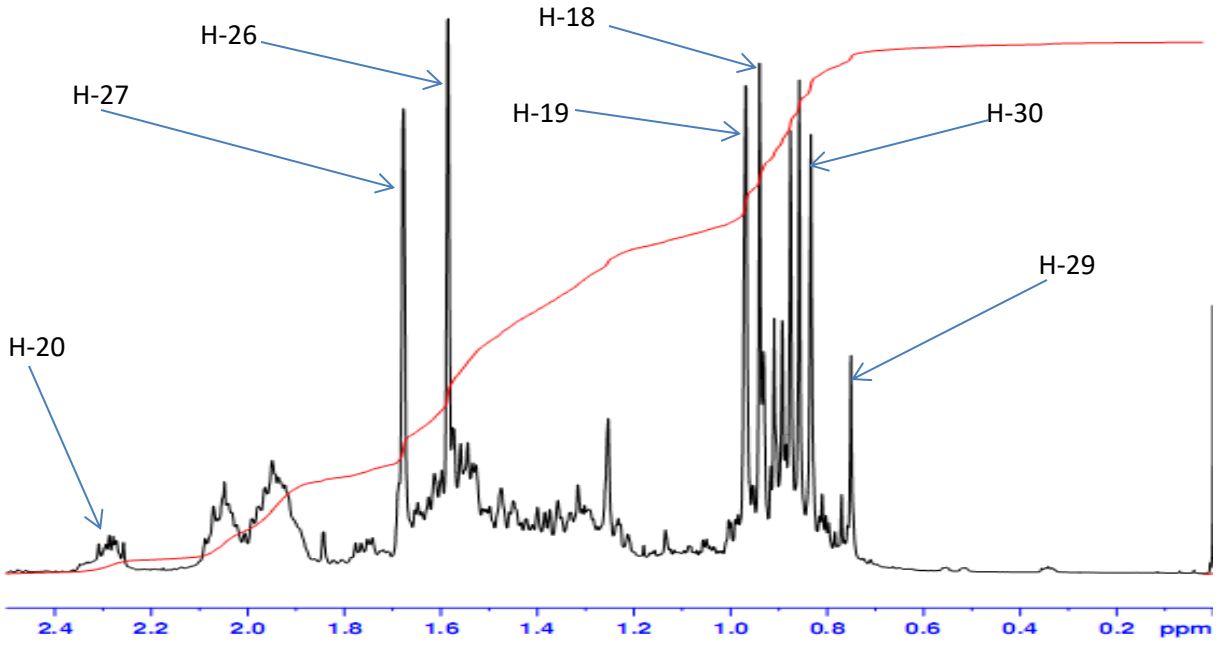


Figure 32: ^{1}H NMR Spectrum PF8 ($CDCl_3$, 500 MHz) enlarged from 0 – 2.5 ppm

^{13}C NMR Spectrum PF8 ($CDCl_3$, 150 MHz) showed 30 carbon resonances. Four olefinic carbon signals at δ_C 123.0 ppm (CH, C-11), δ_C 149.0 ppm (CH, C-9), δ_C 130.5 ppm (CH, C-24) and δ_C 132.0 ppm (CH, C-25) thus confirming the presence of two double bonds at positions 9 and 24 of a lanosteryl triterpene. The carboxylic carbon atom C-21 appears at δ_C 182.5 ppm while the signal of the oxymethine C-3 appears at 77.0 ppm.

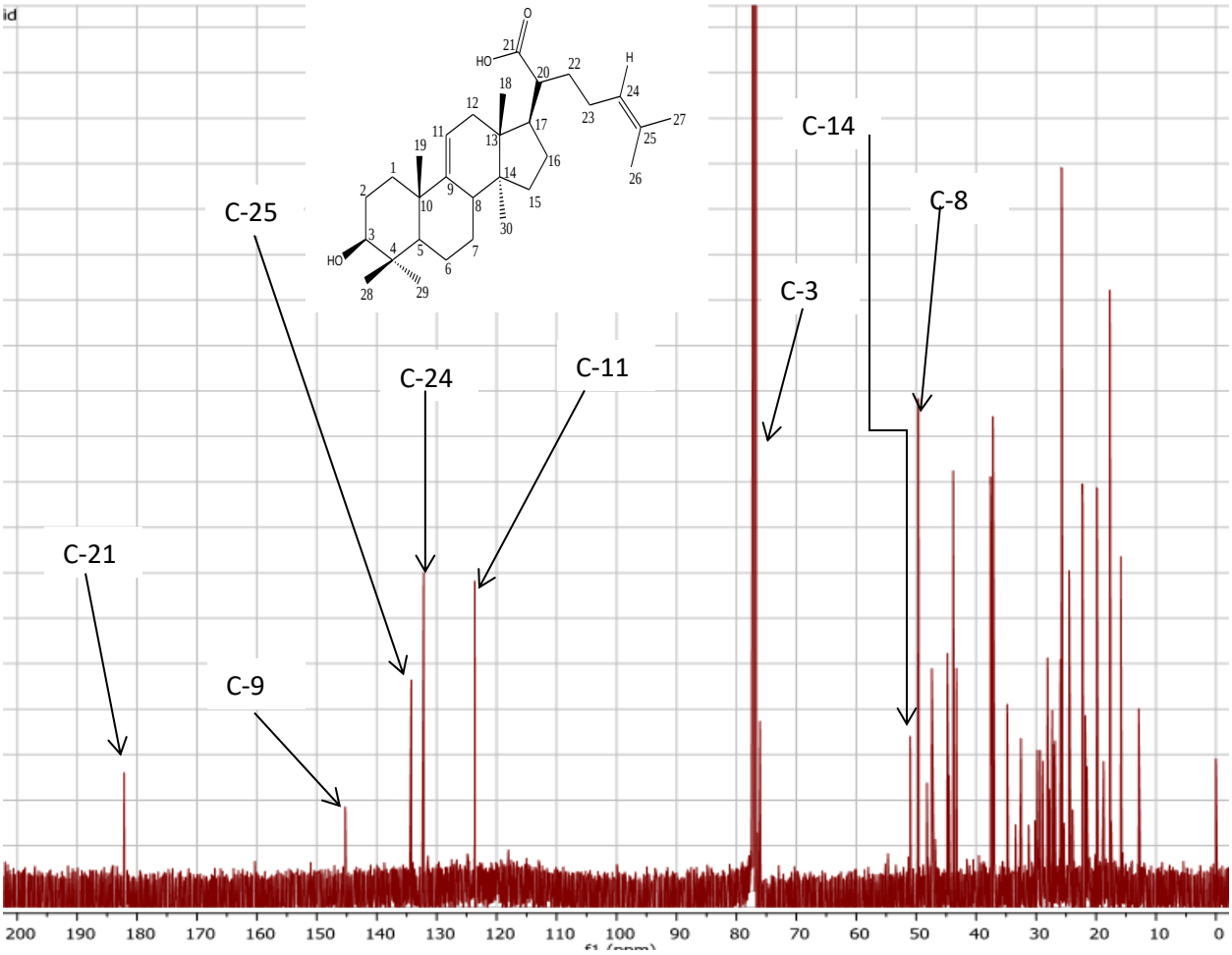


Figure 33: ^{13}C NMR Spectrum PF8 ($CDCl_3$, 150 MHz)

The structure of PF8 is confirmed by 2D-NMR spectra notably HSQC that showed $^{1}J_{C\text{-}H}$ correlations between H-24 and C-24, H-3 and C-3, H-26 and C-26, H-27 and C-27, H-20 and C-20, and H-17 and C-17 amongst others. The HMBC spectra revealed $^{3}J_{C\text{-}H}$ correlations between H-27 and H-26 with C-24 and equally $^{2}J_{C\text{-}H}$ correlations with C-25. H-24 coupled through $^{2}J_{C\text{-}H}$ with C-23 in HMBC. The tertiary methyl protons H-18 and H-19 both showed $^{4}J_{C\text{-}H}$ correlations with the olefinic carbon atom C-11 while in addition H-19 couples through $^{3}J_{C\text{-}H}$ with C-9.

^{1}H-^{1}H COSY spectrum of PF8 showed cross peaks for the following pairs of protons: H-24 and H-23. H-24 also showed long range coupling with H-26 and H-27. Finally, H-20 and H-19 showed cross peaks with each other.

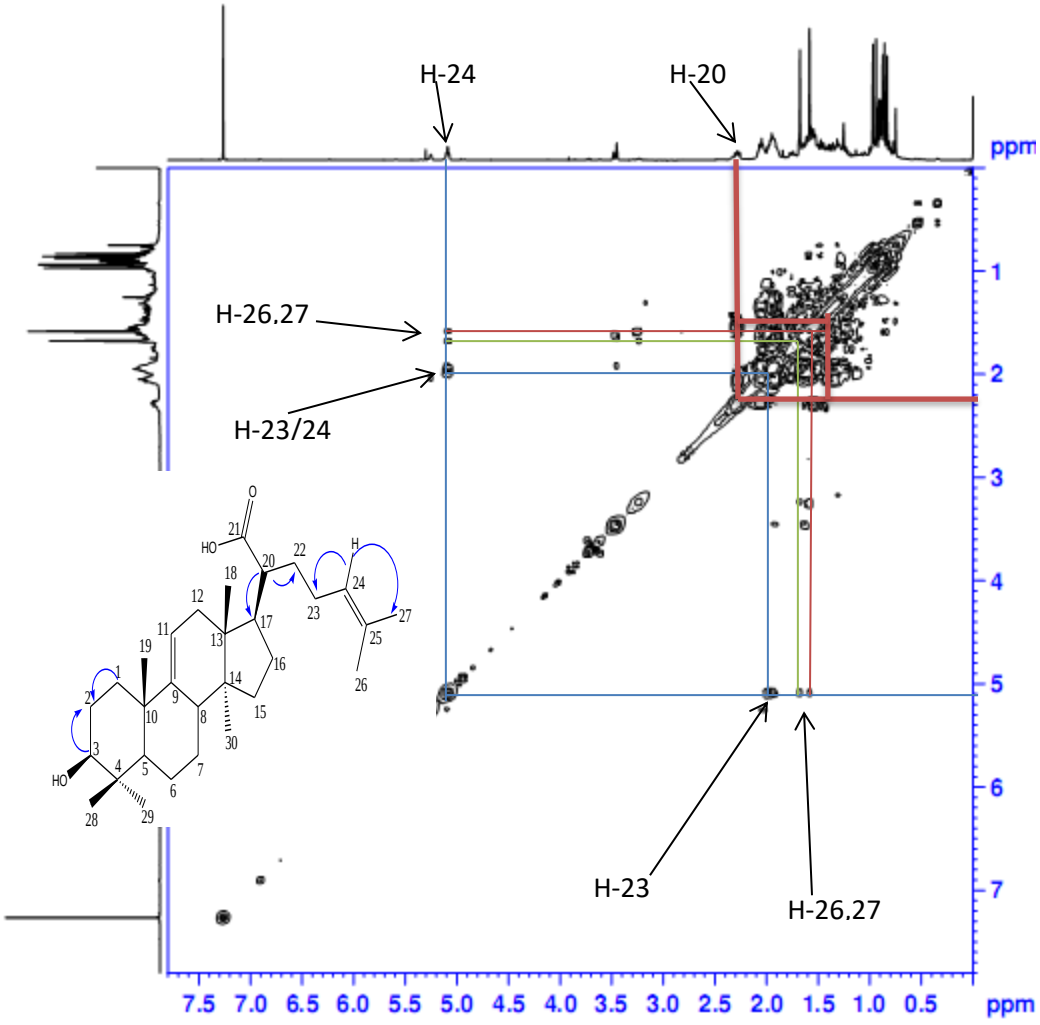
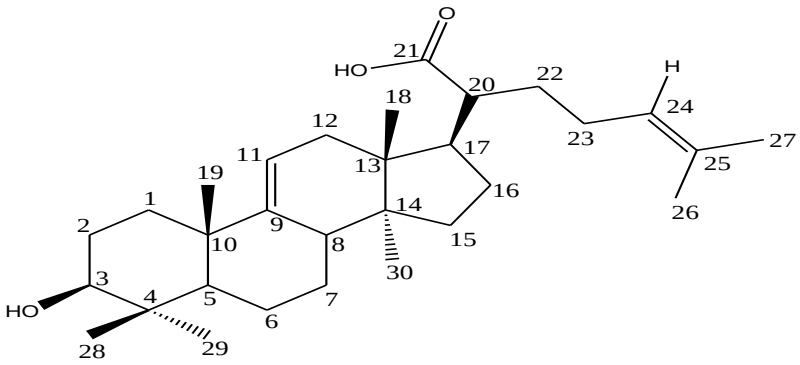


Figure 34: ^{1}H-^{1}H COSY Spectrum PF8

The set of spectral data and by comparison with some data reported elsewhere led to the identification of PF8 as 3β-hydroxylanostan-9,24-dien-21-oic acid isolated previously from *Prothorus longifolia* (**Mosa *et al.*, 2011**) whose structure is given below.



3β-hydroxylanostan-9,24-dien-21-oic acid **(137)**

Table 125: NMR data of 3β-hydroxylanostan-9,24-dien-21-oic acid compared with literature data

PF8			3β-hydroxylanostan-9,24-dien-21-oic acid (Mosa et *al.*, 2011)	PF8			3β-hydroxylanostan-9,24-dien-21-oic acid (Mosa et *al.*, 2011)
Position	**^{13}C**	**^{1}H**	**^{13}C**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**
1	30.5		30.3	**17**	44.0		47.2
2	23.0		23.9	**18**	14.0	0.99, s	16.5
3	77.0	3.5, t	77.2	**19**	18.5	1.00, s	18.5
4	37.3		37.3	**20**	48.2	2.30, m	48.2
5	44.5		44.5	**21**	182.5		181.5
6	15.5		17.7	**22**	32.4		32.4
7	26.0		26.0	**23**	24.5	1.90, m	25.3
8	49.7		49.7	**24**	130.5	5.20, t	128.6
9	149.0		148.7	**25**	132.0		132.2
10	34.9		34.9	**26**	17.5	1.60, s	17.6
11	123.0	5.30, dd	122.0	**27**	25.7	1.70, s	25.7
12	28.3	2.10, m	29.3	**28**	19.5	0.90, s	21.8
13	44.0		43.3	**29**	27.5	0.75, s	27.7
14	51.0		51.0	**30**	19.8	0.88, s	22.7
15	31.2		31.2				
16	27.3		27.3	**-**			

II-4-1-8: Structural identification of PN53

Compound PN53 crystallized in the form of the white crystals in the eluent Hexane/AcOEt (97.5/2.5, v/v). It was found to be soluble in methylene chloride, melted between 181-184ºC and gave a reddish-violet coloration with the Liebermann-Burchard reaction characteristic of triterpenes. Its ESI-TOF MS spectrum enabled us to deduce the molecular formula $C_{30}H_{48}O$ with the molar mass 424 g/mol with 7 double bond equivalences.

The ^{1}H-NMR spectrum displayed a set of AB doublets at δ_H 0.54 ppm and 0.34 ppm (J=4.4 Hz), characteristic of a cyclopropane methylene protons of a cycloartane type triterpenoid Hα-19 and Hβ-19 respectively. Two sets of olefinic proton signals appeared, one in the form of doublet at δ_H 4.20 and δ_H 4.40 ppm corresponding to the exocyclic vinyl protons Hα-26 and Hβ-26 respectively at the end of the cycloartane side chain. Another olefinic proton resonates at signal δ_H = 5.20 ppm in the form of a double doublet attributable to the proton H-12 of a Δ^{12} unsaturated triterpene. The set of angular methyl proton signals appear between 0.70 to 1.05 ppm except for the vinyl methyl which appears as a strong singlet at 1.65 ppm. A hydroxylated methine signal appears at δ_H 3.20 ppm (dd, J = 6.4, 9.2 Hz) indicating the proton H-3 at the foot of a β-hydroxyl group. The above spectral data is characteristic of a 3β-hydroxy-12,25- cyloartan diene.

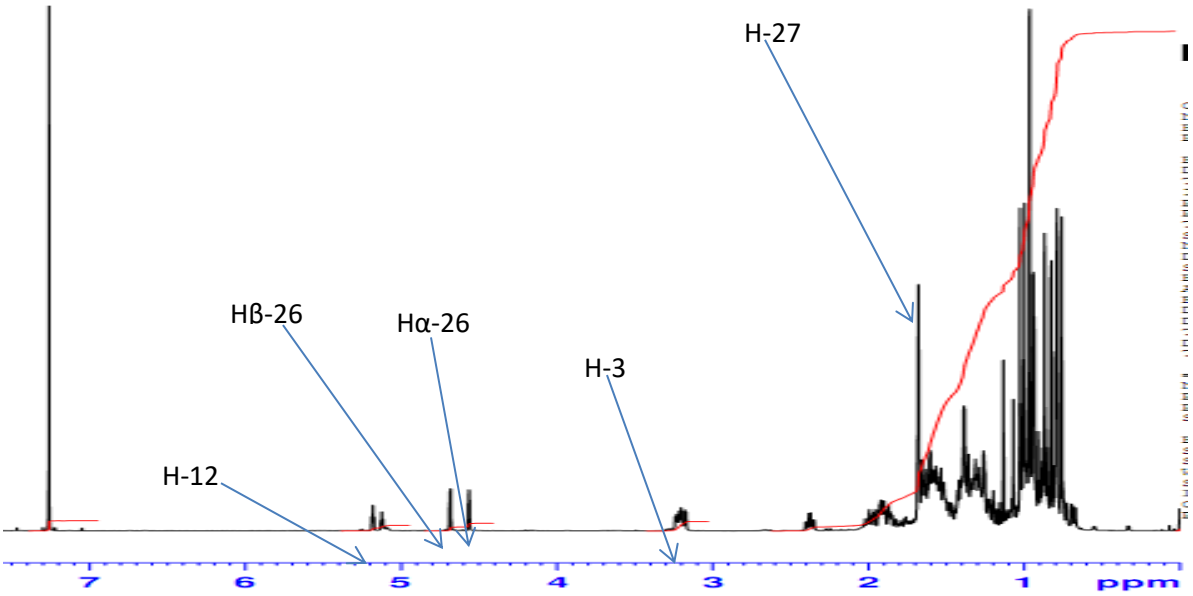


Figure 35: ^{1}H NMR Spectrum PN53 ($CDCl_3$, 500 MHz)

On the ^{13}C NMR Spectrum of PN53 ($CDCl_3$, 500 MHz**)** the signals of characteristic exocyclic olefinic carbon atoms in which 1 is substituted where observed at δ_C 151.0 ppm and 109.3 ppm

corresponding to the substituted C-25 and non-substituted terminal C-26 respectively. A signal of the oxymethine carbon atom C-3 was seen at δ_C 78.0 ppm. The two allylic methylene carbon atoms C-11 and C-24 appeared at δ_C 46.8 ppm and δ_C 48.0 ppm respectively. The set of tertiary angular methyl carbon signals are found between δ_C 14.5 ppm and δ_C 26.9 ppm.

2D NMR experiments were used to confirm the connections between the various atoms. The HSQC showed $^{1}J_{C\text{-}H}$ correlations between H-12 and C-12, H-26 and C-26, H-3 and C-3, H-24 and C-24 and H-11 and C-11.

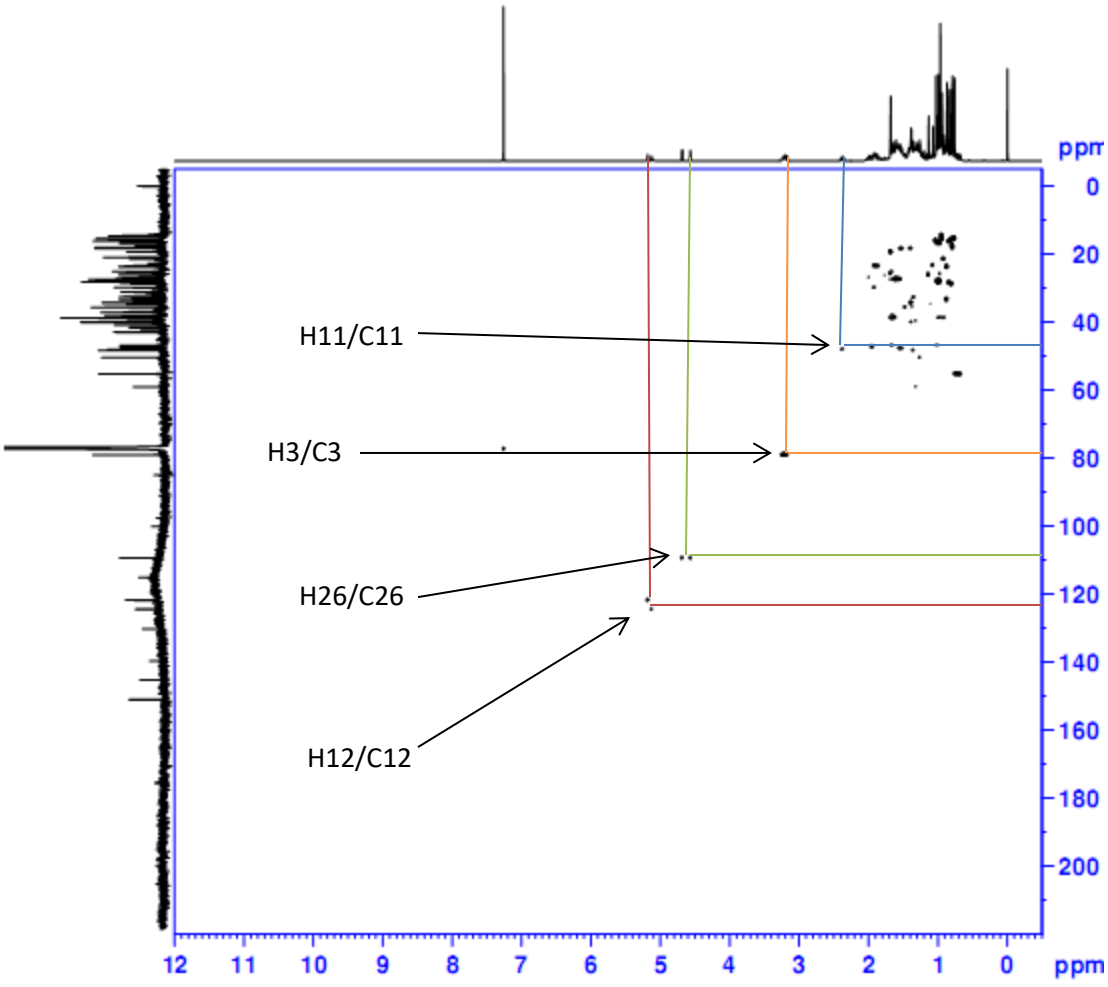


Figure 36: HSQC ($^{1}J_{H\text{-}C}$) spectrum of PN53

The HMBC spectrum of PN53 indicated some major couplings of H-27 with C-24 and C-26 through a $^{3}J_{H\text{-}C}$ and H-27 with C-25 through a $^{2}J_{H\text{-}C}$ coupling. Equally, the angular methyl protons H-28 and H-29 each couples with the oxymethine carbon C-3 through a $^{3}J_{H\text{-}C}$ coupling.

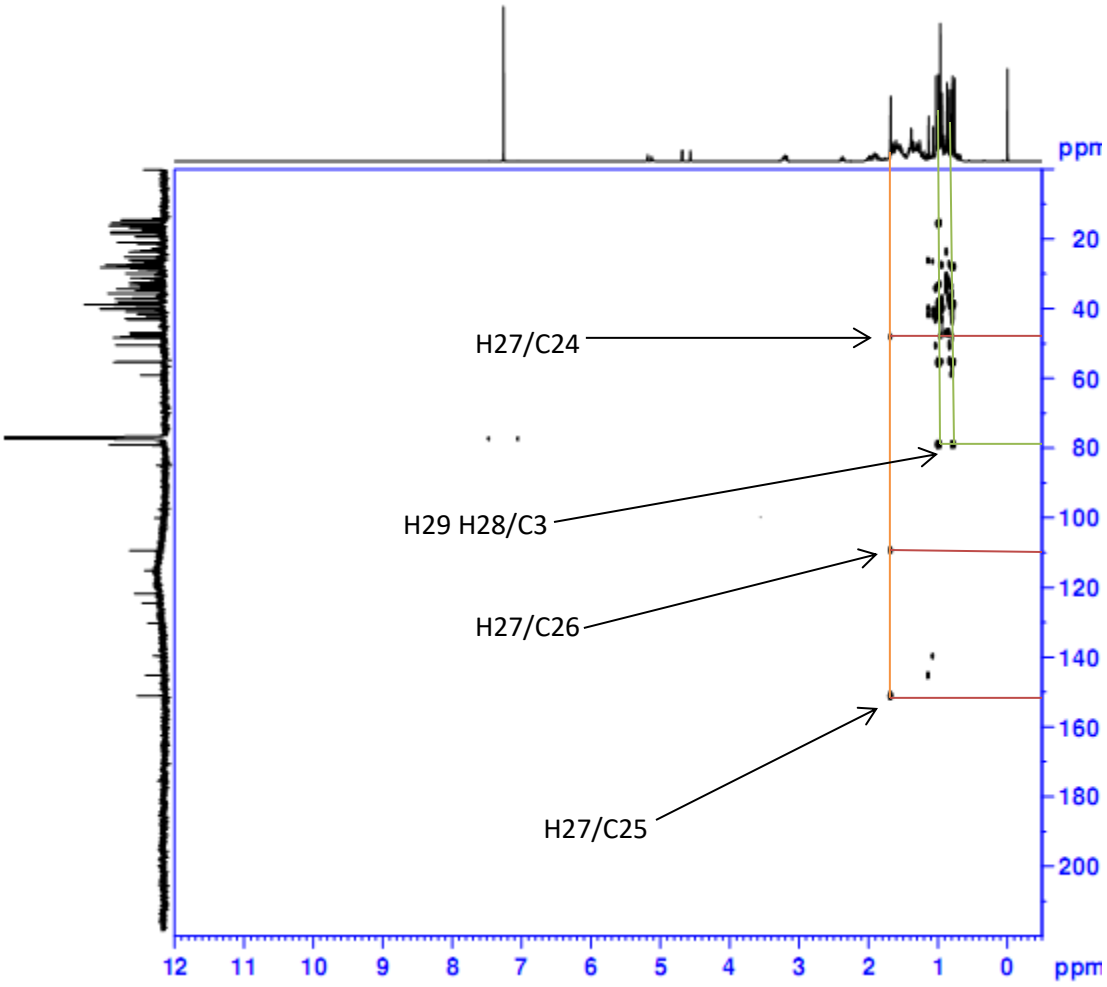


Figure 37: HMBC spectrum of PN53

The ^{1}H-^{1}H COSY spectrum of PN53 showed cross peaks for some pairs of protons which are close in space. Key COSY correlations were observed between the following pairs of adjacent protons: H-12 and H-11, Hα-26 and Hβ-26, H-2 and H-3, H-24 and H-23 in addition to a correlation due to long range coupling between H-26 and H-27.

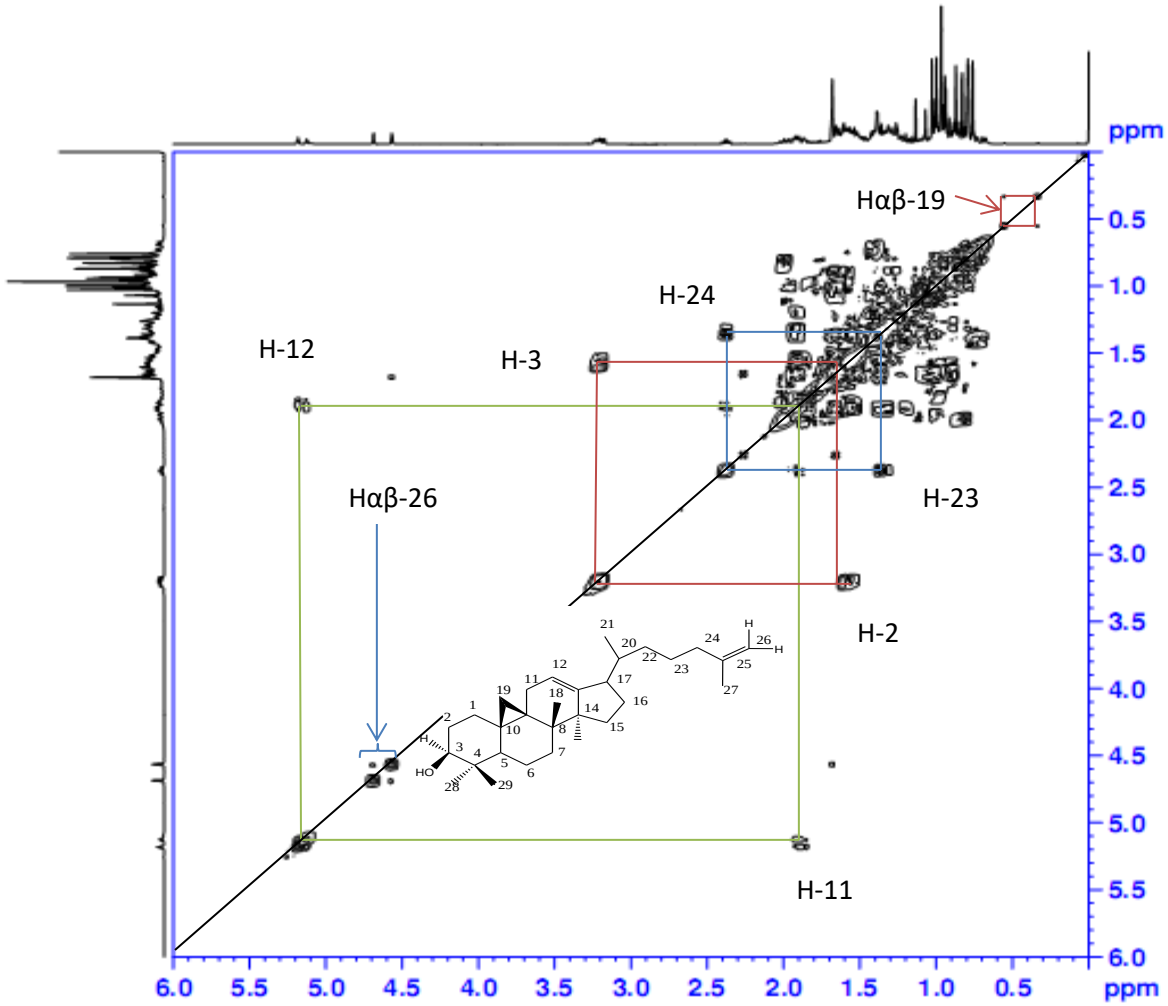
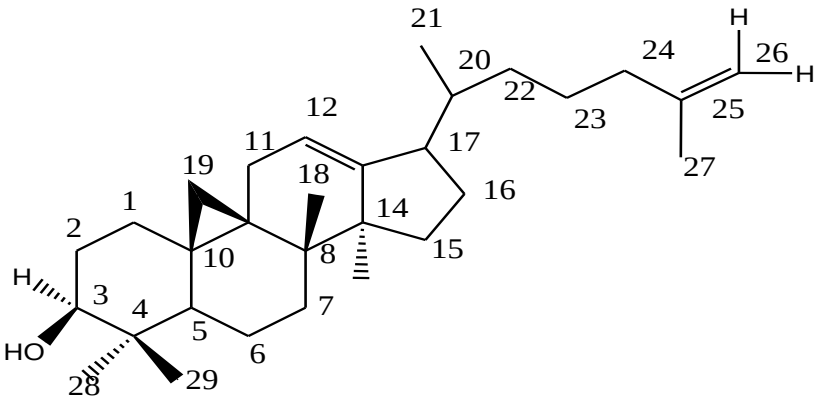


Figure 38: ^{1}H-^{1}H COSY spectrum of PN53

The described spectral together with some data reported in literature led to the identification of PN53 as 3β-hydroxycyloartan-12,25-diene reported previously in Cameroonian propolis (**Sakava *et al*., 2014**).



3β-hydroxycyloartan -12,25-diene **(86)**

Table 26: NMR data of 3β-hydroxycyloartan -12,25-diene compared with literature data

PN53			3β-hydroxycyloartan -12,25-diene (Sakava *et al.*, 2014)	PN53			3β-hydroxycyloartan -12,25-diene (Sakava *et al.*, 2014)
Position	**^{13}C**	**^{1}H**	**^{13}C**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**
1	29.8	1.57, 1.26 m	29.9	**16**	55.2		52.0
2	32.6	1.76, 1.56 m	30.5	**17**	55.3	2.4 m	52.3
3	78.8	3.20, t	78.8	**18**	14.5	0.70	14.0
4	46.8		45.3	**19**	26.9	0.54, 0.34 d	26.4
5	48.3	1.30, dd	48.8	**20**	35.6	1.66 m	35.6
6	18.4		18.3	**21**	19.3	0.89 d	19.3
7	31.1	1.10, 1.37 m	30.0	**22**	38.9		38.8
8	47.9		47.1	**23**	25.1		25.4
9	32.9		32.8	**24**	40.8		40.5
10	26.1		26.0	**25**	151.0		139.3
11	32.5	1.95, 2.10 m	32.0	**26**	109.3	4.2, 4.4 brs	114.0
12	121.7	5.20 m	125.6	**27**	20.9	1.65 s	20.0
13	145.2		129.6	**28**	26.1	0.80 s	26.1
14	36.9		36.4	**29**	18.3	0.78 s	18.1
15	28.1		28.1	**30**	21.4	1.11 s	21.1

II-4-1-9: Structural elucidation of PN306

Compound PN306 crystallized as a white powder in hexane/EtOAc (35/65). Its melting point was found to bebetween 167-169.8ºC and it gave a violet coloration with the Liebermann-Burchard reagent indicating that it is a triterpene. The ESI-TOF MS spectrum of compound PN306 showed a pseudo-molecular ion peak $[M+2Na]^+$ at m/z 532.3 from where the molecular formula of the compound was deduced as $C_{31}H_{50}O_4$ with a double bond equivalence of 7.

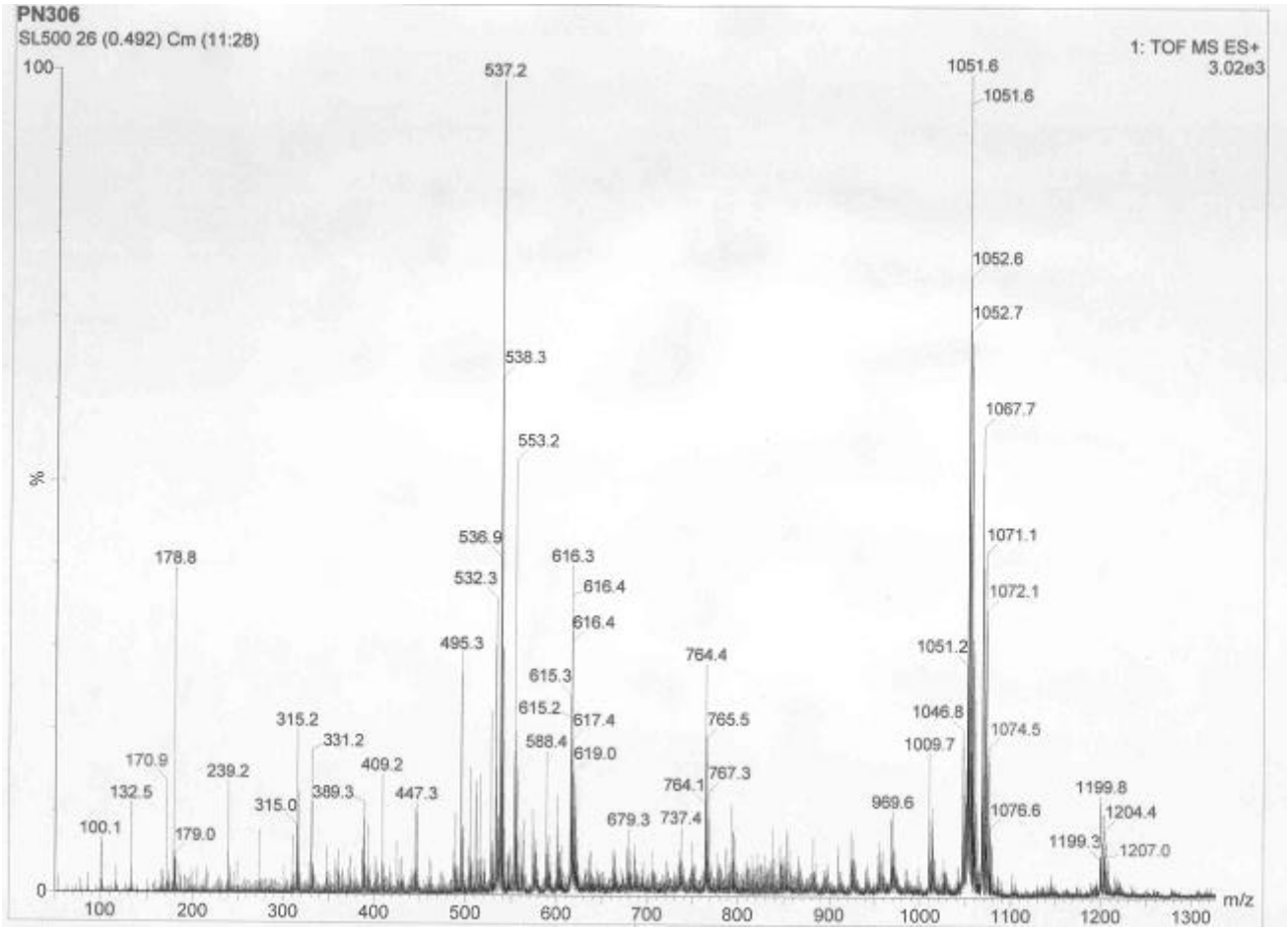


Figure 39: ESI-TOF MS spectrum of PN306

The ^1H NMR (500 MHz) spectrum of PN306 showed characteristic peaks some of which are similar to those of mangiferolic acid. That is, at δ_H 6.80 ppm (1H, t) characteristic of a conjugated olefin corresponding to the methylene proton H-24, δ_H 4.20 ppm (2H, brs) attributable to the protons of the allylic carbon atom bonded to an oxygen atom (H-27), at δ_H 3.05 ppm a triplet (1H, t) attributable to the oxymethine proton in an α-position with the hydroxyl in β-position H-3. A set

of AB doublets at δ_H 0.15 ppm (1H, d, J=4.1 Hz) and 0.35 ppm (1H, d, J=4.1 Hz) characteristic of a cyclopropane methylene protons Hα-19 and Hβ-19 respectively, allylic methylene protons at δ_H 2.10 ppm (1H, m) and 2.25 ppm (1H, m) corresponding to H_α-23 and H_β-23 and finally signals of five tertiary methyls δ_H 0.67 ppm(3H, s) H-29, 0.70 ppm (3H, s) H-30, 0.75 ppm (3H, d, J=6.5 Hz) H-21, 0.80 ppm (3H, s) H-28 and 0.82 ppm (3H, s) H-18 corresponding to the 5 angular methyl groups. In addition to these, was a broad singlet of 3H at 3.24 ppm attributable to methoxyl protons of a methyl ester of a carboxylic acid H-31.

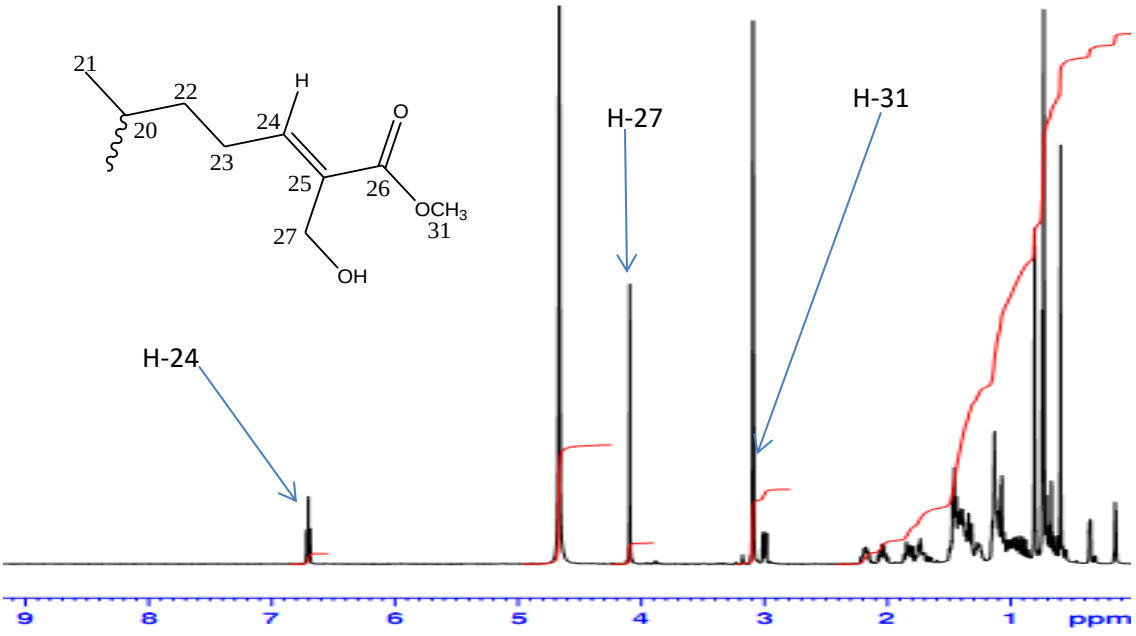


Figure 40: ^{1}H NMR Spectrum PN306 (CD_3OD, 500 MHz)

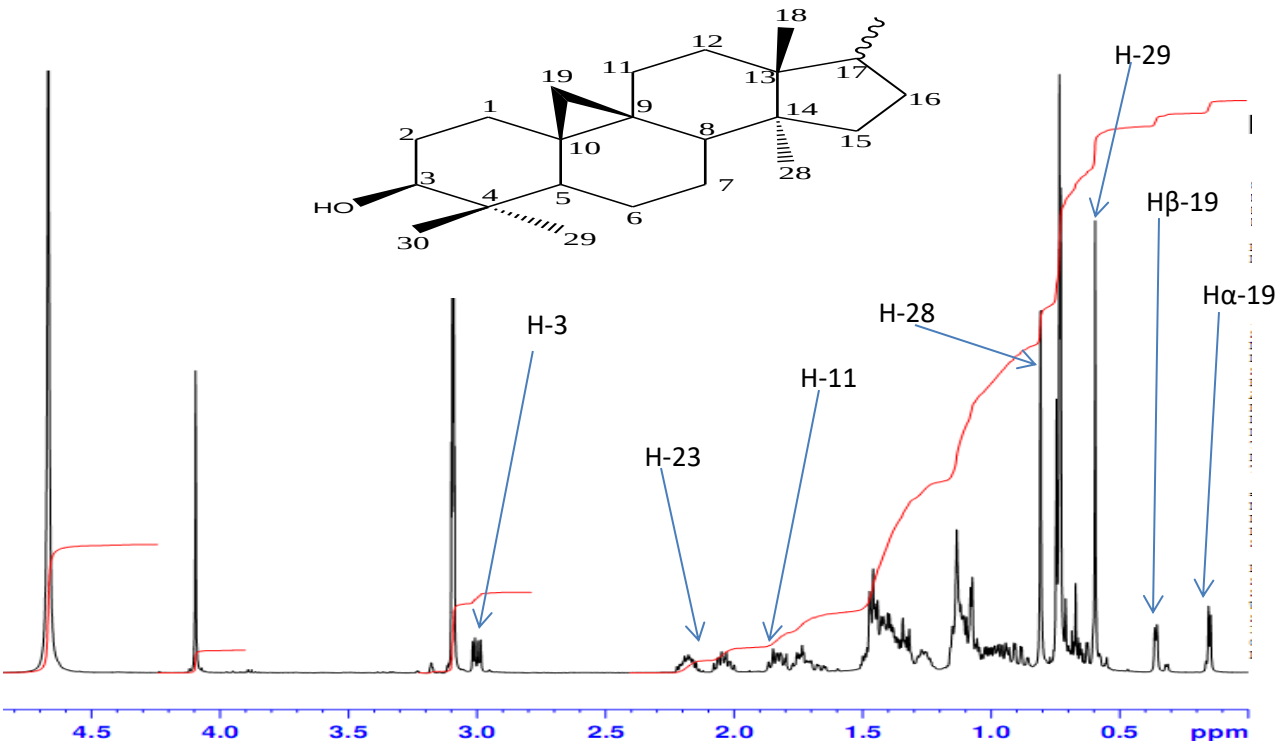


Figure 41: ^{1}H NMR Spectrum PN306 (CD_3OD, 500 MHz) enlarged from 0 - 4.8 ppm.

^{13}C NMR (125 MHz) spectrum of PN306 exhibited the following characteristic signal; five methyls δ_C 14.73 ppm (C-30), 18.65 ppm (C-18), 19.84 ppm (C-21), 21.14 ppm (C-29), 26.08 ppm (C-28), a cyclopropane methylene δ_C 29.19 ppm (C-19), two olefinic carbons δ_C 132.70 ppm (C-25) and 148.21 ppm (C-24), an αβ-unsaturated carbonyl ester carbon δ_C 170.17ppm (C-26), a hydroxyl methylene at δ_C 79.56 ppm (C-3), an alkoxyl carbon at 48.84 ppm (C-31) confirming the presence of a methyl ester seen on the ^{1}H NMR and finally the oxymethylene at δ_C 56.67ppm (C-27). These ^{1}H NMR and ^{13}C NMR data closely resembled those of 28-hydroxymangiferolic acid except for the appearance of the signals due to the allylic oxymethylene (δ_H 4.20 ppm, 2H, brs, H-27; δ_C 56.67ppm, C-27) and the methoxyl group of methylester (δ_H 3.24 ppm, 3H, brs, H-31; δ_C 48.84 ppm, C-31) with the absence of the hydroxyl group on C-28.

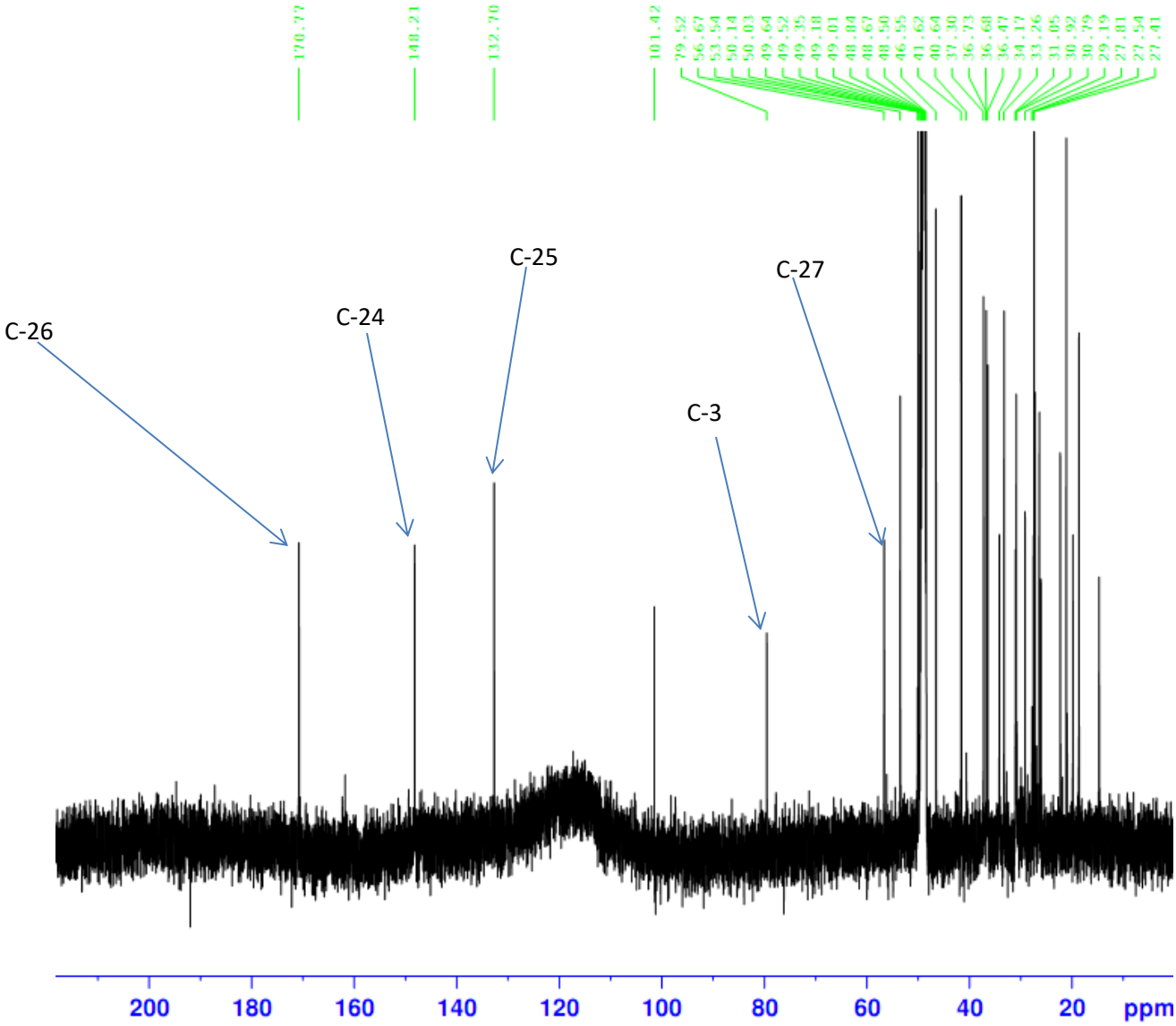


Figure 42: ^{13}C NMR Spectrum PN306 (CD_3OD, 125 MHz)

2D NMR experiments were used to determine the bond connectivities between the various atoms. The HSQC showed key $^{1}J_{C\text{-}H}$ correlations between protons and the respective carbon atoms to which they are bonded as follows: a correlation between the proton at δ_H 4.20 ppm (H-24) and the carbon at δ_C 56.7 ppm (C-24), the proton at δ_H 3.24 ppm (H-31) and the carbon at δ_C 48.8 ppm (C-31), the proton at δ_H 3.05 ppm (H-3) and the carbon at δ_C 79.6 ppm (C-3) and the proton at δ_H 0.15 and 0.35 ppm (H-19) with the carbon at δ_C 29.2 ppm (C-19).

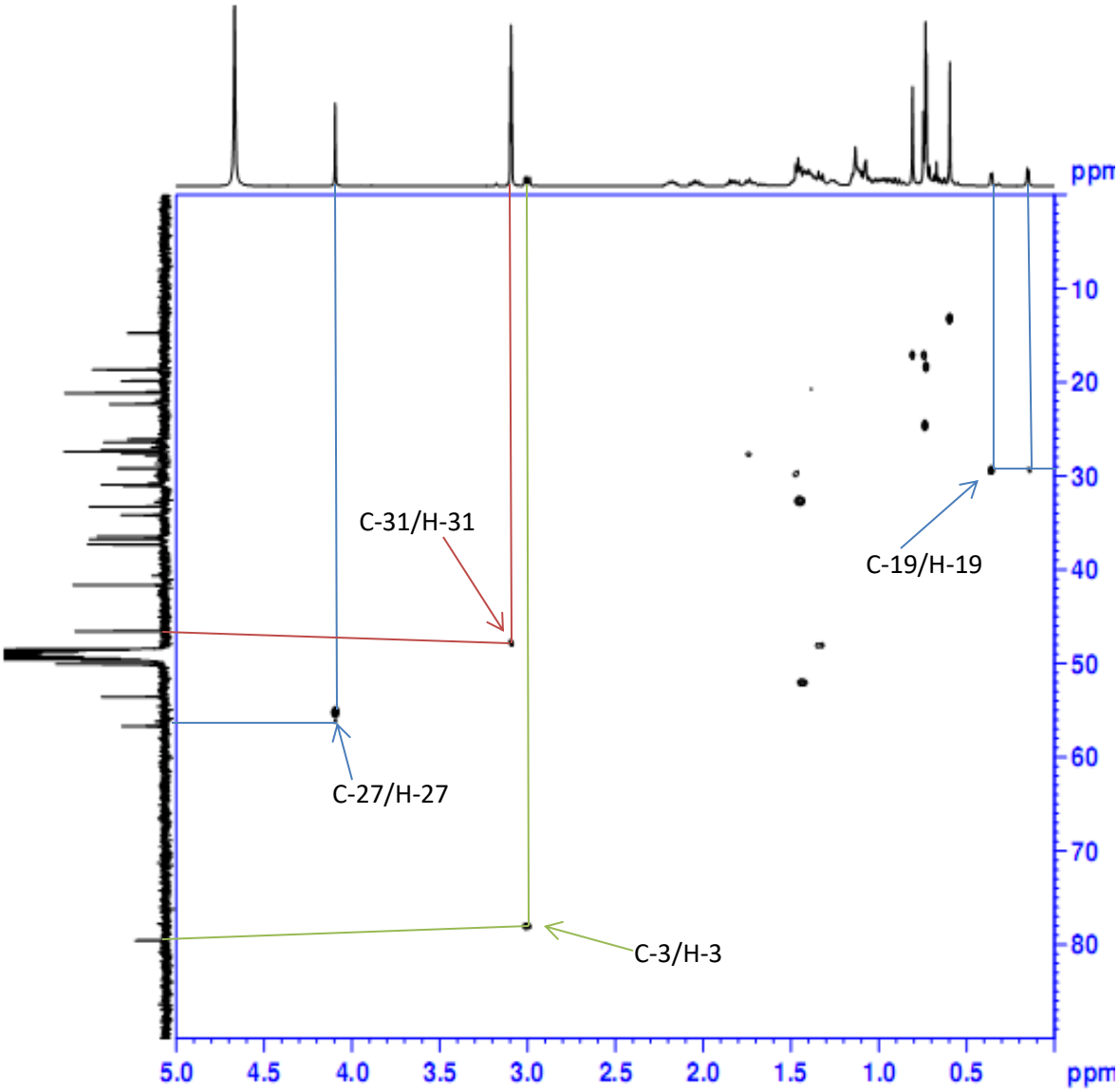


Figure 43: HSQC ($^{1}J_{H-C}$) spectrum of PN306

The HMBC spectrum of PN53 indicated some major couplings ($^{n}J_{H-C}$; $1< n \leq 4$) as follows: H-27 coupled with C-25 through $^{2}J_{H-C}$ and further showed $^{3}J_{H-C}$ couplings with C-24 and C-26. H-30 couples through $^{4}J_{H-C}$ with C-2 while H-29 shows $^{2}J_{H-C}$ and $^{3}J_{H-C}$ couplings with C-4 and C-5 respectively. Equally, the angular methyl protons H-30 and H-29 each couples with the oxymethine carbon C-3 through a $^{3}J_{H-C}$ coupling.

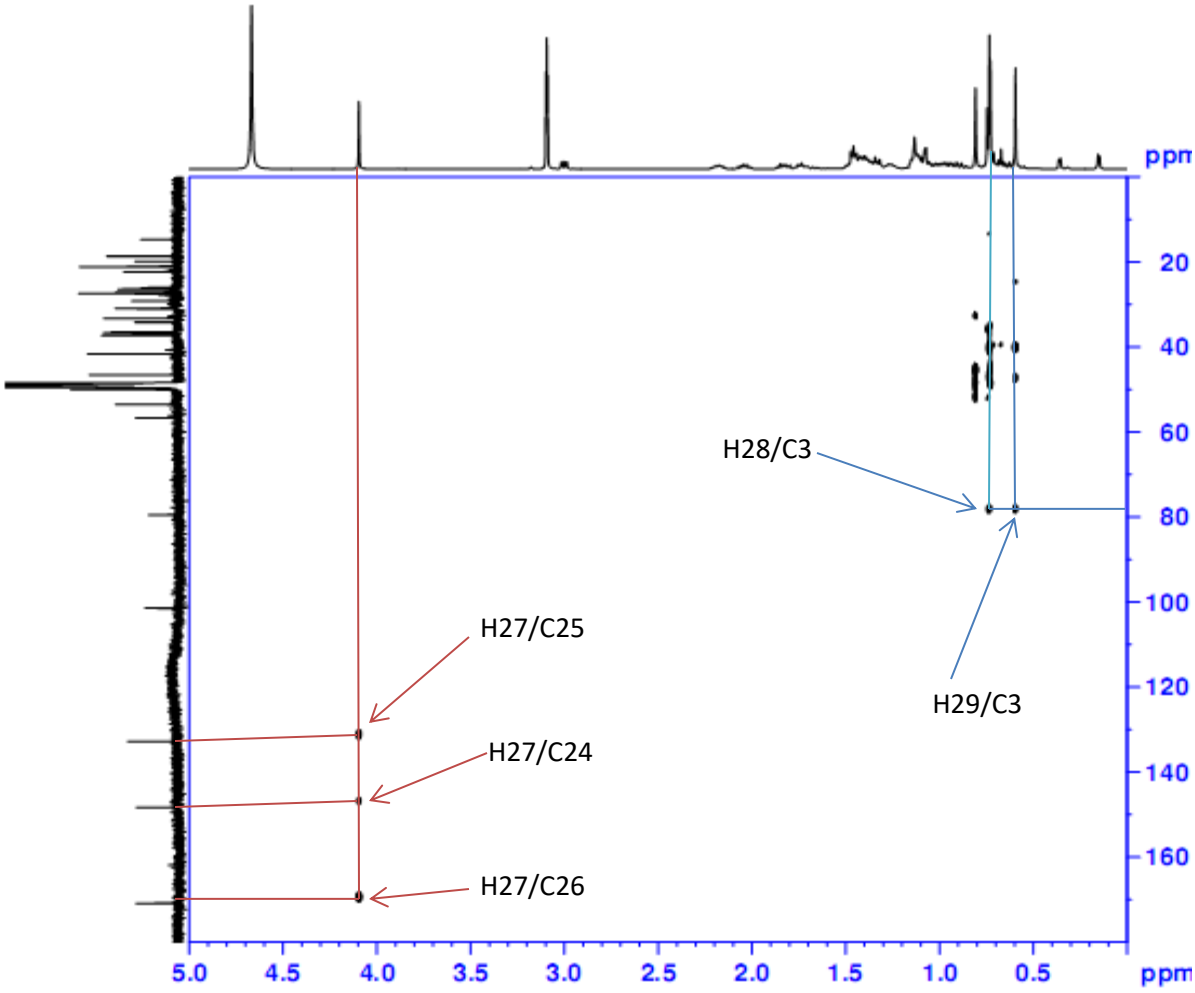


Figure 44: HMBC spectrum of PN306

The ^{1}H-^{1}H COSY spectrum of PN306 showed cross peaks for some pairs of protons which are close in space. Key COSY correlations were observed between the following pairs of protons: H-24 with Hα-23 and Hβ-23, H-23 with H-22 and H-20, H-20 with H-22, H-3 with H-2 and Hα-19 with Hβ-19.

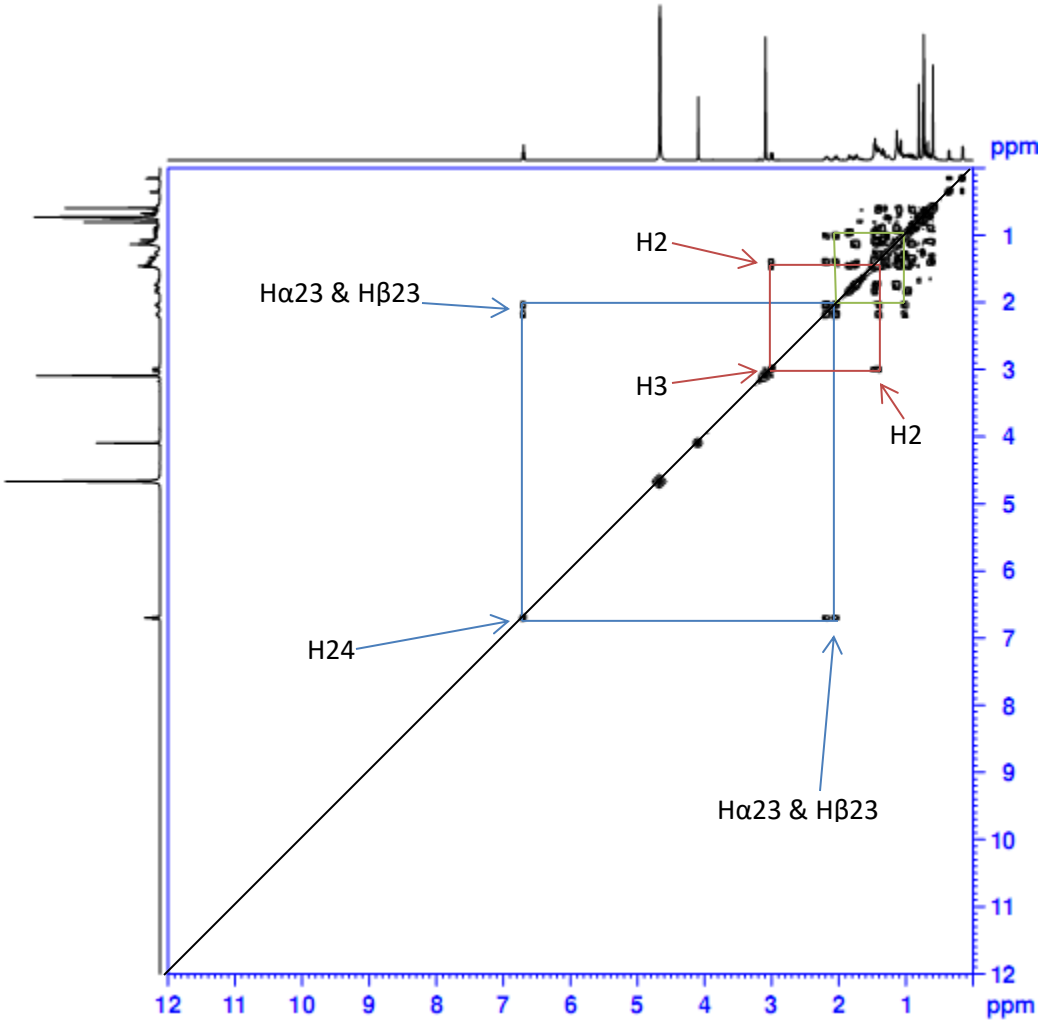
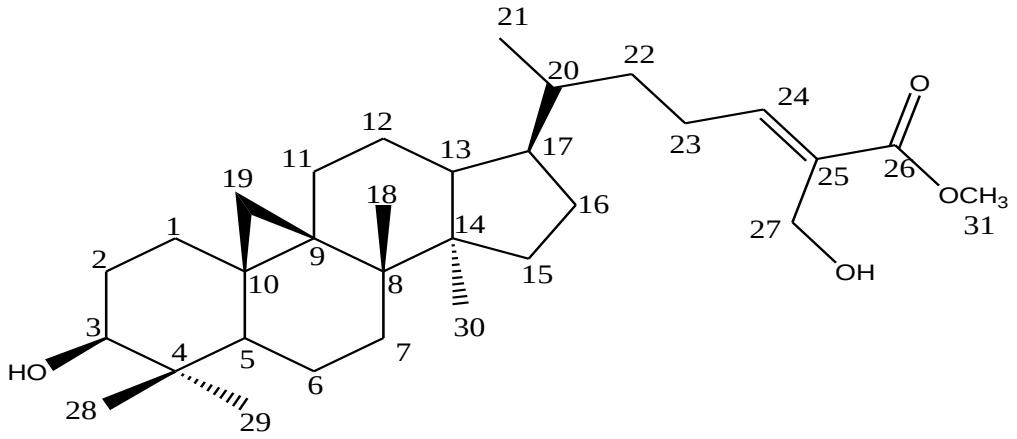


Figure 45: ^{1}H-^{1}H COSY spectrum of PN306

The set of spectral data shows that PN306 is a new derivative of 27-hydroxymangiferolic acid to which we attributed the structure of a 3β,27-dihydroxycycloart-24-en-26-oic acid methyl ester shown below.



Methyl-3β,27-dihydroxycycloart-24-en-26-oate **(138)**

Table 27: NMR data of PN306 (27-hydroxymangiferolic acid methyl ester)

PN306 (27-hydroxymangiferolic acid methyl ester)			PN306 (27-hydroxymangiferolic acid methyl ester)		
Position	**^{13}C**	**^{1}H**	**Position**	**^{13}C**	**^{1}H**
1	31.1	1.57, m	**17**	53.5	1.65, m
2	30.8	1.75, m	**18**	18.7	0.82, s
3	79.6	3.05, t	**19**	29.2	0.15 α ; 0.35 β, d
4	40.6	-	**20**	36.7	1.42, m
5	48.5	1.30, m	**21**	19.8	0.75, m
6	21.1	1.75, m	**22**	34.2	1.67, m
7	27.2	1.38, m	**23**	26.1	2.10 a; 2.25 b, m
8	50.0	1.45, m	**24**	148.2	6.8, t
9	19.8	-	**25**	132.7	-
10	26.4	-	**26**	170.2	-
11	27.4	1.85, t	**27**	56.7	4.20, brs
12	33.3	1.62, m	**28**	26.1	0.80, s
13	46.6	-	**29**	21.1	0.67, s
14	50.1	-	**30**	14.7	0.70, s
15	38.5	1.25, m	**31**	48.8	3.24, s
16	29.6	1.88, m	**-**		

II-4-1-10: Structural identification of TA20

The compound TA20 crystallized from the mixture hexane-CH_2Cl_2 (60/40, v/v) in the form of a white solid. It was found to be soluble in dichloromethane and melted between 216ºC-217ºC. The EI-MS spectrum of TA20 showed a molecular ion peak at m/z 468.3 for the molecular formula $C_{32}H_{52}O_3$. Characteristic base peak of lupane type triterpenoids appeared at m/z 189.

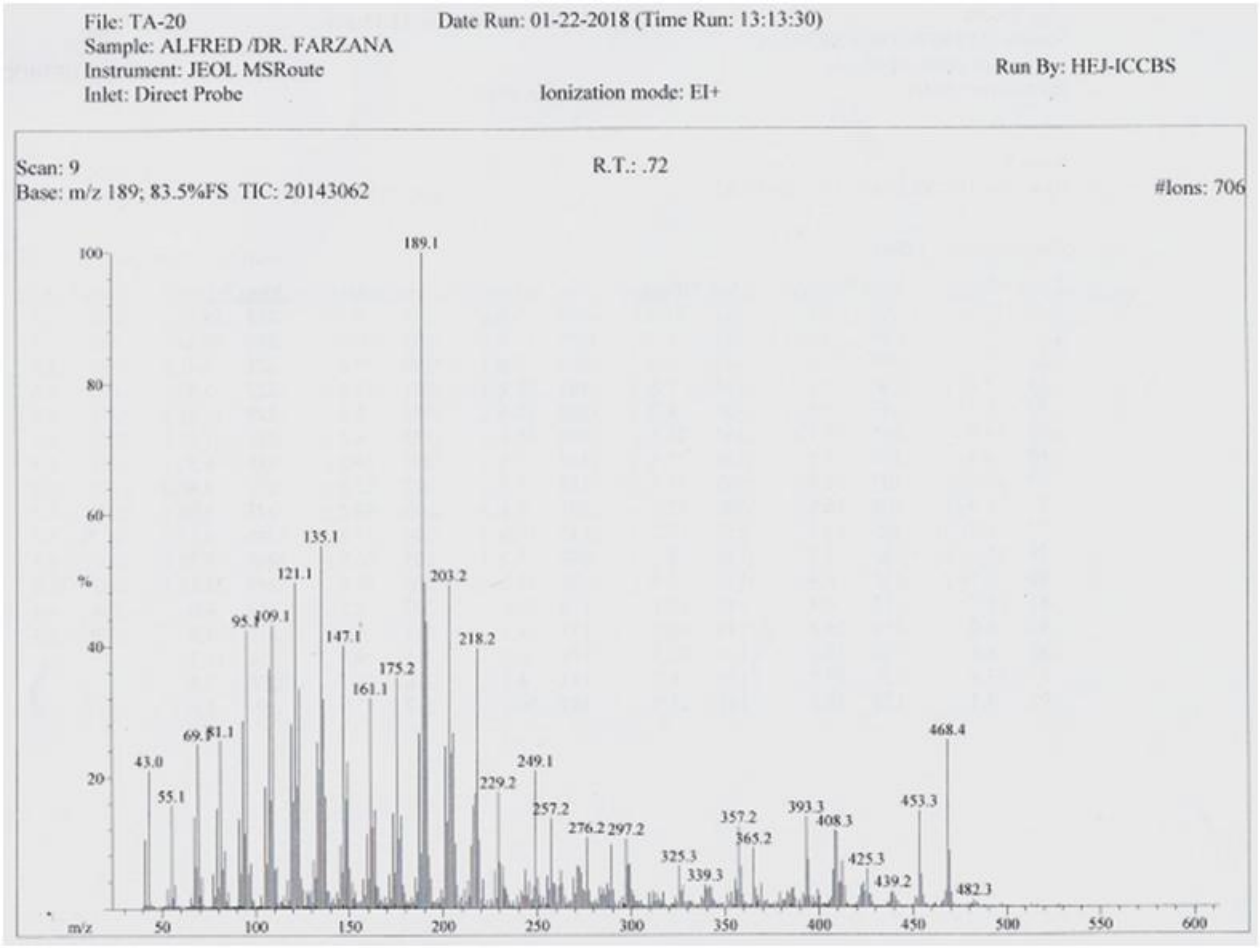


Figure 46: [1]EI-MS Spectrum of TA20

The [1]H-NMR spectrum of TA20 shows the presence of eight methyl singlets with chemical shifts δ_H of 0.77, 0.81, 0.82, 0.83, 0.92, 1.16, 1.66 and 2.03 ppm attributable to protons H-28, H-24, H-23, H-25, H-28, H-26, H-30 and H-2' respectively. Two protons appeared at δ_H 4.66 ppm and 4.55 ppm as doublets indicative of the exomethylene protons H-29a and H-29b of lupane triterpenoid. An oxymethine proton signal ascribable to H-3 occurs at δ_H 4.47 ppm. This confirms that the protons attached to the C-methine (C-3) atom are affected by the electronegativity of the O atoms of the ester group at C-3, causing a large chemical shift value.

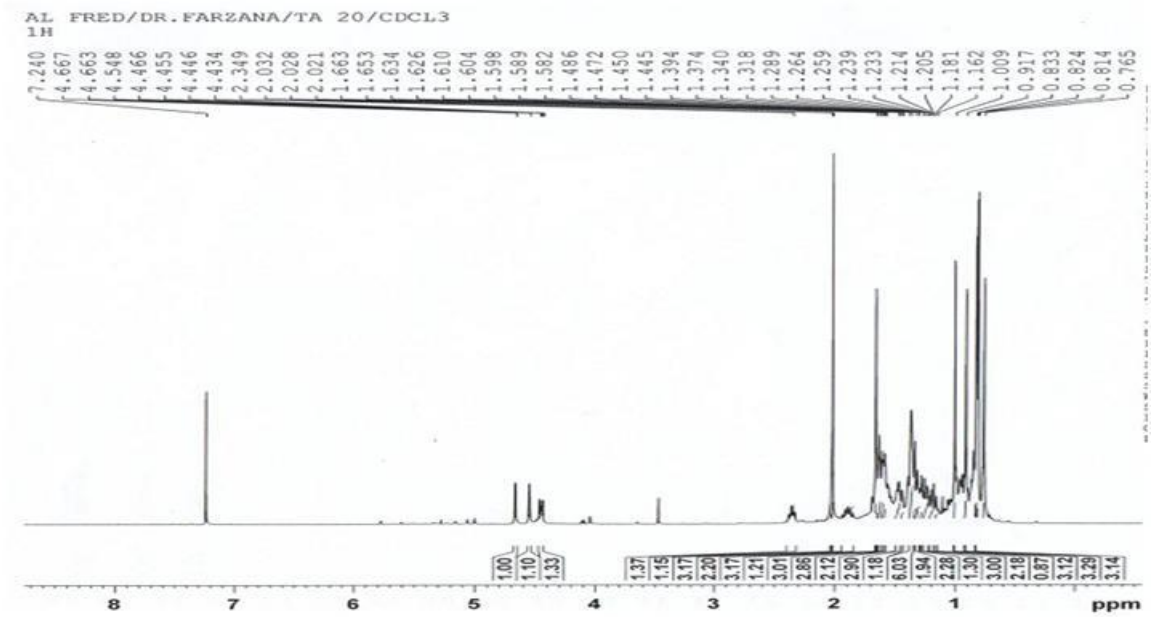


Figure 47: ^{1}H NMR Spectrum of TA20 ($CDCl_3$, 400 MHz)

The ^{13}C-NMR spectrum analysis, showing the signal of a carbonyl carbon of an ester function C-1' at C=170.9 ppm, the oxymethine carbon C-3 whose signal appears at 80.9 ppm. Two sp2 carbons signals at δ 150.9 ppm and 109.3 ppm attributable to the C-20 and C-29 of lupane triterpene respectively. The ^{13}C-NMR signals showed the 30 carbon atoms in total.

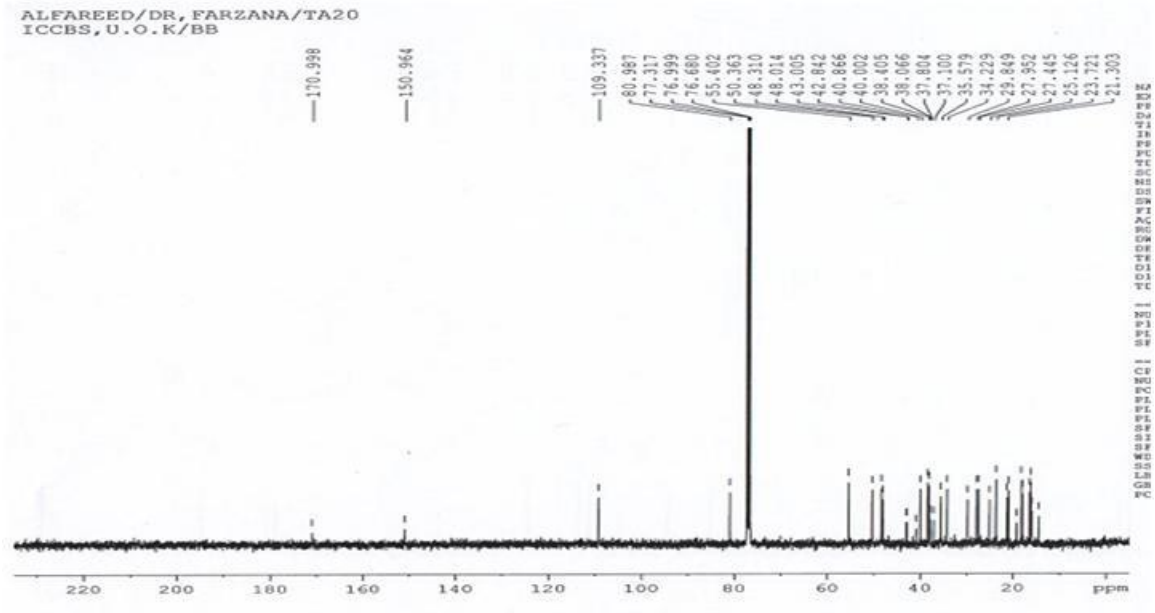
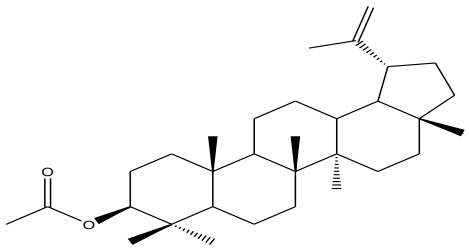


Figure 48: ^{13}C NMR Spectrum TA20 ($CDCl_3$, 150 MHz)

The above spectral information led to the identification of TA20 as lupeol acetate previously described by Riham and co-workers (**Riham *et al.*, 2017**) whose structure is given below.



Lupeol acetate (**82**)

Table 28: NMR data of TA20

TA20			**Lupeol acetate Riham et al., 2017**	**TA20**			**Lupeol acetate Riham et al., 2017**
Position	**^{13}C**	**^{1}H**	**^{13}C**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**
1	38.4		38.4	**17**	43.0		43.0
2	23.7		23.7	**18**	48.3		48.3
3	80.9	4.46,dd, 1H	80.9	**19**	48.0	2.35,m,1 H	48.0
4	37.8		37.8	**20**	150.9		150.9
5	55.4		55.4	**21**	29.8		29.8
6	18.2		18.2	**22**	40.0		40.0
7	34.2		34.2	**23**	27.9	0.82, s,3H	27.9
8	40.8		40.8	**24**	16.5	0.81, s,3H	16.5
9	50.5		50.4	**25**	16.2	0.83, s,3H	16.2
10	37.1		37.1	**26**	15.9	1.16, s,3H	15.9
11	20.9		20.9	**27**	14.5	0.92, s,3H	14.5
12	25.1	1.65,m,2H	25.1	**28**	18.0	0.77,s, 3H	18.01
13	38.1		38.1	**29**	109.3		109.4
14	42.8		42.8	**30**	19.3	1.66,s,3H	19.3
15	27.4		27.4	**1'**	170.9		170.04
16	35.6		35.6	**2'**	21.3	2.03,s,3H	21.3

II-4-1-11: Structural identification of PF4

PF4 crystallized from the mixture hexane-ethyl acetate (87.5/12.5, v/v) in the form of a white solid. It melted between 304ºC-305.5 ºC. The EI-MS spectrum of PF4 showed a molecular ion peak at m/z 456.3 for the molecular formula $C_{30}H_{48}O_3$. Characteristic base peak present at *m/z* 248.3 resulting from the Retro-Diels-Alder fragmentation characteristic of the ursane and oleanane skeletons with a carboxylic (–COOH) group on C-28 corresponding to the base peak. Another prominent peak at m/z 203 results from the loss of the –COOH group from the base peak m/z 248.3. This EI-MS mass spectral search was performed on NIST library and suggested oleanolic acid with a match factor of 99 %.

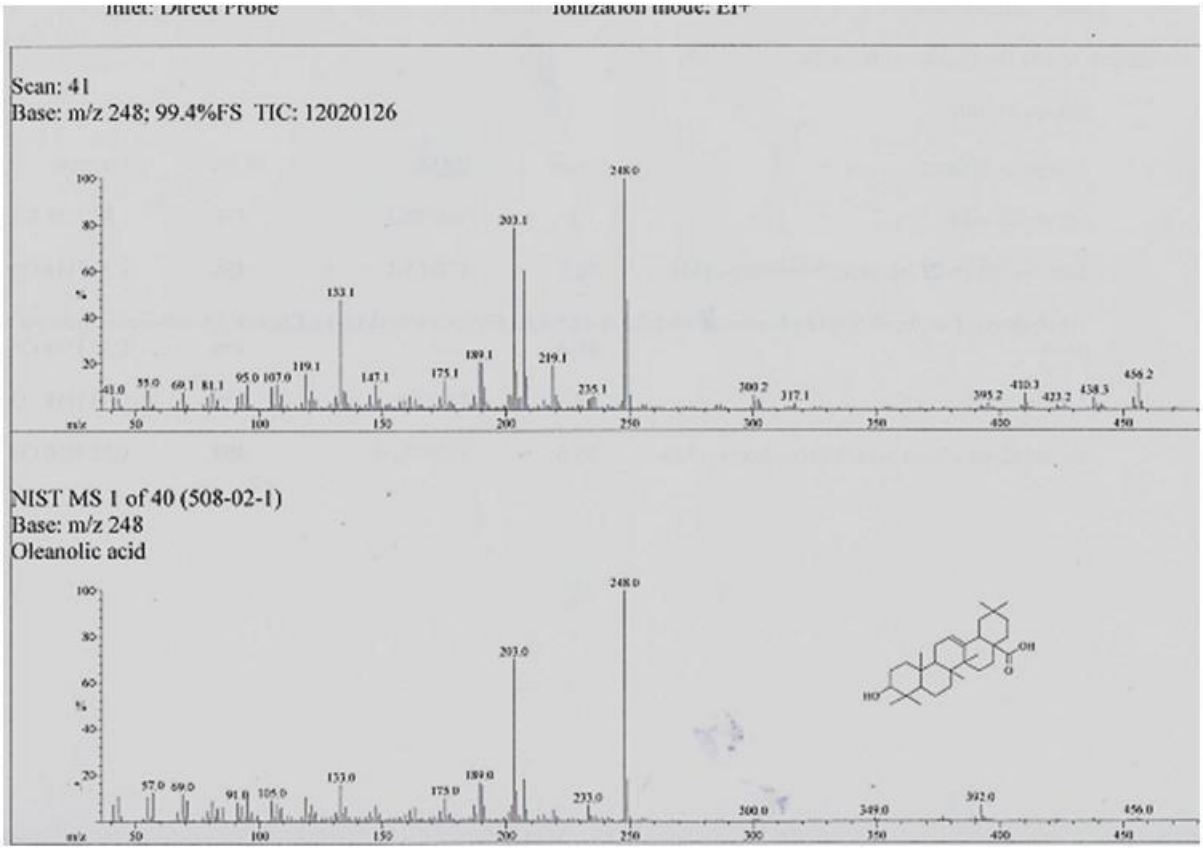


Figure 49: EI-MS Spectrum of PF4

In the 1H NMR spectrum of PF4 showed seven singlet signals of seven angular methyl groups at 1.24 ppm (s, 3H, H-23), 1.04 ppm (s, 3H, H-24), 0.93 ppm (s, 3H, H-25), 1.02 ppm (s, 3H, H-26), 1.30 ppm (s, 3H, H-27), 0.97 ppm (s, 3H, H-29) and1.01 ppm (s, 3H, H-30).The signal of H-18 and the singlet proton signals of H-29 and H-30 permitted the confirmation of the oleanolic acid skeleton. The characteristic signal of H-12 was observed at 5.23 ppm confirming that this

compound is Δ^{12}-unsaturated oleanane triterpenoid while the signal of the oxymethine proton H-3 at the foot of a hydroxyl group appears as a double of doublets at 3.15 ppm.

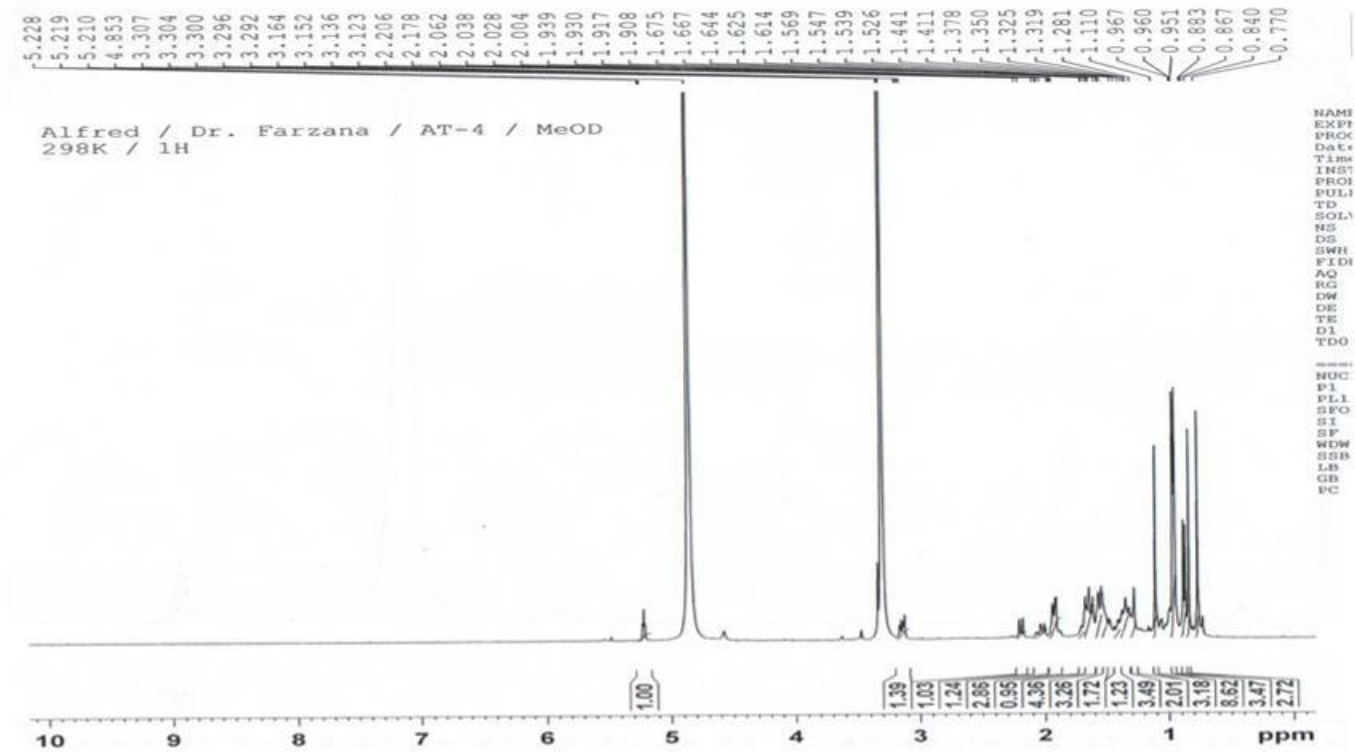


Figure 50: ^{1}H NMR Spectrum of PF4 ($CDCl_3$, 400 MHz)

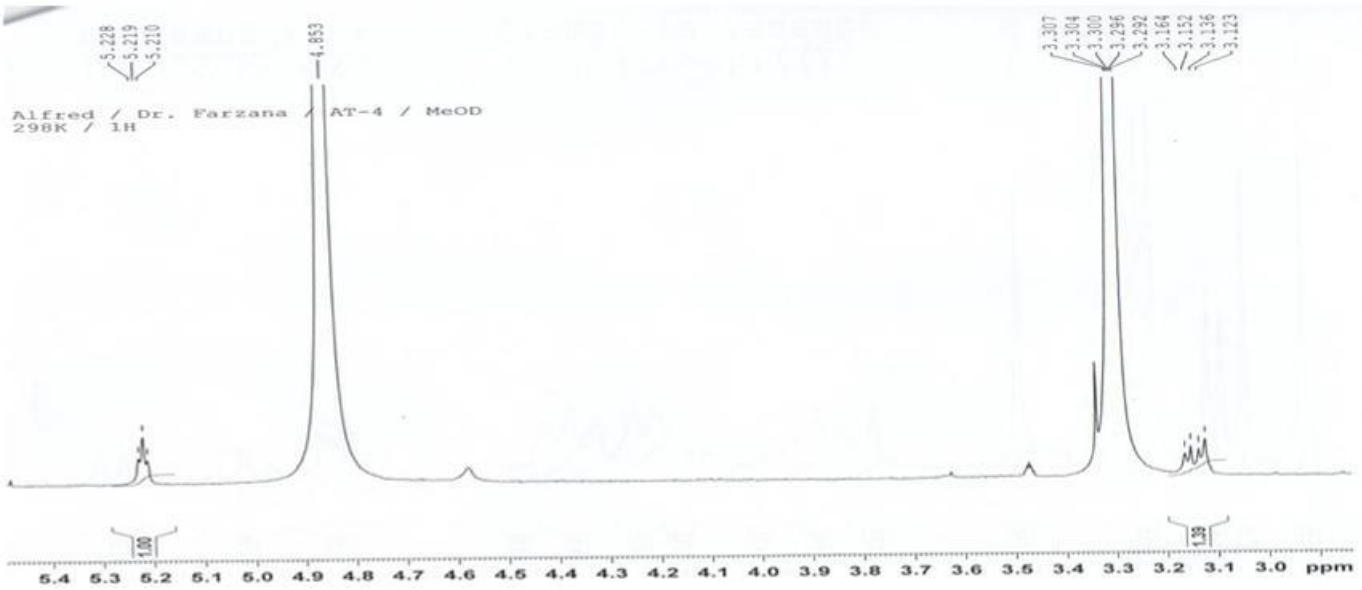
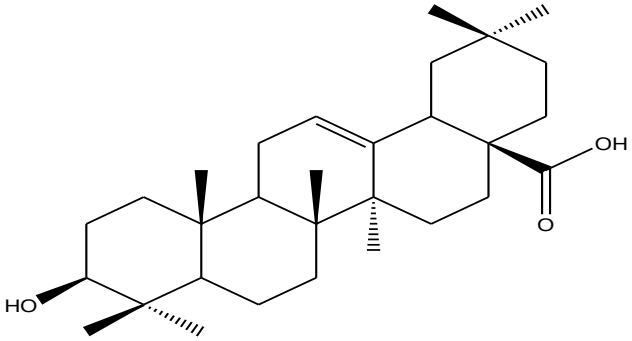


Figure 51: ^{1}H NMR Spectrum of PF4 ($CDCl_3$, 400 MHz) enlarged 3.0 ppm – 5.4 ppm

The above spectral information enabled us to identify AT4 as oleanolic acid by comparison with reported data (**Werner *et al.*, 2003**).



Oleanolic acid **(139)**

Table 29: NMR data of PF4

PF4		**Oleanolic acid (Werner et *al.*, 2003)**		**PF4**		**Oleanolic acid (Werner et *al.*, 2003**	
Position	**^{1}H**	**^{1}H**	**^{13}C**	**Position**	**^{1}H**	**^{1}H**	**^{13}C**
1	1.57	1.57	39.0	**17**	-	-	46.7
2	1.93	1.90	28.1	**18**	1.53, t	1.73, dd	42.1
3	3.15, dd,1H	3.44, dd,1H	78.2	**19**	1.4	1.32	46.6
4	-	-	39.4	**20**	-	-	31.0
5	0.88, d	0.88, d	55.9	**21**	1.44	1.46	34.3
6	1.54, m	1.53, m	18.8	**22**	2.06, m	2.04, m	33.2
7	1.37, m	1.36, m	33.4	**23**	1.33, s	1.24, s	28.8
8	-	-	39.8	**24**	0.97, s	1.04, s	16.5
9	1.67, m	1.71, m	48.2	**25**	0.77, s	0.93, s	15.6
10	-	-	37.4	**26**	0.96, s	0.96, s	17.5
11	1.96, dd	1.96, dd	23.8	**27**	1.11, s	1.30, s	26.2
12	5.22, t	5.49, t	122	**28**	-	-	180
13	-	-	144	**29**	0.84, s	0.97, s	33.4
14	-	-	42.2	**30**	0.95, s	1.01, s	28.8
15	2.18	2.19	28.4				
16	1.94, dd	2.12, dd	23.8	**-**			

II-4-1-12: Structural identification of PF10

The compound PF10 crystallized in the solvent system Hexane: ethyl acetate (60:40) in the form of a whitish-gray solid. The melting point was found to be 297-299 ^{o}C.

The EI-MS of PF10 showed a molecular ion peak at m/z 456.4, giving the molecular formular $C_{30}H_{48}O_3$ and the base peak at m/z 189 characteristic of lupane type triterpenoids, with the peak at m/z 248.2 indicating the presence of –COOH group at position 28.

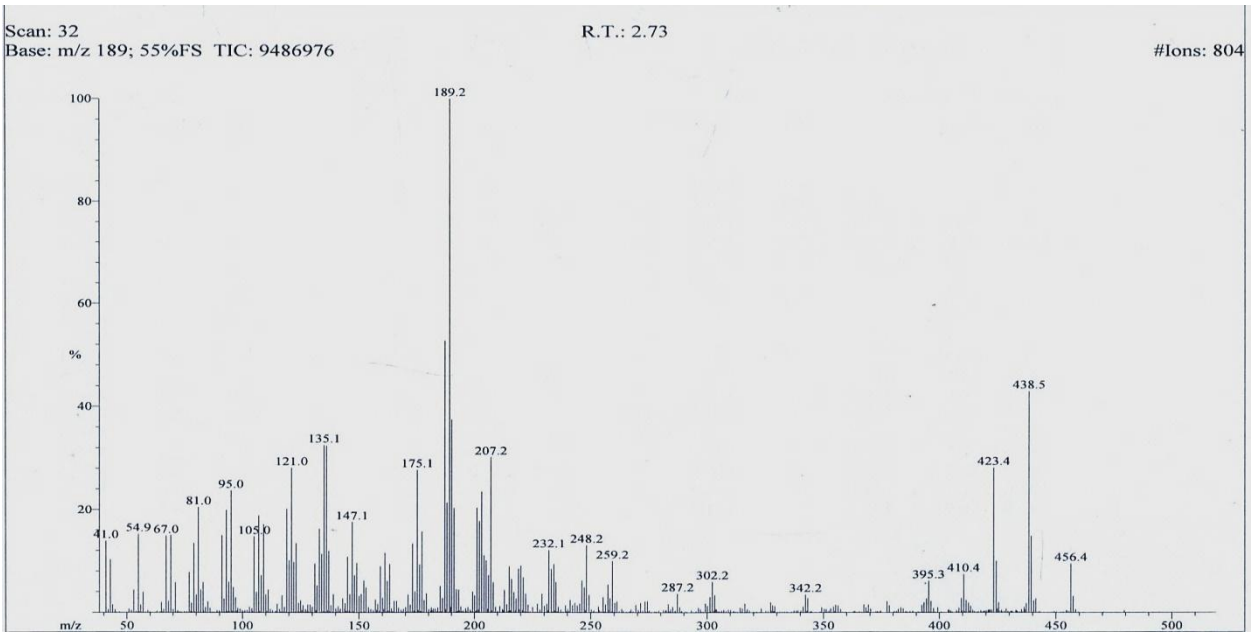


Figure 52: EI-MS Spectrum of PF10

The ^{1}H NMR spectrum of compound PF10 showed resonances for olefinic exocyclic methylene protons at 4.72 ppm (d, H-29a) and 4.59 ppm (d, H-29b) which confirm that PF10 is of a lupane type confirmed by allylic protons of the vinyl methyl singlet at 1.67 ppm indicating the presence of an isopropenyl group of betulinic acid. However, in addition to the vinyl methyl singlet at 1.69 ppm, there are five angular methyl singlets at 1.03 ppm (s, 3-H, H-26), 1.01 ppm (s, 3-H, H-23), 0.91 ppm (s, 3-H, H-27), 0.83 ppm (s, 3-H, H-25) and 0.79 ppm (s, 3-H, H-24) instead of six methyl singlets indicating the presence of –COOH group at position 28. The spectrum also showed a methine signal at 2.20 ppm (ddd, *J*=11.1, 11.1, 5.8 Hz) which was attributed to the allylic proton at H-19. The signal of an oxymethine proton at 3.37 ppm attributable to the H-3 was observed as a multiplet.

The ^{13}C NMR spectrum of PF10 revealed 30 carbon signals, of which some are characteristic namely one oxymethine, one carboxylic acid, and two olefinic carbons. Two vinylic carbon atom

resonances at 150.4 ppm and 109.9 ppm, corresponding to carbon atoms 20 and 29 respectively supported the olefinic methylene protons seen as singlets at 4.72 ppm and 4.59 ppm in the 1H NMR spectrum. The signal at 76.3 ppm was characteristic for the oxymethine carbon at position 3 while a signal at 180.6 ppm was attributed to the carboxylic carbon atom C-28.

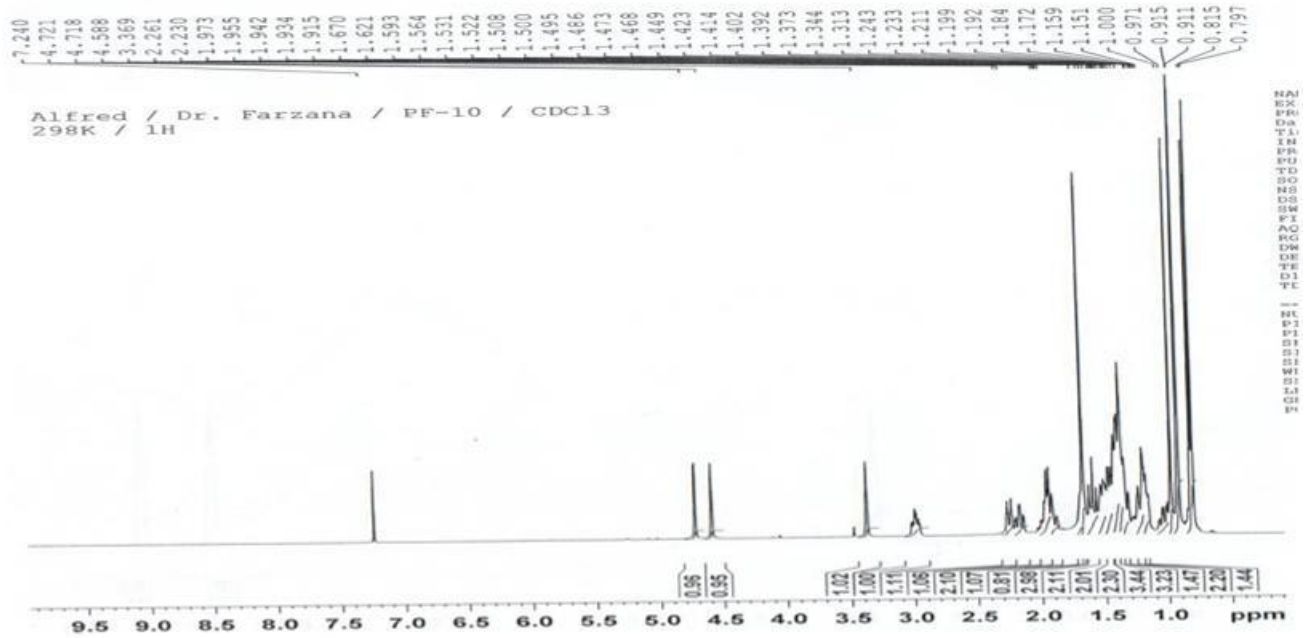


Figure 53: ^{1}H NMR Spectrum of PF10 ($CDCl_3$, 400 MHz)

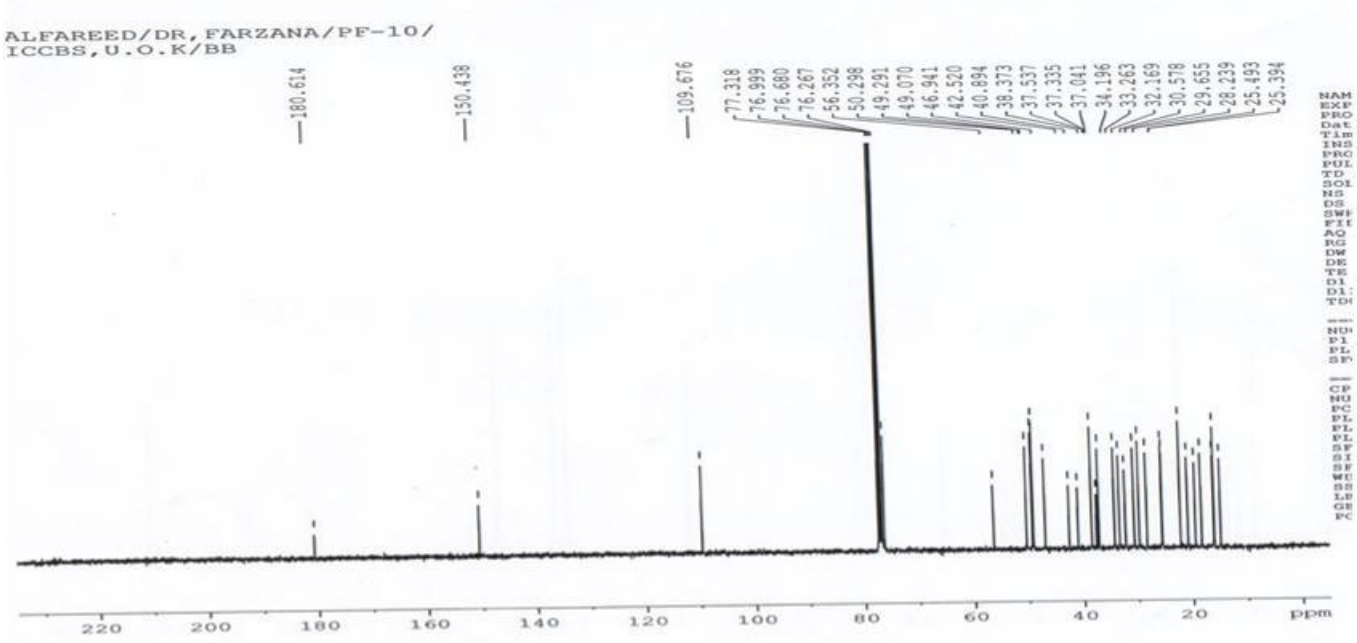
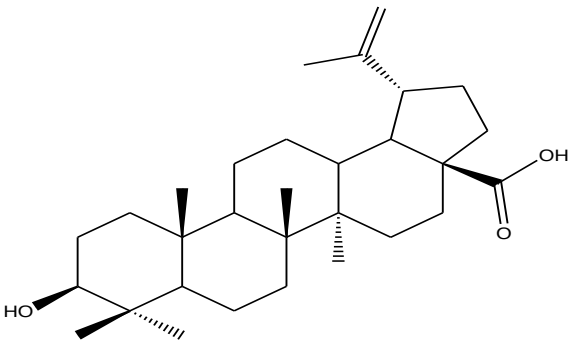


Figure 54: ^{13}C NMR Spectrum of PF10 ($CDCl_3$, 150MHz)

The above spectral information led to the identification of PF10 as betulinic acid previously described by Eun-Hye and co-workers (**Eun-Hye *et al.*, 2015**) whose structure is given below.



Betulinic acid (**140**)

Table 30: NMR data of PF10

PF10			**Betulinic acid (Eun-Hye et al., 2015)**	**PF10**			**Betulinic acid (Eun-Hye et al., 2015)**
Position	**^{13}C**	**^{1}H**	**^{13}C**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**
1	38.4		39.0	**17**	56.4		56.3
2	25.4		27.6	**18**	46.9		47.1
3	76.3	3.36 (m, 1H)	78.2	**19**	49.1	2.20,ddd,1 H	49.4
4	37.5		39.0	**20**	150.4		150.0
5	50.3		55.5	**21**	29.7		29.9
6	18.2		18.4	**22**	37.1		37.3
7	34.2		34.5	**23**	28.2	1.01,s, 3H	27.9
8	40.9		40.8	**24**	15.9	0.79,s, 3H	15.4
9	49.3		50.7	**25**	16.0	0.81,s, 3H	16.2
10	37.3		37.3	**26**	18.2	1.03,s, 3H	16.3
11	22.1		21.0	**27**	14.8	0.91,s, 3H	14.6
12	25.5		25.6	**28**	180.6		180.6
13	38.4		38.2	**29**	109.9	4.72(H-29a),4.59 (H-29b)	108.8
14	42.5		42.5	**30**	19.4	1.67,s, 3H	19.6
15	32.2		30.4				
16	33.3		32.6	**-**			

II-4-1-13: Structural elucidation of TA23

The fraction TA23 crystallized as white crystals in the eluent mixture hexane-CH_2Cl_2 (60:40, v/v). It melted between 192-195ºC and gave a positive test with the Liebermann-Burchard reagent indicating that the compound TA23 is a triterpene.

The ITMS-ESI Full MS mass spectrum showed a quasi-molecular ion peak [M+H] at m/z = 413.6 and retention time 0.99 minutes (RT = 0.99 mins) corresponding to the molecular formula $C_{29}H_{48}O$ and another quasi-molecular peak [M-H] at m/z = 437.6 for the molecular formula $C_{31}H_{50}O$ with retention time 2.41 minutes (RT = 0.93 mins), thereby showing that it is a mixture of two compounds.

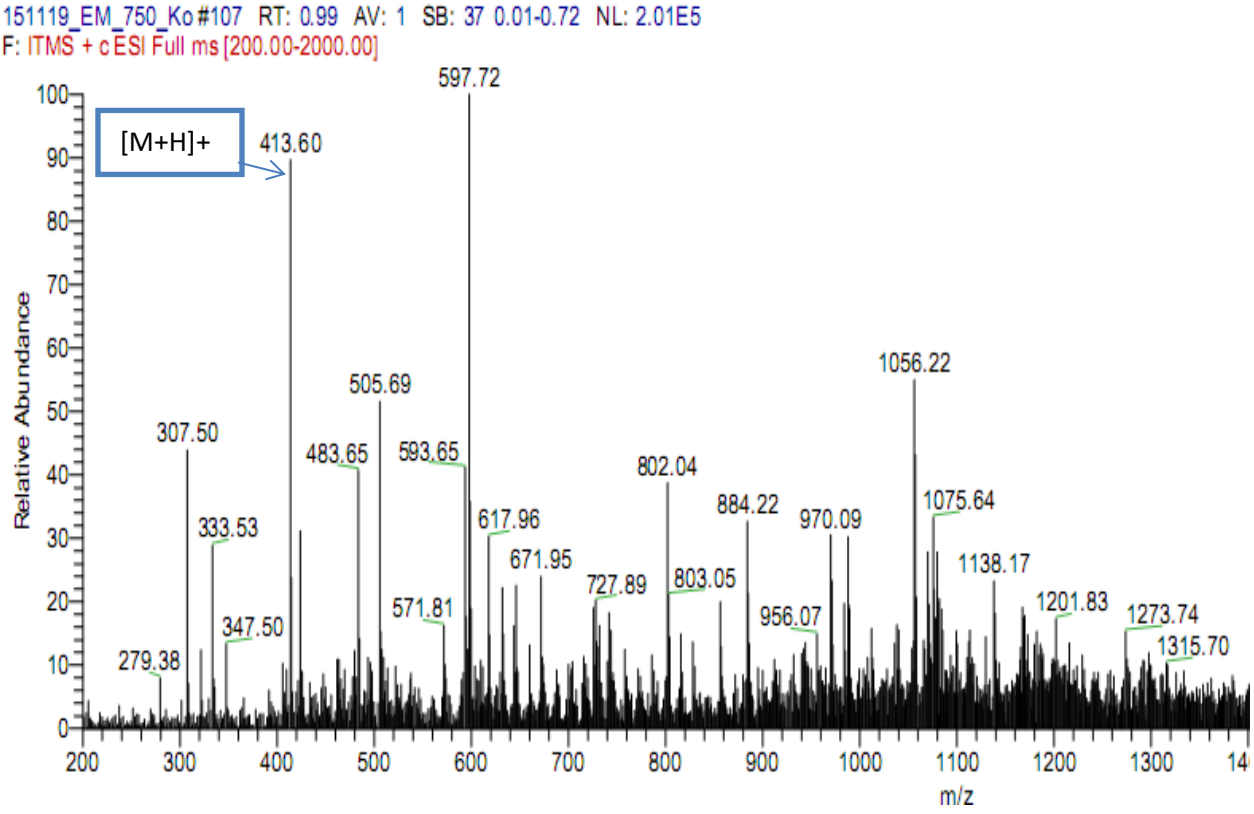


Figure 55: ITMS-ESI Full MS mass spectrum of TA23 in positive mode

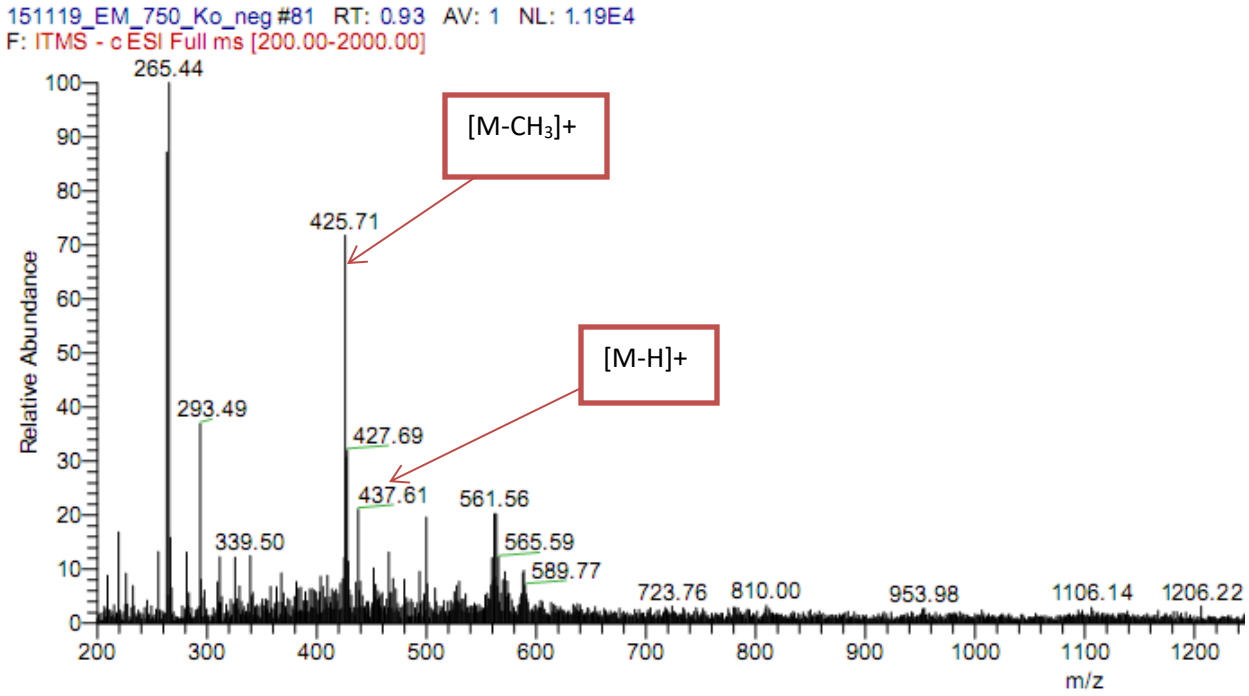


Figure 56: ITMS-ESI Full MS mass spectrum of TA23 in negative mode

The ^{1}H NMR spectra of TAN23 showed the presence of two methyl singlets at δ_H δ 0.71 (H-28) and δ_H 1.03 ppm (H-29); three methyl doublets that appeared at δ_H 0.80 (H-27), 0.82 (H-26), and 0.91 ppm (H-19); and a methyl triplet at δ_H 0.83 ppm (H-24) with other protons at δ_H 4.70, δ_H 5.10 and δ_H 5.20 ppm suggesting the presence of three protons corresponding to that of a trisubstituted and a disubstituted olefinic bond of stigmasterol. The proton corresponding to the H-3 of a sterol moiety was appeared as a triplet of doublet of doublets at δ_H 3.25 ppm. Signals due to a cyclopropane methylene Hα-19, δ_H 0.35 ppm and Hβ-19, δ_H 0.55 ppm both doublets (J = 4.2 Hz) characteristic of a cycloartane triterpenoid. Exocyclic vinylic protons Hα-31 and Hβ-31 atδ_H 4.60 ppm and δ_H 4.70 ppm respectively, H-26 and H-27 both doublets (d, J=7.0 Hz, 3H), a multiplet at δ_H 2.12 ppm H-23 and finally the characteristic multiplet of a 24-methylenecycloartane triterpenoid H-25 at δ_H 2.30 ppm. Singlets of additional tertiary methyls of the cycloartane were seen between 0.80 ppm and 1.0 ppm while the signals of the methyl H-21 appeared at δ_H 0.85 ppm (J = 6.5 Hz).

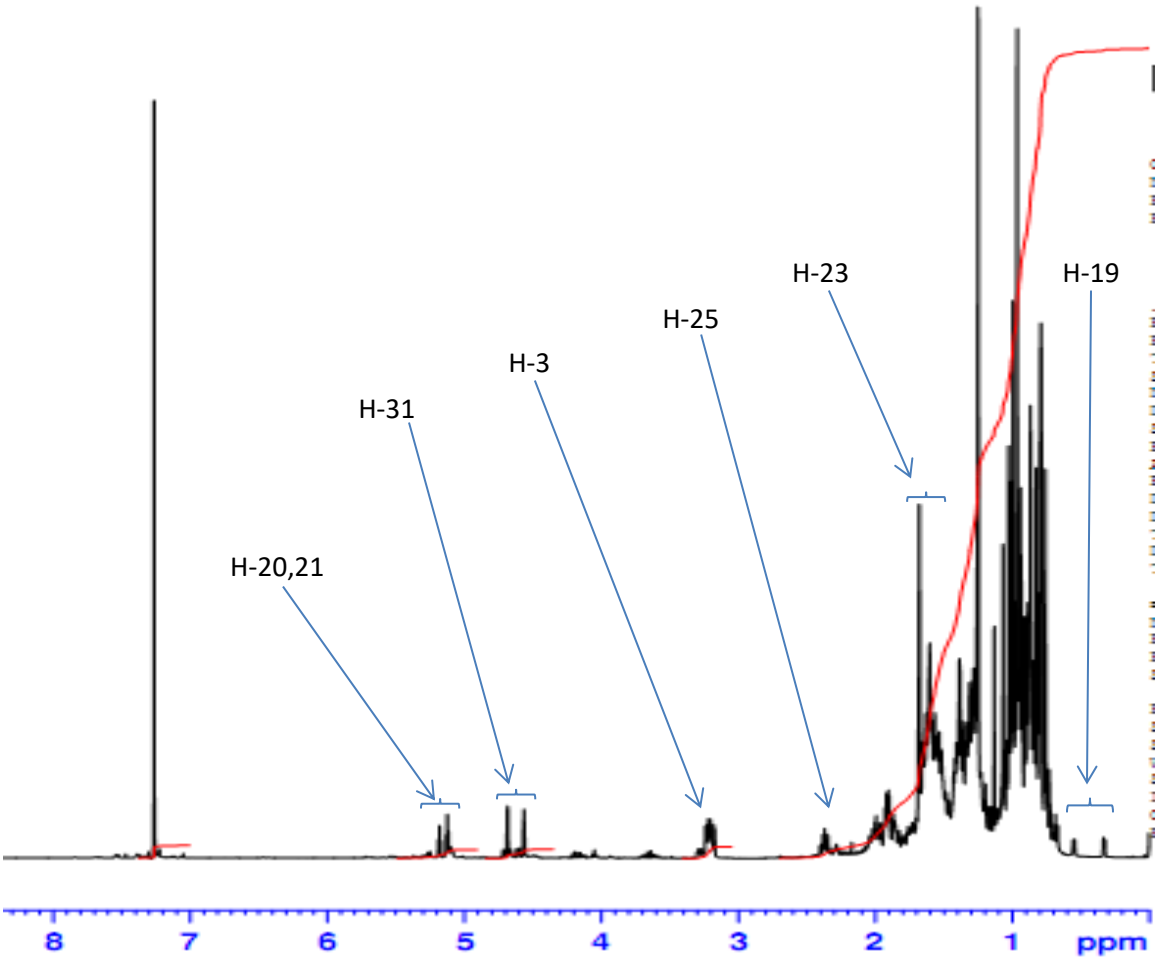


Figure 57: ^{1}H NMR Spectrum TA23 ($CDCl_3$, 500 MHz)

^{13}C NMR (125 MHz) spectrum of TAN23 exhibited characteristic signals for stigmasterol at δ_C 145.2 ppm (C-5), δ_C 139.6 ppm (C-20), δ_C 125.3 ppm (C-21), δ_C 121.7 ppm (C-6) and δ_C 69.8 ppm (C-3). Characteristic signals for 24-methylenecycloartenol were at δ_C 150.9 ppm (C-24), δ_C 109.3 ppm (C-31) and δ_C 79.1 ppm (C-3) in the deshielded zone.

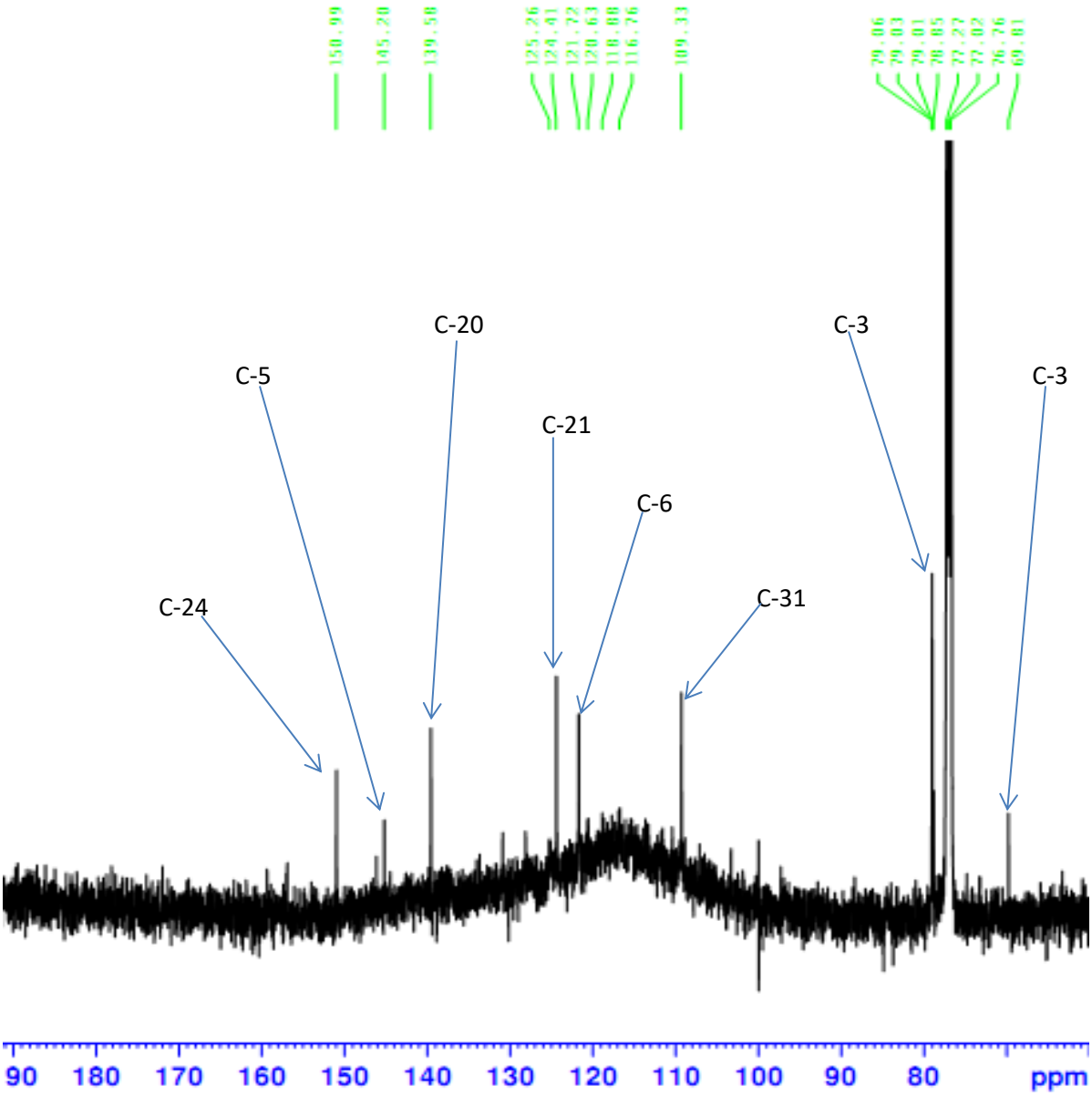


Figure 58: ^{13}C NMR Spectrum TA23 ($CDCl_3$, 125 MHz) enlarged between 60-190 ppm

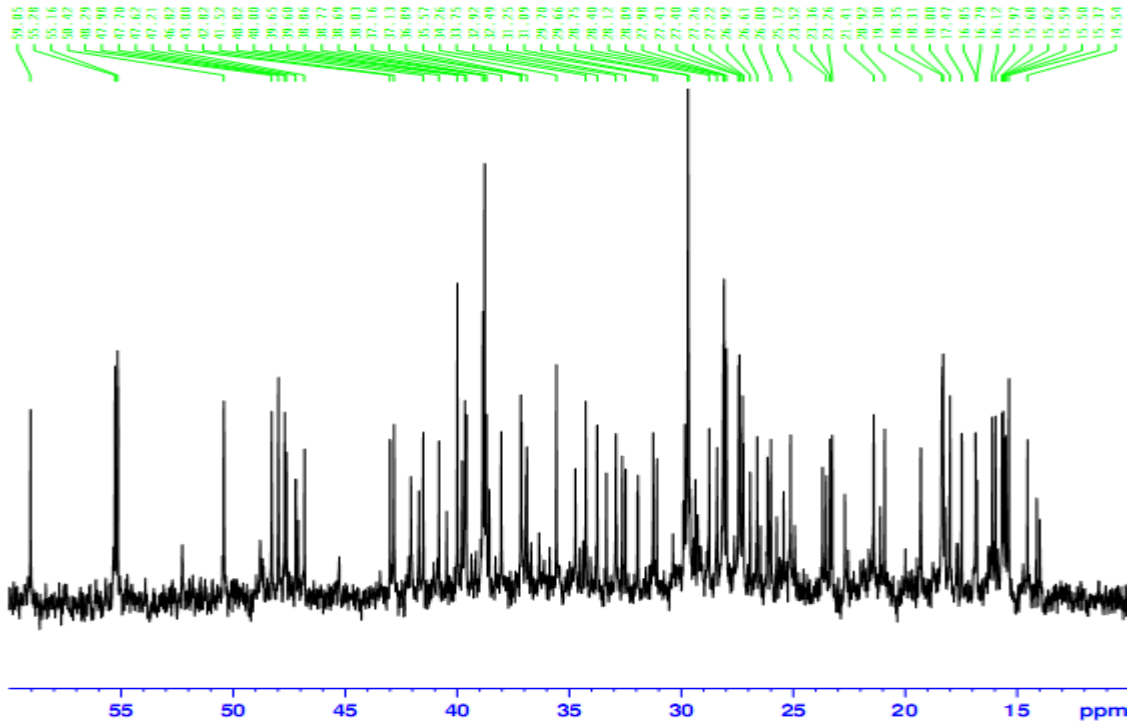
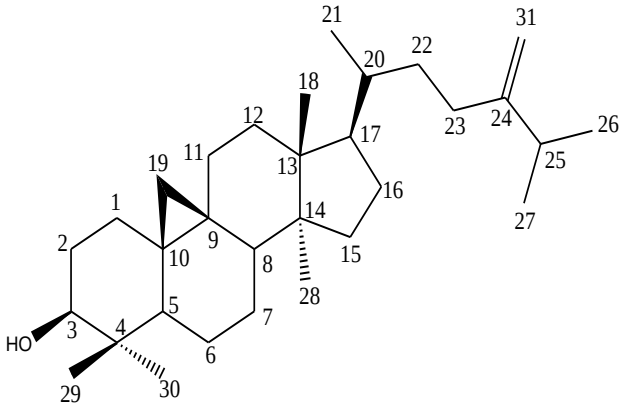
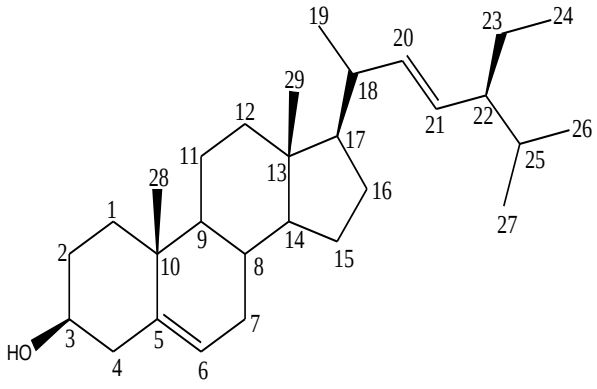


Figure 59: ^{13}C NMR Spectrum TA23 ($CDCl_3$, 125 MHz) enlarged between 10-60 ppm

2D NMR spectra of TAN23 showed key correlations that confirmed the bond connectivities between the various atoms. The spectroscopic data of TA23 compared with data reported in literature (**Chaturvedula *et al.*, 2012**) led to the identification of TA23 as a mixture of stigmasterol and 24-methylenecycloartenol.



24-methylenecycloartenol **(141)**



Stigmasterol **(134)**

Table 31: NMR data of TA23 (mixture of stigmasterol and 24-methylenecycloartenol)

TA23	24-methylenecycloartenol		stigmasterol		TA23	24-methylenecycloartenol		stigmasterol	
Position	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**
1	32.9		38.7		**16**	28.4		31.3	
2	33.7		32.5		**17**	55.1		55.2	
3	79.1	3.25, t	69.8	3.25, m	**18**	21.4		40.5	
4	40.0		43.0		**19**	29.7	0.35α ; 0.55β, d	26.6	0.91, d
5	47.6		145.2	5.30, t	**20**	38.8		139.6	5.20, m
6	23.4		121.7		**21**	18.9	0.85, d	125.3	5.10, m
7	28.1		34.3		**22**	37.1		47.2	
8	48.2		31.1		**23**	35.6	2.12, m	27.9	
9	23.3		50.4		**24**	150.9		14.54	0.83, t
10	28.8		38.7		**25**	37.2	2.30, m	31.3	
11	29.7		23.5		**26**	26.0		20.9	0.82, d
12	35.8		39.7		**27**	25.1		19.3	0.80, d
13	46.8		42.8		**28**	26.9		18.4	0.71, s
14	48.3		59.1		**29**	16.9	0.8, s	15.6	0.71, t
15	38.0		27.3		**30**	20.9	1.0, s	-	
					31	109.3	4.60α ; 4.70β, s	-	

II-4-1-14: Structural identification of TA42

The compound TA42 crystallized as white powder in hexane-ethyl acetate (75/25, v/v). It gave a positive test with the Liebermann-Burchard reagent characteristic of triterpenes.

The ESI-TOF MS spectrum of TA42 showed a pseudo-molecular ion peak $[M+Na]^+$ at m/z = 447.4 from where the molecular formula $C_{30}H_{48}O$ was deduced.

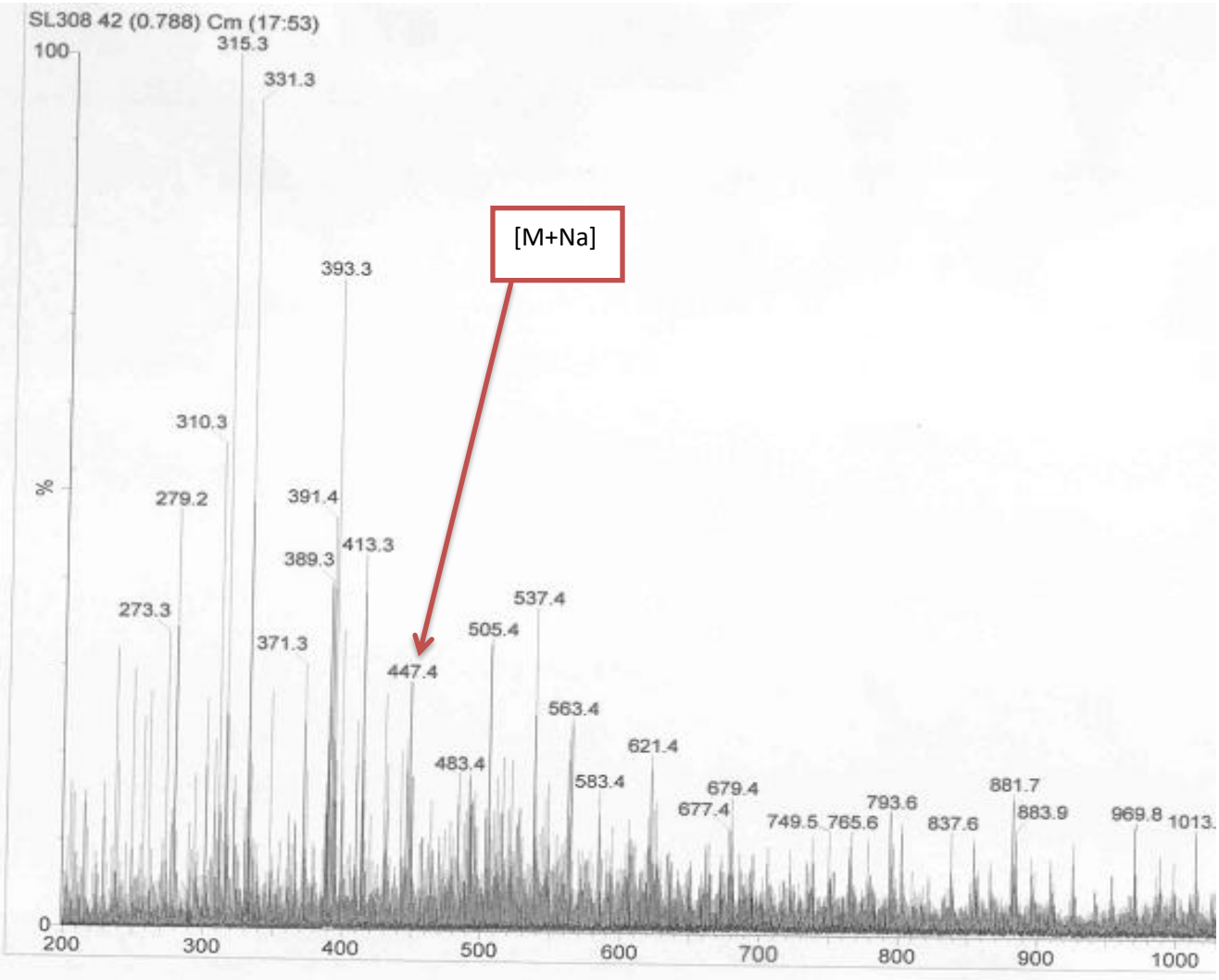


Figure 60: ESI-TOF MS spectrum of TA42

The ^1H NMR spectrum of compound TA42 exhibited signals of one-proton double of doublet at δ_H 5.15 ppm with coupling constants of J = 7.2 Hz and J = 5.3 Hz and assignable to vinyl proton H-12 of a 12,13-unsaturated pentacyclic triterpene which is further confirmed by the allylic proton H-18 whose signal appears in the form of doublet at δ_H 1.95 ppm characteristic of Δ^{12} oleanenes. Two one-protonbroad singlet signals at δ_H 4.65 ppm and 4.80 ppm ascribable to the exocyclic

methylene Hα-29 and Hβ-29 protons respectively of a lupane type triterpenoid. A multiplet due to one-proton at δ_H 3.25 with coupling attributable to the α-oriented oxymethine proton H-3. A three-proton singlet in the deshielded region at δ_H 1.65 ppm was attributed to H-30 allylic methyl protons located on carbon 30 of a 20(29)-unsaturated lupane. The other six three-proton broad signals appeared at δ_H1.05, 1.00, 0.95, 0.90, 0.85 and 0.80 ppm and assigned correspondingly to tertiary H-23, H-27, H-24, H-25, H-26 and H-28 methyl protons.

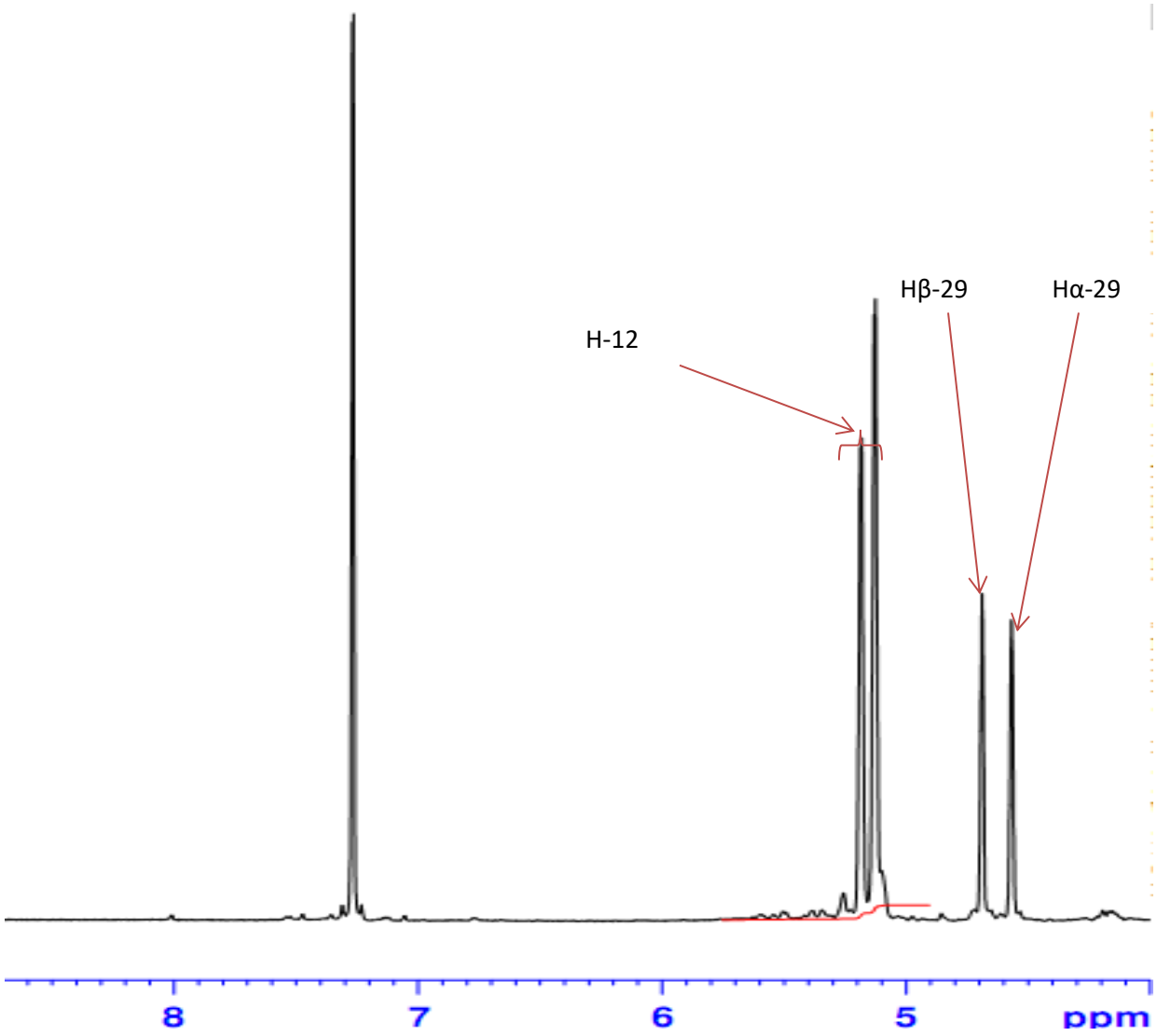


Figure 61: ^{1}H NMR spectra of TA42 enlarged from 4 ppm-8.7 ppm

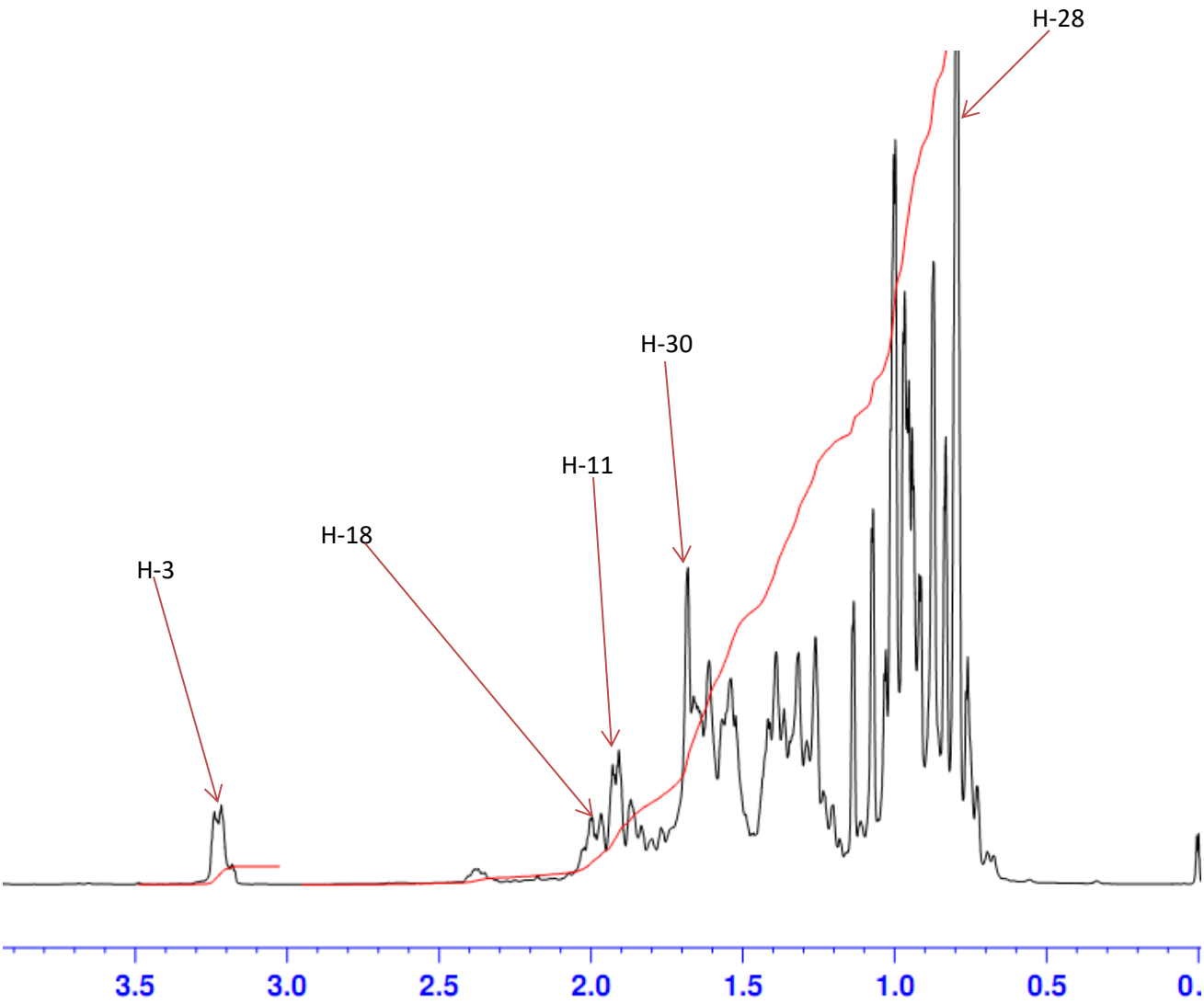


Figure 62: ^{1}H NMR spectra of TA42 enlarged from 0 ppm- 4.0 ppm

HSQC spectrum of TA42 showed $^{1}J_{C\text{-}H}$ correlations between the proton at δ_H 5.15 ppm and a carbon atom at δ_C 122.0 ppm (C-12), the protons at δ_H 4.65 ppm and δ_H 4.80 ppm and a carbon atom at δ_C 109.5 ppm (C-29) and finally the proton at δ_H 3.25 ppm and a carbon atom at δ_C 79.0 ppm (C-3). Key HMBC correlations were observed as follows: δ_H 1.65 ppm (H-30) and a carbon atom at δ_C 109.5 ppm (C-20) and δ_C 151.0 ppm (C-20), δ_H 0.80 ppm (H-23) and δ_H 1.00 ppm (H-24) with δ_C 79.0 ppm (C-3) and δ_H 0.90 ppm (H-27) with δ_C 145.0 ppm (C-13).

However important ^{1}H-^{1}H COSY cross peaks were observed between some protons notably H-12/H-11, H-3/H-2 and Hα-29/Hβ-29/H-30.

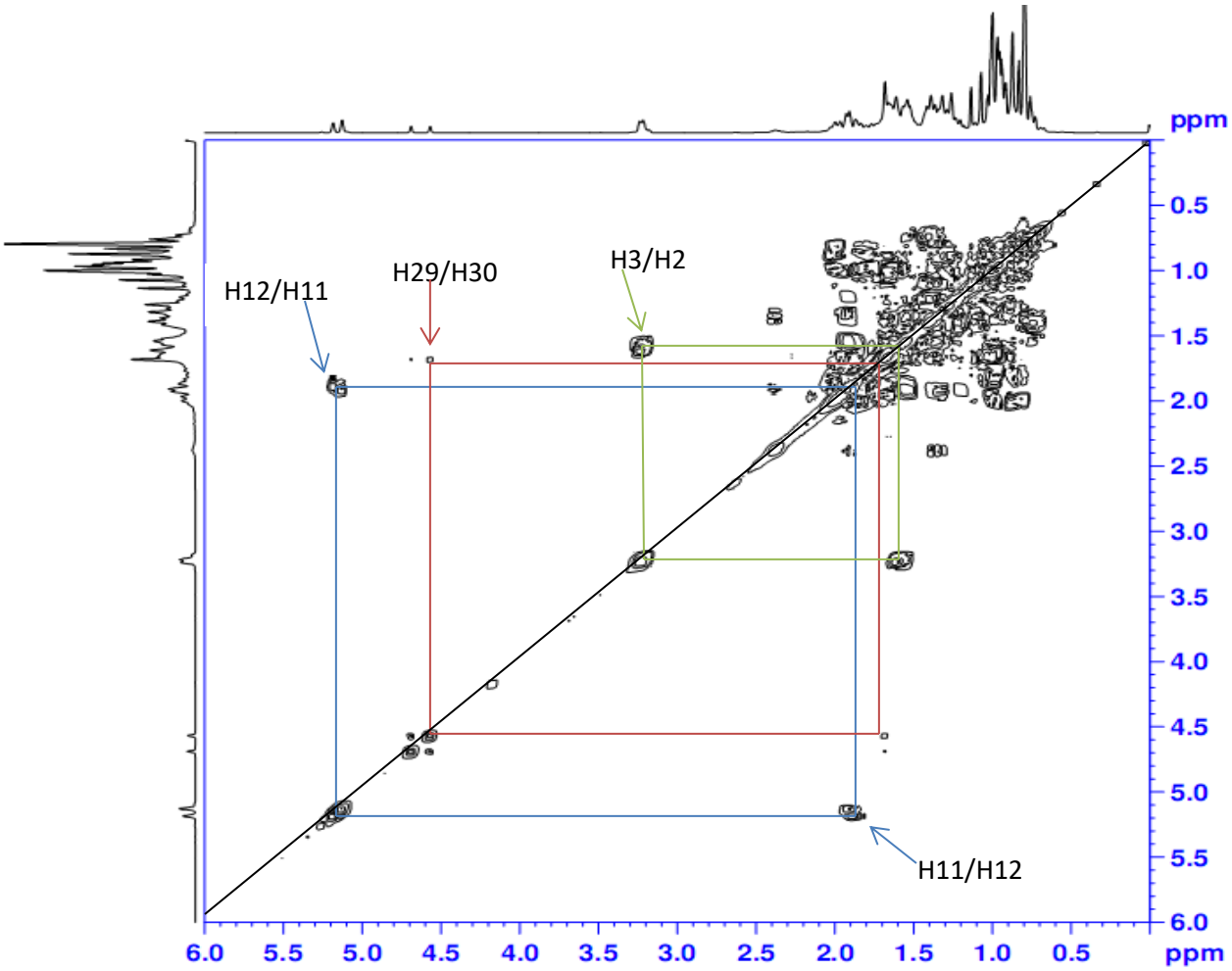
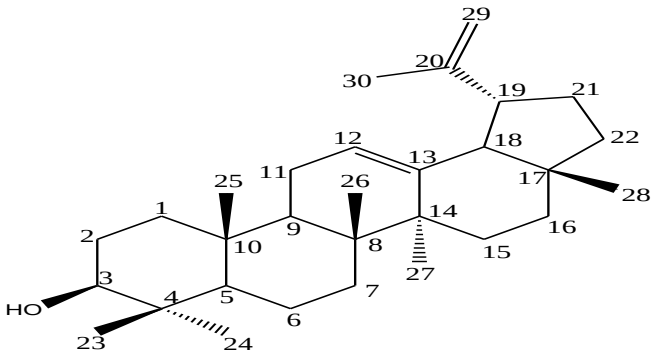


Figure 63: ^{1}H-^{1}H COSY spectrum of TA42

The spectral described above and by comparison with some data reported in literature, TA42 was identified as lup-12,20(29)-dien-3β-ol described previously (**Abreu *et al.*, 2010**).



lup-12,20(29)-dien-3β-ol **(142)**

II-4-1-15: Structural identification of ND22'

Compound ND22 crystallized in the form of the white crystals in system of elution hexane/AcOEt (92.5/7.5, v/v). It melted between 215-217°C, was soluble in methylene chloride and gave a red-violet coloration upon treatment with Liebermann-Burchard reagent indicating that ND22' is a triterpene. Its ESI-TOF mass spectrum showed a pseudo molecular ion peak [M+Na] m/z = 449.3.

The ^{1}H-NMR spectrum of compound ND22' showed signals of seven tertiary methyl at δ_H 0.79 ppm (3H, s, H-27), 0.84 ppm (3H, s, H-26), 0.97 ppm (3H, s, H-24), 1.04 ppm (3H, s, H-23), 1.26 ppm (3H, s, H-28), 1.40 ppm (3H, s, H-25) and 1.69 ppm (3H, s, H-30) for a lupane triterpenoid and 8 methyl singlets at δ_H 0.77 ppm (3H, s, H-25), 0.80 ppm (3H, s, H-23), 0.81 ppm (3H, s, H-30), 0.84 ppm (3H, s, H-29), 0.91 ppm (3H, s, H-24), 0.94 ppm (3H, s, H-26), 1.08 ppm (3H, s, H-28) and 1.15 ppm (3H, s, H-27) for an oleanane pentacyclic triterpene. Also, a pair of singlet signals at δ_H 4.57 and 4.70 ppm (1H, s) indicative of terminal vinyl protons Hα-29 and Hβ-29 of lupane type triterpene respectively further substantiated by a doublet of triplet signal at δ_H 2.4 ppm (1H, dt, H-19). The characteristic signal of the trisubstituted olefinic proton H-12 of oleanane type triterpenes appeared at δ_H 5.18 ppm as a triplet while the allylic protons H-18 and H-11 where observed as multiplet at 1.9 ppm. The presence of a doublet of doublet signal at δ_H 3.20 ppm (dd, J=11.2, 5.2 Hz) typical of the oxymethine proton H-3.

The ^{13}C NMR spectrum of ND22' showed signals of the exomethylene carbon at δ_C 109.3 ppm (C-29), the quaternary carbon attached to the exomethylene at δC 150.9 ppm (C-20) of lupeol and other two olefinic carbon atoms, an olefinic methine at δ_C 121.8 ppm (C-12) and a disubstituted olefin carbon at δ_C 145.3 ppm (C-13). The oxygenated methine carbon signal appeared at δ_C 79.01 ppm (C-3).

These ^{1}H NMR and ^{13}C NMR assignments were confirmed by some 2D NMR experiments amongst them HMBC, HSQC and COSY.

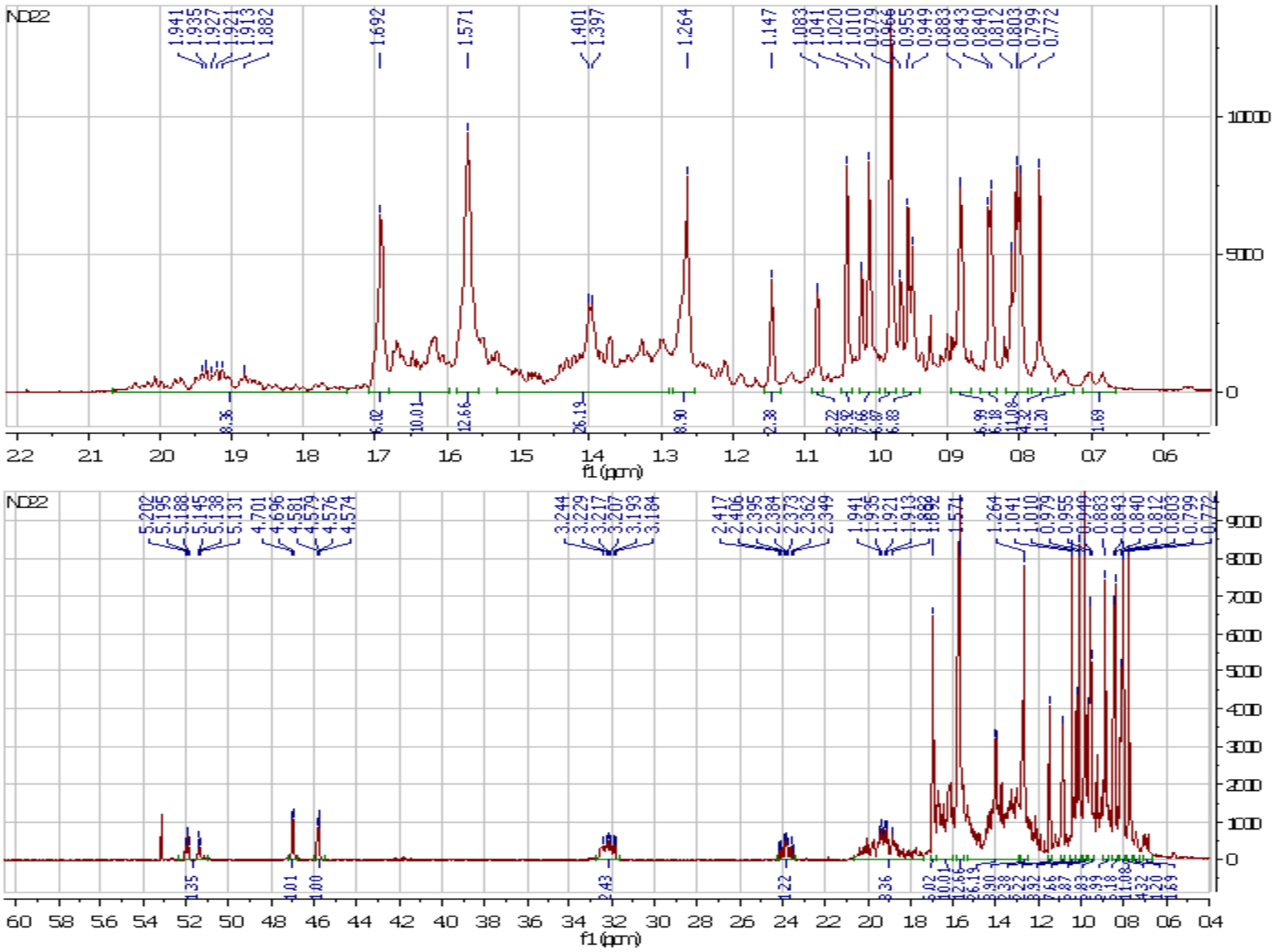


Figure 64: ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of compound ND22'

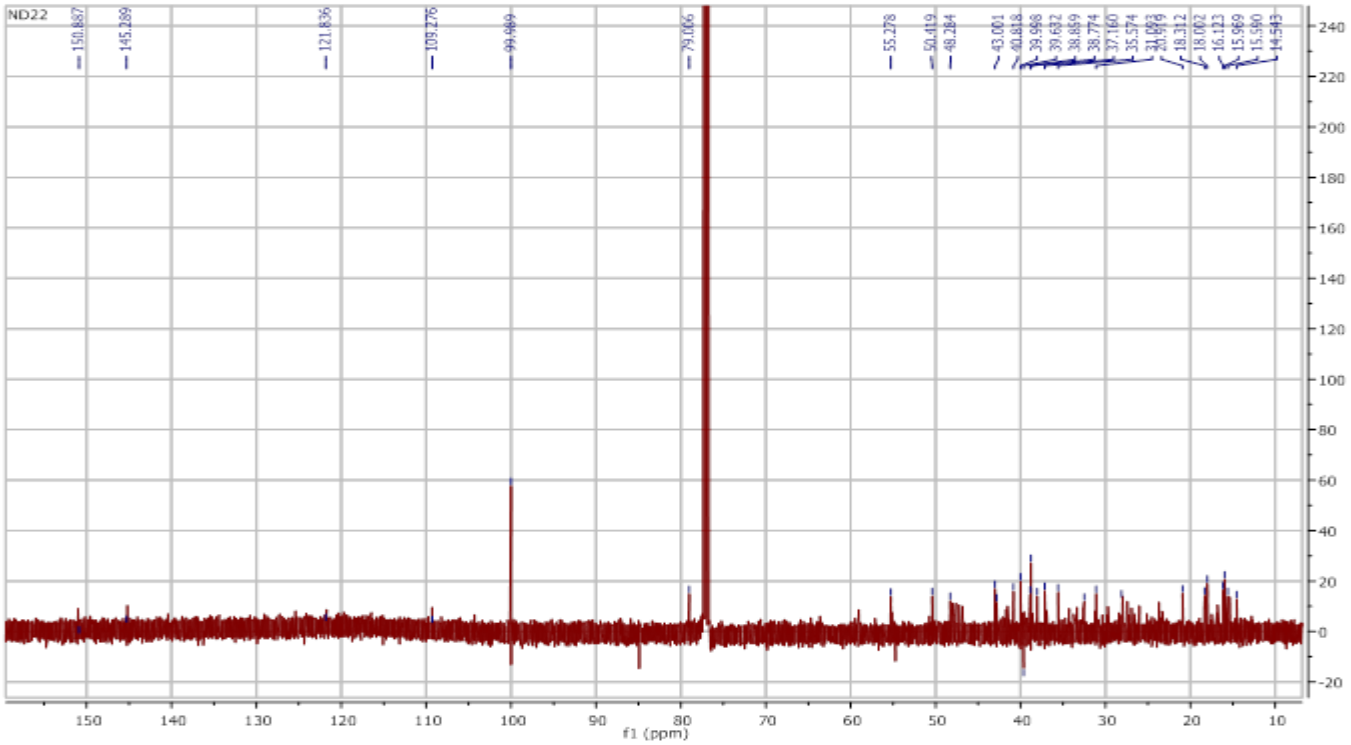
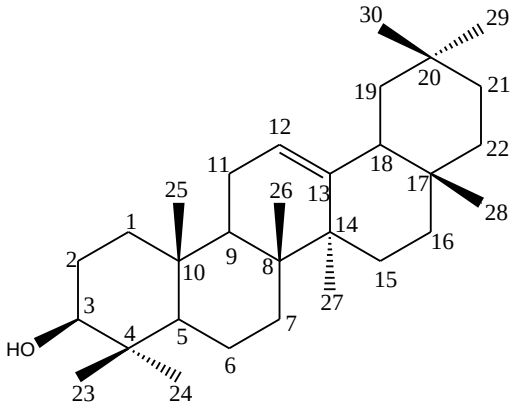
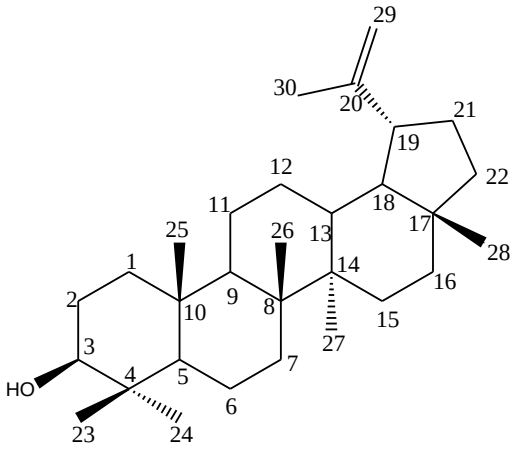


Figure 65: ^{13}C-NMR spectrum ($CDCl_3$, 500 MHz) of compound ND22'

The above spectral information led to the identification of ND22'as a mixture of β-amyrine and lupeol previously isolated form Peruvian Viagra, *Corynaea crassa* (**Gonzalo *et al.*, 2015**).



β-amyrine **(143)**



Lupeol **(88)**

Table 32: NMR data of ND22' (β-amyrine and lupeol)

ND22'	Lupeol		β-amyrine		ND22'	lupeol		β-amyrine	
Position	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**	**Position**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**
1	38.8		38.7	1.91, dd	**16**	35.6		31.1	
2	27.5		23.6		**17**	43.0		40.8	
3	79.0	3.19, t	79.01	3.24, t	**18**	48.3		50.5	1.94, m
4	39.9		37.2		**19**	47.8	2.38, m	28.1	
5	55.3		55.3		**20**	150.9		33.7	
6	19.3		18.0		**21**	30.1		39.6	
7	34.2		32.8	1.57, m	**22**	40.3		39.7	
8	41.1		41.5		**23**	28.4	1.04, s	28.1	0.80, s
9	50.5		47.6		**24**	15.6	0.97, s	16.7	0.91, s
10	37.2		36.8		**25**	16.1	1.40, s	15.6	0.77, s
11	21.2	1.40, m	28.1	1.94, m	**26**	16.0	0.84, s	16.8	0.94, s
12	25.3	1.88, m	121.8	5.18 m	**27**	14.5	0.79, s	23.2	1.15, s
13	38.5		145.3		**28**	18.1	1.26, s	17.5	1.08, s
14	42.8		42.1		**29**	109.3	4.56α ; 4.65β, s	18.7	0.84, s
15	27.2		26.6		**30**	20.2	1.69	21.3	0.81, s

II-4-1-16: Structural identification of PN6

Compound PN6 was isolated as white crystalline needles on elution with hexane-ethyl acetate (97.5/2.5, v/v) mixture. It melted between 209-211°C and gave a positive test with the Liebermann-Burchard reagent characteristic of triterpenes.

The ESI-TOF MS spectrum of compound PN6 showed a pseudo-molecular ion peak $[2M+H]^+$ at m/z = 969.6 for the molecular formula $C_{32}H_{52}O_3$ with a double bond equivalence of 7.

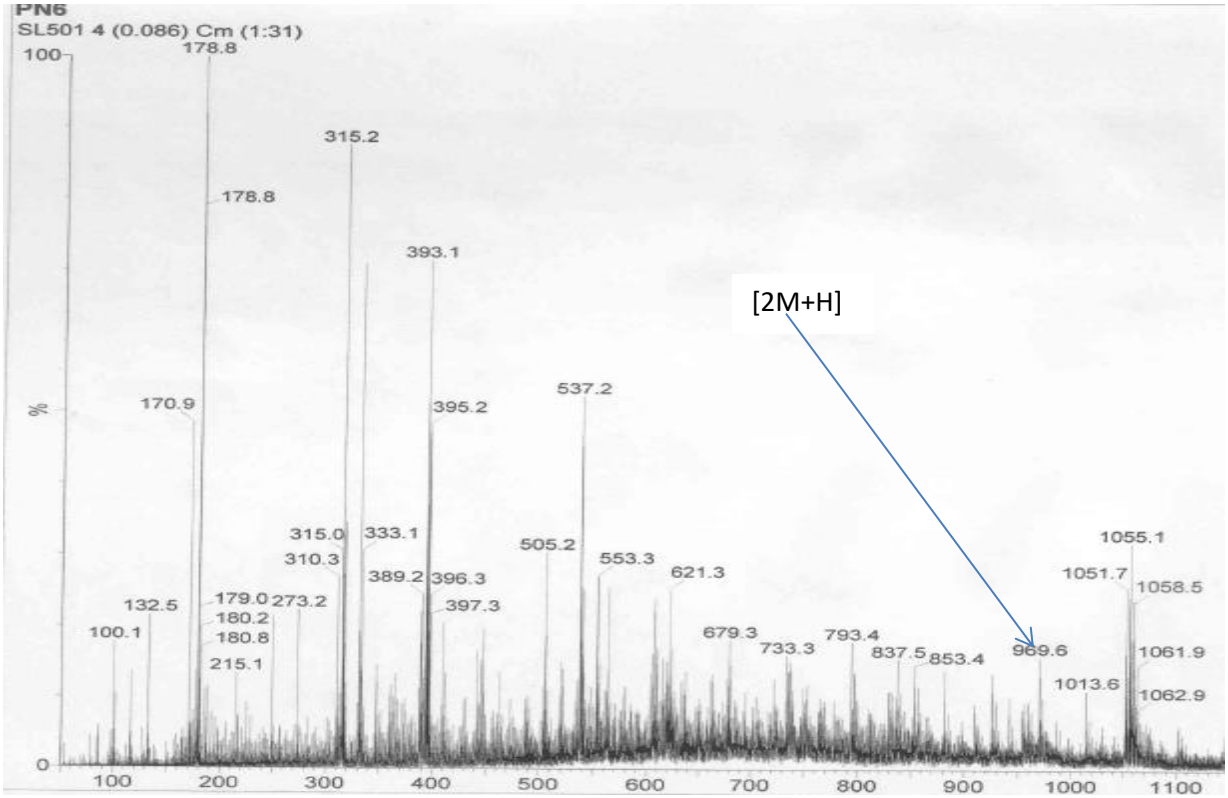


Figure 65: ESI-TOF MS spectrum of PN6

The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of PN6 showed the presence of six methyls at δ_H 1.40 ppm (3H, *s*, H-25), 1.05 ppm (3H, *s*, H-23), 0.95 ppm (3H, *s*, H-24), 0.86 ppm (3H, m, H-26), 0.80 ppm (3H, *s*, H-27) and the vinylic methyl at δ_H 1.65 ppm (3H, *s*, H-30). An acetate methyl at δ_H 2.05 ppm (s, 3H, H-2'). A pair of broad singlet protons appeared at δ_H 4.60 ppm and δ_H 4.70 ppm which were indicative of the exomethylene protons Hα-29 and Hβ-29 while the doublet of doublet at δ_H 4.45 ppm (dd, 1H, J=10.4, 7.6 Hz) was indicative of the proton attached to the carbon bonded to the acetoxy group. The oxymethylene H-28 appears as a singlet at δ_H 3.65 ppm. A sextet proton

at δ_H 2.30 ppm (dt, 1H, J = 10.8, 5.8 Hz) attributable to 19β-H. This ^{1}H NMR data is characteristic of lupane type triterpenoid.

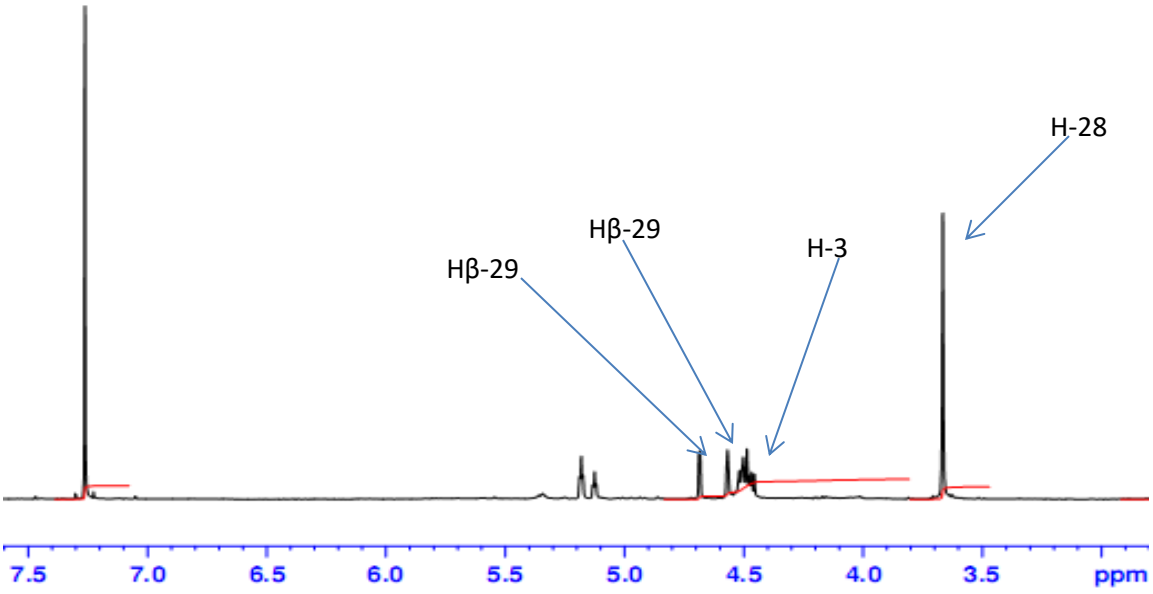


Figure 66: ^{1}H NMR Spectrum PN6 ($CDCl_3$, 500 MHz) enlarged between 3-7.5 ppm

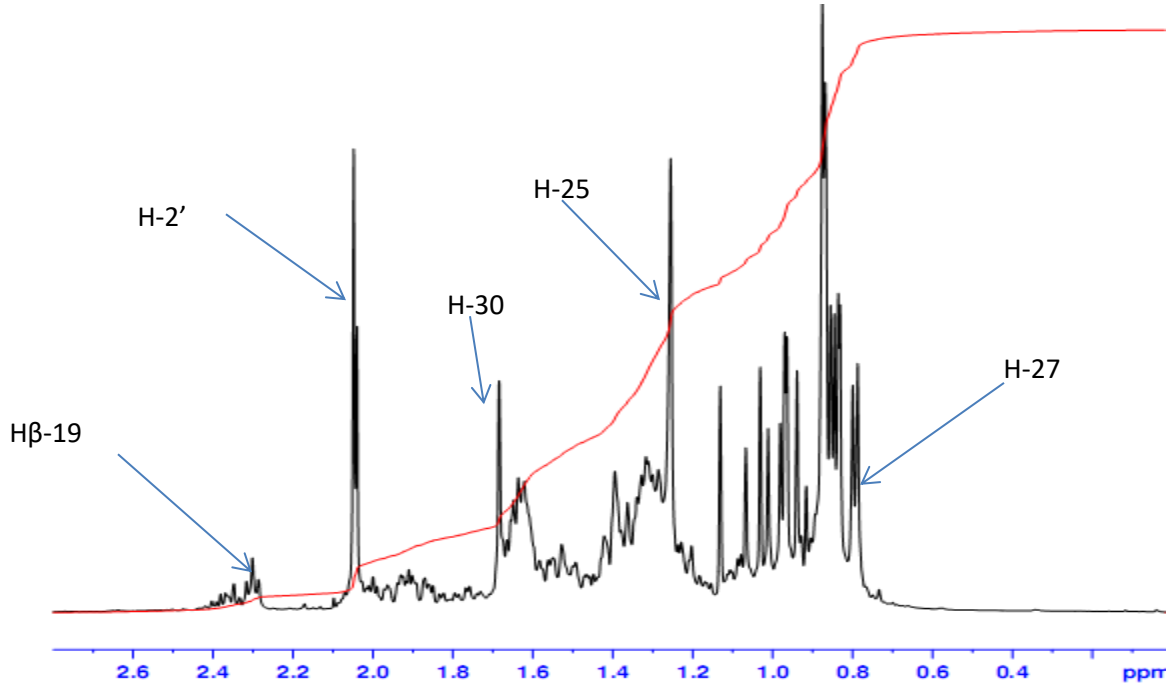


Figure 67: ^{1}H NMR Spectrum PN6 ($CDCl_3$, 500 MHz) enlarged between 0-3 ppm

^{13}C-NMR spectrum of PN6 showed the presence of a carbonyl ester at δ_C 171.1 ppm (C-1') and two sp2 olefinic carbons at δ_C 150.1 ppm (C-20) and δ_C 109.4 ppm (C-21). An oxymethine at δ_C 81.1 ppm (C-3) indicating that it is bonded to the oxygen atom of acetate and an oxymethylene at δ_C 59.1 ppm (C-28). Peaks of sp3 methyl carbons were also observed as follows: δ_C 14.5 ppm (C-27), δ_C 15.5 ppm (C-24), δ_C 15.9 ppm (C-25), δ_C 16.2 ppm (C-26), δ_C 20.9 ppm (C-30), δ_C 27.9 ppm (C-23) and the acetoxy methyl carbon at δ_C 21.32 ppm (C-2'). The carbon assignments took into consideration the $^1J_{C-H}$ correlations observed on the HSQC spectrum.

The major HMBC couplings of PN6 are as follows: H-30 coupled with C-25 through $^2J_{H-C}$ and $^3J_{H-C}$ couplings with C-20 and C-29. H-2' couples through $^2J_{H-C}$ with C-3. The ^{1}H-^{1}H COSY spectrum of PN6 showed cross peaks between the following pairs of protons: Hβ-19 with H-21 and H-18, H-3 with H-2 which in turn showed cross peaks with H-1.

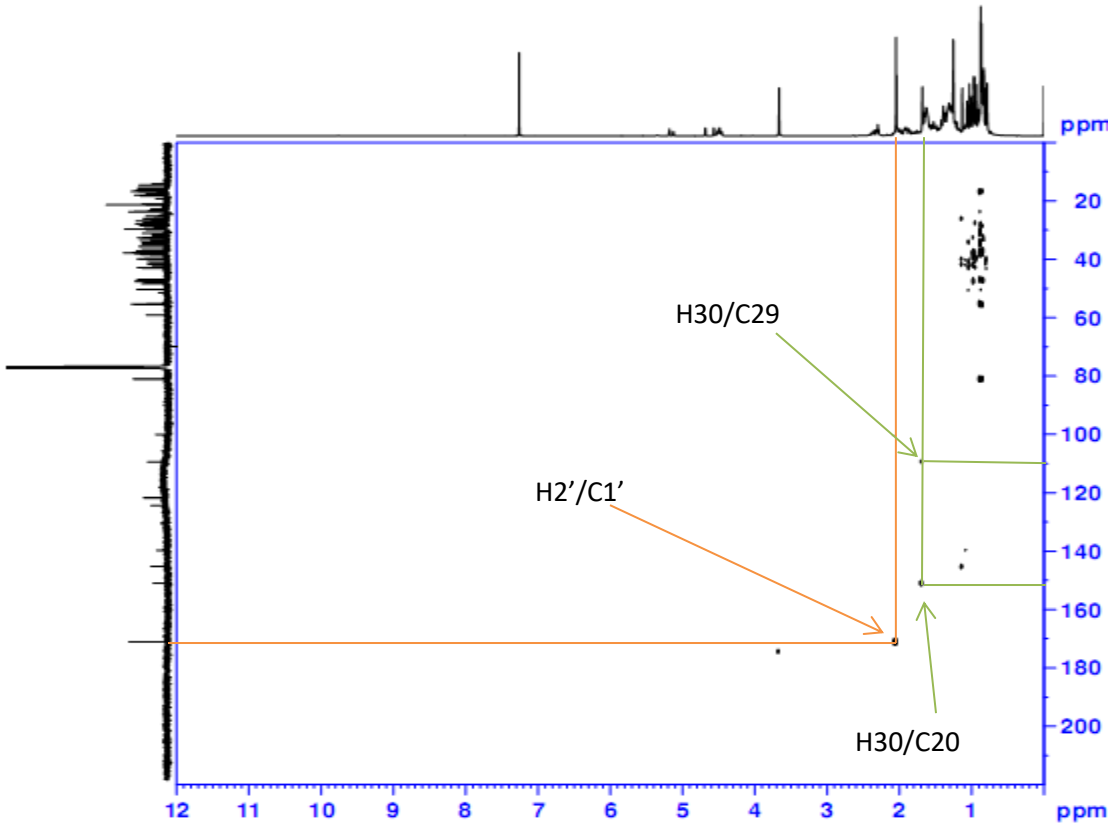
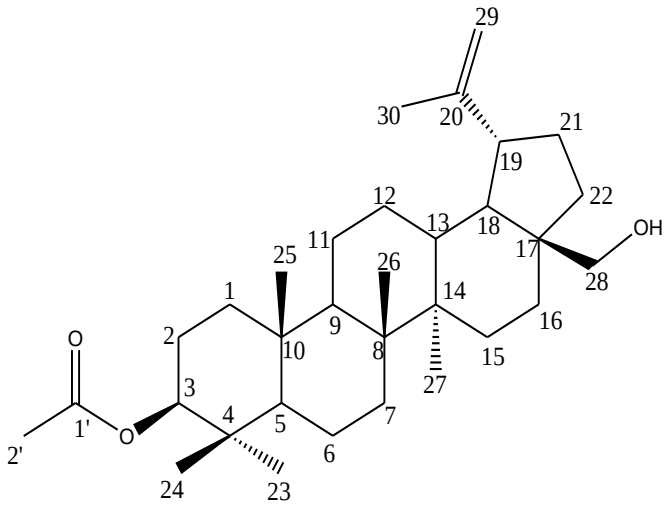


Figure 68: HMBC spectrum of PN6

Comparison of the spectral data of PN6 with published data (**Prachayasittikul *et al.*, 2010**) enabled PN6 to be identified as of as 3-O-acetyl betulin.



3-O-acetyl betulin **(144)**

Table 33: NMR data of PN6 (3-O-acetyl betulin)

PN6 (3-O-acetylbetulin; Prachayasittikul *et al.*, 2010)			PN6 (3-O-acetylbetulin; Prachayasittikul *et al.*, 2010)		
Position	**^{13}C**	**^{1}H**	**Position**	**^{13}C**	**^{1}H**
1	38.7		**17**	46.3	
2	27.3		**18**	47.6	
3	81.1	4.45, dd; J=10.4, 7.6 Hz	**19**	48.7	2.3, dt; J=10.8, 5.8 Hz
4	38.8		**20**	150.1	
5	55.2	0.65, d	**21**	29.7	
6	18.2		**22**	34.1	
7	34.5		**23**	27.9	1.05, s
8	40.8		**24**	15.5	0.95, s
9	50.0		**25**	15.9	1.40, s
10	38.7		**26**	16.2	0.86, m
11	20.7		**27**	14.5	0.8, s
12	25.1		**28**	59.1	3.65, s
13	37.5		**29**	109.4	4.62α & 4.70β, s
14	42.6		**30**	20.9	1.65, s
15	27.0		**1'**	171.1	-
16	29.5		**2'**	21.32	2.05, s

II-4-2: Characterisation of fatty compounds

II-4-2-1: Structural elucidation of ISGV

The compound ISGV crystallized in the form of white crystals in hexane-ethyl acetate (20/80, v/v) gradient system. TLC of ISGV eluted in hexane/CH_2Cl_2 (50/50) had a retardation factor, Rf = 0.2 and eluted with hexane/ethyl acetate (20/80) gave Rf=0.5. Its melting point is between 68-69.2ºC The ESI-TOF MS spectrum of ISGV (positive mode) showed a pseudo-molecular ion peak of [M+Na]+ at m/z =395.4 and another diagnostic peak [2M+Na]+ at m/z = 767.5 from which the molecular mass of the compound ISGV was deduced as 372.4 g/mol for $C_{23}H_{48}O_3$ with a double bond equivalence of zero.

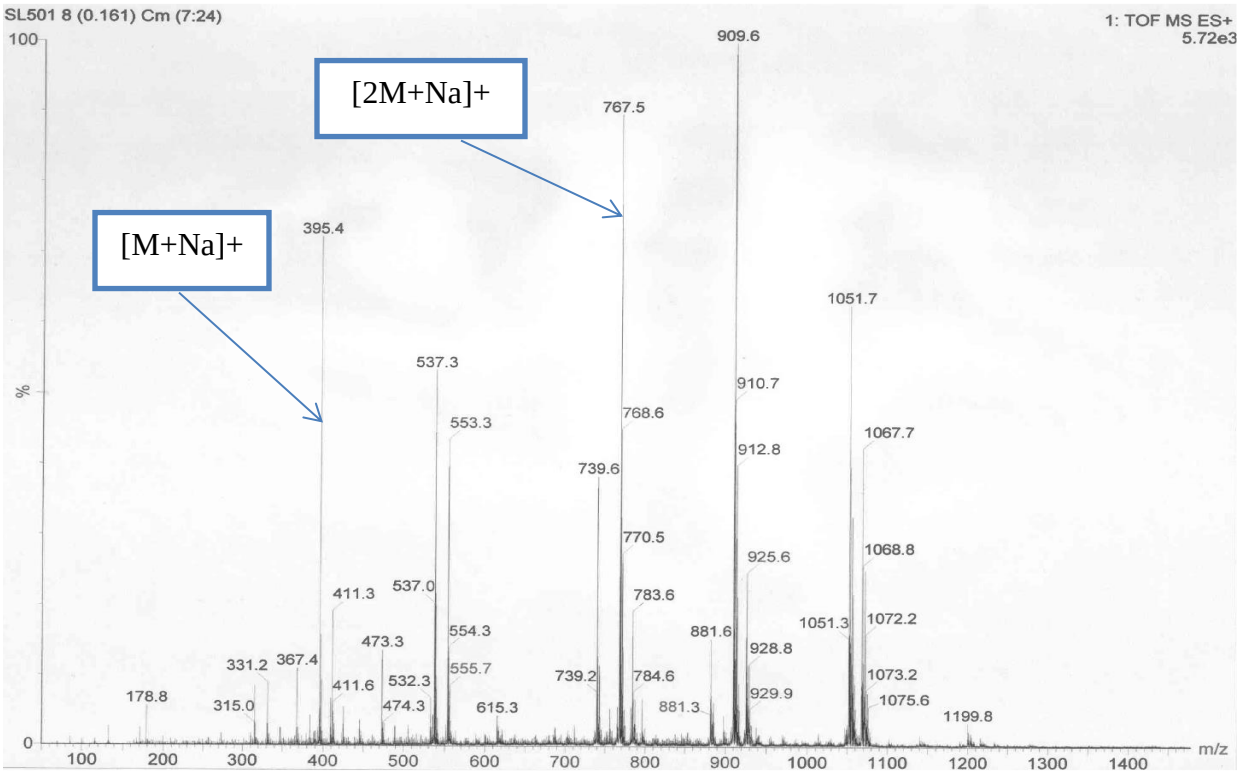


Figure 69: ESI-TOF MS spectrum of ISGV

The ^1H NMR ($CDCl_3$, 600.13 MHz) spectrum showed chemical shifts of oxymethine and oxymethylene protons at δ_H 3.88, 3.55, 3.53 and 3.7 ppm. Thus, at δ_H = 3.53 ppm (1H, t) and δ_H = 3.88 ppm (2H, d) corresponding to the methylene protons at α-positions of either side of an ether functional group. Their multiplicities suggest that the proton at δ_H = 3.88 ppm (2H, d) is linked to a secondary hydroxymethine while that at δ_H = 3.53 ppm (2H, t) is bonded to a methylene. The peaks at δ_H = 3.55 ppm (1H, m) and δ_H = 3.67 ppm (2H, dd) are attributable to the hydroxymethine

proton and two diastereotopic hydroxymethylene protons of a glycerol moiety respectively. The following information suggests that we have an aliphatic chain and a glycerol moiety bonded to each other through an ether function. The peak at δ_H = 1.27 ppm is the peak of several methylene groups of a long chain whereas that at δ_H = 0.96 ppm (3H, t) corresponds to a terminal methyl group at the end of the aliphatic chain.

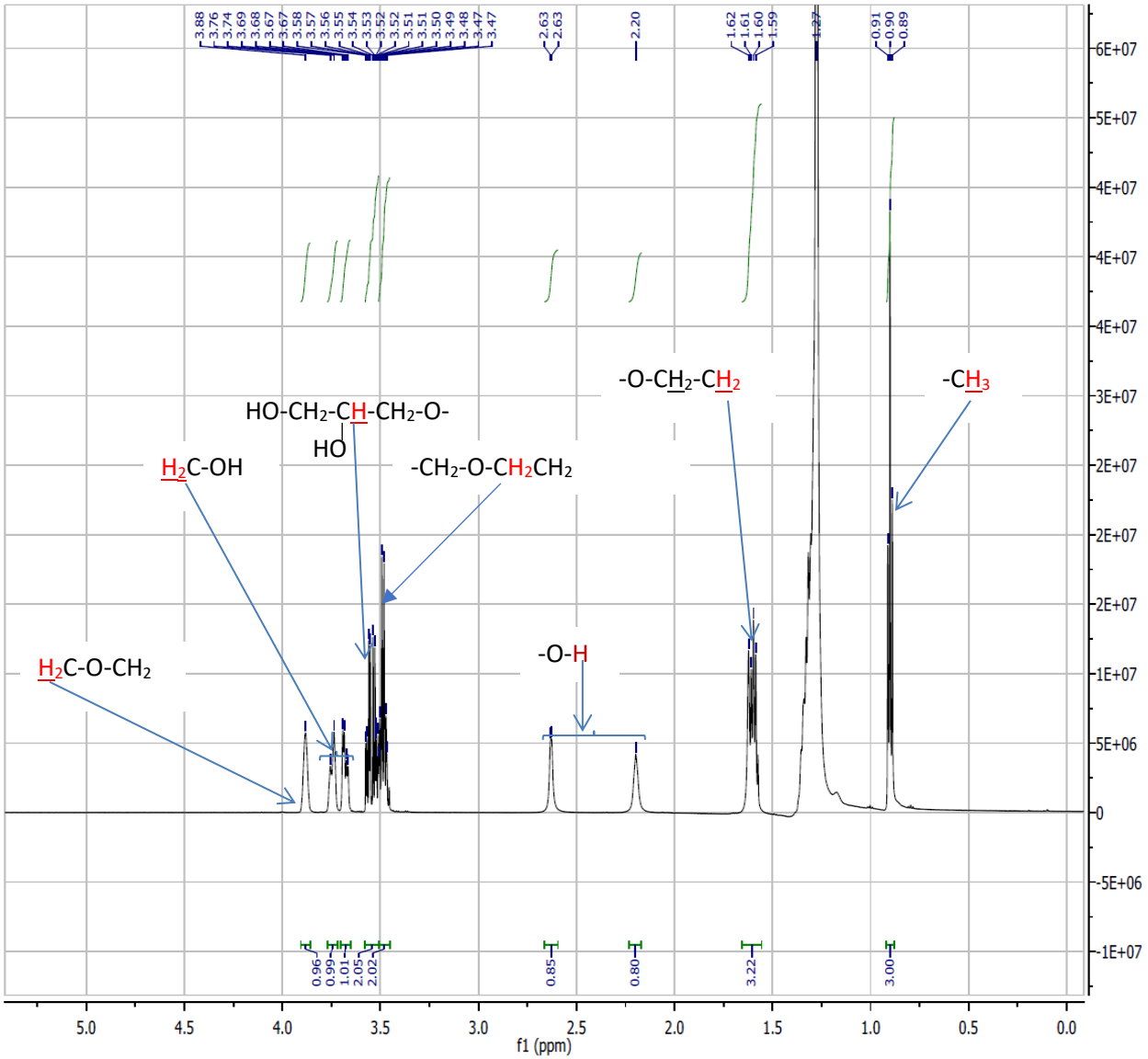
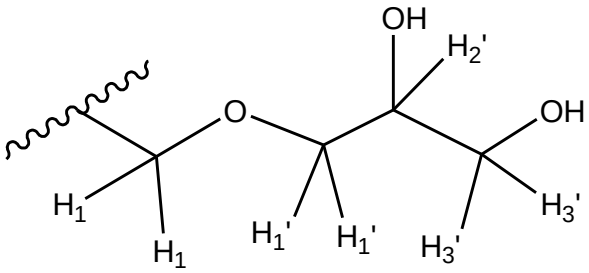


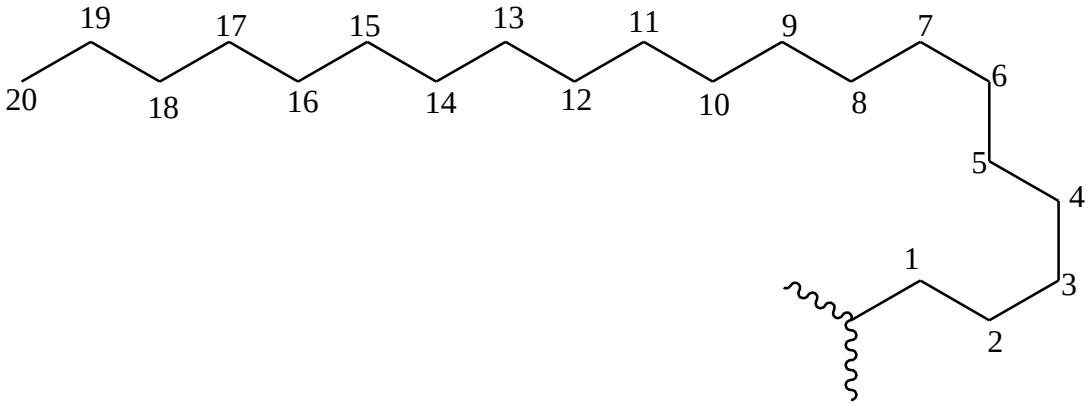
Figure 70: ^{1}H NMR spectra ($CDCl_3$, 600.13 MHz) of ISGV

On the ^{13}C NMR spectrum, two peaks appear at δ_C 70.37 ppm and δ_C 71.88 ppm attributable to the α-carbon atoms of an ether function the former is linked to a glycerol moiety while the latter is linked to an aliphatic chain. Other signals at δ_C 72.57 ppm and δ_C 64.35 ppm suggest two

oxygenated carbon atoms, a hydroxyl methine and the terminal hydroxyl methylene of a glycerol moiety respectively. This information together with the ^{1}H NMR data suggests the substructure below.



The signal of a terminal methyl of a long chain appears at δ_C 14.14 ppm while a broad signal of the connecting methylene chain is seen around δ_C 29.72-29.77 ppm thereby suggesting a long aliphatic chain as given in the substructure below and whose length is justified by the molecular mass deduced from the mass spectra.



Summarily the ^{13}C NMR (150.92 MHz) spectrum of ISGV reveals carbon atoms of the long aliphatic chain linked through an ether function to a molecule of glycerol as follows: δ_C 72.57 ppm (C-2'), 71.88 ppm (C-1), 70.37 ppm (C-1'), 64.35 ppm (C-3'), 31.95 ppm (C-2), between δ_C 29.72-29.77 ppm (C-3 to C-19) and δ_C 14.14 ppm (C-20).

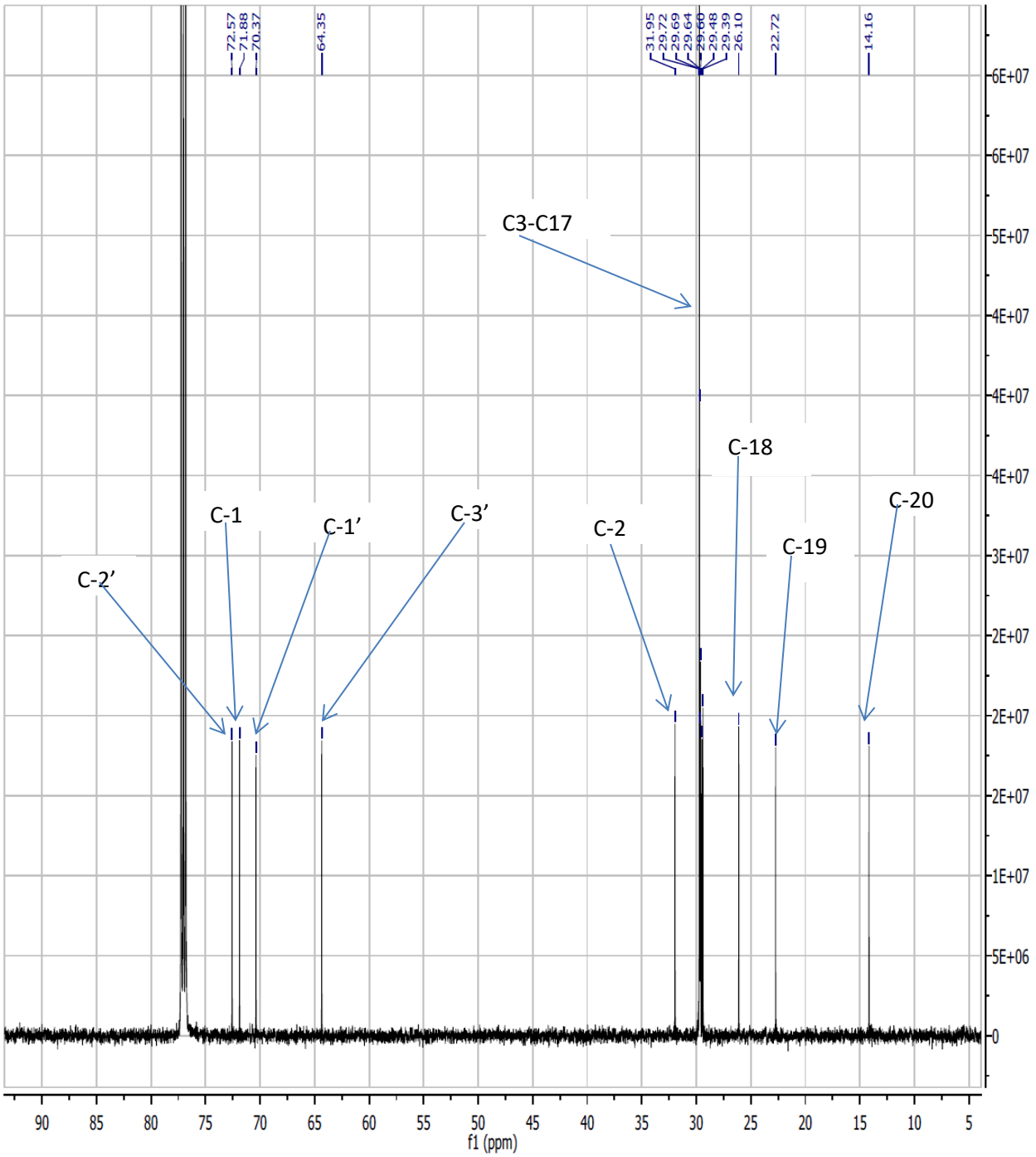


Figure 71: ^{13}C NMR spectrum ($CDCl_3$, 150.92 MHz) of ISG V

The connectivities of aliphatic and glycerol moieties were elucidated as follows: HSQC ($^1J_{H-C}$) showed correlation between some protons and their respective carbon atoms to which they are bonded as follows: δ_C 14.14 ppm (C-20) and δ_H 0.8 ppm (H-20); δ_C 29.72 ppm (C-3) δ_H 1.2 ; δ_C 71.88 ppm (C-1) and δ_H 3.53 (H-1), δ_C 64.35 ppm (C-3') and δ_H 3.67 (H-3').

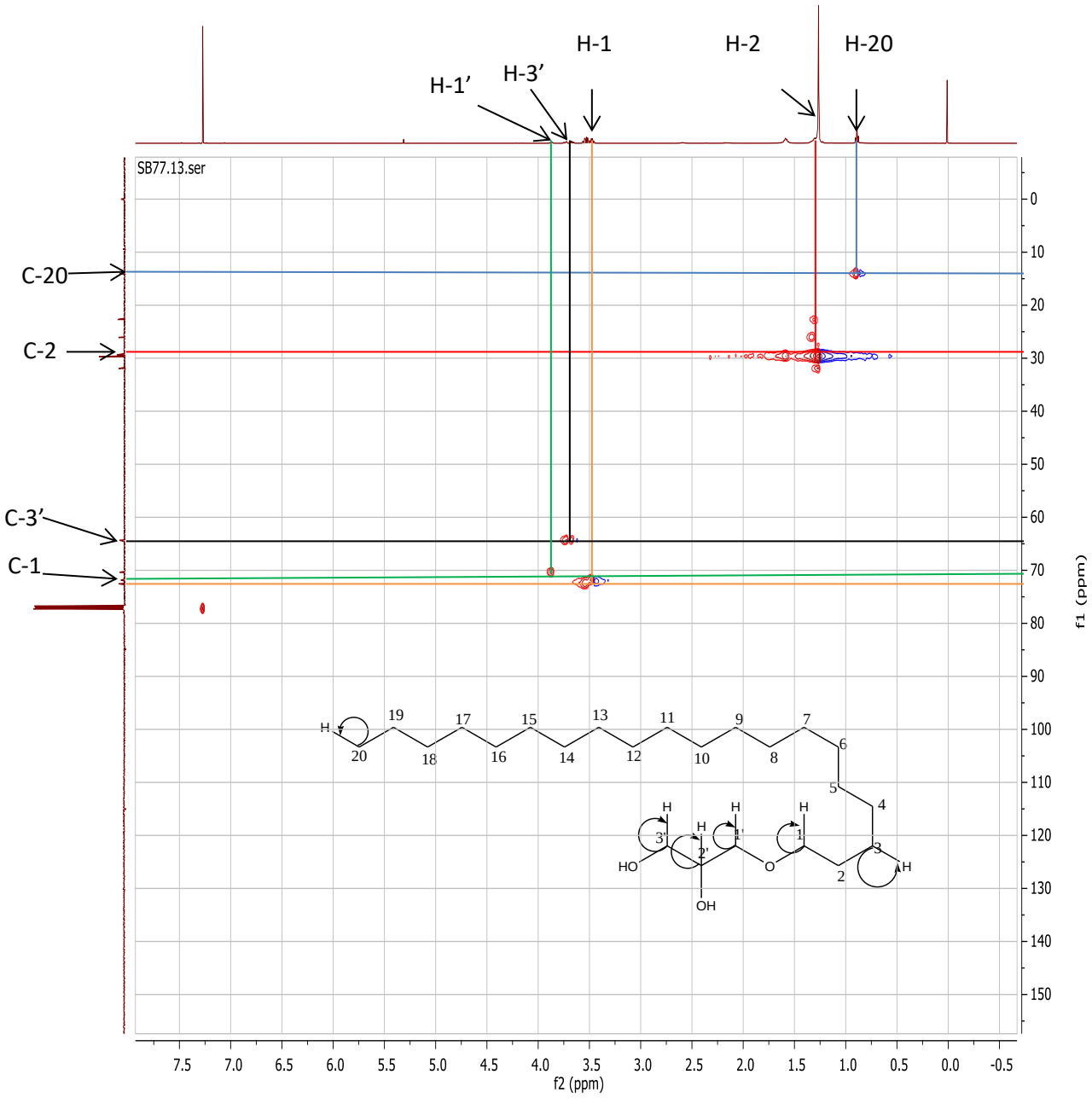


Figure 72: HSQC ($^{1}J_{H\text{-}C}$) spectrum of ISGV

HMBC showed long range correlations between H-20 and C-19, H-3 and C-2.

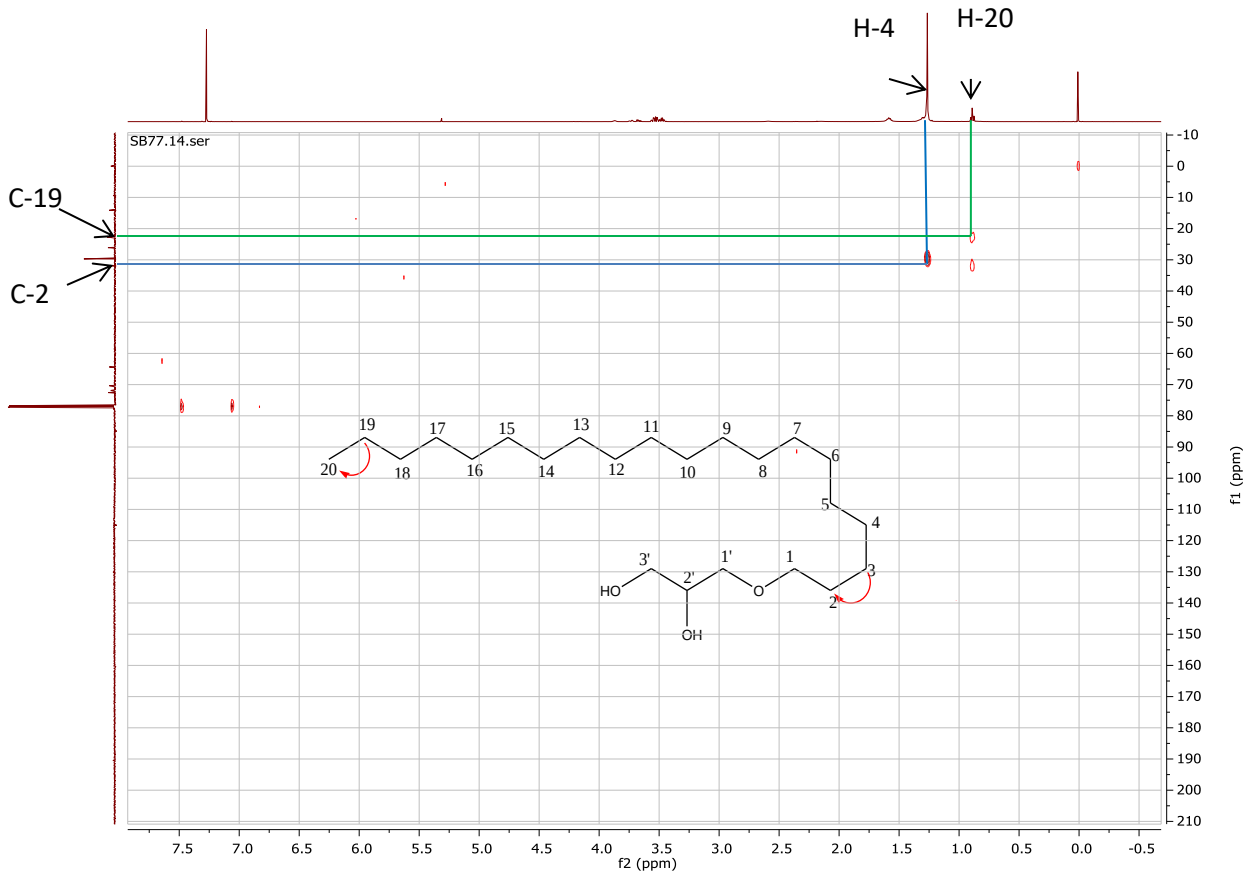


Figure 73: HMBC spectrum of ISGV

Important COSY (^{1}H-^{1}H) correlations were also observed between the following pairs of protons: H-19 and H-20, H-1 and H-2, H-2 and H-3, H-1'and H-2', H-2' and H-3' indicating that they are located on adjacent carbon atoms.

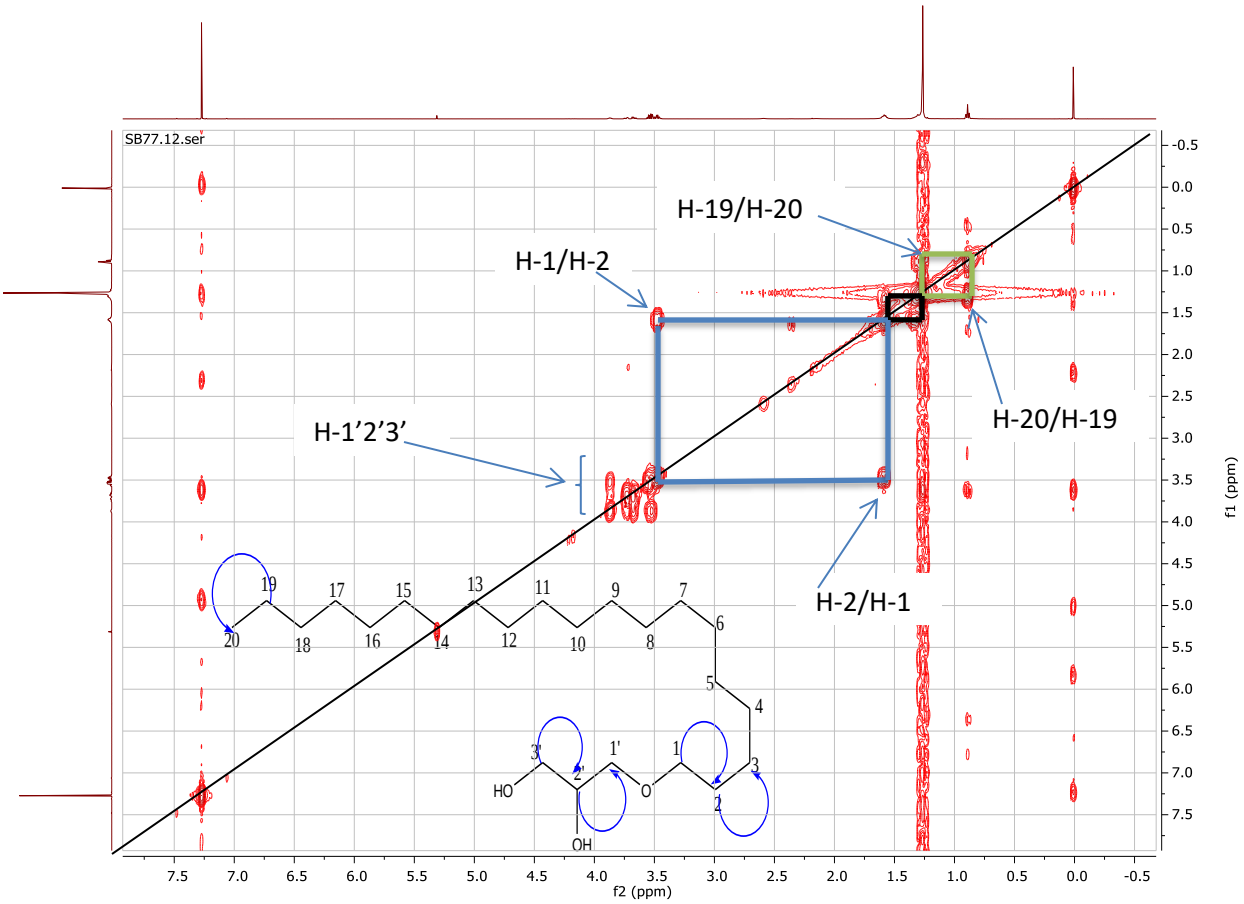
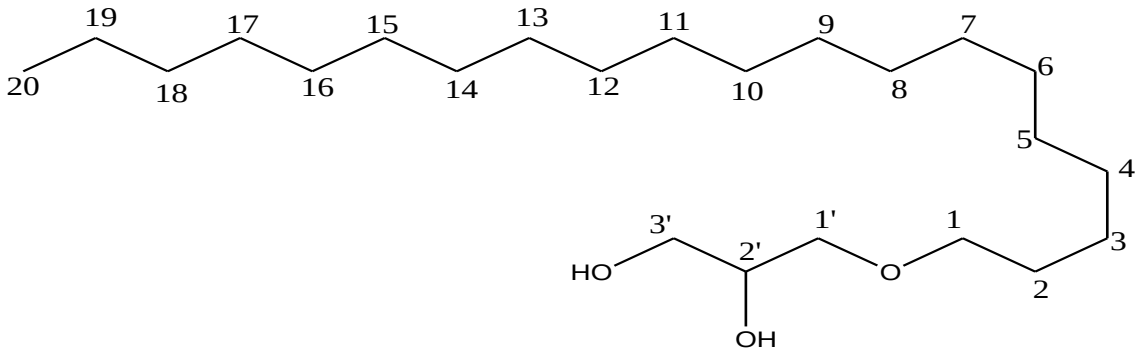


Figure 74: ^{1}H-^{1}H COSY spectra of ISGV

The foregoing spectral data and information enabled us to attribute to ISGV the structure below and gave the name 1'-O-eicosanyl glycerol which is a new compound.



1'-O-eicosanyl glycerol **(145)**

Table 34: NMR data of 1'-O-eicosanyl glycerol

Position	δH, multiplicity	δC/ type	HMBC	COSY
1	3.53, t	71.88 /CH_2	/	H-2
2	1.6, m	31.95 /CH_2	C-1, C-3	H-3
3	1.27, m	29.72 /CH_2	C-2	H-2
4-18	1.25-1.27, m	29.75 /CH_2	/	/
19	1.26, m	29.77 /CH_2	C-20	H-20
20	0.96, t	14.14 /CH_3	C-19	H-19
1'	3.88, dd, J=6.4, 4.0 Hz	70.37 /CH_2	/	H-2'
2'	3.55, m	72.57 /CH	/	H-2', H-1'
3'	3.67, dd, J=6.4, 4.5 Hz	64.35 /CH_2	/	H-2'

II-4-2-2: Structural identificationof TA5

The compound TA5 was obtained as a white powder and crystallized in hex-AcOEt (60/40) solvent mixture. It melted between 98-102ºC. The ESI-TOF MS spectrum of this compound showed a pseudo-molecular ion peak at $[M+5H]^+$ at m/z 331.3 and $[M+6H]^+$ at m/z 332.3 (calculated 326.3 for $C_{21}H_{42}O_2$) with a calculated double bond equivalent of 1.

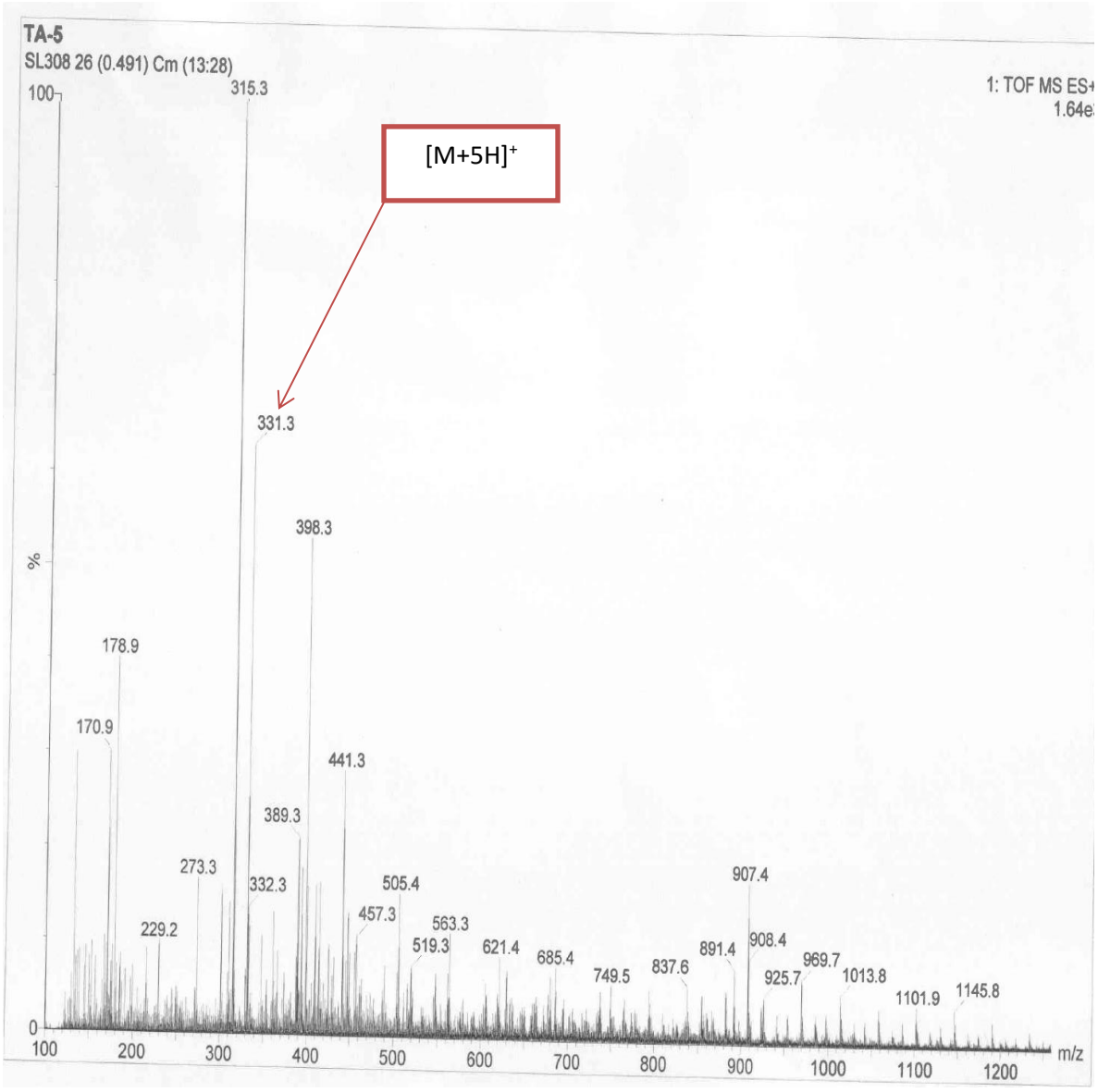


Figure 75: ESI-TOF MS spectrum of TA5

The ^1H NMR spectrum of TA5 shows five characteristic peaks of an ester of fatty acid and long chain alcohol at δ_H 4.50 ppm, 2.30 ppm, 1.60 ppm, 1.30 ppm, and 0.90 ppm. The triplets at δ_H 4.10 ppm and 2.30 ppm are diagnostic for methylene groups ($-CH_2-O-CO-CH_2-$) bound to the O- atom

and carbonyl C-atom of an ester group respectively while the strong peak at 1.30 ppm indicates the presence of a long hydrocarbon chain. Finally, the characteristic peaks at 1.60 ppm represents connector CH_2 groups linking the long hydrocarbon chains to the methylene bound to the ester function. The peak at δ_H 0.9 ppm represents the terminal methylene protons of the long chain. Summarily, the following characteristic protons were observed: δ_H 4.50 ppm (t, 2H, H-1'), δ_H 2.30 ppm (t, 2H, H-2), δ_H 1.60 ppm (m, 2H, H-3), δ_H 1.30 ppm (t, 3H, H-4), δ_H 1.60 ppm (m, 2H, H-2'), δ_H 1.30 ppm (m, 2H, H-15'), δ_H 1.20 ppm (m, 2H, H-16') and δ_H 0.90 ppm (t, 3H, H-17').

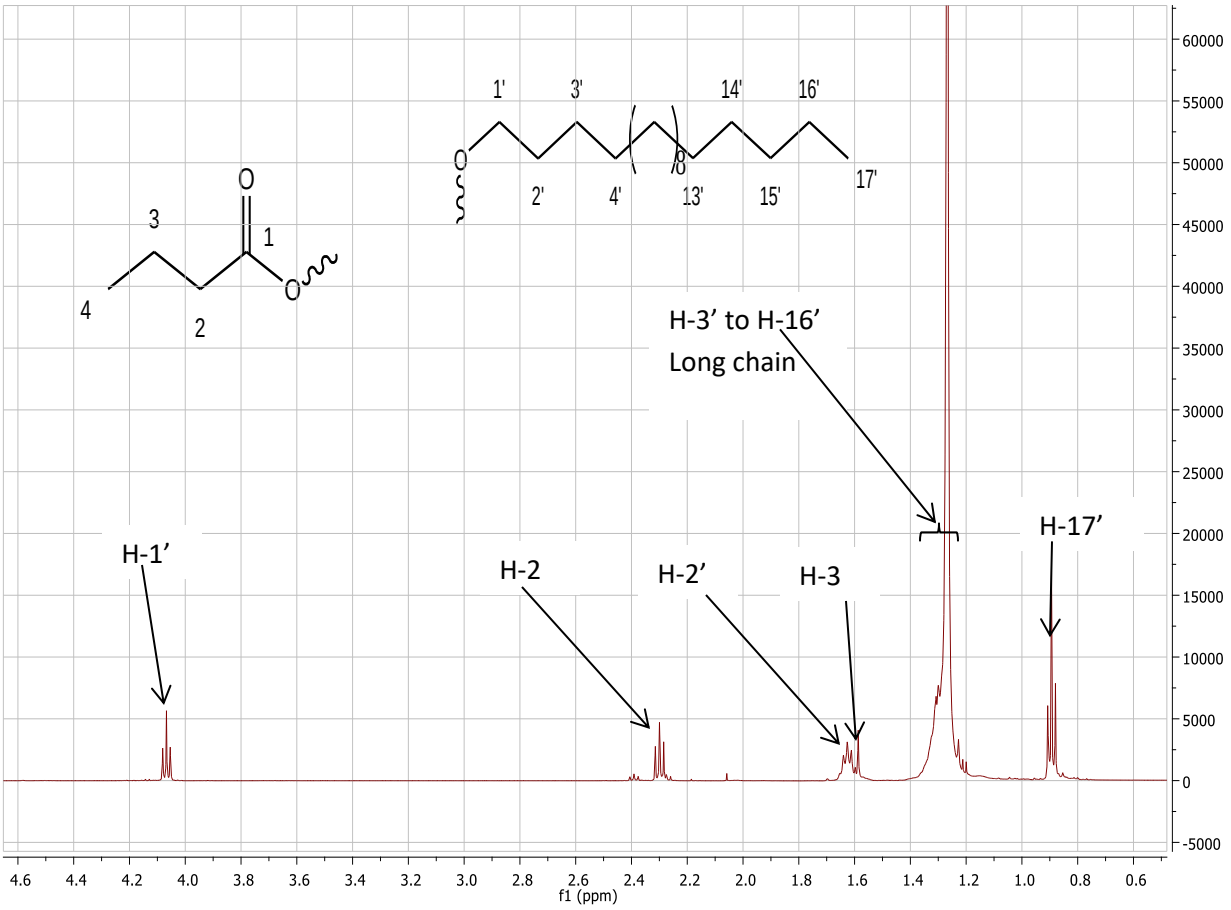


Figure 76: ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of TA5

On the ^{13}C NMR spectrum of TA5, a peak at δ_C 174.0 ppm (C-1) characteristic of the carbonyl function of an ester group is observed. The peak at δ_C 64.5 ppm (C-1') is attributable to the oxymethine (-CH_2-O-) C-atom of an ester group. Other characteristic peaks where observed at δ_C 34.0 ppm, 29.0 ppm and 25.0 ppm at positions α-, β- and γ- positions to the carbonyl atom respectively corresponding to C-2, C-3 and C-4. Also, C-2' and the terminal methyl carbon C-17'

appeared at δ_C 27.0 ppm and 14.1 ppm respectively. The sp3 carbon signals corresponding to the carbon atoms of the long aliphatic chain were observed as a massive peak at δ_C 27-29.9 ppm.

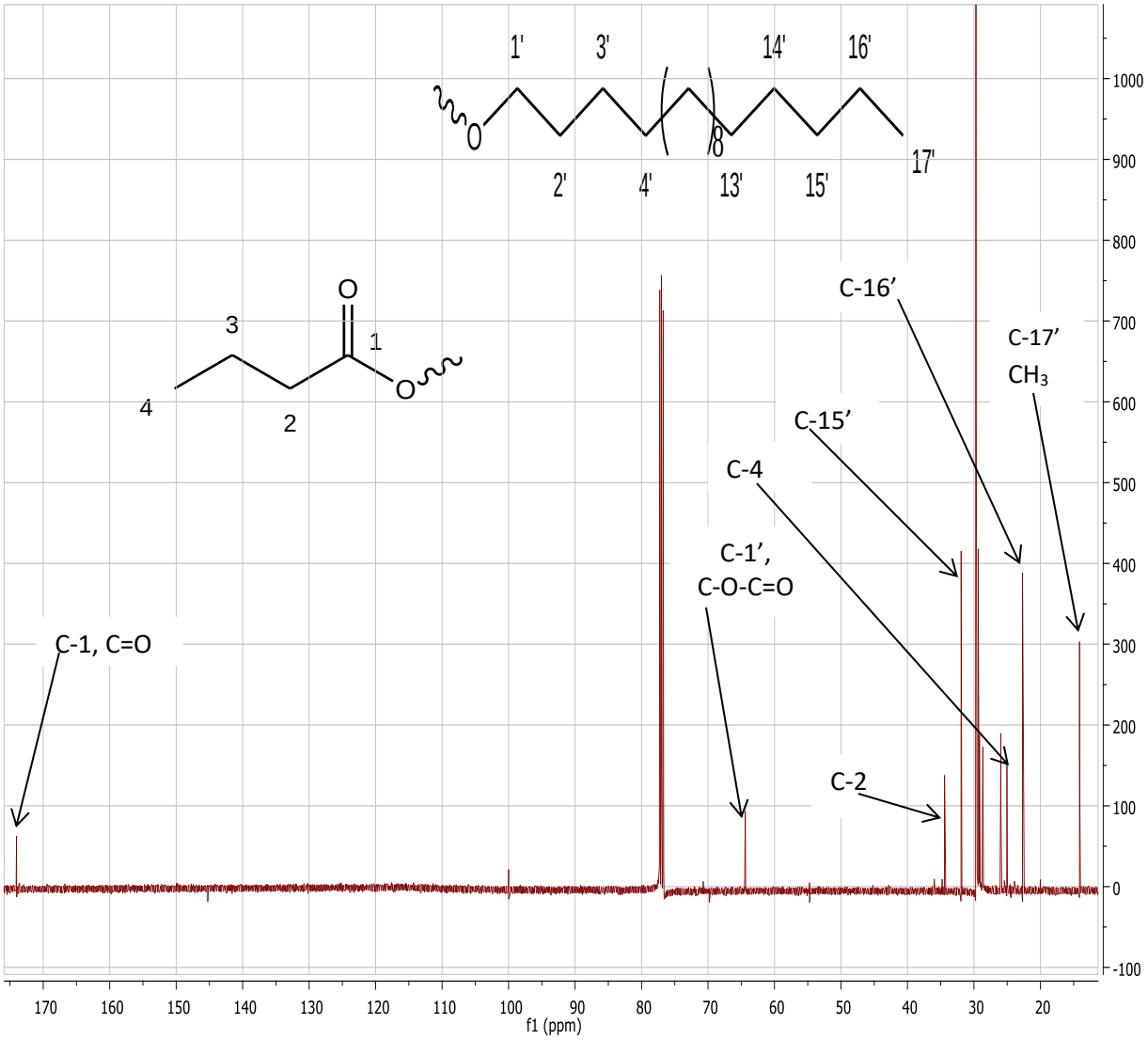


Figure 77: ^{13}C NMR spectrum (150 MHz, $CDCl_3$) of TA5

The HSQC ($^1J_{C-H}$) spectrum of TA5 showed correlation spots between the following pairs of protons and carbon atoms: the protons at δ_H 4.50 ppm (H-1') and the carbon atom at δ_C 64.5 ppm (C-1'), the protons at δ_H 2.30 ppm (H-2) and the carbon atom at δ_C 34.0 ppm (C-2), the protons at δ_H 0.90 ppm (H-17') and the carbon atom at δ_C 14.1 ppm (C-17') and finally the protons at δ_H 1.60 ppm (H-2' and H-3) and the carbon atom at δ_C 25.0 ppm (C-4) and δ_C 25.0 ppm (C-2') respectively.

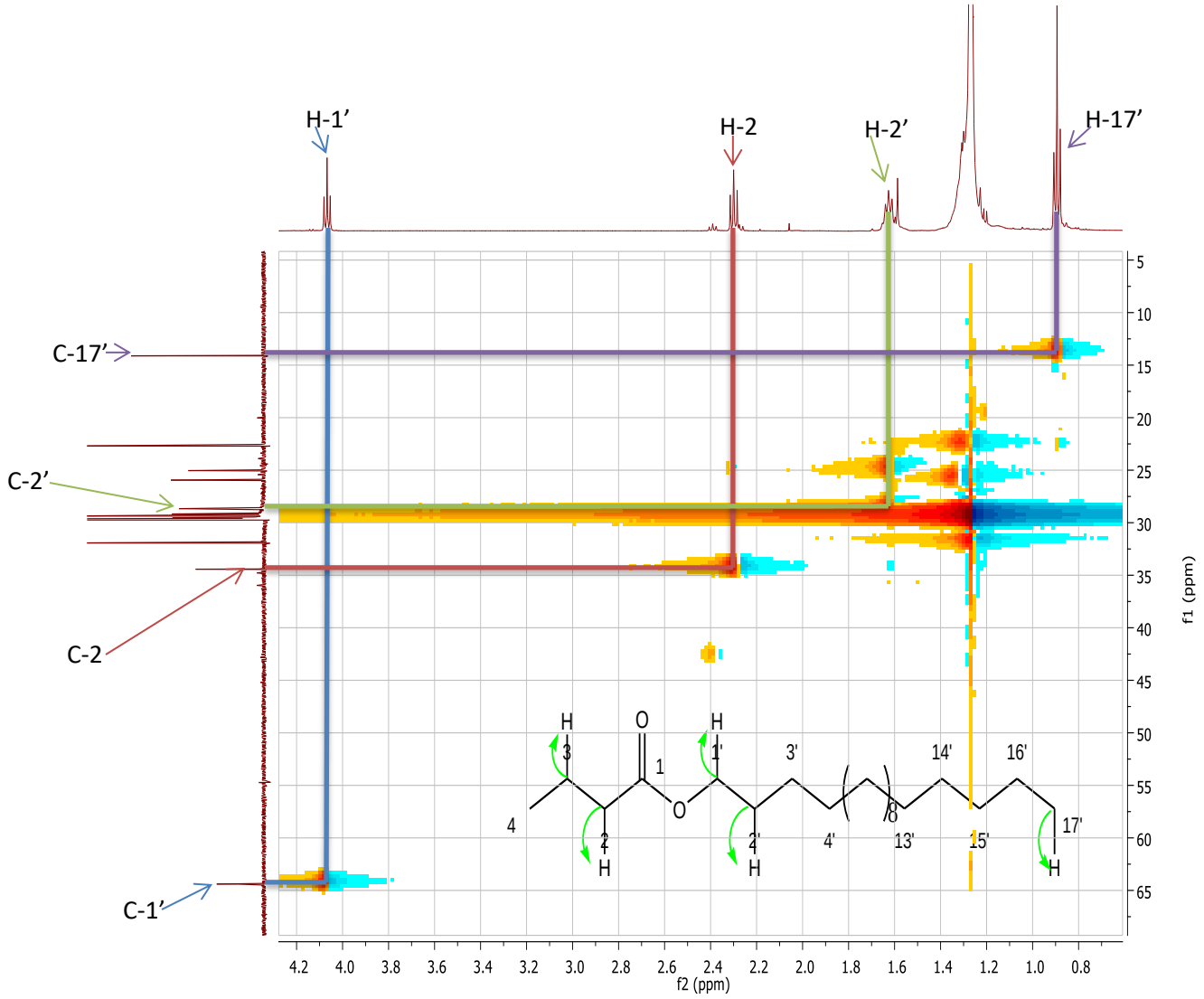


Figure 78: HSQC spectrum ($CDCl_3$) of TA5

On the HMBC spectrum of TA5, some key correlations were observed as follows: between the protons H-1' (δ_H 4.50 ppm) and the carbon atoms C-1 (δ_C 174.0 ppm) and C-2' (δ_C 27.0 ppm), protons H-2 (δ_H 2.30 ppm) and the carbon atoms C-1 (δ_C 174.0 ppm), C-3 (δ_C 29.0 ppm) and C-4 (δ_C 25.0 ppm), protons H-2' (δ_H 1.60 ppm) and the carbon atoms C-1' (δ_C 64.5 ppm) and C-3' (δ_C 29.0 ppm) and finally between protons H-17' (δ_H 0.90 ppm) and the carbon atoms C-16' (δ_C 22.5 ppm) and C-15' (δ_C 31.9 ppm).

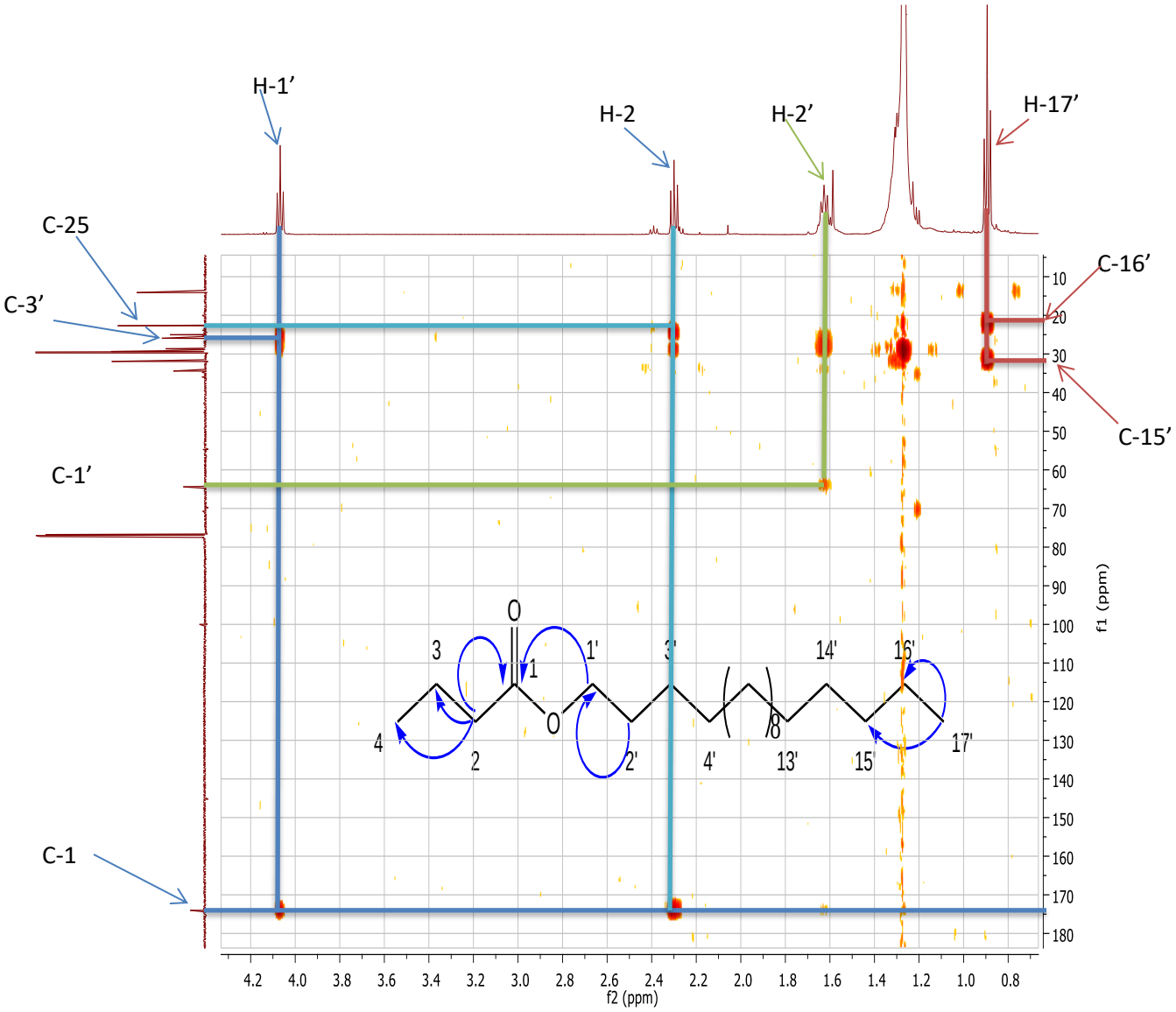


Figure 79: HMBC spectrum ($CDCl_3$) of TA5

[1] H-[1]H COSY spectrum of TA5 showed cross peaks between the following pairs of protons: H-2 (δ_H 2.3 ppm, t) and H-3 (1.60 ppm, m), H-1' (δ_H 4.50 ppm, t) and H-2' (1.60 ppm, m) and H-16' (δ_H 1.20 ppm, m) and H-17' (0.90 ppm, t)

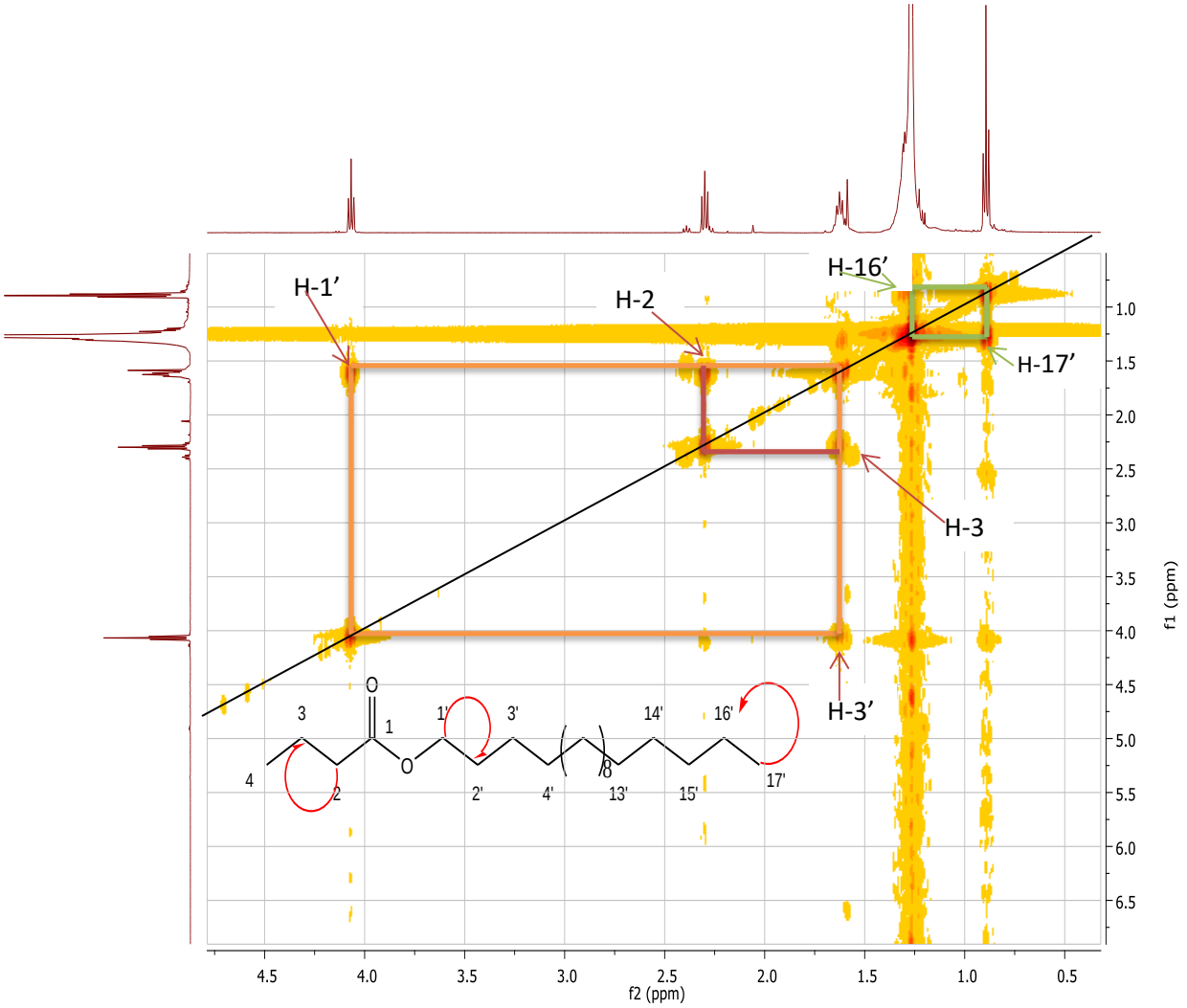
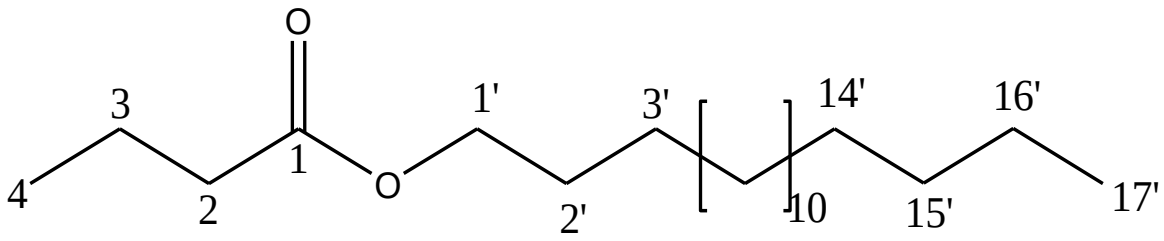


Figure 80: ^{1}H-^{1}H COSY spectrum ($CDCl_3$) of TA5

All the spectral information put together led to the identification of TA5 as heptadecyl butanoate previously described (**Kavita *et al.*, 2009**) whose structure is given below.



Heptadecyl butanoate **(146)**

Table 35: NMR data of TA5

Position	δH, multiplicity	δC/ type	HMBC	COSY
1	/	174 /C	/	/
2	2.3, t	34.2 /CH_2	C-1, C-2, C-3	H-3
3	1.6, m	29 /CH_2	/	H-2
4	1.3, t	25 /CH_3	C-3	/
1’	4.5, t	64.5 /CH_2	C-1, C’-2	H-2’
2’	1.6, m	27 /CH_2	C’-1, C’-3	H-1’
15’	1.3, m	31.9 /CH_2	/	/
16’	1.2, m	22.5 /CH2	/	H-17’
17’	0.9, t	14.1 /CH_3	C’-15, C’-16	H-16’

II-4-2-3: Structural identificationof TA14

The compound TA14 was obtained in the form of white plates (almost transparent) in the eluent mixture hexane-ethyl acetate (80/20, v/v) and had a melting point of 90-91°C. It was tested with a few reagents for qualitative analysis but failed to show any important result.

The ESI-TOF MS spectrum of TAN14 showed a quasi-molecular ion peak of [M+H]+ at m/z= 537.3 from which the molecular formula $C_{37}H_{76}O$ is deduced. The double bond equivalence of the compound was calculated as zero.

The ^{1}H NMR Spectrum TA14 ($CDCl_3$, 500 MHz) showed four characteristic sets of protons as follows: at δ_H 3.65 ppm (t, 2H, H-1), δ_H1.50 ppm (m, 2H, H-2), δ_H 1.25 ppm (br m, 68H, H-3 to H-36) and finally at δ_H 0.85 ppm (t, 3H, H-37). This data enabled us to identify TA14 as a fatty alcohol with its protons distributed according to the ^{1}H NMR Spectrum given below.

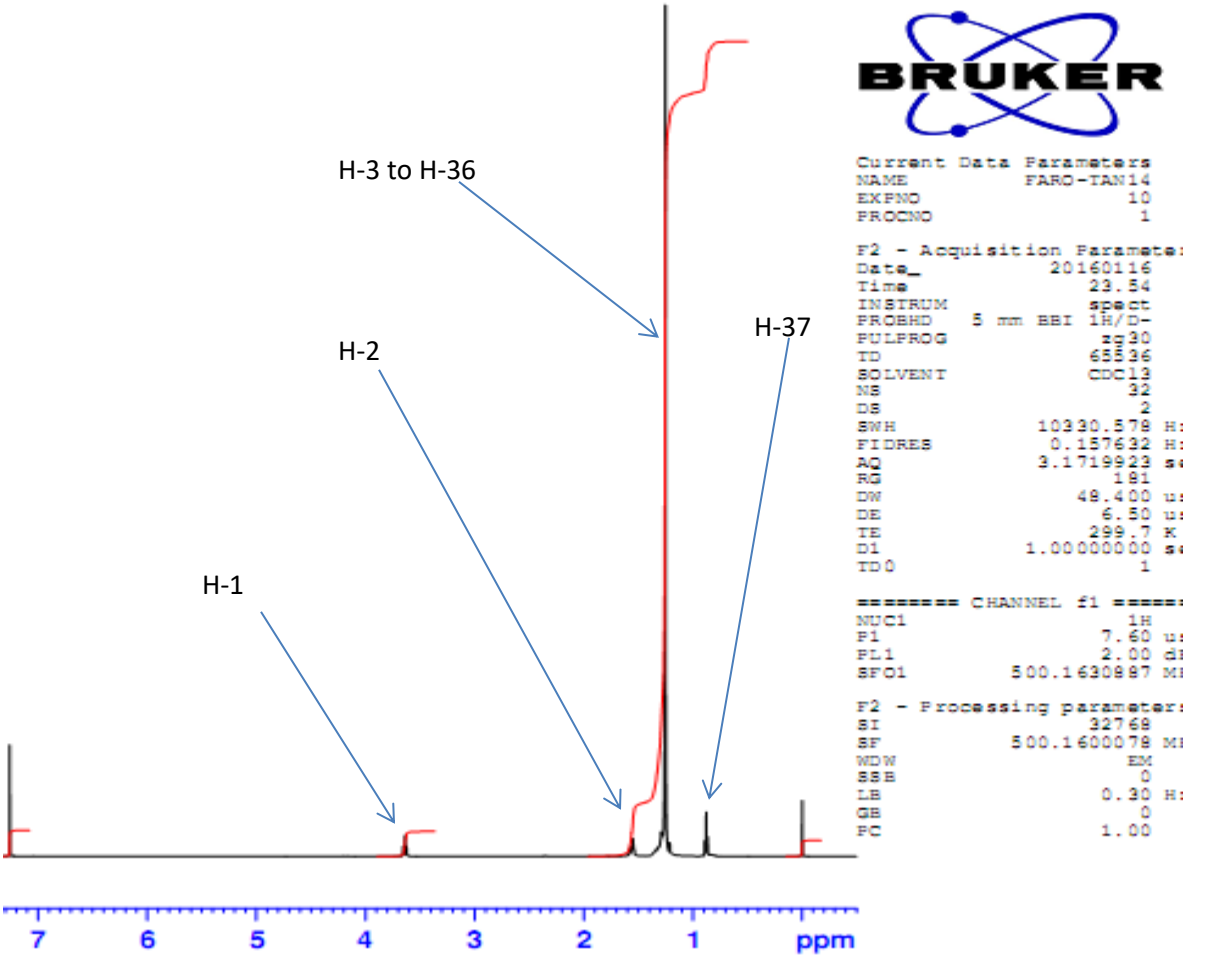


Figure 81: ^{1}H NMR Spectrum TA14 ($CDCl_3$, 500 MHz)

On the ^{13}C NMR Spectrum of TA14 ($CDCl_3$, 150 MHz) the following signals are observed. The hydroxylated methylene carbon C-1 at δ_C 63.1 ppm, C-2 at δ_C 32.8 ppm, C-3 at δ_C 31.9 ppm, C-4 to C-32 between 29.7 ppm and 29.6 ppm, C-33 at δ_C 29.4 ppm, C-34 at δ_C 25.4 ppm, C-35 at δ_C 24.5 ppm, C-36 at δ_C 22.7 ppm and lastly the terminal methyl C-37 at δ_C 14.12 ppm.

2D NMR techniques showed some major bond connections. HSQC showed correlations between C-1/H-1, C-2/H-2 and C-37/H-37 while HMBC showed correlation between H-2 and C-3. The ^1H-^1H COSY showed cross peaks for the following pairs of adjacent protons: H-1 and H-2, H-2 and H-3 and finally H-36 and H-37.

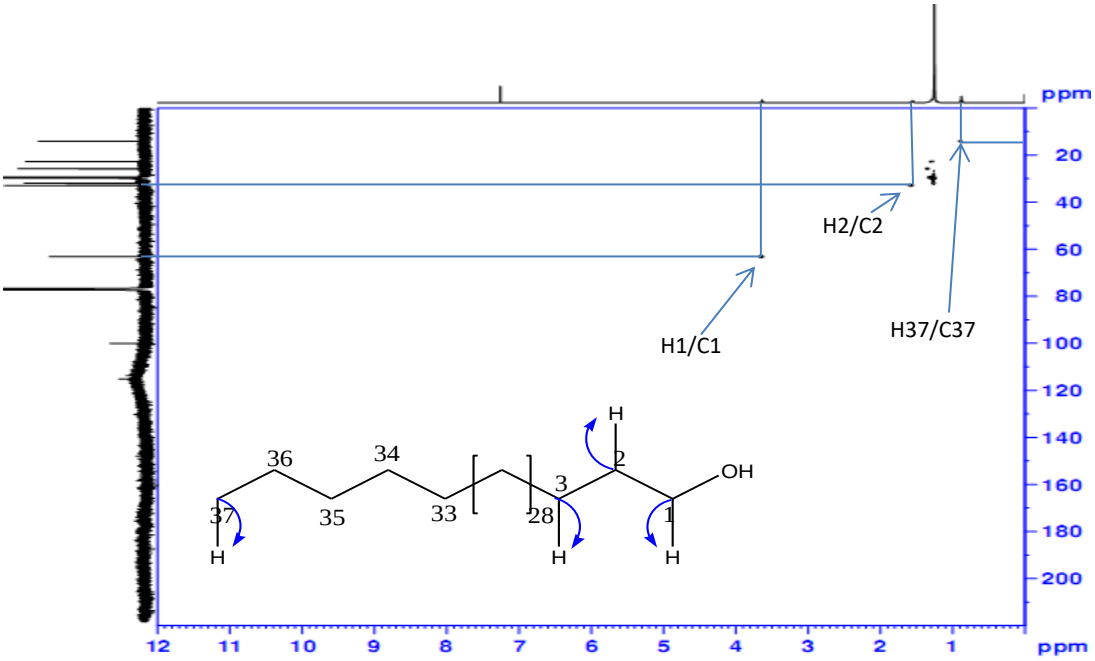


Figure 82: HSQC spectrum of TA14

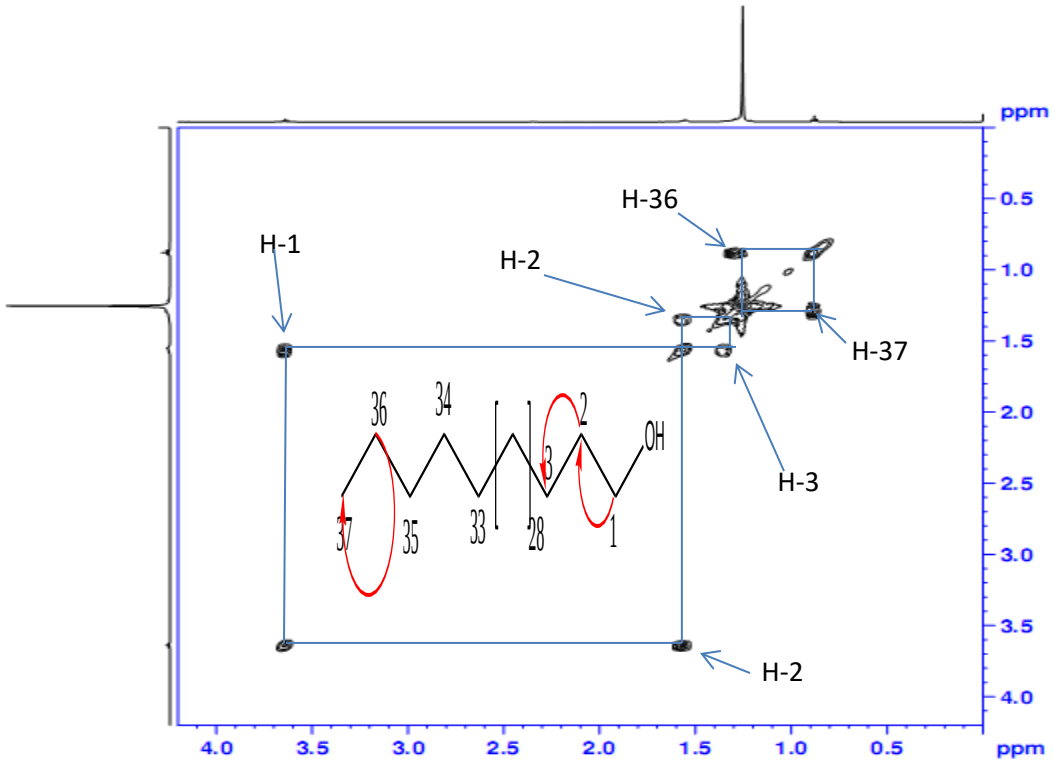
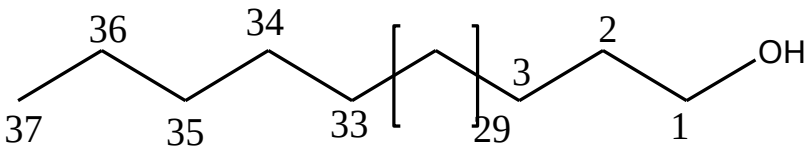


Figure 83: ^{1}H-^{1}H COSY spectrum of TA14

The above spectral data conforms with that of n-heptatriacontanol isolated previously from *Halochris hispida* (**Ahmed Gohar, 2001**) having the structure given below.



n-heptatriacontanol **(147)**

Table 36: NMR data of TA14 n-heptatriacontanol

Position	**δH, multiplicity**	**δC/ type**	**HMBC**	**COSY**
1	3.65, t (2H)	63.1 /CH_2	C-2, C-3	H-2
2	1.50, m (2H)	32.8 /CH_2	C-1, C-3	H-3, H-1
3-35	1.27-1.25, m (66H)	29.7-24.5 /CH_2	C-2,C-36, C-37	H-3/H-2
36	1.25, m (2H)	22.7 /CH_2	C-37	H-37
37	0.85, t (3H)	14.1 /CH_3	C-36	H-36

II-4-2-4: Structural elucidation of CFB3

The compound CFB3 crystallized as white powder in pure hexane. The ESI-TOF MS spectrum of CFB3 showed a pseudo-molecular ion peak $[M+Na]^+$ at m/z=391.4 from which the molecular formula $C_{24}H_{48}O_2$ was deduces and the double bond equivalence was calculated as 2.

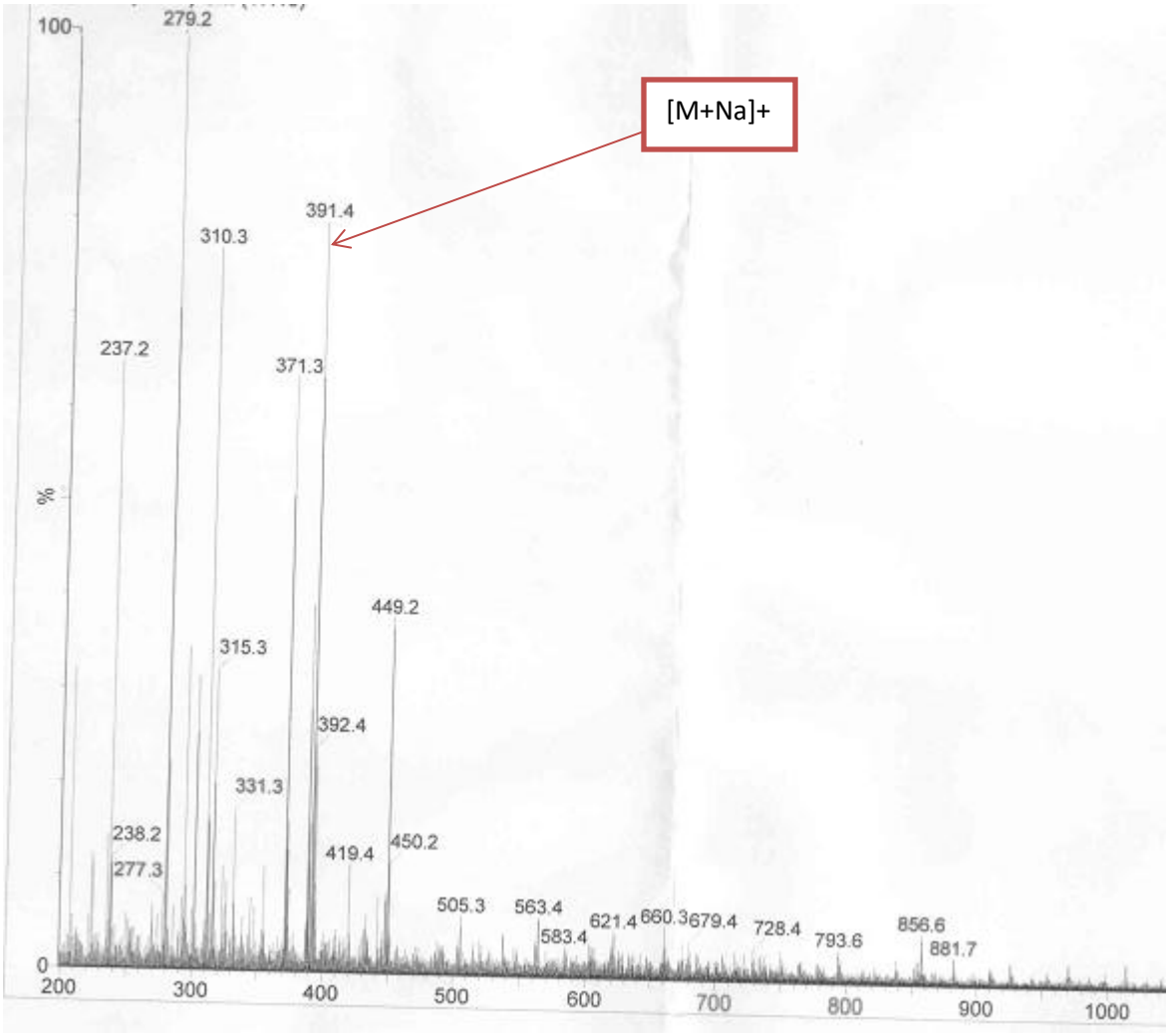


Figure 84: ESI-TOF MS spectrum of CFB3

The ^1H NMR spectrum of CFB3 shows characteristic peaks of a fatty acid ester at δ_H 4.10 ppm (t, 2H, H-1), 2.30 ppm (t, 2H, H-2'), 1.65 ppm (m, 2H, H-2 and H-3'), sp3 methylene protons of the long chain 1.25-1.35 ppm (H-3 to H-19) and 0.90 ppm the sp3 terminal methyl. The triplets at δ_H 4.10 ppm and 2.30 ppm are diagnostic for methylene groups ($-CH_2-O-CO-CH_2-$) bound to the O- atom and carbonyl C-atom of an ester group respectively.

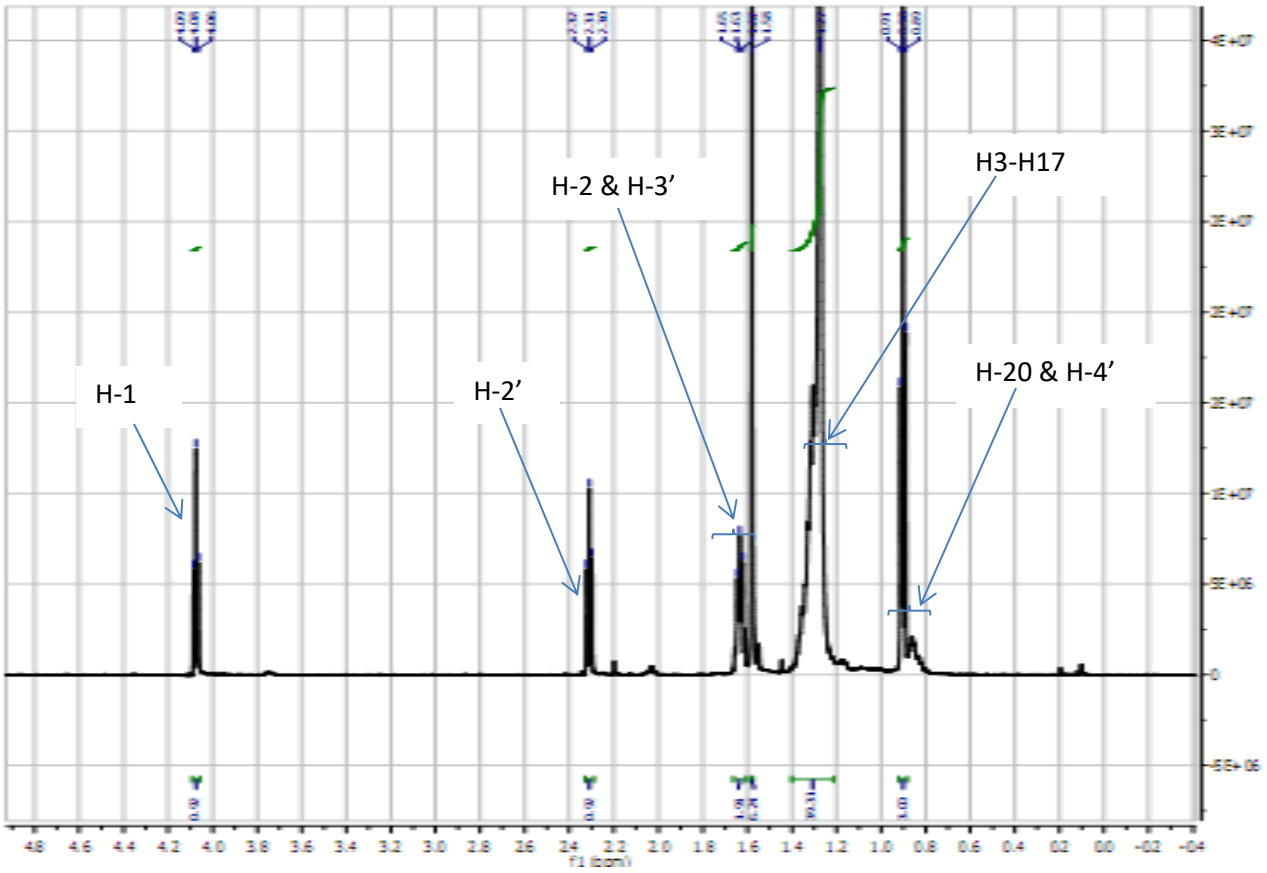
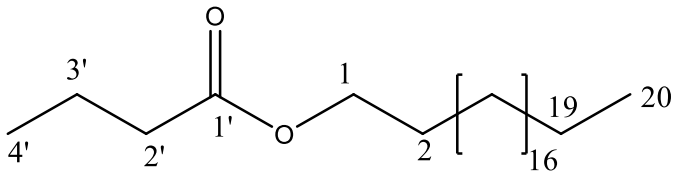


Figure 85: ^{1}H NMR Spectrum CFB3 ($CDCl_3$, 600.13 MHz)

This spectral data in combination with data reported literature permits us to attribute the structure of eicosanyl butanoate to CFB3 which is a known compound (**Kavita *et al.*, 2009**).



Eicosanyl butanoate **(148)**

II-4-3: Characterisation of compounds obtained from chemical derivatization

Lupeol and β-amyrine obtained by column chromatography from the identical mixtures ND22 and AT3 described above were used for chemical transformations.

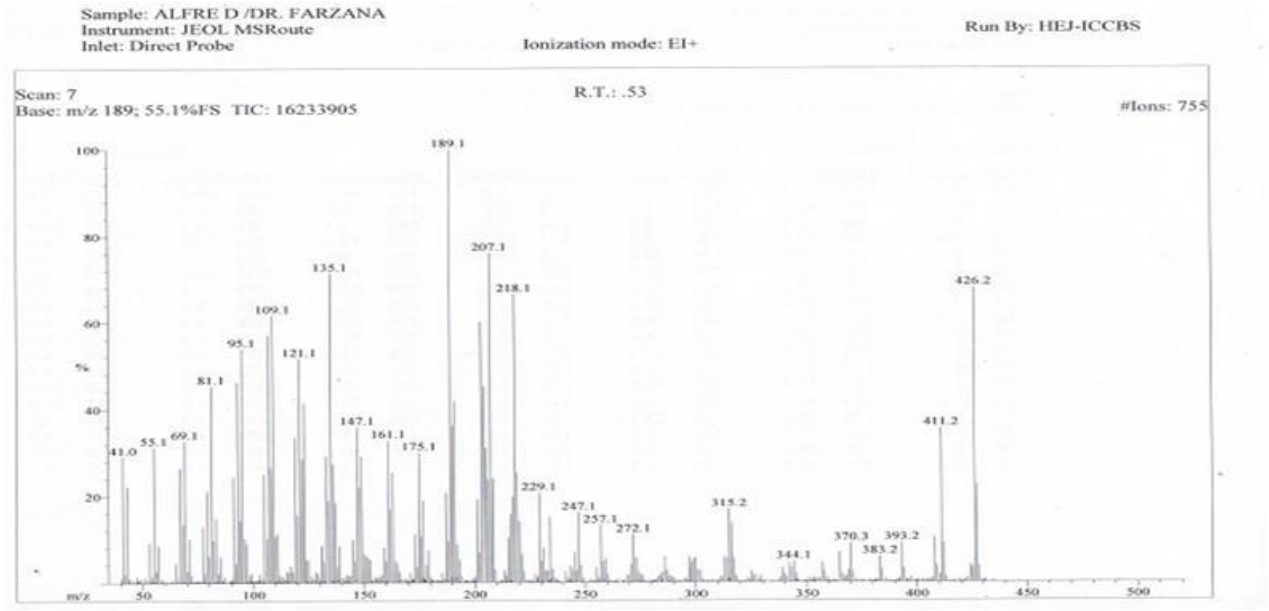


Figure 86: EI-MS Spectrum lupeol

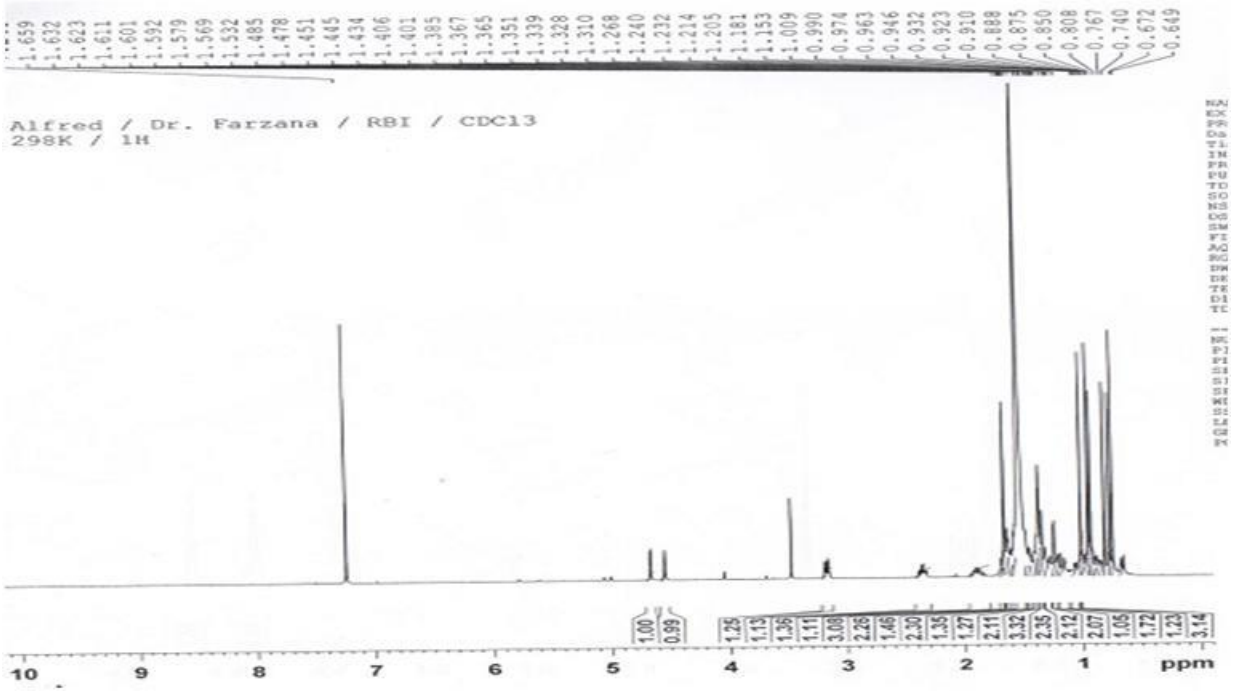


Figure 87: ^{1}H NMR Spectrum Lupeol ($CDCl_3$, 600.13 MHz)

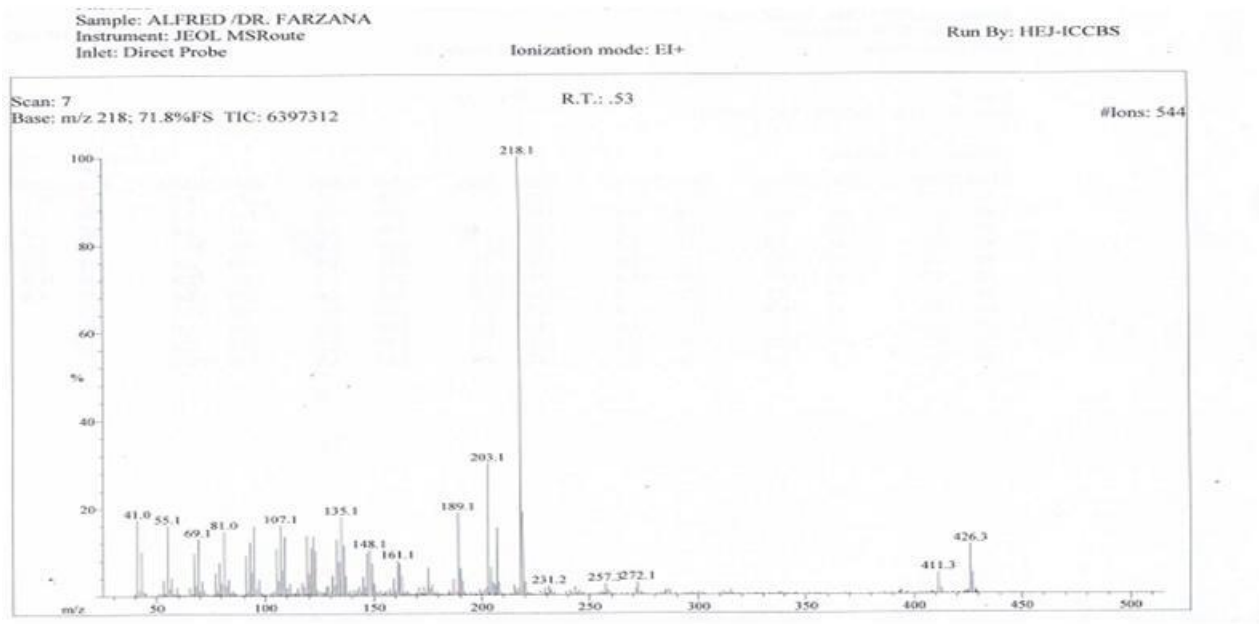


Figure 88: EI-MS Spectrum β-amyrine

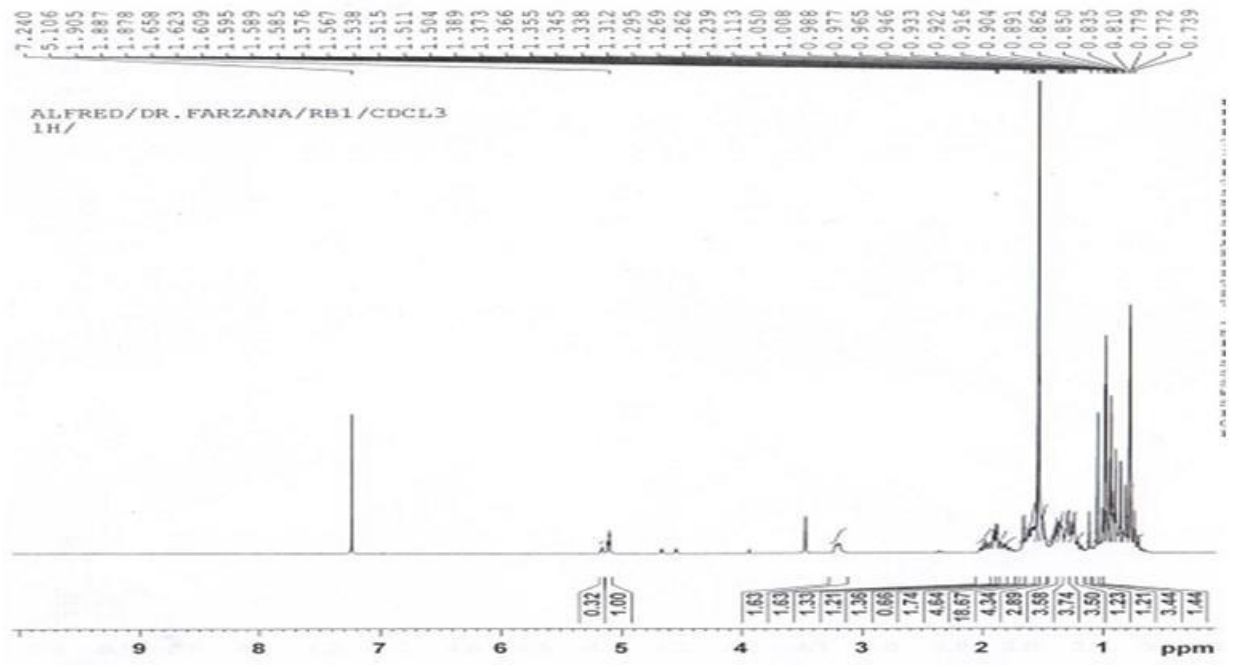
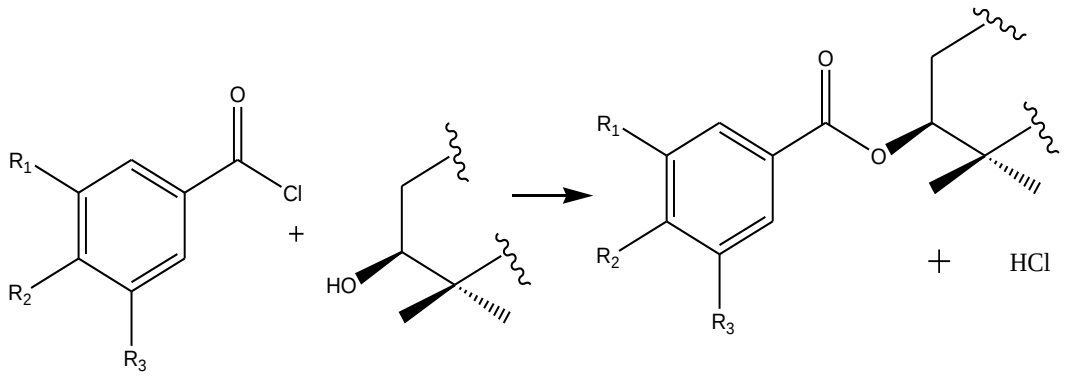


Figure 89: ^{1}H NMR Spectrum of β-amyrine ($CDCl_3$, 400 MHz)

Firstly, esters were prepared form lupeol and benzoyl chloride, p-methoxy benzoyl chloride and 3,4,5-trimethoxybenzoyl chloride. Equally, esters from β- amyrine and benzoyl chloride, p-methoxy benzoyl chloride and 3,4,5-trimethoxybenzoyl chloride were prepared and β- amyrine was oxidized to amyrenone.

II-4-3-1: Characterisation of benzoyl derivative esters of lupeol and β-amyrine

Esterification of lupeol and amyrine was done using suitable benzoyl chlorides with N, N′-diisopropylcarbodiimide (DIC) as condensing agent and 4-dimethylaminopyridine (DMAP) as catalyst under mild conditions at room temperature in anhydrous dichloromethane (**Weijie and Yeyu, 2017**). The general equation for the reaction is as given below with the same reaction conditions (DIC, DMAP, CH_2Cl_2, room temperature, 24 hours) but different R substituents on benzene ring (R_1, R_2, R_3 either H or CH_3O-).



II-4-3-1-1: Identification of 3,4,5-trimethoxy benzoyl esters of lupeol and β-amyrine

The compound RBK was obtained as white powder after liquid-liquid extraction with DCM-H_2O in 62% yield. This compound was soluble in chloroform. The IR spectrum of this compound showed absorption frequencies at 2943.9, 1785.7, 1685.4, 1588.8, 1506.6, 1465.3, 1417.0, 1328.3, 1268.9, 1228.3, 1124.9, 998.9, 935.3, 856.4, 759.9 and 714.4 cm^{-1}.

The EI-MS of RBK gave a molecular ion peak at m/z 620 confirming the theoretical molecular formula $C_{40}H_{60}O_5$ consistent with eleven degrees of unsaturation.

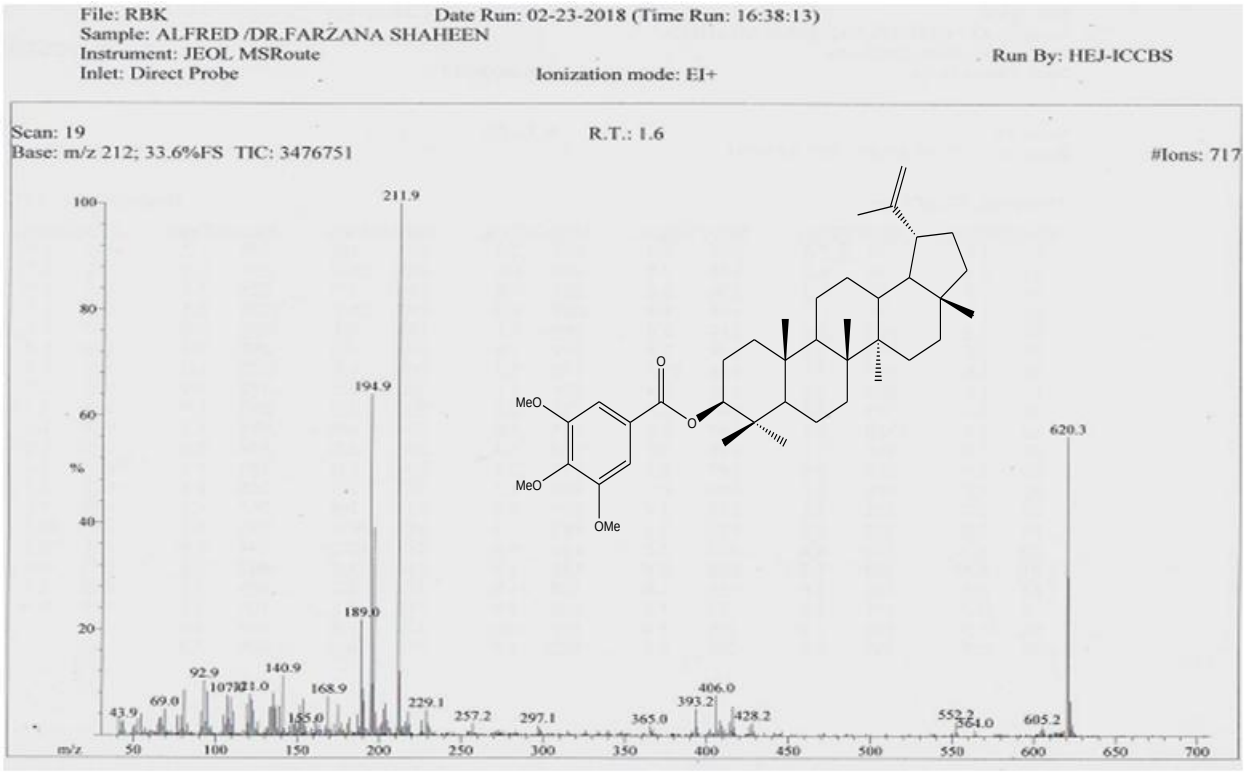


Figure 90: EI-MS Spectrum of RBK

On the ^{1}H NMR spectrum of RBK, there are characteristic proton signals as follows:

Two olefinic protons at δ 4.75 ppm (1H, s) and 4.55 ppm (1H, s) of terminal methylene protons on C-29 of lup-20(29)-ene triterpenoids. One oxymethine proton at 4.09 ppm (1H, t) attributable to H-3 and this confirmed the success of the chemical transformation. Singlet signals attributable to the methoxyl protons on the aromatic ring are present at 3.91 ppm (3H, s), 3.72 ppm (3H, s) and 3.40 ppm (3H, s). The normal angular methyl signals appearing as singlets are observable. Signals appearing at δ_H 7.28 ppm (1H, s) and 7.24 ppm (1H, s) are attributable to the protons at the two ortho positions of the 3,4,5-trimethoxybenzyl ring.

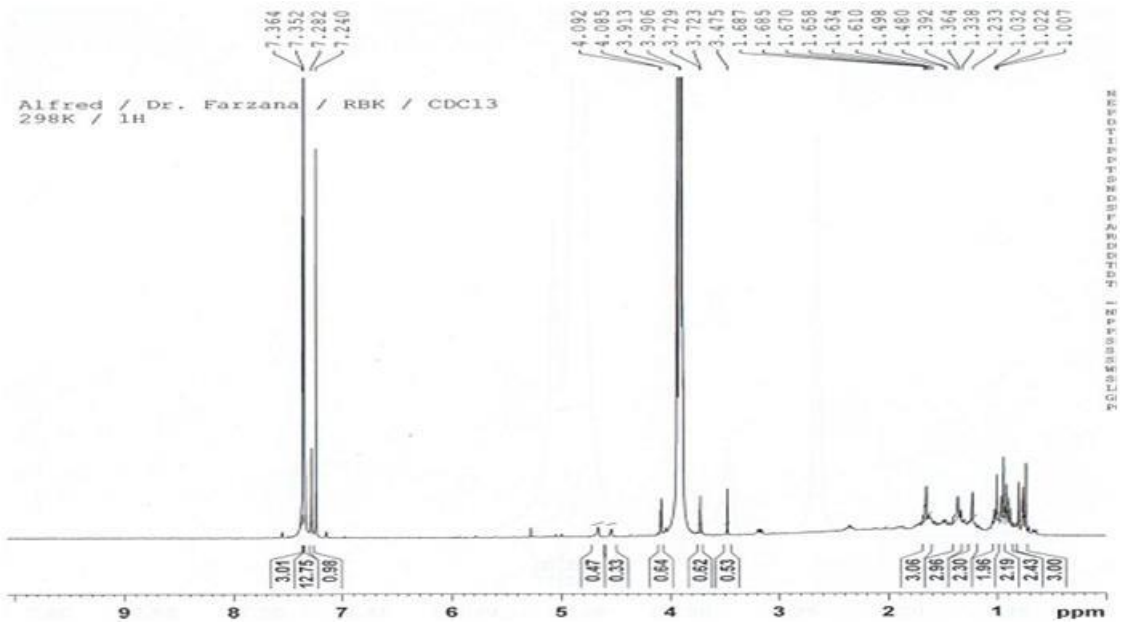
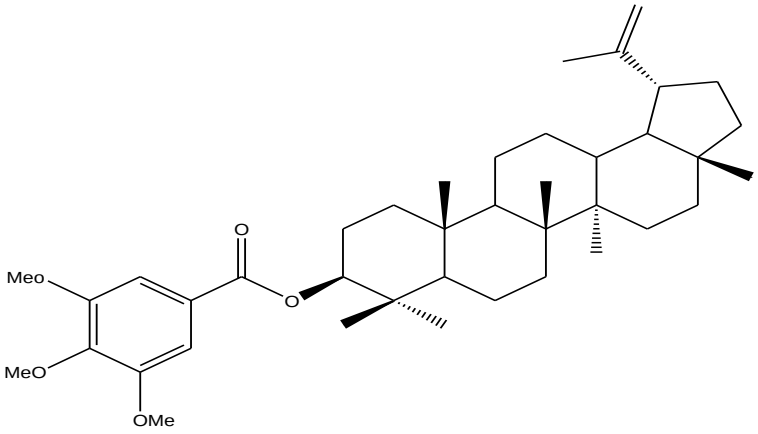


Figure 91: ^{1}H NMR Spectrum RBK ($CDCl_3$, 400 MHz)

Based on the EI-MS spectrum and fragmentation pattern of RBK and in conformity with the ^{1}H NMR spectrum of RBK, it is evident that the reaction took place and the 3,4,5-trimethoxy benzoyl ester of lupeol was formed.



Lupeol-3,4,5-trimethoxy benzoate (**149**)

The compound RBB was obtained as white powder after liquid-liquid extraction with DCM-H_2O in 55 % yield. This compound was soluble in chloroform. The IR spectrum of this compound showed absorption frequencies at 2945.5, 1786.5, 1685.0, 1589.4, 1506.6, 1463.5, 1417.3, 1330.4, 1229.1, 1125.4, 997.9, 857.3, 760.0 and 715.3 cm^{-1}. The EI-MS of RBK gave a molecular ion peak at m/z 620 confirming the theoretical molecular formula $C_{40}H_{60}O_5$ consistent with eleven degrees

of unsaturation. The MS fragmentation pattern was characteristic of an oleanane derivative giving the base peak at m/z 218.2 and another important peak at m/z 402.2 resulting from the RDA fragmentation.

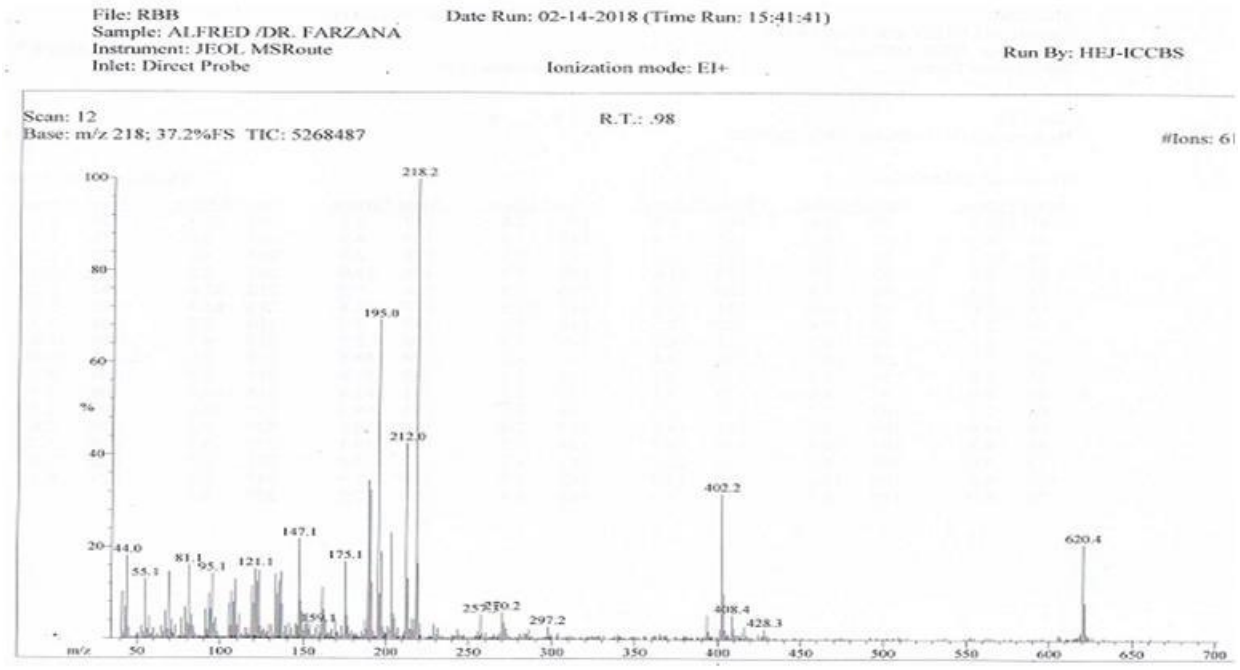
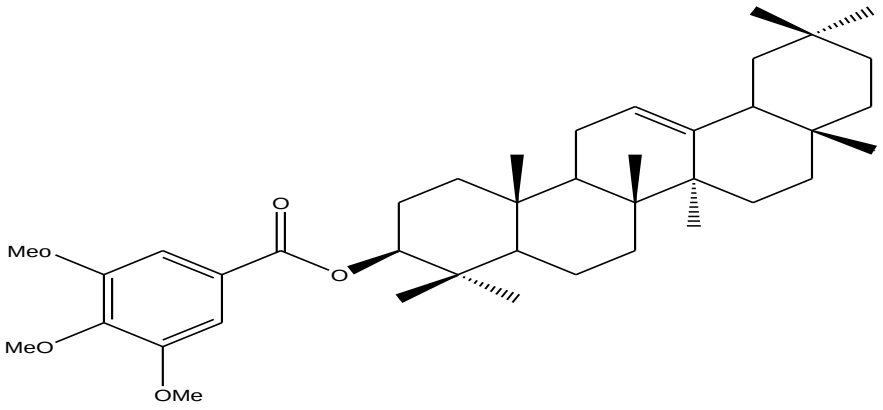
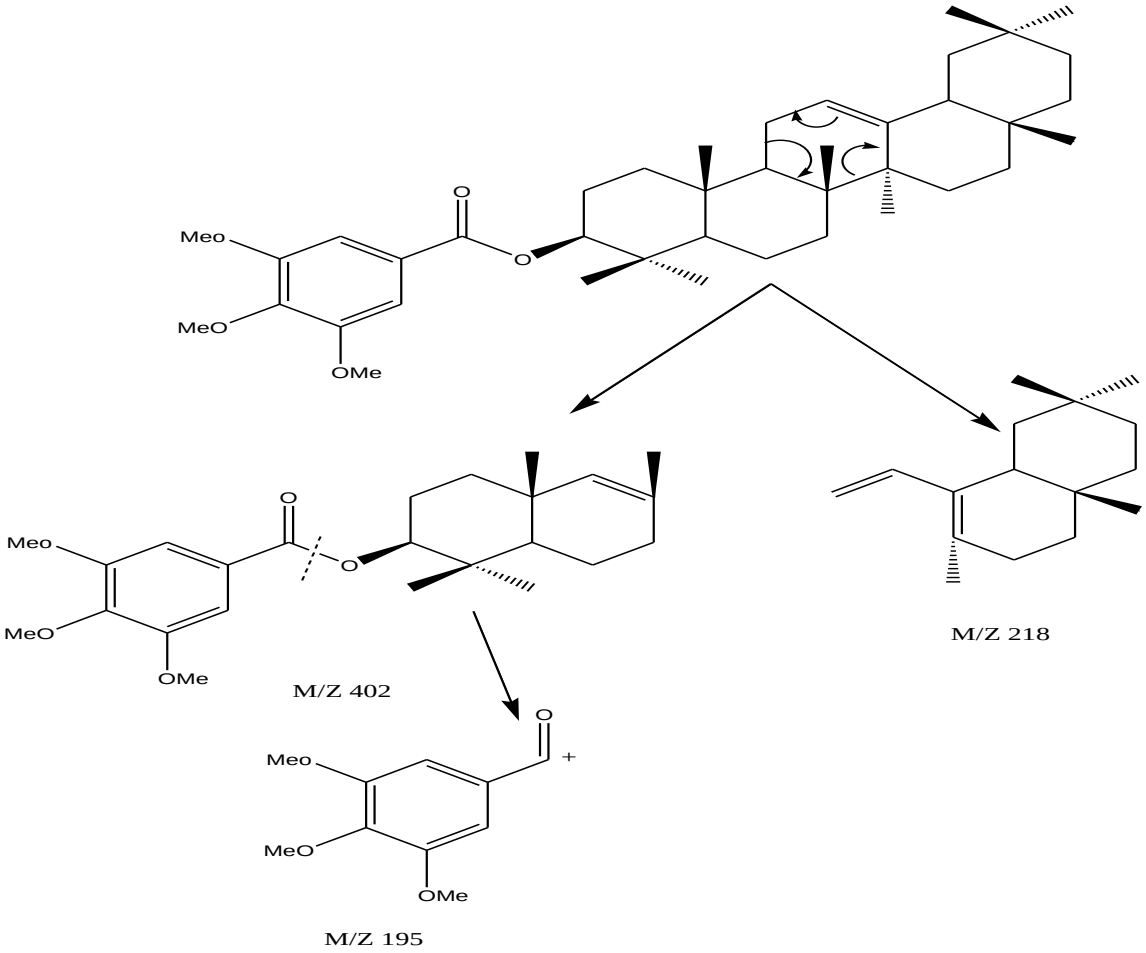


Figure 92: EI-MS Spectrum of RBB

The mass spectrum above is in with the structure below attributable to RBB.



β-amyrine-3,4,5-trimethoxy benzoate **(150)**



Scheme 7: Fragmentation pattern of β-amyrine-3,4,5-trimethoxy benzoate

II-4-3-1-2: Identification of 4-methoxy benzoyl (p-anisoyl) esters of lupeol and β-amyrine

The compound RBJ was obtained as white powder after liquid-liquid extraction with DCM-H_2O in 33% yield. This compound was soluble in chloroform. The IR spectrum of this compound showed absorption frequencies at 2939.1, 1772.4, 1685.0, 1604.7, 1428.5, 1302.5, 1263.6, 1169.3, 1106.5, 1025.0, 924.6, 844.8 and 771.4 cm^{-1}. The EI-MS of RBJ gave a molecular ion peak at m/z 560 confirming the theoretical molecular formula $C_{38}H_{60}O_3$ consistent with eleven degrees of unsaturation. Other peaks at m/z 545.2 (M-CH3) which subsequently loses the isopropenyl group to give the peak at m/z 504.2 and the characteristic peak of lupane type triterpenoids derivatives at m/z 189.2 are present.

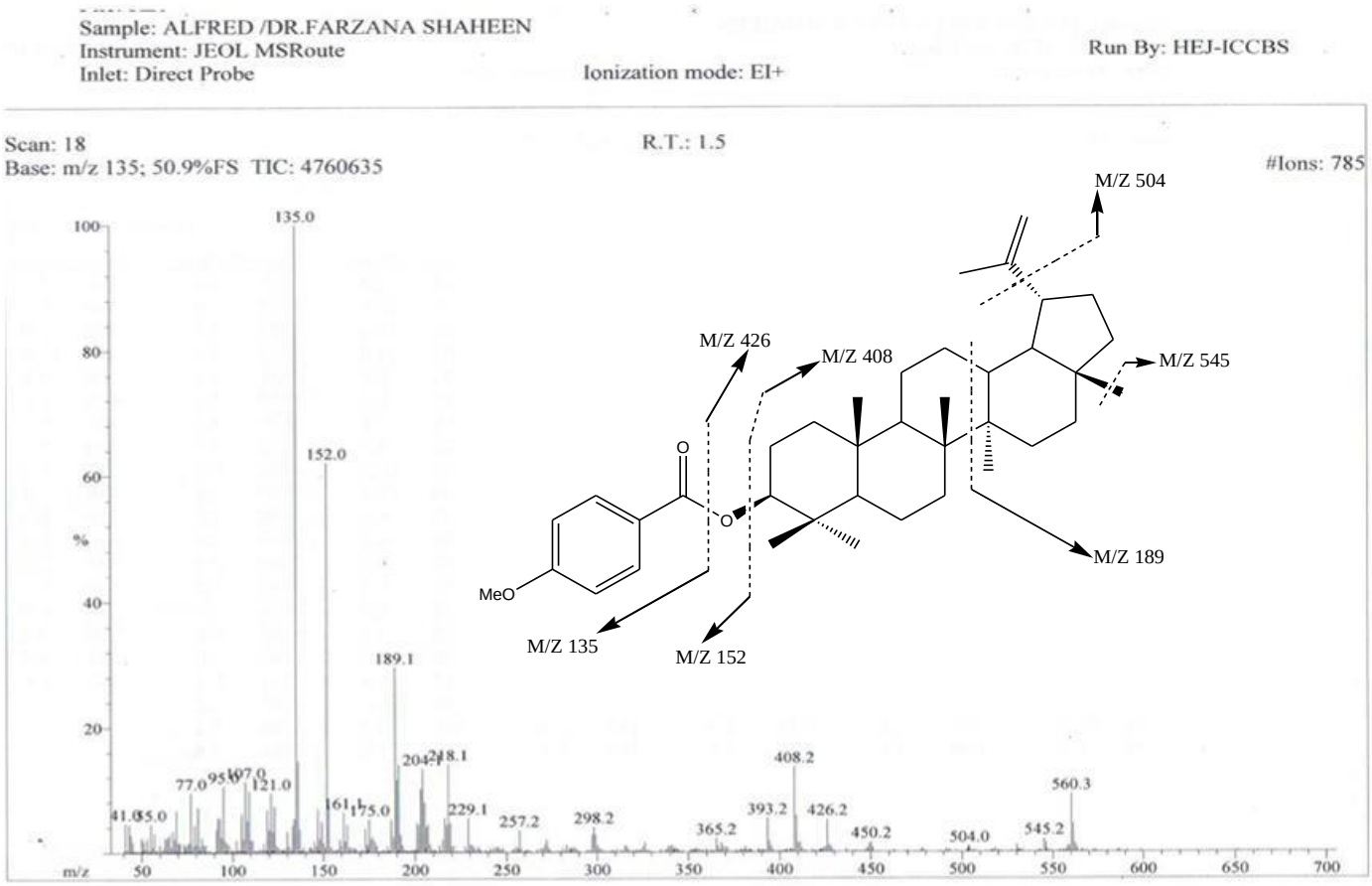
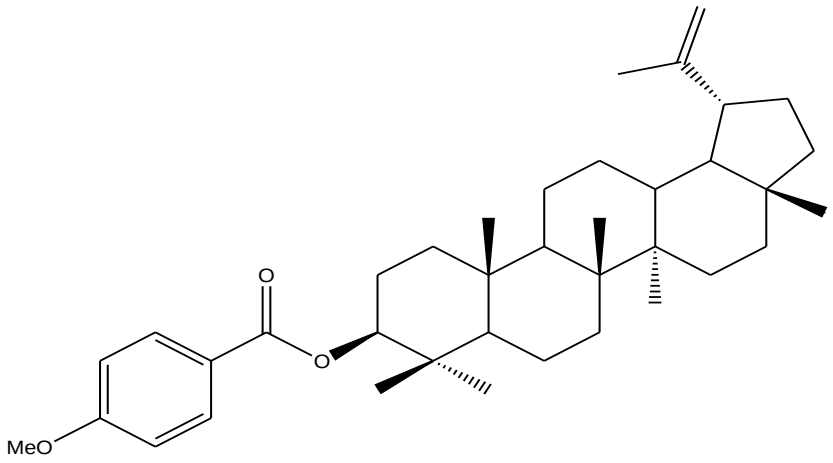


Figure 93: EI-MS Spectrum of RBJ

The EI-MS data and fragmentation pattern of RBJ enabled us confirm that the p-anisoyl ester of lupeol whose structure is given below is formed.



lupeol-4-methoxy benzoate **(151)**

The compound RBA was obtained as white powder after liquid-liquid extraction with DCM-H_2O in 35% yield. This compound was soluble in chloroform. The IR spectrum of this compound showed absorption frequencies at 2942.6, 1792.3, 1684.8, 1649.3, 1605.0, 1567.6, 1428.0, 1301.8, 1261.3, 1218.1, 1167.5, 1024.5, 924.9, 844.7 and 770.7 cm^{-1}. The EI-MS of RBJ gave a molecular ion peak at m/z 560 confirming the theoretical molecular formula $C_{38}H_{60}O_3$ consistent with eleven degrees of unsaturation. The characteristic basepeak of oleanane type triterpenes appears at m/z 218.2 resulting from RDA fragmentation which subsequently loses a methyl group (218.2-CH_3) to give the peak at m/z 203 or an ethyl group to give the peak at m/z 189.2. The peak at m/z 135 results from the p-methoxybenzoyl fragment is also prominent.

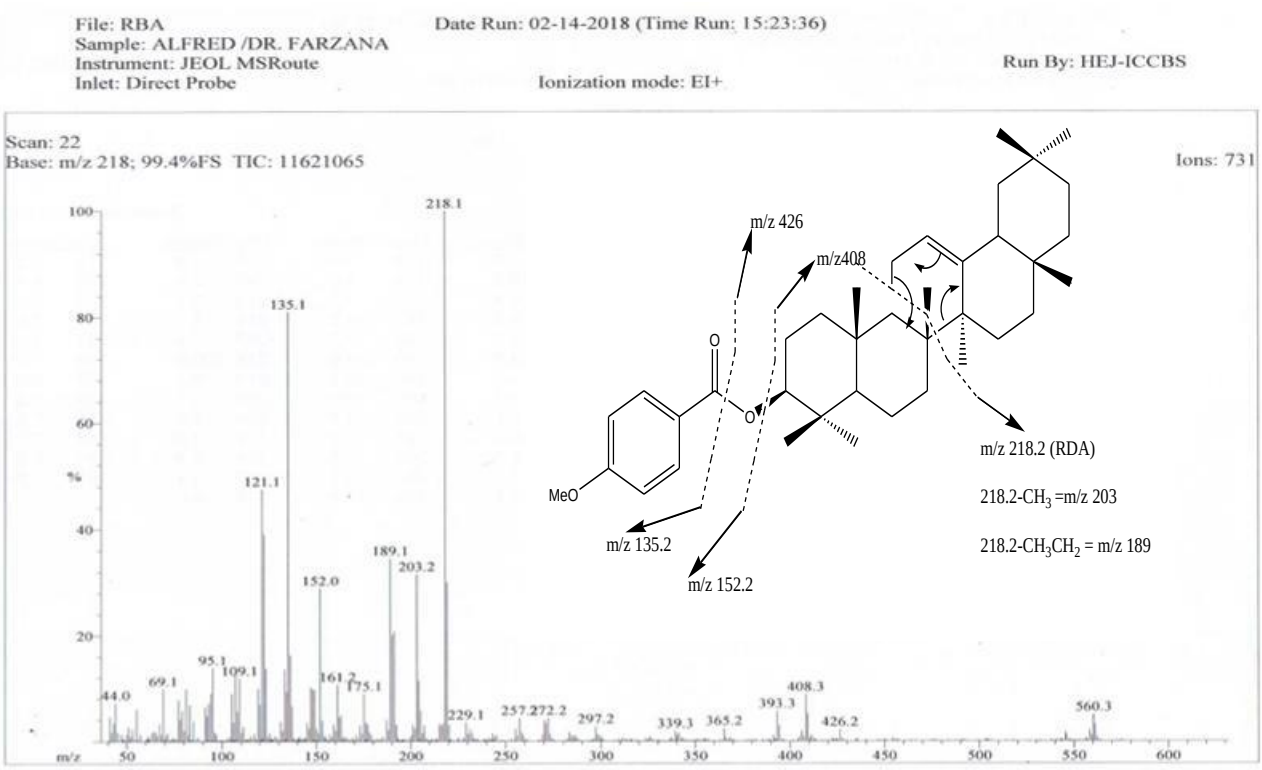
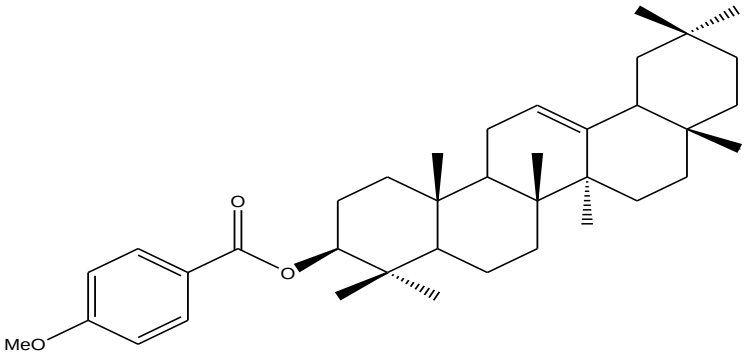


Figure 94: EI-MS Spectrum RBA

The EI-MS data and fragmentation pattern of RBA enabled us confirm that the p-anisoyl ester of β-amyrine whose structure is given below is formed.



β-amyrine-4-methoxy benzoate **(152)**

II-4-3-1-3: Identification of benzoyl esters of lupeol and β-amyrine

The compound RBP was obtained as white powder after liquid-liquid extraction with DCM-H_2O in 20% yield. This compound was soluble in chloroform. The IR spectrum of this compound showed absorption frequencies at 2925.5, 1699.9, 1646.2, 1457.2, 1382.4, 1289.1, 1039.1, 979.9, 880.1, 768.8 and 743.0 cm^{-1}. The EI-MS of RBJ gave a molecular ion peak at m/z 530 confirming the theoretical molecular formula $C_{37}H_{54}O_3$ consistent with eleven degrees of unsaturation. Other peaks at m/z 189.1 characteristic of lupane type triterpenes which subsequently loses the isopropenyl group to give the prominent peak at m/z 147. The base peak at m/z 129.9 results from the fragmentation of the C_3-C_4 and C_3-C_2 bonds.

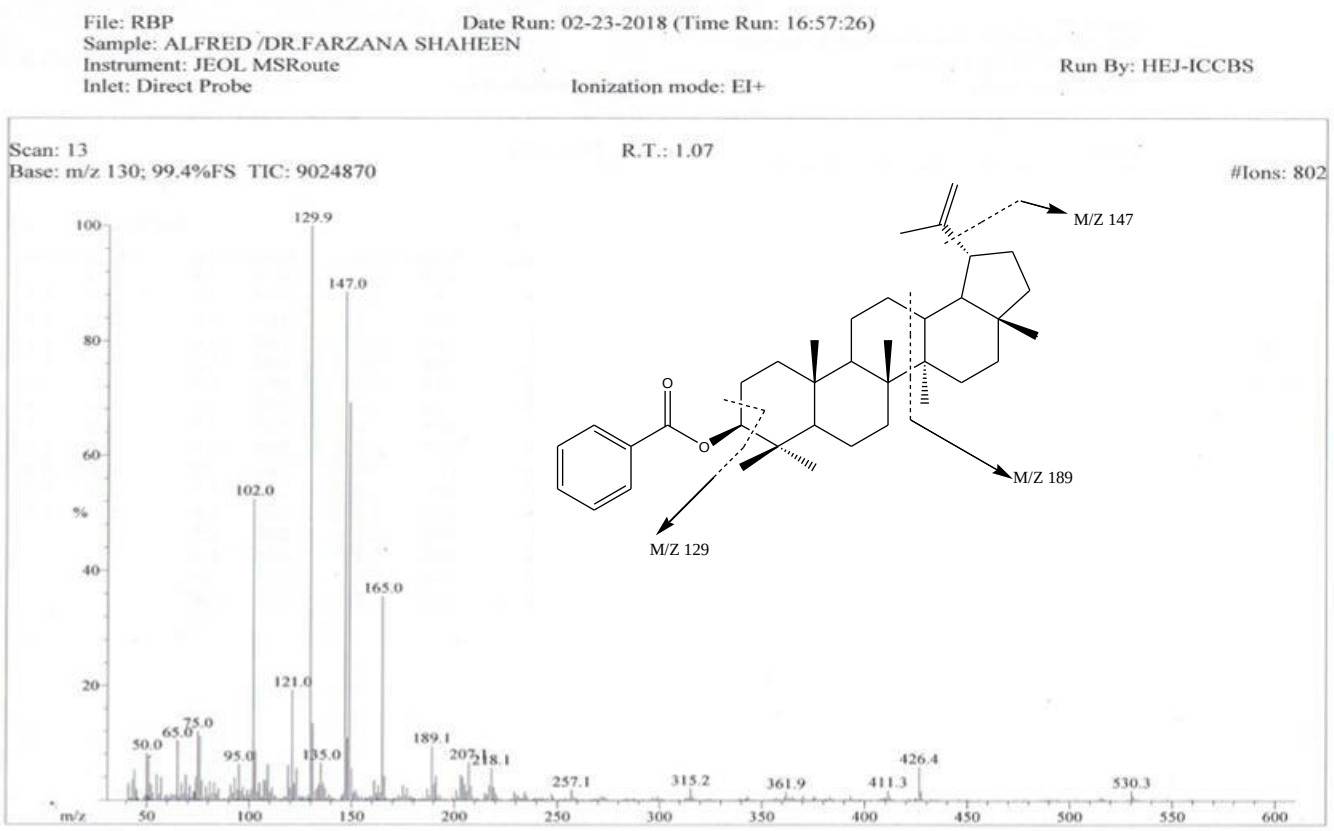
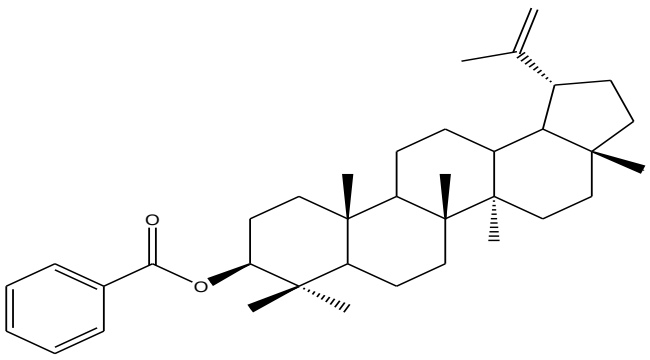


Figure 95: EI-MS Spectrum RBP

This enabled us to attribute the structure below to RBP.



Lupeol benzoate **(153)**

The compound RBP was obtained as white powder after liquid-liquid extraction with DCM-H_2O in 20% yield. This compound was soluble in chloroform. The IR spectrum of this compound showed absorption frequencies at 2925.7, 1696.0, 1557.6, 1426.0, 1286.0, 1117.7, 993.3, 864.0, 766.5 and 744.2 cm^{-1}. The EI-MS of RBJ gave a molecular ion peak at m/z 530 confirming the theoretical molecular formula $C_{37}H_{54}O_3$ consistent with eleven degrees of unsaturation. Other

peaks at m/z 218.1 resulting from RDA characteristic of oleanane type triterpenes which subsequently loses a methyl group to give the prominent peak at m/z 203.

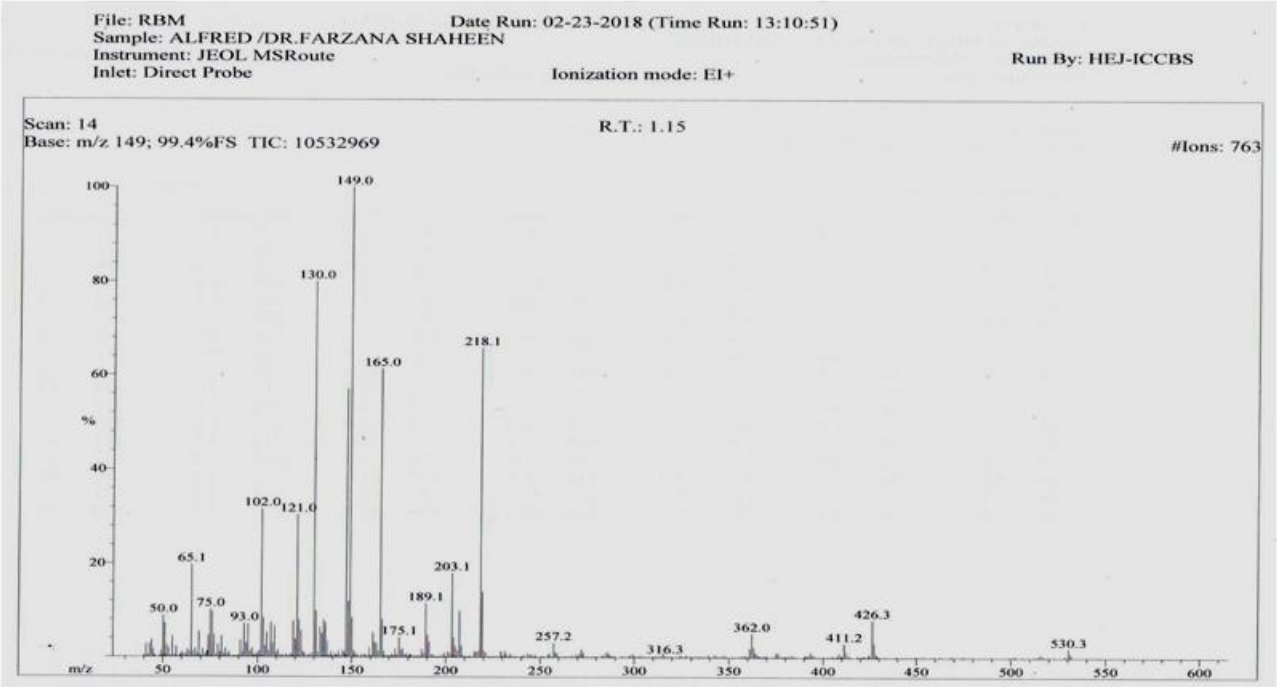
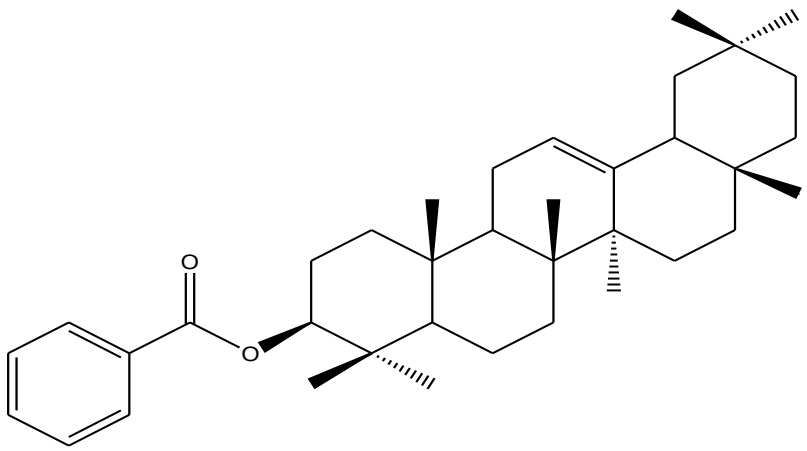


Figure 962: EI-MS spectrum of RBM

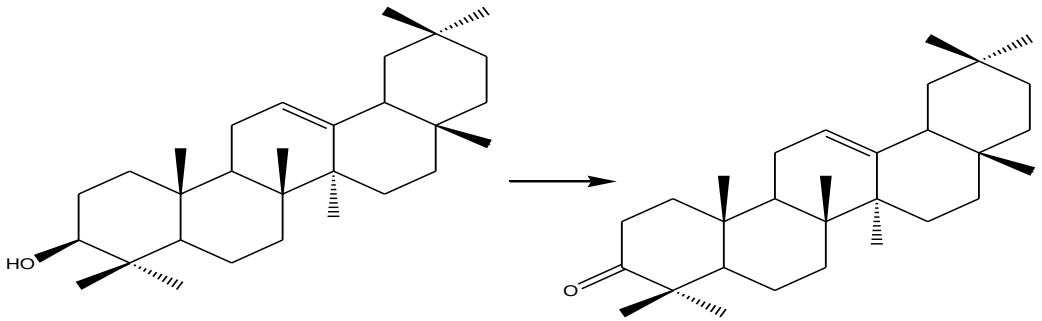
This enabled us to attribute the structure below to RBM



β-amyrine benzoate **(154)**

II-4-3-2: Identification of β-amyrenone

Oxidation of β-amyrine to β-amyrenone was done by using PCC (Pyridinium chloro chromate) in dichloromethane at room temperature according to the equation below.



The compound RBC was obtained as white powder after purification on column chromatography using an isocratic system hexane: ethyl acetate (85:15) in 60% yield. This compound was soluble in chloroform and melts at 149-151°C. The IR spectrum of this compound showed absorption frequencies at 2947.4, 2868.1, 1707.3, 1460.3, 1389.9 and 1244.8 cm^{-1}. The EI-MS of RBE gave a molecular ion peak at m/z 424 from where the molecular formula $C_{30}H_{48}O$ is obtained, consistent with seven degrees of unsaturation. The characteristic base peak of oleanane type triterpenes occurs at m/z 218.2 and the base peak at m/z 205.2 both resulting from RDA fragmentation. Other prominent peaks appear at m/z 409.3, 313.2, 203, 189.2, 121.1, 109 and 82.9.

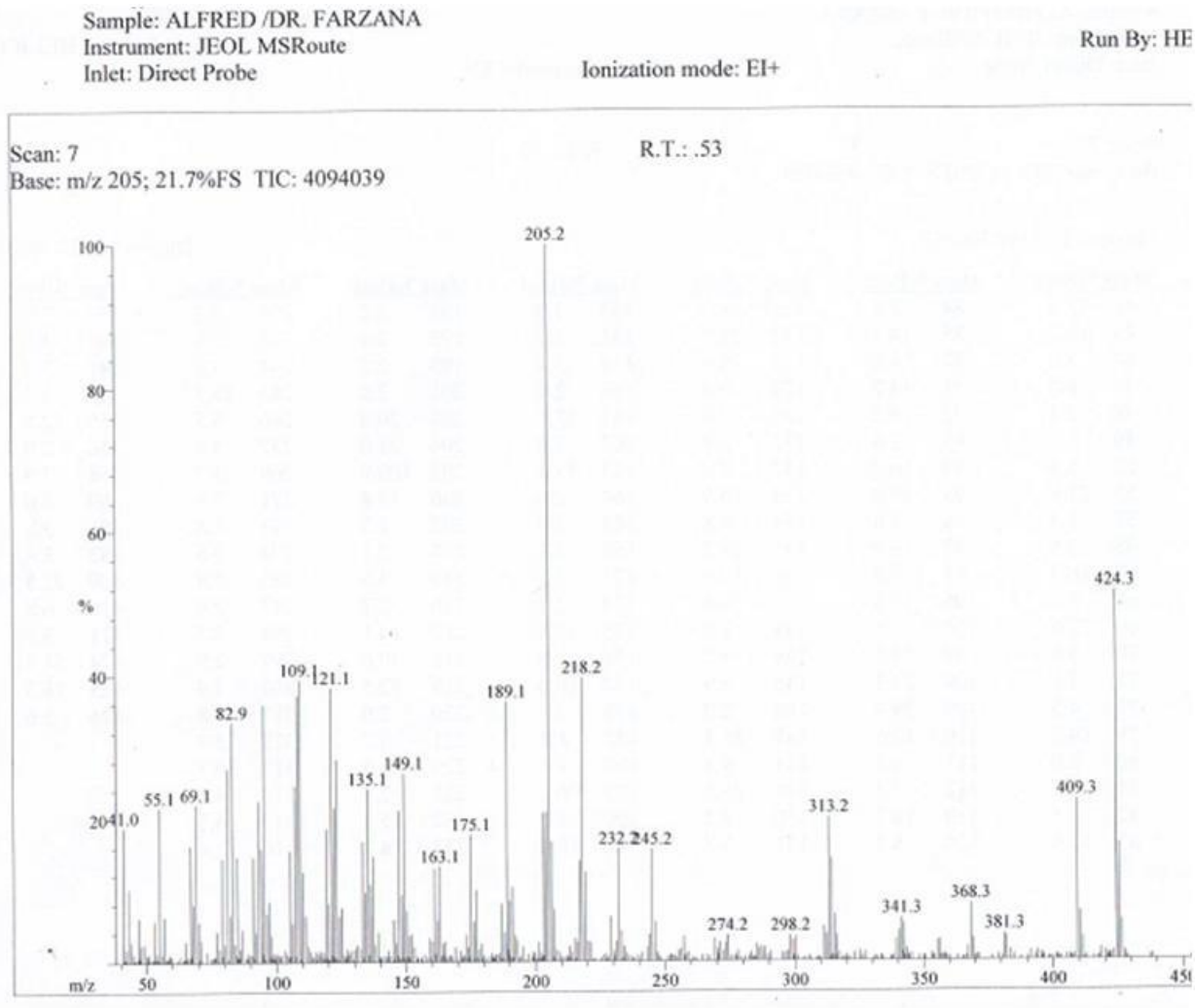


Figure 973: EI-MS Spectrum of RBC

The NMR spectrum showed the presence of eight methyl singlets, one olefinic proton signal at 5.20 ppm attributable to the proton H-12. The disappearance of the signal of the oxygenated proton at H-3 and the appearance of the protons at 2.75 ppm attributable to the protons in α-position to a carbonyl function confirms that the reaction took place.

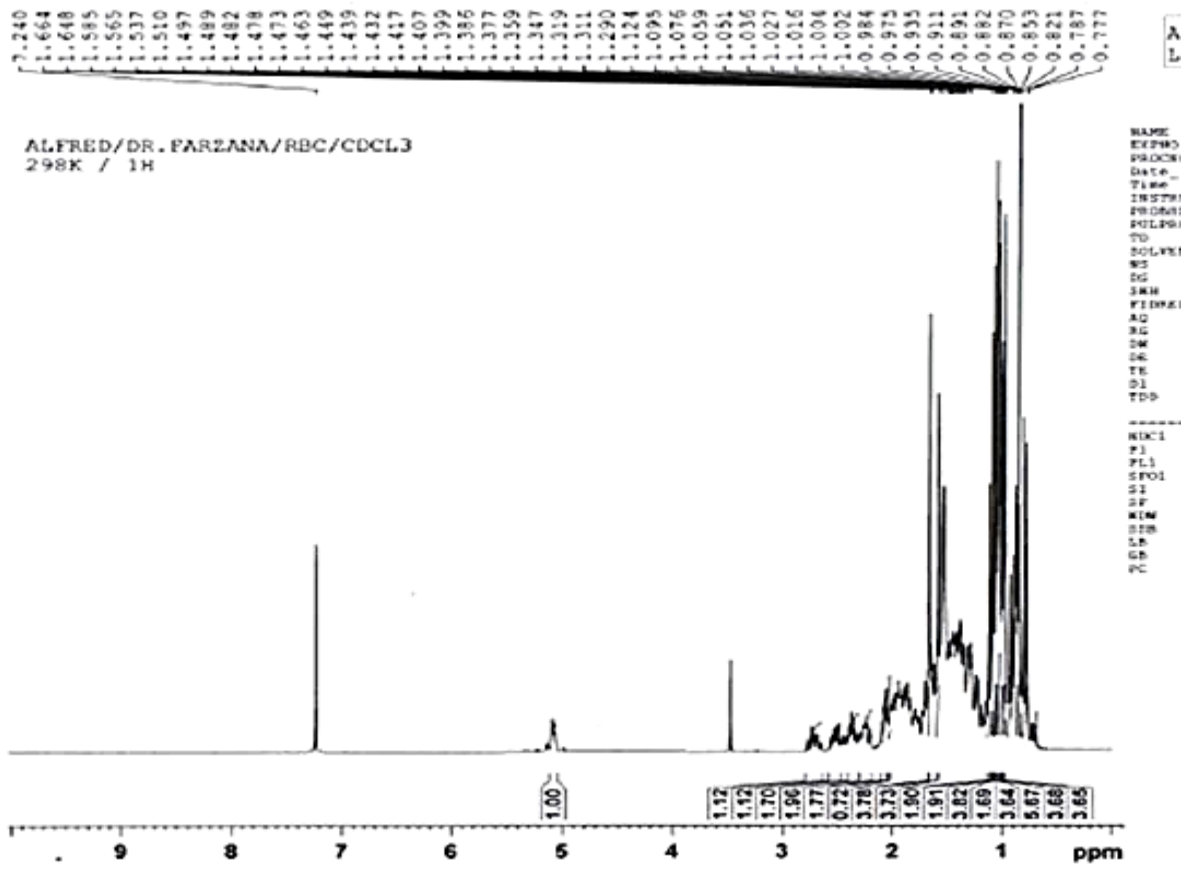
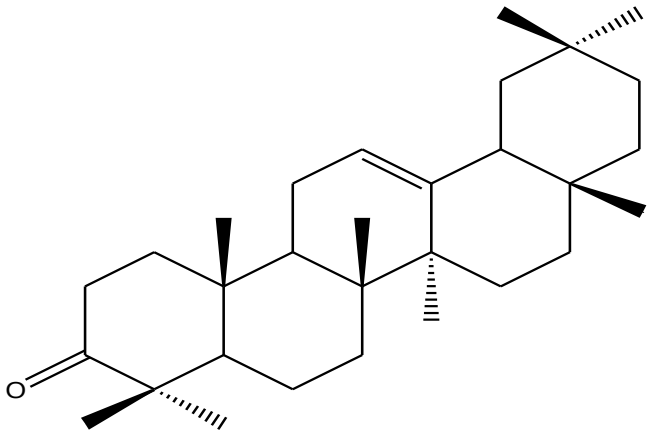


Figure 98: ^{1}H NMR Spectrum of RBC ($CDCl_3$, 400 MHz)

The spectra above indicate that reaction was successful and that the product below was formed.



β-amyrenone **(155)**

II-4-4: Identification of components by GC-MS

II-4-4-1: Identification of constituents of ANT46 by GC-MS

The fraction ANT46 crystallized in the form of a white powder and gave a violet coloration when reacted with Liebermann-Burchard reagent characteristic of triterpenes. The ^{1}H NMR of ANT46 revealed that it was a mixture of triterpenes. This fraction was then analyzed by GC-MS and its constituent compounds identified from their retention times, MS fragmentation patterns and comparison with internal standards and MS data bases (Wiley—Wiley Registry of Mass Spectral Data; NIST—Mass Spectral Library) and other data reported in literature. The TIC of ANT46 and the MS spectra of the silylated constituents are given below.

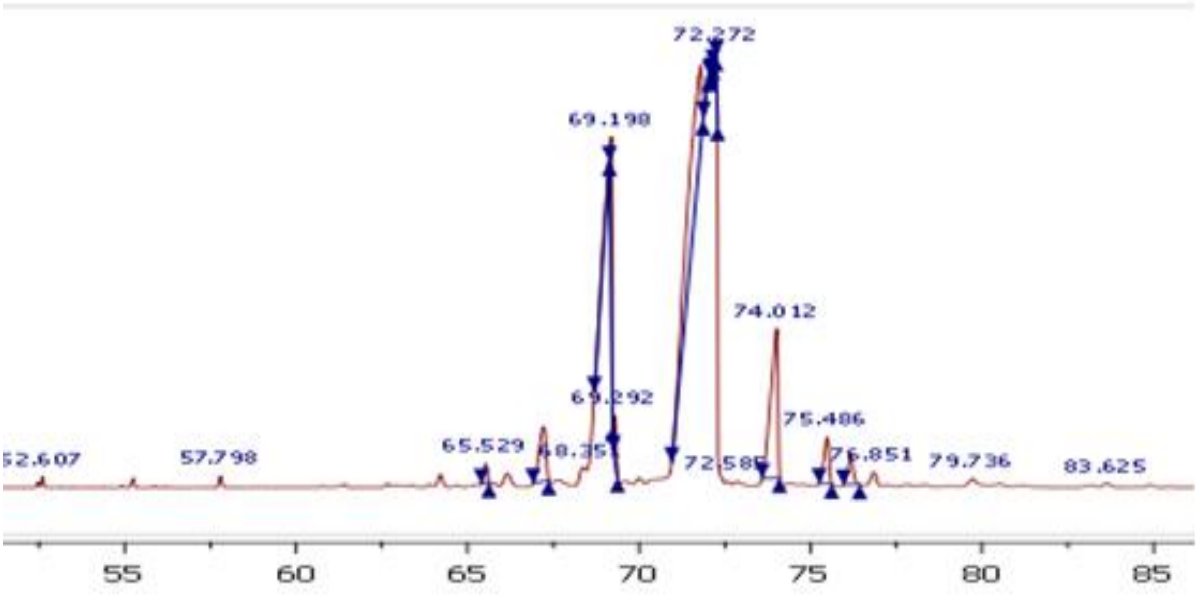


Figure 99: TIC of ANT46 silylated sample

Sample processing for GC-MS involves solubilization, concentration to dryness and consecutive derivatization often carried out in a two-step procedure. In the first step is achieved by a reaction of sample components with diluted pyridine to stabilize thermolabile compounds. In the second step, extracted metabolites are derivatized with silylating reagents *N*-[dimethyl-(2-methyl-2-propyl) silyl]-2,2,2-trifluoro-*N*-methylacetamide (MTBSTFA), *N*,*O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) which substitute protons bound to heteroatoms in functional groups such as -OH, -COOH, -NH_2, -NH, -SH etc. and generate trimethylsilyl (TMS) and *tert*-butyldimethylsilyl (TBS) derivatives. The latter step is crucial for the adequate derivatization of non-volatile compounds in order to capture a huge

variety of metabolites with polar characteristics and high boiling points on a GC-MS system. The retention times, %TIC and some key fragment ions of the triterpene constituents of ANT46 are given in the table below.

Table 37: GC-MS data showing silylated constituents of ANT46 with their key fragment ions

RT	Compound	Key ion fragments of silylated sample (m/z and relative abundance)	% of TIC
67.2	lanosterol	M+TMS = 498.6 (40.9%), 393.5 (100%), 69.2 (40.45), 394.5 (31%), 109.2 (27.5%), 189.2 (17.6%)	2.4
69.2	α-amyrine	M+TMS = 498.6 (4.5%), 218.3 (100%), 203.3 (36.2%), 189.3 (16.3), 219.3 (18.5%), 135.2 (6.7%)	20.5
69.3	28-norolean-12-en-3-ol	M+TMS = 498.6 (30.5%), 204.3 (100%), 177.2 (70.9%), 190.3 (43.2%), 109.2 (31.3%), 231.3 (30.2%)	0.9
71.8	Cycloartenol	M+TMS = 498.6 (16.9%), 218.3 (100%), 408.5 (45.1%), 365.4 (35.2%), 189.3 (37.1%), 135.2 (26.9%), 175.2 (17.8%), 109.2 (23.7%)	36.3
72.3	3-epi-α-amyrine	M+TMS = 498.6 (19.3%), 218.3 (100%), 189.3 (39.5%), 203.3 (25.3%), 191.3 (12.8%), 135.3 (19.1%), 109.2 (17.3%)	13.45
72.6	lupeol	M+TMS = 498.6 (60.6%), 189.3 (100%), 218.3 (59.3%), 109.2 (55.9%), 135.2 (49.3%), 369.5 (40.9%), 107.2 (39.9%)	13.45
74.0	24-methylenecycloartenol	497.6 (9.7%), 422.5 (100%), 379.4 (89.9%), 353.4 (26.9%), 380.4 (27.9%), 203.4 (24.6%), 175.2 (34.8%), 107.2 (40.3%)	6.3

The individual mass spectra of the constituents of ANT46 are given below. The peak corresponding to TMS group appears at 73.1 on all the spectra while molecular masses of the silylated compound corresponds to M+TMS appearing at 498.55 for the triterpenes of molecular mass 426 g/mol.

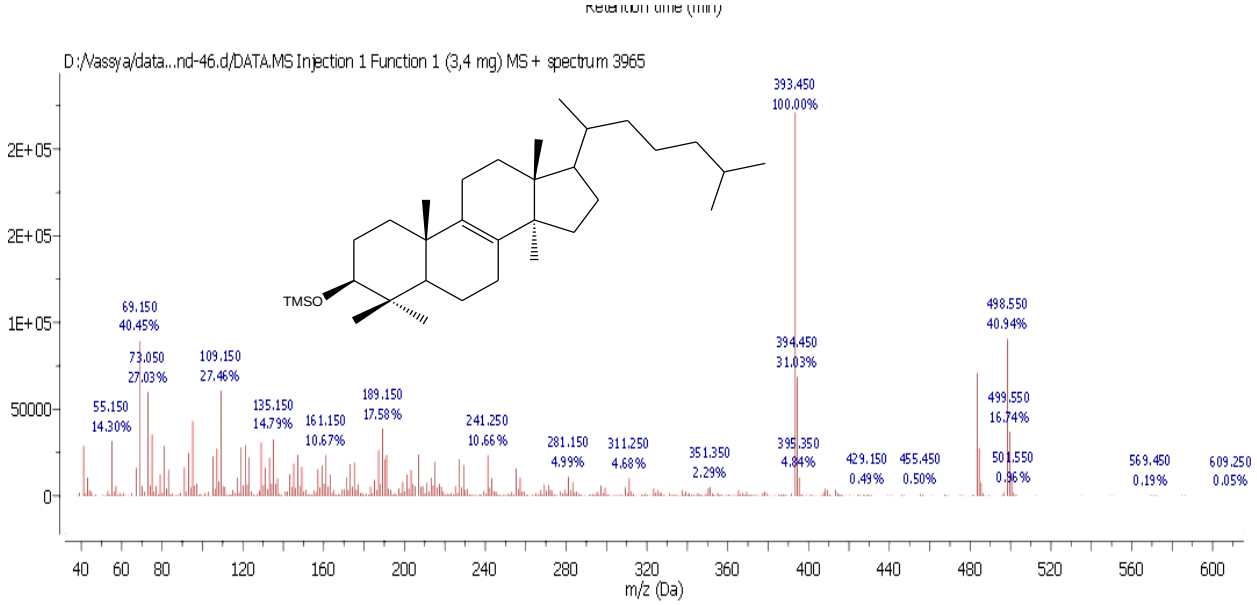


Figure 100: Mass spectrum of silylated Lanosterol

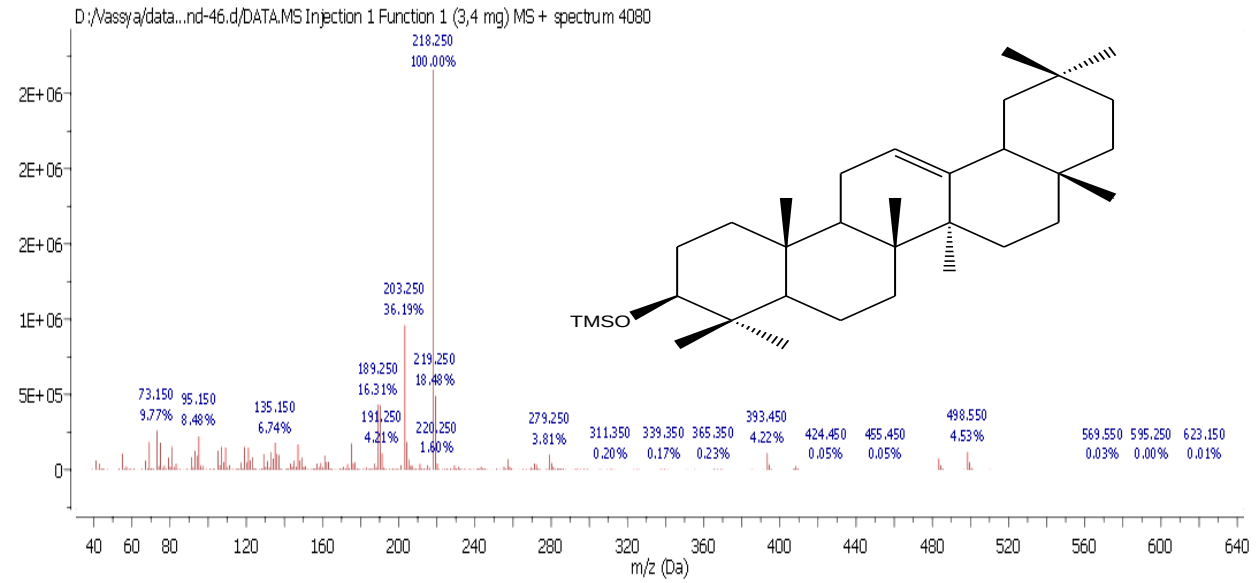


Figure 101: Mass spectrum of silylated β-amyrine

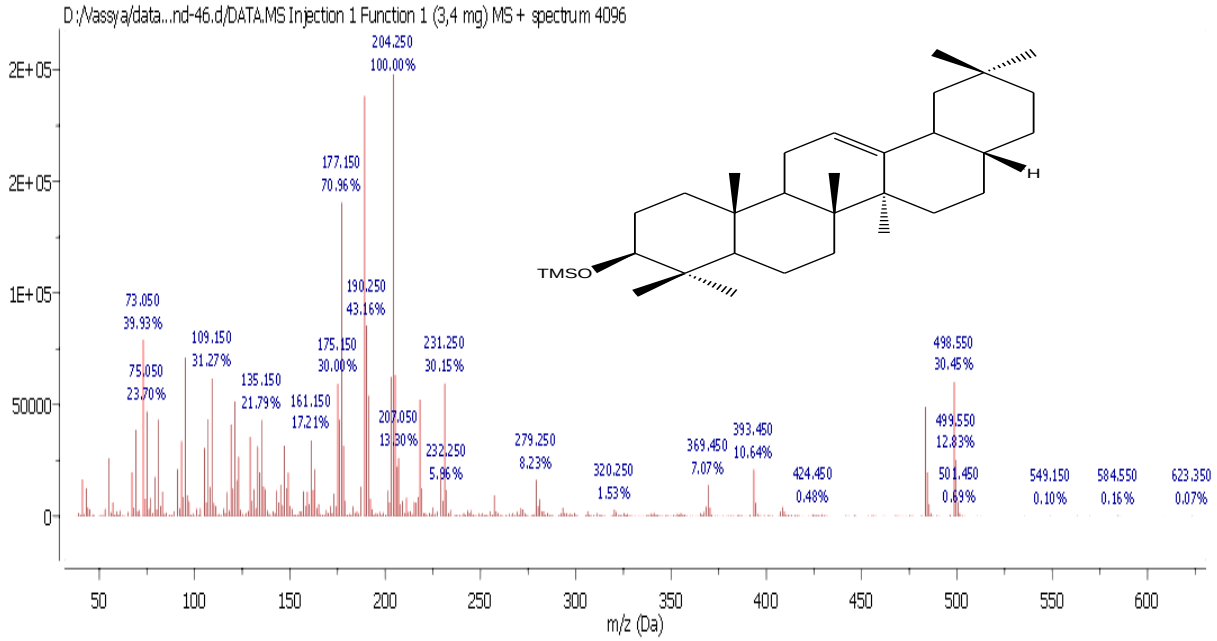


Figure 102: Mass spectrum of silylated 28-norolean-12-en-3-ol

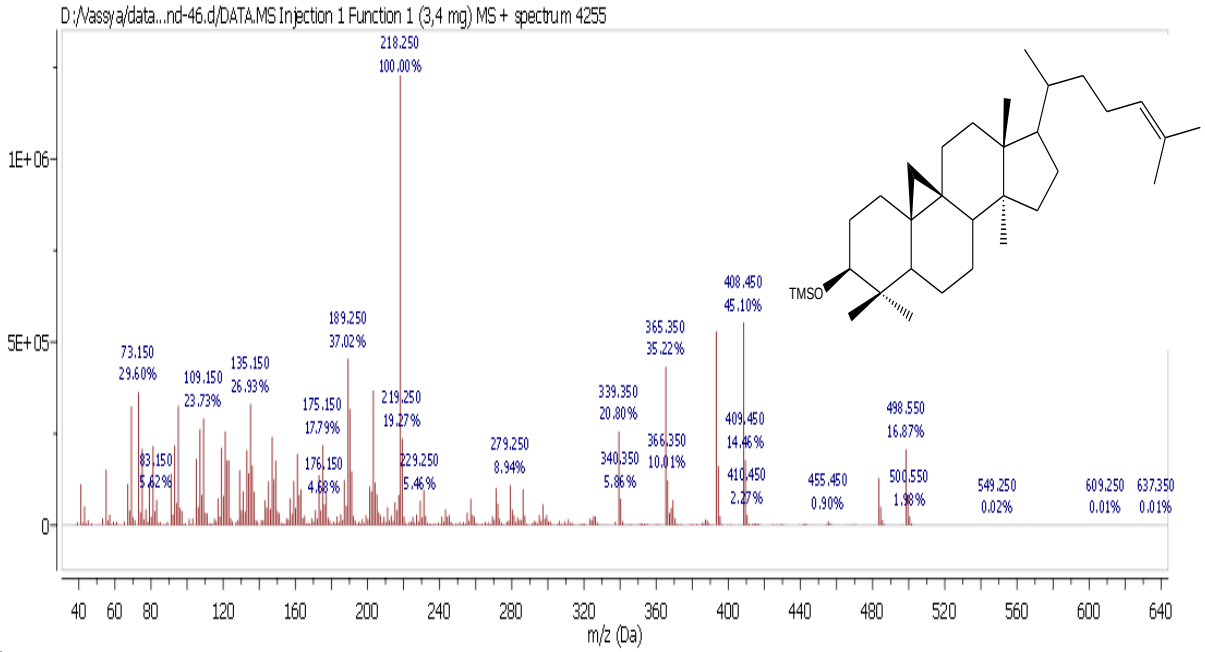


Figure 103: Mass spectrum of silylated cycloartenol

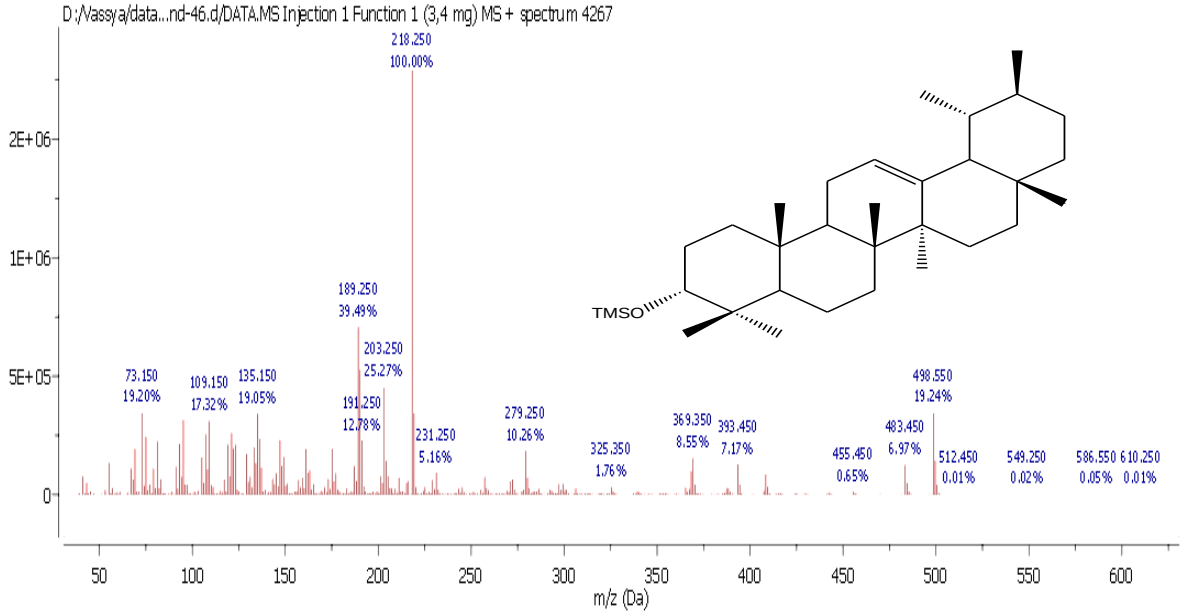


Figure 1044: Mass spectrum of silylated 3-epi-α-amyrin

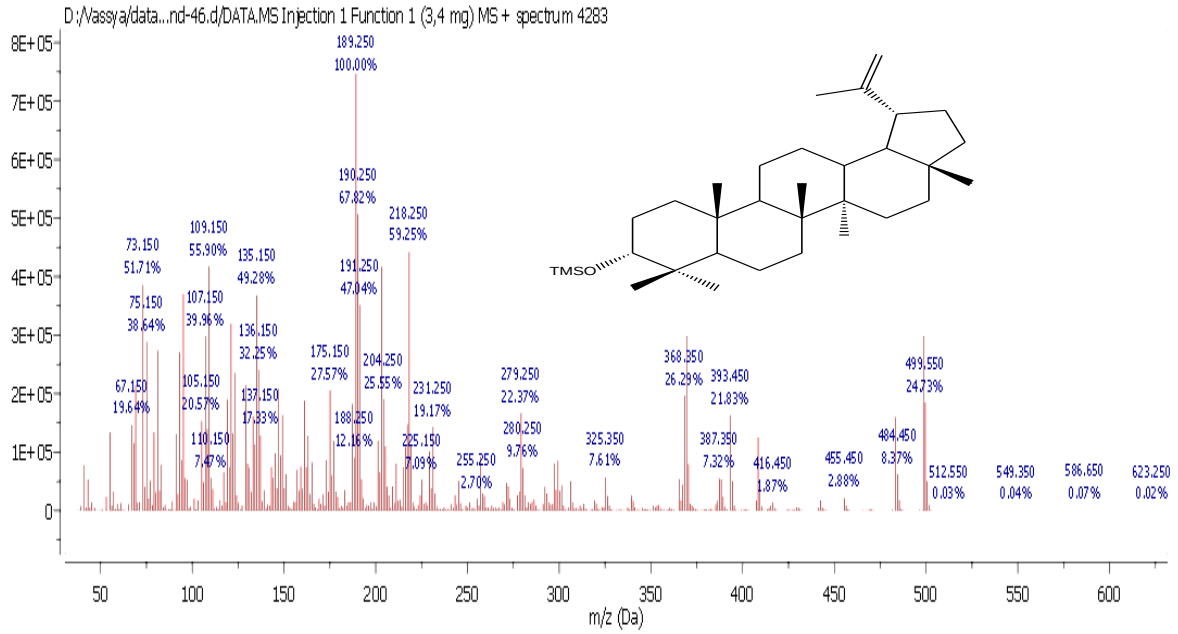


Figure 105: Mass spectrum of silylated lupeol

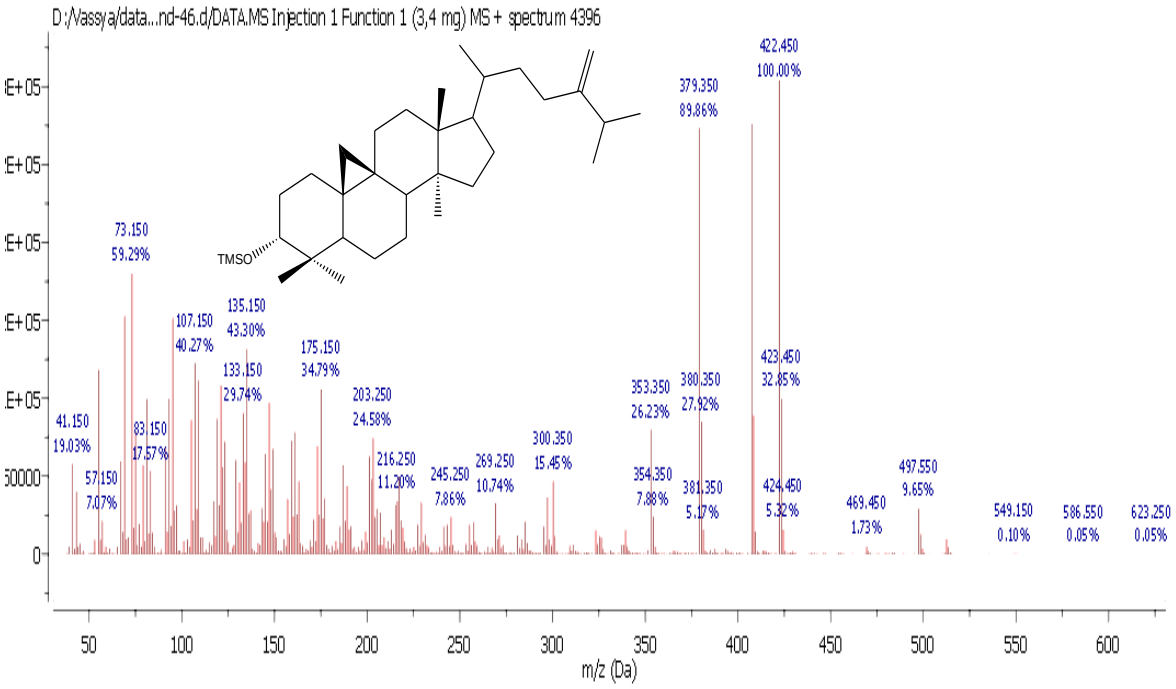


Figure 106: Mass spectrum of silylated 24-methylenecycloartenol

GC-MS is an important and powerful analytical method and the time-of-flight mass spectrometry (GC-TOF-MS) analyzer used provides fast scanning, high sensitivity and mass accuracy. Not only terpenoids are detectable by this method but also compounds such as sugars, sugar alcohols/acids, amino and fatty acids, phosphorylated intermediates and many plant secondary metabolites such as phenolics, steroids and alkaloids. Regarding the high number of metabolites analyzable after derivatization, GC-MS profiling is gaining grounds as a very sensitive method for the characterization of complex profiles of compounds found in plant and food samples. For this reason, the chemical profiles of the various propolis samples were established using this method.

II-5: GC-MS PROFILING OF SOME PROPOLIS SAMPLES

GC-MS techniques have been proven to be suitable for chemical profiling of propolis (**Sforcin and Bankova, 2012**). Even though these techniques provide a sufficient profile and identification of the compounds analyzed (**Gómez-Caravaca *et al.*, 2006),** the propolis has to be derivatised in order to increase the concentration of volatile compounds for detection. The preliminary GC-MS investigation of different extracts of propolis from three Geographical regions of Cameroon revealed the presence of over 30 compounds belonging to a variety of classes of natural products. Identification was performed based on the mass-spectral fragmentation, in such cases for some compounds only tentative structures were proposed while some constituents remained unidentified because of the lack of relevant references and information (none of them major constituent). The identification of mixtures of natural products by mass spectral analysis alone might be difficult because of the number of isomers and minor differences can be observed in their mass spectra. The relative concentrations of these compounds vary greatly from one extract to another and from one region to another. The variability of constituents of propolis in the different samples shows that they were collected by the honeybee from different plants sources. This might also depend on the method of extraction used for each propolis sample. The results showing the types of major compounds in the complex composition of the different samples identified are listed in Table 28 below together with the retention time of the major peaks. Their percentages are also given in the tables and refer to percent of the Total Ion Current, TIC which are semi-quantitative since the ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation. Alkanes (Pentacosane, Heptacosane, Nonacosane and Hentriacontane), triterpene ketones (Bauerenone, α-amyrenone and Lupenone) and triterpene diols like Olean-12-ene-3,11-diol are found in all the propolis extracts except Boyo 3. Boyo 3 which only contain traces of alkanes and Tala-Mokolo 2 extract with a small number of alkanes is an indication that the propolis samples from North-west region are poor in alkanes and lack lacceroic acid while those of Adamawa region Djerem 5 and Mbere 6, Mbere 7 and West region Mbouda 8 are rich in alkanes and contain lacceroic acid. The propolis from North-West Boyo is the richest in aromatic acids such as hydroxybenzoic acid. gallic acid, caffeic acid, chlorogenic acid and chlorogenic acid isomer and shows a slight resemblance with the propolis of West region Mbouda 8 both containing caffeic acid and glycerol exclusively. However, triterpene O-acetates, fatty acids such as Myrisric acid, Hexadecenoic acid and lacceroic acid (C32) and triterpene alcohols like α-amyrine,

lupeol,24-methylene, cycloartenol and epi-lupeol are present in all propolis samples from the different regions indicating that there is something common in the botanical origin of propolis from the three regions. Other compounds which are also present in some of the extracts include glycerol, quinic acid, eicosanyl glycerol and trihydroxymonomethoxy flavone. The flavonoid trihydroxymonomethoxy flavone is present only in the propolis from West Region Noun 8.

Table 38: GC-MS profiles of propolis samples from different regions showing the major components, their retention times and % TIC

Compound	**RT**	**Tala-Mokolo 2**	**Boyo 3**	**Boyo 4**	**Djerem 5**	**Mbere 6**	**Mbere 7**	**Mbouda 8**
Alkanes		**0.2**	**tr**	**tr**	**4.7**	**7.6**	**2.8**	**1.2**
Pentacosane	37.68	tr	-	-	0.8	0.3	0.3	0.2
Heptacosane	40.70	tr	tr	tr	1.6	1.7	0.9	0.5
Nonacosane	43.49	0.1	-	tr	0.9	1.2	0.6	0.2
Hentriacontane	46.12	0.1	-	-	1.4	4.4	1.0	0.3
Aromatic acids		**tr**	**8.8**	**tr**	**-**	**tr**	**tr**	**0.2**
Hydroxybenzoic acid	21.15	tr	0.1	-	-	tr	tr	tr
Gallic acid	25.33	tr	0.4	tr	-	tr	tr	tr
Caffeic acid	31.51	-	0.2	-	-	-	-	0.2
Chlorogenic acid	46.21	-	7.3	-	-	-	-	-
Chlorogenic acid isomere	49.32	-	0.8	-	-	-	-	-
Fatty acids		**0.5**	**0.7**	**0.5**	**4.0**	**0.8**	**2.0**	**2.6**
Myrisric acid	29.9	0.4	0.4	0.4	1.1	1.4	1.0	1.5
Hexadecenoic acid	32.95	0.1	0.3	0.1	0.7	0.9	0.4	0.4
Lacceroic acid (C32)	42.61	tr	tr	-	2.2	0.5	0.6	0.7
Triterpene ketones		**32.0**	**-**	**44.6**	**8.5**	**29.5**	**32.6**	**25.5**
Bauerenone	48.66	2.0	-	0.6	0.8	4.0	2.1	1.3
α-amyrenone	48.96	11.2	-	11.5	3.0	9.4	10.5	8.3
Lupenone	49.60	18.8	-	32.5	4.7	16.1	20.0	15.9
Triterpene alcohols		**21.5**	**1.3**	**20.2**	**13.4**	**26.7**	**26.4**	**20.3**
α-amyrine	49.23	4.9	1.3	4.9	2.2	6.8	5.2	1.8
Lupeol	49.78	12.7	-	12.9	9.3	17.4	18.6	16.2

Table 38: continuation

24-methylene cycloartenol	50.43	2.6	-	1.4	1.9	1.6	2.6	2.3
Epi-lupeol	50.80	1.3	-	1.0	tr	0.9	1.4	0.6
Triterpene O-acetates		**6.4**	**18.7**	**10.6**	**43.4**	**5.8**	**8.4**	**6.0**
β-amyrine acetate	50.34	2.1	6.7	2.5	9.3	1.7	2.1	1.8
α-amyrine acetate	50.90	4.3	12.0	8.1	33.3	4.1	5.6	4.2
Lupeol acetate	51.96	tr	-	tr	0.8	tr	0.7	tr
Triterpene diols		**27.1**	**-**	**10.9**	**5.7**	**6.8**	**5.5**	**5.5**
Triterpene diol	48.89	6.4	-	4.0	0.2	0.5	2.0	0.6
Triterpene diol	49.52	16.2	-	2.6	2.4	3.8	2.1	3.1
Triterpene diol	50.20	1.5	-	2.6	3.1	2.5	-	-
Olean-12-ene-3,11-diol	51.79	3.0	-	1.7	-	tr	1.4	1.8
Others		**0.1**	**12.5**	**tr**	**0.3**	**0.8**	**0.3**	**2.4**
Glycerol	12.28	tr	10.9	tr	tr	tr	tr	1.8
Quinic acid	26.06	-	1.6	-	-	-	-	-
Eicosanyl glycerol	43.03	0.1	tr	tr	0.3	0.8	0.3	0.4
Trihydroxymonomethoxy flavone	44.80	-	-	-	-	-	-	0.2

tr= compound present in traces. - = compound not present. Values are percentages of Total Ion Current (TIC) and are semi-quantitative.

Due to the uncharacteristic nature of the chemical profiles of the propolis samples reported in the table above, other propolis samples from different localities were again sampled and subjected to GC-MS profiling. This phase involved the propolis samples from Nkambe (North-West Region) and Foumban (West Region) areas.

The GC-MS analysis of the silylated samples of the three extracts of propolis (PHEN, PAEN and PMEN) led to the identification of over 40 compounds belonging to various classes of natural products such as triterpenoids, alkenyl phenols and alkenyl resorcinols, fatty acids, sugars and anarcadic acids. The most abundant compounds in the acetone and hexane extracts are triterpenes and triterpene derivatives. α-amyrenone, α-amyrine, 24-methylenecycloartenol, cycloartenol, α-amyrine acetate and lupenone were the most abundant in the PAEN while lupenone, α-amyrenone,

α-amyrine acetate and lupeol were the most abundant in the PHEN, based on the %TIC values. The hexane and acetone extracts (PHEN and PAEN), are similar in that they all contain α-amyrenone, β-amyrine, lupenone, lupeol, α-amyrine, α-amyrine acetate and β-amyrine acetate, but the PHEN contains germanicone and β-amyrine exclusively. Propolis samples from tropical and subtropical regions such as Cameroon have been proven to be rich in triterpenes and almost deprived of or contain only traces of other constituents (**Savka *et al.*, 2015**). Many studies with African propolis from different regions, like Kenya, Cameroon, Congo and Ethiopia, showed that triterpenoids are major chemical components (**Rushdi *et al.*, 2014; Papachroni *et al.*, 2015; Ricardo *et al.*, 2015**) and phytochemical studies of Cameroonian and some African propolis samples led to the isolation of or identification of a significant number of triterpenes (**Talla *et al.*, 2013; Sakava *et al.*, 2014; Kardar *et al.*, 2014; Almutairi *et al.*, 2014; Zhang *et al.*, 2014; Papachroni *et al.*, 2015**). Triterpenoids including β-amyrin, β-amyrone, lupeol, and lupenone, and polyprenyl benzophenones such as 7- epi-nemorosone, 7-epi-clusianone, xanthochymol and gambogenone have been detected in propolis samples from the Brazilian Amazon (**de Castro Ishida *et al.*, 2011**) and triterpenoids with major diterpenoids together with caffeate esters were reported in the propolis samples from Egypt but no aromatic acids and flavonoids (**El-Hady and Hegazi, 2002**). The major constituents of the PHEN and PAEN are triterpenoids, and triterpenes were found to be predominant in the hexanic and EtOAc phases of some Cameroonian propolis samples (**Kardar *et al.*, 2014**). Therefore, the major source of triterpenoids is terrestrial vegetation (**Hernández-Vázquez *et al.*, 2010**). Generally, the main constituents of propolis are resins and volatiles, which are substances obtained from a variety of botanical processes in different parts of plants found in the site of collection of the propolis samples and beeswax secreted by the bees. Besides triterpenes, a number of fatty acids, Hexadecanoic acid, Octadecenoic acid, Octadecanoic acid, Eicosanoic acid and Tetracosanoic acid were also identified in the PAEN. A good number of fatty acids and organic acids have been identified in Turkish propolis (**Kartal *et al.*, 2002; Baykara *et al.*, 2015**) and also fatty acids have been revealed in Omani propolis (**Popova *et al.*, 2013**). To the best of our knowledge, this is the first time that fatty acids are reported in important amounts in Cameroonian propolis and tropical propolis although methyl esters of these acids have been reported as major constituents of Ethiopian propolis (**Rushdi *et al.*, 2014**). An alkenyl phenol, Pentadecylphenol together with alkenyl resorcinols, Pentadecenyl resorcinol, Heptadecyl resorcinol, Heptadecadienyl resorcinol, Heptadecatrienyl resorcinol, Heptadecenyl resorcinol and

Nonadecenyl resorcinol were also identified in the PAEN. These compounds were reported previously in Cameroonian propolis (**Kardar *et al.*, 2014**) with the exception of Heptadecadienyl resorcinol identified in Brazilian geopropolis (**Araújo *et al.*, 2015**) and Heptadecatrienyl resorcinol. An inseparable mixture of four alk(en)yl resorcinols, (5-pentadecyl resorcinol, 5-(8'Z,11'Z-heptadecadienyl)-resorcinol, 5-(11'Z-heptadecenyl)-resorcinol, and 5-heptadecyl resorcinol), was isolated and characterized from Indonesian propolis together with three cycloartane type triterpenes, mangiferolic acid, isomangiferolic acid and 27-hydroxyisomangiferolic acid (**Trusheva *et al.*, 2011**). Kardar and co-workers attributed some characterized triterpenes with cycloartenol inclusive, mangiferonic acid, mangiferolic acid and isomangiferolic acid inclusive and some alk(en)ylresorcinols, 5-Pentadecylresorcinol, 5-Heptadecylresorcinol, 5-(11'Z-Heptadecenyl)-resorcinol and 5-(12'Z-Heptadecenyl)-resorcinol in Cameroonian propolis as known constituents of mango (*Mangifera indica,* Anacardiaceae) a resin-producing plant widely used in honey production in Cameroon and throughout tropical Africa (**Nguemo Dongock *et al.*, 2004; Focho *et al.*, 2009**). Therefore, mango could be a possible plant source of resin used by bees for the manufacture of propolis from the site of collection in Njap-Nkambe, a hypothesis that might require further verification. This fact is supported by the presence of anacardic acids; Anacardic acid (C15:1), Anacardic acid (C17:2), Anacardic acid (C17:1) and Anacardic acid (C19:1) in the PAEN. Popova and co-workers identified alkylphenol (Cardanol), alk(en)yl resorcinols (cardols) and anacardic acids in Omani propolis (**Popova *et al.*, 2013**) and documented that these three related compound types which have been found in propolis samples from Brazil, and cardols which have been detected in propolis from Thailand and Indonesia (**Silva *et al.*, 2008; Trusheva *et al.*, 2011; Teerasripreecha *et al.*, 2012**), most probably originate from *Mangifera indica* fruit bark and are known antifungal substances (**Trusheva *et al.*, 2011; Popova *et al.*, 2013**). Glycerol was identified in both PAEN and PMEN. Glycerol has been detected in a good number of propolis samples from different regions around the world for example in Turkish propolis, Canadian propolis and Brazilian geopropolis (**Kartal *et al.*, 2002; Araújo *et al.*, 2015**). The PMEN is exclusively rich in sugars with Glucose %TIC=10.2, Fructose %TIC=8.2, Pinitol %TIC=7.0 and Quinic acid %TIC=6.3 as predominant constituents. Monosaccharides such as glucose, fructose, ribose, rhamnose, talose, gulose, and saccharose are commonly present in propolis (**Kurek-Gorecka *et al.*, 2014**). Sugars were also found in Turkish propolis and Omani propolis (**Kartal *et al.*, 2002; Popova *et al.*, 2013**) and geopropolis from Northeast Brazil (**Araújo**

***et al.*, 2015**). Some of the polyols alcohols identified in the PMEN such as Pinitol, Glucitol, Inositol and Quinic acid, are known to possess good biological activities.

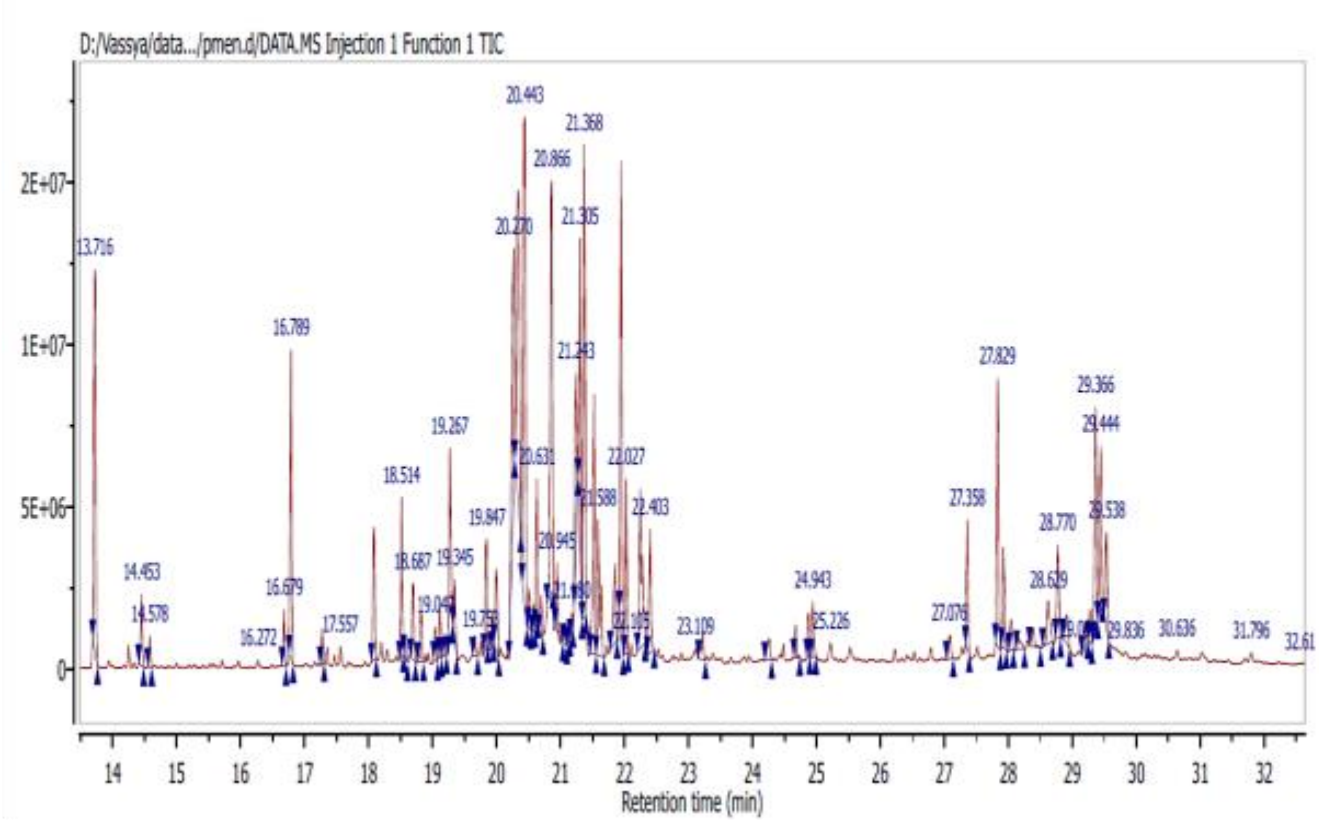


Figure 107: TIC of methanol extract (PMEN) of Nkambe propolis

Table 39: GC-MS profile of methanol extract (PMEN) Nkambe sample (silylated sample)

RT	Compound	% of TIC
13.73	Glycerol	4.0
20.44	Pinitol	7.0
20.86	Quinic acid	6.3
21.52	Glucitol	2.3
21.85	Inositol	1.2
	Monosaccharides	31.5
27.35	Pallatinose	1.2
28.06	sucrose	2.8

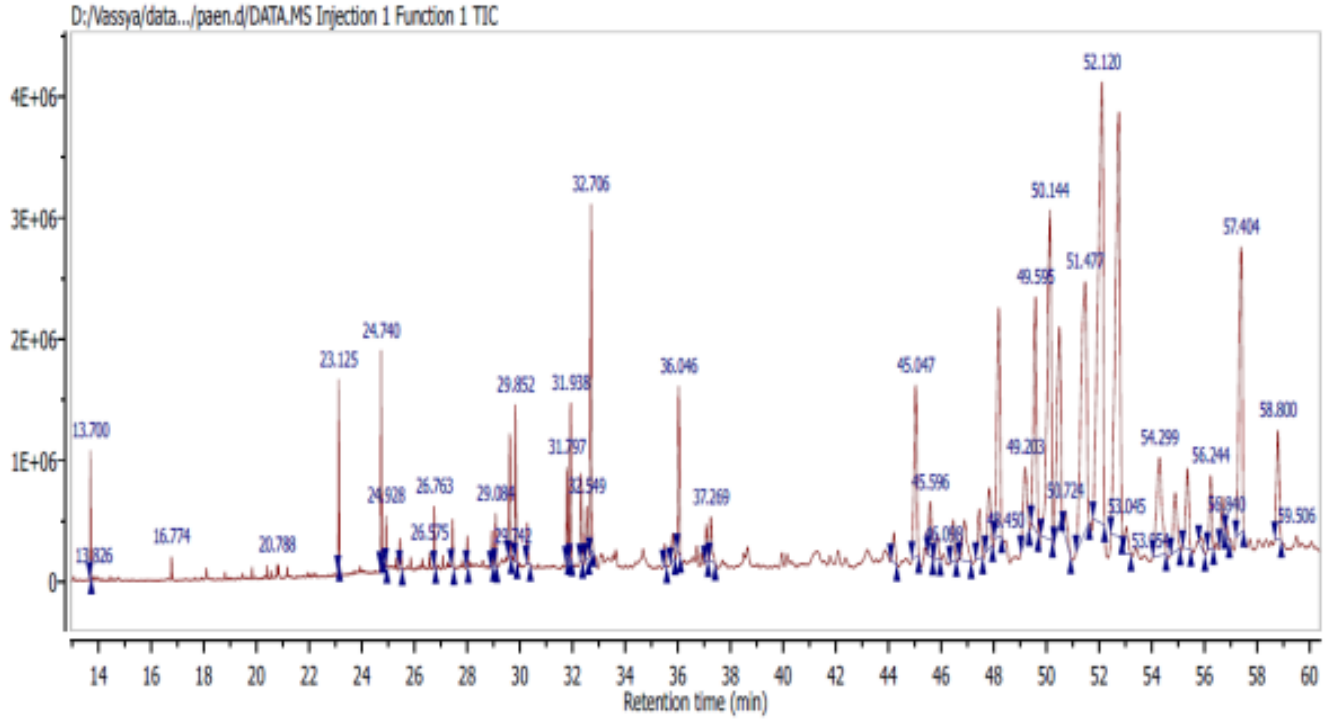


Figure 108: TIC of acetone extract (PAEN) of Nkambe propolis

Table 40: GC-MS profile of Acetone extract (PAEN) Nkambe sample (silylated sample)

RT	Compound	% of TIC
13.70	Glycerol	0.4
23.14	Hexadecanoic acid	0.6
24.74	Octadecenoic acid	0.8
24.93	Octadecanoic acid	0.2
27.45	Pentadecylphenol	0.2
28.94	Eicosanoic acid	0.2
29.63	Pentadecenylresorcinol	0.9
29.85	Heptadecylphenol	0.8
31.81	Tetracosanoic acid	0.6
31.94	Anacardic acid ($C_{15:1}$)	1.0
32.34	Heptadecadienylresorcinol	0.6
32.55	Heptadecatrienylresorcinol	0.6
32.72	Heptadecylresorcinol	1.4
32.87	Heptadecylresorcinol (isomere)	0.1
35.53	Anacardic acid ($C_{17:2}$)	0.2
36.04	Anacardic acid ($C_{17:1}$)	1.5
37.09	Nonadecenylresorcinol	0.4
42.10	Anacardic acid ($C_{19:1}$)	0.3
50.14	Alpha-amyrenone	7.1
51.48	Cycloartenol	8.2
52.12	Alpha-amyrine	12.3
52.76	Lupenone	10.4
54.30	24-methylene cycloartenol	2.7
55.37	Alpha-amyrine acetate	1.6
56.26	Lupeol	1.2
57.40	Beta-amyrine acetate	5.6
58.80	3-hydroxydammarene	1.9

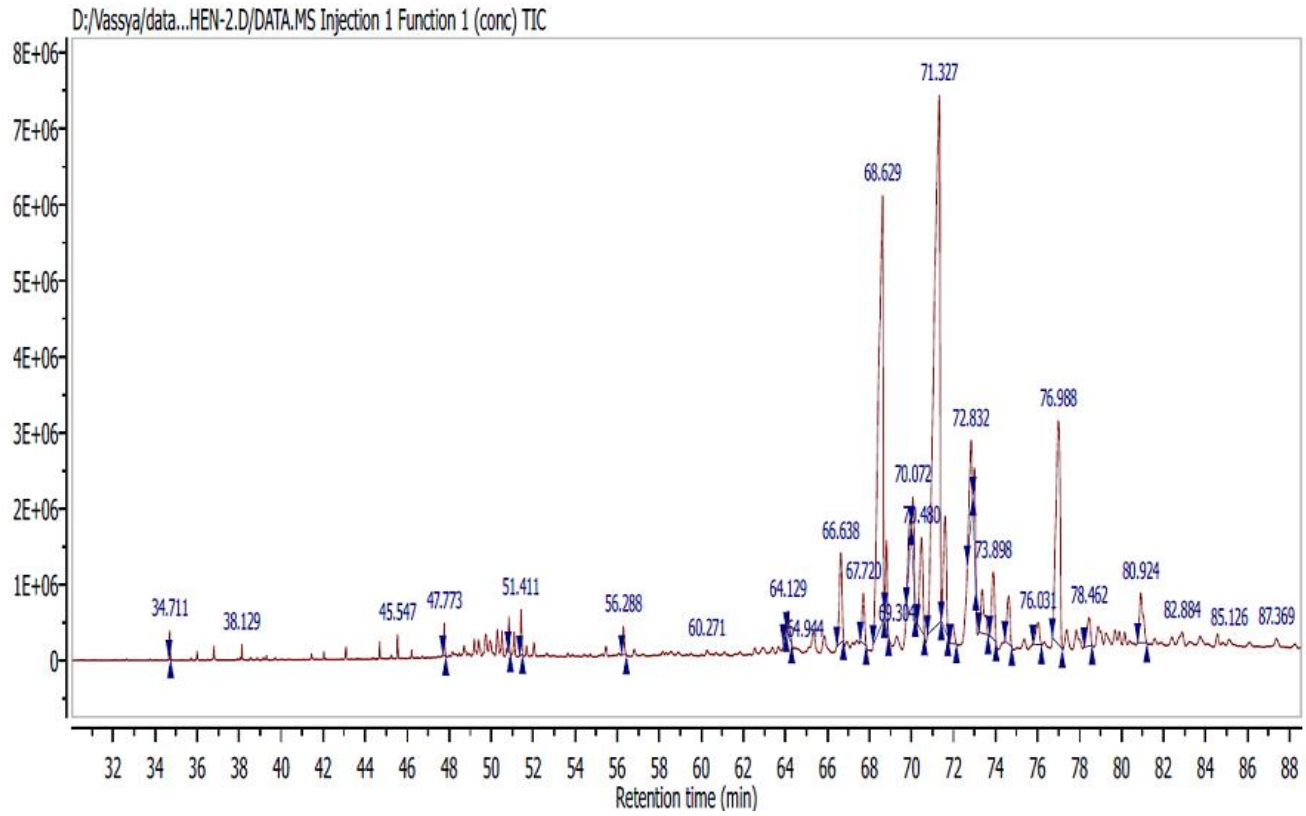


Figure 109: TIC of Hexane extract (PHEN) of Nkambe propolis

Table 41: GC-MS profile of Hexane extract (PHEN) Nkambe sample (silylated sample)

RT	**Compound**	**% of TIC**
68.61	α-amyrenone	16.0
68.82	germanicone	2.0
70.07	β-amyrine	3.4
71.31	lupenone	24.7
72.84	lupeol	7.1
72.98	α-amyrine	3.2
73.89	β-amyrine acetate	1.9
77.00	α-amyrine acetate	7.3

The GC-MS investigation of different extracts of propolis of Foumban revealed the presence of over 30 compounds belonging to a variety of classes of natural products. The ethyl acetate extract

of Foumban propolis contained mainly cycloartenol %TIC=12 and amyrenone %TIC=13.6 together with a number of alkenyl phenols and resorcinols. The methanol extract of propolis of Foumban was a sugar rich extract with glycerol %TIC=13.3 and monosaccharides %TIC=21.3 alongside with some pentacyclic triterpenes. The hexane extract of propolis of Foumban on its part contained triterpenes that is, lanostane type ketones %TIC=31.2, β-amyrenone %TIC=17.3, α-amyrenone %TIC=9.1 and amyrine %TIC=10.

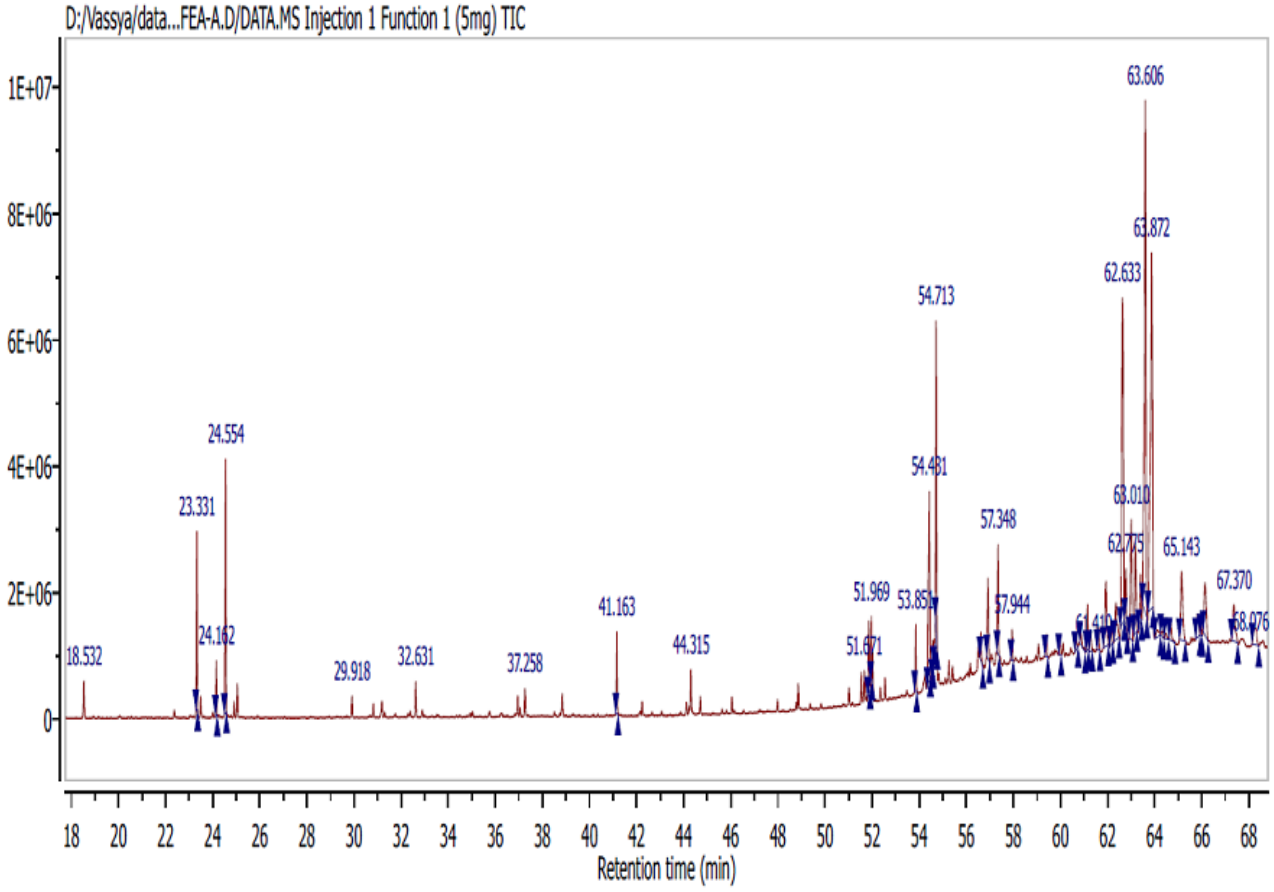


Figure 110: TIC of ethyl acetate extract (PEFEA) of Foumban propolis

Table 42: GC-MS profile of Ethyl Acetate extract of Foumban propolis (silylated sample)

RT	Compound	% of TIC
23.33	Glycerol	1.9
35.73	Vanillinic acid	0.1
32.63	Hydroxybenzoic acid	0.4
36.96	Dihydroxybenzoic acid	0.3
41.16	Hexadecanoid acid	0.9
44.31	Oleic acid	0.6
44.72	Octadecanoic acid	0.2
48.88	Pentadecyl phenol	0.3
51.03	Docosanoic acid	0.2
51.54	Heptadecenyl phenol	0.4
51.86	Heptadecyl phenol	1.3
51.96	Pentadecenyl resorcinol	1.0
52.03	Pentadecyl resorcinol	0.6
53.85	Fatty acid 440	0.8
54.43	Heptadecadienyl resorcinol	3.5
54.57	Heptadecatrienyl resorconol	0.7
54.73	Heptadecyl resorcinol	5.7
56.63	Anacardic acid (C17:2)	0.7
56.92	Anacardic acid(C17:1)	1.6
57.34	Nonadecyl resorcinol	1.8
61.92	Triterpenic ketone	1.8
62.35	lanosterol	1.8
62.65	Amyrenone	8.9
63.01	β-amyrin	3.0
63.18	Triterpenic ketone	2.6
63.60	Amyrenone + Triterpene [496 (100%), 481 (21), 253 (18), 223 (48), 170 (70)]	13.6
63.87	Cycloartenol	12.0
66.14	Amyrin acetate	2.3
	Monosaccharides (sum)	0.6

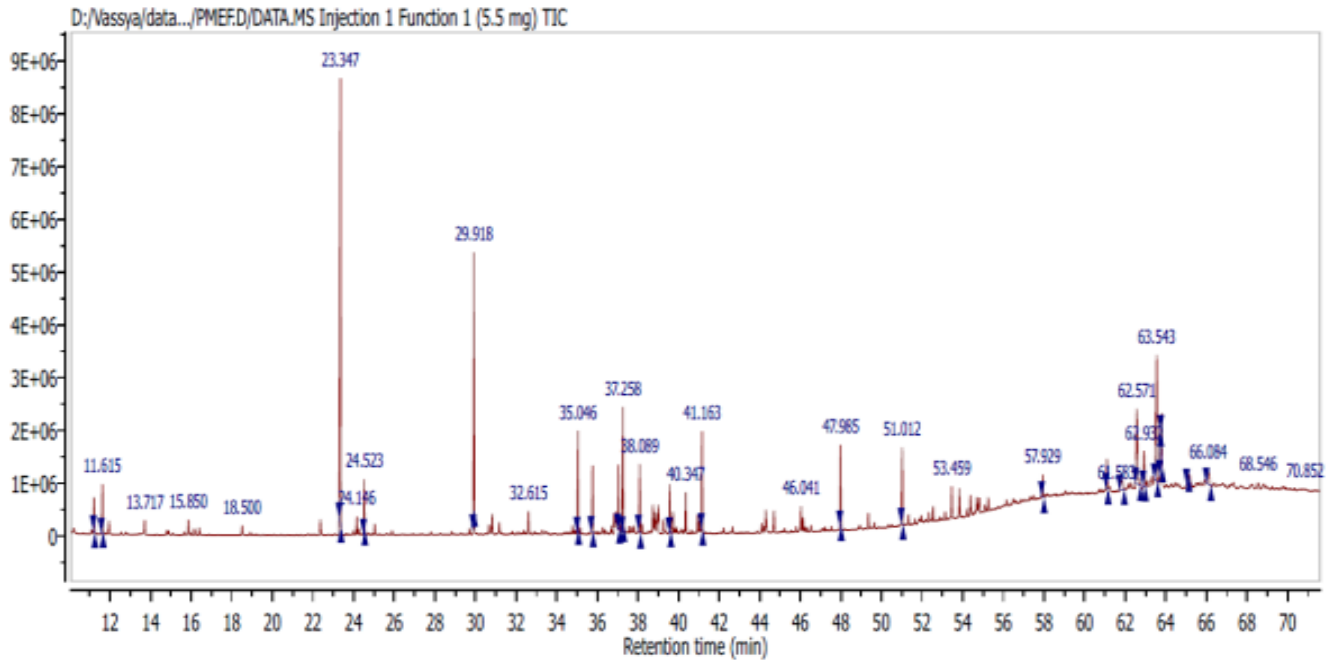


Figure 111: TIC of methanol extract (PMEF) of Foumban propolis

Table 43: GC-MS profile of Methanol extract of Foumban propolis (silylated sample)

RT	Compound	% of TIC
23.34	Glycerol	13.3
30.67	Cinnamic acid	0.4
32.61	Hydroxybenzoic acid	0.6
37.27	Pinitol	3.7
41.16	Hexadecanoic acid (palmitic)	2.6
44.30	Octadecenoic acid	0.7
44.71	Octadecanoic acid (stearic)	0.6
47.98	Eicosanoic acid (arahidic)	2.3
51.03	Docosanoid acid (behenic)	2.2
54.38	Hepdadecanienyl resorcinol	0.3
54.69	Heptadecenyl resorcinol	0.7
62.58	Triterpene [496 (100%), 481 (21), 253 (18), 223 (48), 170 (70)]	3.8
62.93	β-amyrine	1.8
63.73	α-amyrine	2.6
63.79	cycloartenol	3.7
	Monosaccharides	21.3
	Disaccharides	1.4

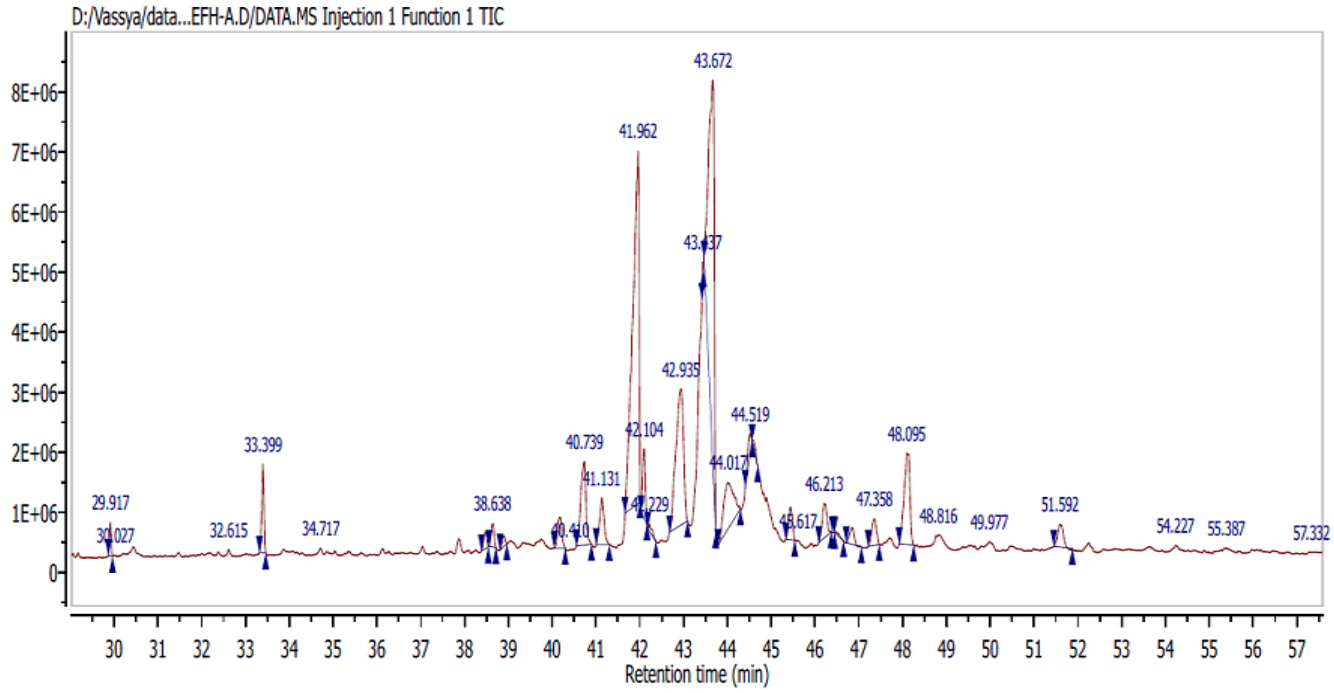


Figure 112: TIC of Hexane extract (PEFH) of Foumban propolis

Table 44: GC-MS profile of Hexane extract of Foumban propolis (silylated sample)

RT	Compound	% of TIC
38.65	Triterpenic ketone	0.7
40.19	Triterpenic ketone	1.1
40.73	Triterpenic ketone	3.3
41.14	Triterpenic ketone	1.9
41.96	β-Amyrenone	17.3
42.95	α-Amyrenone	9.1
43.67	Triterpenic ketone (lanostane type)	31.2
44.01	Cycloartenol	5.4
44.52	amyrine	10.0
46.21	β-Amyrine acetate	1.5
48.09	α-Amyrine acetate	3.5
51.60	Triterpenic aldehyde	1.1

Summarily, the extracts of Foumban propolis are rich principally in triterpenes, fatty acids, alkenyl phenols and resorcinols showed resemblance with propolis from Nkambe, although the constituents common to both samples are present in different amounts in the different samples. The acetone extract of Nkambe propolis resembled the ethyl acetate extract of Foumban propolis but for the fact that ethyl acetate extract of Foumban propolis contained vanillic acid, hydroxybenzoic acid, dihydroxybenzoic acid, hexadecanoic acid, oleic acid, docosanoic acid, heptadecenyl phenol, pentadecyl resorcinol, fatty acid 440, lanosterol, and monosaccharides exclusively while acetone extract of Nkambe propolis contains octadecenoic acid, eicosanoic acid, tetracosanoic acid, anarcadic acid (C15:1), 24-methylene cycloartenol, lupeol and 3-hydroxydammarane exclusively. Similarly, the methanol extracts of both Nkambe and Foumban propolis are rich in sugars but methanol extract of Foumban propolis contained hydroxybenzoic acid, cinnamic acid, with fatty acids and triterpenes exclusively. The hexane extracts of both Nkambe and Foumban propolis contained triterpenic ketones alongside amyrine acetates and triterpenes alcohols. Cameroonian propolis just like propolis from tropical areas, have shown rich triterpene profile. In addition to the triterpenes, Cameroonian propolis is equally rich in and fatty acids, alkenyl phenols and alkenyl resorcinols (**Kardar *et al.*, 2014; Zhang *et al.*, 2014**) which can this far be considered as characteristic in the GC-MS chemical profile of Cameroonian propolis.

The rich chemical profiles of the propolis samples are an indication of the possible biological properties that these propolis samples can possess.

II-6: RESULTS OF TESTED BIOLOGICAL ACTIVITIES

The biological activities of some propolis samples and compounds were tested against gastric ulcers in rat models and the possible antioxidant effect involved in ulceration investigated. Also, DPPH antiradical activity, antimicrobial and antiinflamatory potentials were tested and the results from these findings are discussed below.

II-6-1: Anti-ulcer and in vivo antioxidant activity of extracts of propolis from the Nkambe

Peptic ulcers are a deep gastrointestinal erosion disorder that involves the entire mucosal thickness, penetrating the muscular mucosa. An estimated 15.000 deaths occur each year as a consequence of peptic ulcer diseases (**Valle *et al.*, 2005**) and as the prevalence of this disease increases over time, one would expect peptic ulcers to continue to have a significant global impact in the basic health and economic systems and in patients' life quality (**Yuan *et al.*, 2006**). For decades it was believed that gastrointestinal ulcerations were caused by the excessive secretion of gastric acid, but many patients presenting such ulcerations had normal acid secretion rates (**Wallace and Granger, 1996**). Then some researchers reported that peptic ulcers are caused when the balance between aggressive factors (such as acid and pepsin) and defense mechanisms (such as mucus, bicarbonate, blood flow and mucosal turnover) are shifted in favour of the former (**Lima *et al.*, 2006**). Exogenous aggressive factors such as cigarette smoke, non-steroidal anti-inflammatory drugs (NSAIDs), alcohol, stress, fatty foods and *Helicobacter pylori* infections trigger tissue necrosis through mucosal ischemia, free radical generation and cessation of nutrient delivery. Hydrochloric acid together with pepsin, pancreatic enzymes and bile decrease the defense mechanisms of gastrointestinal mucosa such as the intercellular junctions, local blood flow, mucus/bicarbonate secretion and cellular growth (**Bandyopadhyay *et al.*, 2001**). Although histamine H_2-receptor blockers (for example, ranitidine and famotidine), proton-pump inhibitors (for example omeprazole and lansoprazole), antibiotics (for example metronidazole, amoxicillin, clarithromycin, and tetracycline) and other drugs are extensively used in the management of peptic ulcers, there are reports of adverse effects and relapse within one year (**Wolfe and Sachs, 2000**) and also a number of side effects. For example, proton pump inhibitors (omeprazole, lansoprazole) may cause nausea, abdominal pain, constipation, diarrhea, and H_2-receptor antagonists (cimetidine) may cause gynaecomastia, loss of libido. Due to the occurrence of many side effects triggered by use of synthetic drugs for many diseases, medicinal plants are considered as the main source of new drugs as they are believed to have less or no side effects. Herbal medicines are considered as safe for the treatment of ulcers, with less adverse effects. Drugs for the treatment of gastric ulcers might be very expensive and unaffordable by many. Also in poor countries, not everyone can have access to conventional and modern drugs and so they tend to recourse to medicinal plants and other natural products for treatment of various ailments. In addition to being economical, plant sources are effective and relatively less toxic and extensive research is currently

being carried out in the search for potent antiulcer agents of plant origin (**Vinay *et al.*, 2005; Srivastava *et al.*, 2011; Lakshmi *et al.*, 2013**).

The possible therapeutic usefulness of the rich chemical profiles of the three extracts of Nkambe propolis were tested using well known experimental methods of gastric ulcer, namely, absolute ethanol-, HCl/ethanol-, HCl/ethanol pretreated with indomethacin- and pylorus ligation-induced gastric ulcer. When the extracts were screened for cytoprotective activity against the highly corrosive absolute ethanol solution, control rats developed hemorrhagic lesions in the glandular portions of their stomachs 1 hour after induction of the lesions. The methanol, acetone and hexane extracts (200-600 mg/kg) dose-dependently prevented the formation of gastric lesions, % inhibition attaining 61, 54 and 55%, respectively, at the dose of 600 mg/kg. Sucralfate (100 mg/kg) prevented lesion formation by 30.5%. Mucus production increased from 74.6 mg in the controls to 288.8, 375.8 and 375.2 mg, respectively, for the methanol, acetone and hexane extracts compared with 77.4 for sucralfate (Table 45). The highly corrosive nature of absolute ethanol to the gastric mucosa is well known. Absolute ethanol causes gastric mucosal lesions through the release of tissue-derived mediators such as histamine and leukotriene C_4 as well as by superficial aggressive cellular necrosis. The action of these mediators on gastric microvasculature results in both mucosal and sub mucosal gastric tissue destruction (**Oates and Hakkinen, 1985**). The significant cytoprotection offered by the propolis extracts against absolute ethanol (54 – 61% inhibition) was accompanied by highly significant increases in mucus production, suggesting important inhibitory effects by extracts on the generation of the destructive tissue-derived mediators, or inhibition of their action on the gastric micro-vasculature (**Tulassay and Herszenyi, 2010**).

Table 45: Effects of propolis from Nkambe on gastric ulcers induced by absolute alcohol in rats

Treatment	Dose (mg/kg)	N	% ulcerated surface	Ulcer index	Mucus production (mg)	% Inhibition
Control		5	14.4	5.22 ± 0.40	74.63 ± 6.31	-
Methanol extract	200	5	11.1	3.37 ± 1.40	138.46 ± 19.65*	35.4
	400	5	1 .9	2.32 ± 0.60*	228.77 ± 11.01***	54.9
	600	5	0.4	2.00 ± 0.55*	230.34 ± 17.11***	61.7
Acetone extract	200	5	11.3	4.16 ± 1.20	128.13 ± 19.53*	20.1
	400	5	3.9	3.02 ± 0.18	371.81 ± 52.13***	40.6
	600	5	0.7	2.36 ± 0.64*	375.84 ± 17.56***	54.8
Hexane extract	200	5	15.9	4.76 ± 0.70*	215.60 ± 32.64***	32.6
	400	5	4.9	2.66 ± 0.44*	271.49 ± 50.86***	47.8
	600	5	2.7	2.34 ± 0.62*	275.16 ± 8.95***	55.2
Sucralfate	100	5	1.4	2.04 ± 0.47*	77.44 ± 10.32	60.9

*Statistically different relative to control; **p<0.05; ***p<0.001; N, number of rats. The values are expressed as mean± SEM.*

Table 46: Effects of propolis extracts on gastric ulcers induced by pylorus ligation in rats

Treatment	Dose (mg/kg)	N	% ulcerated surface	Ulcer index	Mucus production (mg)	% Inhibition
Control	-	5	7.48	3.46 ± 0.34	38.00 ± 5.19	-
Methanol extract	400	5	3.44	2.33± 0.66	63.00 ± 5.20*	32.66
	600	5	2.86	1.23 ± 0.53*	63.20 ± 4.39*	64.45
Acetone extract	400	5	1.73	1.50 ± 0.38*	63.80 ± 4.20*	56.65
	600	5	0.57	0.93 ±0.41**	71.00 ± 8.73**	73.12
Hexane extract	400	5	5.26	3.20 ± 0.33	15.60 ± 1.40**	7.51
	600	5	5.60	2.90 ± 0.60	81.00 ± 6.81***	16.18
Cimetidine	50	5	0.31	1.32 ±0.61*	88.81 ± 0.13***	61.8

*Statistically different relative to control; *p<0.05; **p<0.01; ***p<0.001; N, number of rats. The values are expressed as mean± SEM.*

Table 47: Effect of propolis extracts on gastric secretion in pylorus-ligated rats

Treatment	Dose (mg/kg)	N	Gastric contents (ml)	Gastric pH	Gastric acidity (mEq/L	% reduction of gastric acidity
Control		5	6.40 ± 0.70	1.93 ± 0.09	84.20 ± 7.07	-
Methanol extract	400	5	4.28 ± 1.32	2.11 ± 0.05	55.80 ± 12.09*	45.6
	600	5	5.54 ± 0.73	2.23 ± 0.07	78.00 ± 4.05	7.4
Acetone extract	400	5	4.28 ± 0.92	2.07 ± 0.10	65.60 ± 12.16*	34.0
	600	5	2.88 ± 0.83**	3.14 ± 0.44**	75.80 ± 8.90	10.0
Hexane extract	400	5	5.96 ± 0.68	2.31 ± 0.15	91.00 ± 4.49	-8.1
	600	5	4.80 ± 0.38	2.24 ± 0.11	97.80 ± 2.15	-16.2
Cimetidine	50	5		4.30 ± 0.34	35.75 ± 0.58**	57.5

*Statistically different relative to control; **p<0.05; **p<0.01; N, number of rats. The values are expressed as mean± SEM.*

Table 48: Effects of propolis extracts on gastric ulcers induced by HCl/ethanol solution in rats

Treatment	Dose (mg /kg)	N	% ulcerated surface	Ulcer Index	Mucus Production (mg)	% Inhibition
Control	-	5	5.61	4.33 ± 0.32	104.00 ± 8.38	-
Acetone extract	400	5	0.49	1.25 ± 0.53***	109.20 ± 3.01	71.1
Acetone extract	600	5	0.07	0.60 ± 0.40***	152.00 ± 11.85*	86.1
Sucralfate	100	5	1.13	2.80 ± 0.97*	105.9 ± 12.17*	35.3

*Statistically different relative to control; **p<0.05; ***p<0.001; N, number of rats. The values are expressed as mean± SEM.*

Table 49: Effects of propolis extracts on HCl/ethanol-induced gastric lesions in rats pre-treated with indomethacin

Treatment	Dose (mg /kg)	N	% ulcerated surface	Ulcer Index	Mucus Production (mg)	% Inhibition
Control	-	5	25.5	5.55 ± 0.73	50.60 ± 4.63	-
Acetone extract	400	5	3.2	2.15 ± 0.20***	120.80 ± 9.46**	62.3
Acetone extract	600	5	2.2	1.89 ± 0.15***	153.60 ± 17.93***	65.9
Sucralfate	100	5	3.1	2.80 ± 0.97*	59.40 ± 6.81	49.5

*Statistically different relative to control; **p<0.05; ***p<0.001; N, number of rats. The values are expressed as mean± SEM.*

Table 50: Effect of propolis extract on oxidative stress parameters in stomach tissues of rats subjected to HCL/Ethanol/Indomethacin-induced gastric lesions

Treatment	Dose (mg/kg)	*N*	GSH (µmol/g of tissue)	MDA (µmol/g of tissue)
Normal rats	-	5	6.99 ±0.12	2.26 ± 0.19
Control	-	5	4.09 ± 0,45	4.70 ± 0,49
Acetone extract	400	5	3.70 ± 0,06	6.11 ± 0,95
Acetone extract	600	5	3.65 ± 0,05	6.35 ± 1,07

Since gastric acid and pepsin secretion are very important precursors for the creation of ulcers, the extracts were further screened for their ability to prevent gastric acid secretion using the pylorus ligation technique. In the control rats subjected to pyloric ligature alone, gastric lesion indices were 3.46 ± 0.34. Increasing doses of the methanol, acetone and hexane propolis extracts inhibited lesion formation by 64.5, 73.1 and 16.2 per cent, respectively, for the highest dose of extracts compared with 61.8 % for cimetidine. Cytoprotection was highest for the acetone extract and lowest for the

hexane extract. Although the cytoprotection was accompanied by significant increases in mucus production (Table 46), none of the extracts showed antisecretory activity compared with the controls (Table 47). Even though gastric acidity for the methanol and acetone extracts (55.8 and 65.6 mEq/L respectively) were statistically low compared with the controls, previous studies show that gastric acid levels of this magnitude are known to be highly ulcerogenic (**Tan *et al.*, 1996; Amang *et al.*, 2014**). Unlike the methanol and acetone extracts which had slight tendencies to reduce gastric acidity at 400 mg/kg (45.6 and 34.0% reduction), the hexane extract (400-600 mg/kg), increased gastric acid levels by 8.1 and 16% compared with the controls (Table 47). Acid substances like hydrochloric acid, acetyl salicylic acid (aspirin) and glacial acetic acid are well known for their ulcerogenic effects on the gastric mucosa. The chemical profiles of the propolis extracts revealed the presence of 11 sugars and 1 acid in the methanol extract, 10 acids, 6 alkenyl resorcinols and phenols, 9 triterpenes and 1 sugar in the acetone extract, and 8 triterpenes in the hexane extract. The presence of organic acids in the extracts (especially the methanol and acetone extracts) would have been expected to provide an additive ulcerogenic effect to the pylorus ligation-induced hyperacidity. This was apparently not the case since the methanol and acetone extracts significantly ($P< 0.01$) prevented the formation of gastric lesions (64.5 and 73.1% inhibition, respectively. see table 47) in spite of the highly acidic gastric environments (78.0 and 75.8 mE/L, respectively). The quantification of individual organic constituents of the extracts was done based on internal normalization, and gave percentage values of Total Ion Current (% TIC) for each compound. Although % TIC values are semi-quantitative, they may be useful in explaining the cytoprotective actions observed. The cocktail of 11 sugar molecules in the methanol extract represent 43.2 % TIC compared with 6.3 % TIC for quinic acid. Intragastric administration of a mannitol, glucose-fructose-sucrose-maltose mixture to pylorus ligated rats prevented the formation of mucosal lesions in an osmolality-dependent manner. The effect occurs by luminal dilution of the necrotising agent and acid without affecting acid content (**Gharzouli *et al.*, 2001**). When **Gharzouli *et al.*, 2002** obtained significant cytoprotection (87 -100%) by a glucose-fructose-sucrose-maltose mixture against ethanol-, indomethacin-, and acidified aspirin-induced lesions in the rat, they concluded that the sugar-rich solutions may prevent gastric damage by a mechanism involving the release of some protective agents. Carbohydrates at high concentrations behave as mild irritants that can induce adaptive cytoprotection (**Gharzouli *et al.*, 1999**). Hexoses, which are present in the methanol extract are major structural components of mucins which in turn

are the major components of the protective gastric mucus. The 6 alkenyl resorcinols and alkenyl phenol compounds and 9 triterpenes in the acetone extract together account for 56.9 % TIC compared with 5.6% TIC for the 10 acids. The possible ulcerogenic actions of the acid compounds may therefore be masked by the quantitative superiority of the triterpenes, sugars and phenolic compounds. Moreover, the quinic acid present in the methanol extract may well have cytoprotective effects since caffeoylquinic acids from *Ligularia* species possess peroynitrite-scavenging activity and showed antiulcer activity against HCl/ethanol- and indomethacin/bethanechol, and reduced the volume of gastric juice (**Lee *et al.*, 2010**). In addition, the cytoprotective actions of phenolic compounds and triterpenes are well known. A review of antiulcer drugs of plant origin shows that triterpenes because of their ability to strengthen defencive factors such as stimulation of mucous synthesis or maintenance of the prostaglandins content of gastric mucosa at high levels, are potentially the compounds with antiulcer activity (**Lewis and Hanson, 1991**).These compounds exert cytoprotective actions through increased mucosal blood flow; increased mucus, bicarbonate and prostaglandin secretion, and enhancement of the *in vivo* antioxidant status (**Morikawa *et al.*, 2006; Sun *et al.*, 2006**). Polyphenolic compounds possess antioxidant activity often attributed to their redox properties which enable them to act like reducing agents and metals chelators, and they scavenge free radicals (**Rice-Evans *et al.*, 1996**). Most effective medicinal plants are rich in polyphenols and possess high antioxidant potentials (**Akinmoladun *et al.*, 2007**). Although all the three propolis extracts had no antisecretory activity, the acetone extract had the most significant cytoprotection (56.6-73.1 % inhibition under highly acidic gastric environments). In addition, 5 out of the 8 triterpenes (α-amyrenone, α-amyrine, lupenone, lupeol and α-amyrineacetate) were present in both the hexane and acetone extracts. The acetone extract was therefore judged to be the most active and further tests were carried out to elucidate its possible mode of action. Table 48 shows the antiulcer actions of the acetone extract against HCl/ethanol solution. The extract significantly ($P<0.01$) reduced ulcer index scores from 4.33 ± 0.32 in the controls to 1.25 ± 0.53 and 0.6 ± 0.04 at the dose of 400 and 600 mg/kg, respectively (% inhibition: 71-86%). Furthermore, pretreatment of the rats with indomethacin prior to HCl/ethanol raised ulcer index scores to 5.55 ± 0.73 compared with 4.33 ± 0.32 for the HCl/ethanol controls. In response, the acetone extract significantly ($P<0.001$) decreased ulcer index from 5.55 ± 0.73 to 1.89 ± 0.15 at the dose of 600 mg/k (Table 49). Inhibition of ulcer formation was accompanied by highly significant ($P<0.001$) increase in mucus production.

Although pretreatment with indomethacin reduced protective effect of the acetone extract by 23 to 27%, cytoprotection remained high (62-66% inhibition) (Table 49). Indomethacin is a prostaglandin inhibitor which suppresses gastro-duodenal bicarbonate secretion, disrupts the mucosal barrier and reduces endogenous prostaglandin synthesis as well as gastric mucosa blood flow in animals (**Flemstrom *et al.*, 1982**). On the other hand, prostaglandins synthesized in large quantities by the gastrointestinal mucosa are known to prevent experimentally-induced ulcers caused by various ulcerogens. The role of Prostaglandins in cytoprotection has been well discussed (**Robert *et al.*, 1983**). When the cytoprotective action of an antiulcer agent is significantly decreased by pre-treatment with indomethacin, it can be interpreted that the cytoprotection is occurring through the mediation of endogenous prostaglandins (**Yamamoto *et al.*, 1992**). This may well be the case for the acetone extract of propolis used in our experiment. Table 50 shows that subjection of the rats to the HCl/ethanol/indomethacin treatment significantly decreased antioxidant enzyme concentration (GSH) and increased the MDA concentration compared with controls. Treatment with acetone extract did not prevent the drop in the concentration of GSH. The high MDA concentrations (4.70 ± 0.49 mmol/g) created by the ulceration procedure were not reversed in the extract-treated groups. These results suggest that antioxidant effects may not be involved in the mode of antiulcer activity of the propolis extract.

II-6-2: Antimicrobial activity of some propolis samples

II-6-2-1: Comparative antimicrobial activity of propolis samples from different regions

All the propolis extracts demonstrated activity against *staphylococcus aureus* except the extract Tala-Mokolo 2 which showed no activity against *staphylococcus aureus*, taking into consideration that natural products which produce minimum inhibitory concentrations (MIC) in the range 100–1000 mg mL^{-1} in in-vitro susceptibility tests can be classified as antimicrobials (**Abreu *et al.*, 2012**). Globally, of the active extracts against *staphylococcus aureus*, Tala-Mokolo 1, Boyo 3 Boyo 4, Mbere 7 and Mbouda 8 have relatively low antimicrobial activity with MIC 175-184 mg/mL than that of the propolis samples from Adamawa Djerem 5 and Mbere 6 having MICs 156 mg/mL and 159 mg/mL respectively. However, some extracts Boyo 4, Djerem 5 and Mbere 6 showed antifungal activities against *Candida albicans* with the propolis extract from North-West Region Boyo 4 showing lowest activity while all the Adamawa extracts are active with Djerem 5 showing higher activity MIC 78 mg/mL than Mbere 6 with MIC 80 mg/mL. Of the extracts

showing both antibacterial activity against *staphylococcus aureus* and antifungal activity against *Candida albicans*, the propolis from North-West Boyo 4 has lowest MIC while the propolis from Adamawa Mbere 6 shows higher MIC than Djerem 5. None of the samples showed antibacterial activity against *Escherichia coli*. Our results agree with those of Cretan propolis and some European propolis samples which are active against *staphylococcus aureus* and *Candida albicans* (**Hegazi *et al*., 2000; Popova *et al.*, 2009**) and Sonoran propolis active on *staphylococcus aureus* (**Velazquez *et al*., 2007**) but contrasts in the fact that Cretan propolis and European propolis are active on *Escherichia coli* while the extracts used in this study are not. A study of the antibacterial activity of certain Cameroonian propolis samples from Adamawa region and West region showed that the extracts were active on *staphylococcus aureus* but not on *Escherichia coli* (**Mbawala *et al*., 2010**). However, propolis from Meiganga Adamawa region of Cameroon has been proven active on *Escherichia coli* (**Sakava *et al*., 2014**). Data from studies concerning antibacterial properties of propolis support the fact that propolis is active mainly against Gram-positive bacteria and shows lower activity against the Gram-negative ones or inactive at all at small quantity. Such results can be seen in the work of Kujumgiev and co-workers who tested propolis samples from different geographic regions (tropical and temperate zones) against *Staphylococcus aureus* and *Escherichia coli*. All the extracts displayed significant antibacterial activity against *S. aureus* but none was active against *E. coli* (**Kujumgiev *et al.*, 1999**). The differences and variations found in the antibacterial activity of the different propolis extracts evaluated could be because of qualitative and quantitative differences in the chemical composition of the propolis samples. Bankova and co-workers reported the antibacterial activity of different fractions of Brazilian propolis towards *S. aureus*, and observed that the antibacterial activity is mainly due to polar phenolic compounds (**Bankova *et al.*, 1995**). The GC-MS profiles of the extracts used in this study show that the extracts are poor in phenolic compounds but show rich triterpene and triterpene derivatives in their chemical profiles. It is possible that some of these triterpenes and their derivatives are accountable for the observation and variation in antimicrobial activity of the tested extracts since antimicrobial activities of triterpenes and propolis have been reported previously (**Sakava *et al*., 2014; Popova *et al.*, 2009**).

Table 51: Antimicrobial activity of various extracts of propolis from different regions

Sample	MIC (mg/mL)		
	***S. aureus* 209**	***E. coli* WF+**	***Candida albicans* 62I**
Tala-Mokolo 1	181	-	**-**
Tala-Mokolo 2	-	-	**-**
Boyo 3	175	-	**-**
Boyo 4	184	-	**92**
Djerem 5	156	-	**78**
Mbere 6	159	-	**80**
Mbere 7	178	-	**-**
Mbouda 8	**175**	**-**	**-**

II-6-2-2: Antibacterial activity of Ndian propolis

The method of micro-dilution made it possible to determine the activity of the extracts of our propolis on the various bacterial stocks. This led to the determination of the MIC and the MBC which varied from one bacterial strain to another and from one extract to another. The table below shows the MIC and MBC of two extracts of propolis and ceftriaxone determined by micro-dilution method on different microbial stocks.

Table 52: MIC and MBC of extracts of Ndian propolis and ceftriaxone expressed in µg /mL

Micro-organism	AcOEt		H. E.		Ceftriaxone	
	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	6000	>12000	>12000	NT	12.5	>100
Klebsiella pneunoniae	6000	>12000	>12000	NT	3.13	>100
Proteus mirabilis	12000	>12000	>12000	NT	6.25	>100
Pseudomonas aeruginosa	>12000	NT	>12000	NT	6.25	>100
Staphylococcus aureus	12000	>12000	>12000	NT	1.56	100
Bacillus cireus	6000	>12000	>12000	NT	50	>100

NT=not tested

These results showed that the H.E does not present any activity on the six bacterial strains tested. On the other hand, the ethyl acetate extract is active on almost all the bacterial strains tested but inhibiting the bacterial strains to different extents. However, this extract did not show any activity

against *Pseudomonas aeruginosa*. Three of the bacterial strains *Escherichia coli, Bacillus cereus and Klebsiella pneumoniae* have the same level of sensitivity with ethyl acetate extract with MIC=6000 µg/mL. Although the ethyl acetate extract shows an inhibiting activity, this activity is quite low compared with that of the reference antibiotic ceftriaxone used in the study. Indeed, the MIC of ceftriaxone which lies between 1.56 and 50.00 µg/mL shows that ceftriaxone more active than ethyl acetate extract. The strain *pseudomonas aeruginisa* did not show any sensitivity with the ethyl acetate extract. At the concentrations tested, the two extracts did not show any bactericidal activity. The resistance of *Pseudomonas aeruginosa* is not surprising, it is in relation to the nature of the external membrane which confers to it resistance to a majority of antimicrobial agents (**Carson *et al.*, 2006**). Segueni and co-workers evaluated the antibacterial activity of ethanol extract of the Algerian propolis on four bacteria *escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa* and their results obtained from this study showed that the ethanol extract has an inhibiting effect on all the bacteria studied with the MIC varying from 1000 to 1200 µg/mL. These results showed that our extracts are approximately six times less active than those collected in Algeria on similar bacterial strains (**Segueni *et al.*, 2014**). Our result relatively corroborates with the works of Papachroni andco-workers who evaluated the antibacterial activity of ethanol extracts of three samples of Cameroonian propolis (Oku, Ngaoundal, Tekel) on four bacterial strains (*Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli*) and obtained MIC varying between 13.80 and 22.44 mg/mL (**Papachroni *et al.*, 2015**). Some Cameroonian propolis has shown inhibiting activity against *Staphylococcusaureus, Escherichia coli* and *Pseudomonas aeruginosa* (**Mbawala *et al.*, 2010; Sakava *et al.*, 2014**). This difference can be explained by the chemical composition of the propolis related to the local flora and the method of extraction used. The antimicrobial activity of the propolis was attributed to the presence of the secondary metabolites such as the phenolic compounds, flavonoids, alkaloids, terpenoids and tannins (**Preeti *et al.*, 2013**) and preliminary screening of our extracts showed the presence of some of these secondary metabolites.

II-6-3: Anti-inflammatory activity of extracts of Ndian propolis

Edema induced by xylene on the ears of rats is useful for tracing and evaluation of anti-inflammatory activity of extracts at the acute phase of inflammation (**Dulcetti *et al.*, 2004**). This inflammatory reaction results to the formation of prostaglandins which causes a vasodilation, reinforces the action of histamine, bradykinine and leukotrienes which are involved in the process

of the inflammation. Moreover, the protein kinase C (PKC) also supports the secretion and the activation of several immunizing mediators like cytokines and chemokines which increase and maintain an inflammatory response. The anti-edema effect of hexane and ethyl acetate extract was investigated using edema of the ear induced by xylene in rat model and the results are given in the table below.

Table 53: The Effect of extracts of Ndian propolis on the edema induced by xylene in the rats

Sample	Doses	AR (g)	AL(g)	AR-AL (g)	%inhibition
Distilled H_2O	10 mL/Kg	0.018±0.001	0.011±0.001	0.007±0.001	-
Dexamethasone	1 mg/Kg	0.013±0.001	0.010±0.001	0.002±0.001***	71.42
Hexane Extract	200 mg/Kg	0.013±0.001	0.010±0.002	0.003±0.001***	57.14
Hexane Extract	400 mg/Kg	0.013±0.001	0.010±0.001	0.002±0.001***	71.42
AcOEt Extract	200 mg/Kg	0.013±0.001	0.010±0.001	0.004±0.001***	42.86
AcOEt Extract	400 mg/Kg	0.014±0.001	0.010±0.001	0.003±0.002***	57.14

*AR=average Area of Right ear, AL=average Area of Left ear, The values are average ± standard deviation, *** P < 0.05 regarded as significant.*

The rats of the negative control group (NC) having received 1 ml of xylene locally administered on the internal and external faces of the right ear developed at the end of 30 minutes an edema characterized by an increase in weight of the ear of 0.007±0.001 g. The animals of the positive control (PC) group received dexamethasone of a dose 1 mg/Kg presented a total inhibition of about 71.42 %. Those of the groups treated with the ethyl acetate extract at the doses of 200 and 400 mg/kg presented an inhibition 42.86% and 57.14% respectively. In addition, those treated with hexane extract showed an inhibition of 57.14% and 71.42% at doses of 200 and 400 mg/Kg respectively. From these results, it is noted that the extracts of propolis present dose-dependent anti-inflammatory effects. The effects of the propolis extracts on the inflammation of the ear of the rats induced by xylene could be explained by the inhibition of the synthesis of pro-inflammatory substances. Moreover, many studies indicate that triterpenes have anti-inflammatory

properties and also intervene in the functioning of the immune system by modulating the adhesion of monocytes during the inflammation. They inhibit the expression of the inflammatory mediators such as prostaglandins, histamine, bradykinine and leukotrienes (**Vivek *et al.*, 2010**).

II-6-4: DPPH radical scavenging activity of some sampled propolis extracts

II-6-4-1: Comparative DPPH radical scavenging activity of propolis from different regions

Of the antioxidant evaluation methods used, the DPPH assay, based on the reaction of the stable free-radical (2,2- diphenyl-1-picrylhydrazyl) with components of the sample, evaluating a change in the color of the solution, is the most frequently used (**Sawaya *et al.*, 2011**). It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability. Hence, DPPH radical was scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H•. The color changed from purple to yellow after reduction and the decrease of absorbance at wavelength 517 nm was quantified as percentage DPPH radical scavenging activity and plotted against concentration of test sample as given in figure 113 below.

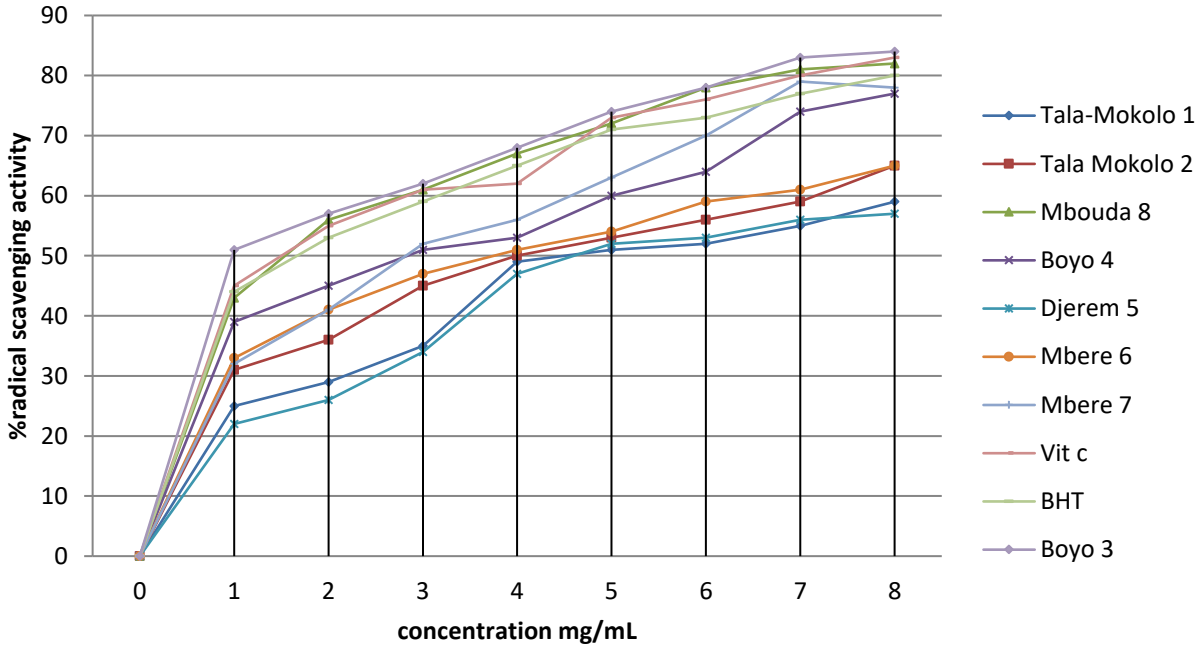


Figure 113: A plot of variation of %radical scavenging activity against concentration of propolis extracts from different regions

From figure 113 above, the percentage radical activity increases with an increase in the concentration of the test sample. However, at the same concentration, the samples showed different

%radical activity indicating that this activity differs from one extract to another. Two standard antioxidants, butylated hydroxytoluene (BHT) and vitamin C (Vit C), known to possess antiradical activity were used as positive control and their activities were compared with those of potentially active extracts. In this test, the highest radical scavenging activities were observed for the two extracts Boyo 3 second by Mbouda 8, both extracts having higher radical scavenging activities than the standard antioxidant, Vit C and BHT. All the propolis extracts showed positive DPPH radical scavenging activity. A comprehensive means of interpreting the activity of each extract was to determine the IC_{50} of each extract which is the concentration at which the extract will have a percentage radical scavenging activity of 50%. The curves of percentage inhibition against concentrations for extracts were drawn individually and the IC_{50} values were determined for each extract. The IC_{50} is inversely proportional to % radical scavenging activity, meaning that the greater the IC_{50} value the lower the antiradical activity and vice versa. The order of ascending antiradical activity of the test samples based on their IC_{50} values is given in figure 114 below.

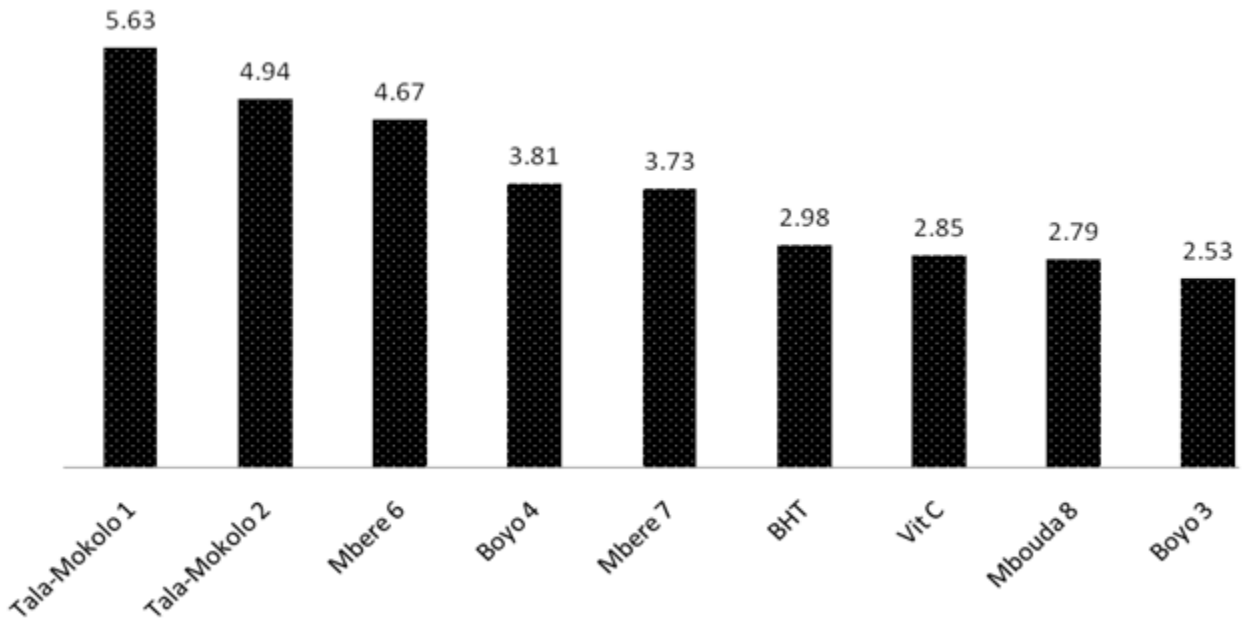


Figure 114: Decreasing order of IC_{50} values in mg/mL or increasing order of radical scavenging activity of tested samples

From figure 114 above, the propolis samples from Nothern regions show lowest radical scavenging activity while those of West Region Mbouda 8 and North-West Region Boyo 3 show highest antiradical activity. Also, this high radical activity of Boyo 3 and Mbouda 8 might also be reflected

by the presence of aromatic acids and other components revealed in their GC-MS profiles. The presence of Caffeic acid, a metabolite of caffeoylquinic acid, in the two extracts Boyo 3 and Mbouda 8 is undoubtedly responsible for its high radical scavenging activity. This caffeic acid has been proven to exhibit powerful DPPH radical scavenging activity and has been found to have IC_{50} value for DPPH radical scavenging activity to be lower than that of ascorbic acid and trolox (**Izuta *et al.*, 2009**). In addition to caffeic acid, there is the presence of hydroxybenzoic, chlorogenic and gallic acids in Mbouda 3 and trihydroxymonomethoxy flavone in Mbouda 8 extracts which can also foster the antiradical activity of the extracts. Similarly, Sonoran propolis showed potent free radical scavenging activity similar to those of the well-known antioxidants BHT and vitamin C and some propolis constituents like rutin and caffeic acid phenyl ester showed higher activity than antioxidant BHT (**Velazquez *et al.*, 2007**). The results of the radical scavenging activity show a regional variation and it has been proven that the antioxidant activity also differs with the region in Brazil (**Kumazawa *et al.*, 2004**). Based on their radical scavenging activities, there is a relative resemblance between the propolis samples from Mbouda in the West region and Boyo in the North-West region (high activity) and between Djerem and Mbere in Adamawa and Tala-Mokolo in the Far-North (low activity).

II-6-4-2: The DPPH• free radical scavenging activities of various extracts of propolis of Tekel-Ngaoundal

The DPPH• free radical scavenging activities of the various extracts of propolis of Ngaoundal at concentrations varying from 1-8 mg/mL were determined with BHT (butylated hydroxytoluene) and Vitamin C as references. The results showing the variation of percentage inhibition as a function of concentration of the extracts and references are shown in figure 115 below.

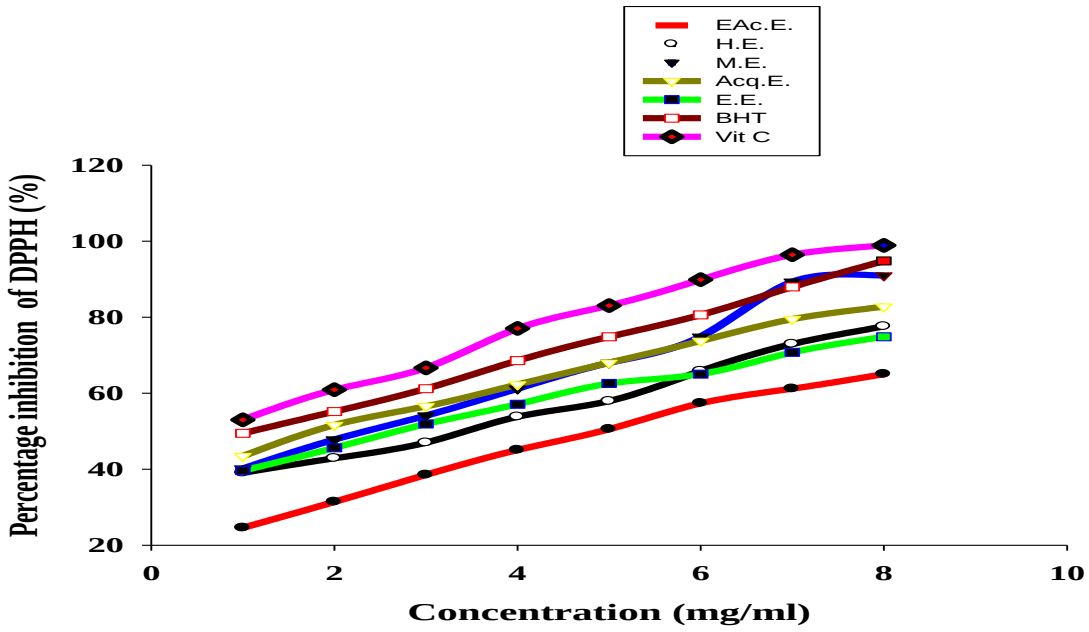


Figure 115: Plot of percentage inhibition against concentration of various extracts of propolis of Tekel-Ngaoundal

Generally, the percentage inhibition and hence the antiradical activity increases with increase in the concentration of the extracts. Vitamin C has the highest antiradical activity while the ethyl acetate extract has the lowest. For the extracts only, methanol extract has the highest antiradical activity closely followed by aqueous extract. It should be noted that the aqueous extract has higher antiradical activity than methanol extract below 5 mg/mL while methanol extract has higher antiradical activity than aqueous extract above 5 mg/mL but both extracts have the show the same free radical scavenging activity at 5 mg/mL. Correlation is also observed between hexane extract and ethanol extract at 6 mg/mL, below which ethanol extract shows greater antiradical activity and above which hexane extract surpasses the ethanol extract in its radical scavenging activity. The methanol extract has the same value of percentage inhibition with BHT at 7 mg/mL. However, the curves of percentage inhibition against concentrations for extracts were drawn individually and IC_{50} determined for each extract.

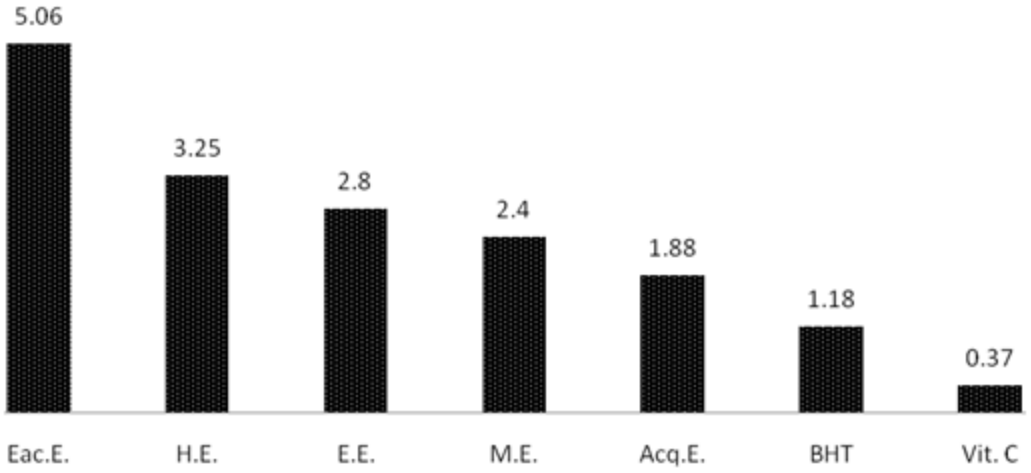


Figure 116: Decreasing order of IC_{50} values in mg/mL of extracts of Tekel-Ngaoundal propolis

This value is termed IC_{50} and is defined as the concentration of extract or compound which inhibits the formation of 50% of $DPPH^{\bullet}$ free radical. All the values of IC_{50} were determined by graphical methods as 5.06mg/mL, 3.25 mg/mL, 2.80 mg/mL, 2.40 mg/mL, 1.88 mg/mL, 1.18 mg/mL and 0.37 mg/mL for ethyl acetate extract, hexane extract, ethanol extract, methanol extract, aqueous extract, BHT and Vitamin C respectively.

II-6-4-3: The DPPH• free radical scavenging activities of some extracts and compounds from propolis of Ndian and Foumban

The ability of some extracts and compounds from propolis of Ndian and Foumban to scavenge DPPH radical were determined and recorded in table 54 below. It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability. Hence, DPPH radical was scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H•. A comprehensive means of interpreting radical scavenging activity of each extract was to determine the IC_{50}. The IC_{50} is inversely proportional to % radical scavenging activity.

Table 54: The IC_{50} mg/mL of some extracts and compounds from propolis of Ndian and Foumban

sample	IC_{50} mg/mL
Hexane extract of Foumban propolis	5.60
Ethyl acetate extract of Foumban propolis	1.40
Methanol extract of Foumban propolis	1.07
Ethyl acetate extract of Ndian propolis	1.65
Hexane extract of Ndian propolis	4.00
1'-O-eicosanyl glycerol	0.93
3β-hydroxylanostan-9,24-dien-21-oic acid	1.22
mangiferonic acid	1.09
methyl-3β,27-dihydroxycycloart-24-en-26-oate	0.98
Vitamin C	0.80
Gallic acid	0.30

Therefore the order of decreasing radical scavenging activity is Hexane extract of Foumban propolis (IC_{50} = 5.6 mg/mL), Hexane extract of Ndian propolis (IC_{50} = 4.00 mg/mL), Ethyl acetate extract of Ndian propolis (IC_{50} = 1.65 mg/mL), Ethyl acetate extract of Foumban propolis (IC_{50} = 1.40 mg/mL), 3β-hydroxylanostan-9,24-dien-21-oic acid (IC_{50} = 1.22 mg/mL) Methanol extract of Foumban propolis (IC_{50} = 1.07 mg/mL), mangiferonic acid (IC_{50} = 1.09 mg/mL) methyl-3β,27-dihydroxycycloart-24-en-26-oate (IC_{50} = 0.98 mg/mL) 1'-O-eicosanyl glycerol (IC_{50} = 0.93 mg/mL), Vitamin C (IC_{50} = 0.80 mg/mL) and Gallic acid (IC_{50} = 0.30 mg/mL). Although none of the samples showed antiradical activity greater than that of the standards, their activities remained nevertheless closer to those of the standard antioxidants Gallic acid and vitamin C. It was observed that the pure compounds showed higher DPPH$^{\bullet}$ radical scavenging activity than the extracts except for methanol extractof Foumban propolis that was more active thanmangiferonic acid and 3β-hydroxylanostan-9,24-dien-21-oic acid. The order of decreasing antiradical activity above implies that a smaller amount of the extract with high radical activity is required to inhibit 50% of DPPH$^{\bullet}$ free radical while a greater amount is required of the lower antiradically active extract to have the same effect. However, it should be noted that all the extracts have free radical scavenging effect on DPPH$^{\bullet}$ and hence antiradical activities. They can neutralize free radicals to give stable compounds.

II-6-5: Ferrous ion chelating capacity of various extracts of propolis of Ngaoundal

The chelating of Fe^{2+} by extracts was estimated and the extent to which an extract can for complexes with the ferrous ion reflects its antioxidant activity. Propolis extracts can quantitatively form complexes with Fe^{2+}. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals (**Aboul-Enein *et al*., 2003**). The main strategy to avoid reactive oxygen species generation that is associated with redox active metal catalysis involves chelating of the metal ions. Ebrahimzadeh and co-workers showed that extracts with highest phenol and flavonoids content had the highest chelating activity of ferrous ion although no total correlation between flavonoids and phenolic content with chelating capacity was found (**Ebrahimzadeh *et al*., 2008**). A plot of percentage ferrous ion chelating capacity against the concentrations of the extracts is shown in figure below.

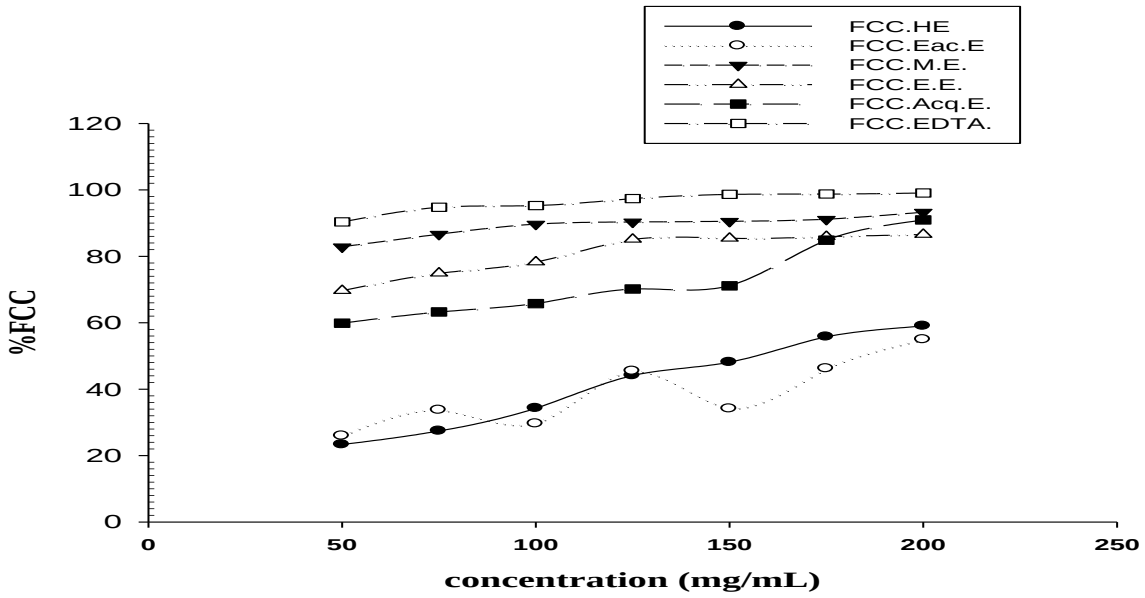


Figure 117: Plot of percentage ferrous iron chelating capacities

From figure 117 above, the positive control test using EDTA has the highest %FCC followed by the methanol extract (M.E) then the ethanol extract (E.E) which is closely followed by the aqueous extract (Acq.E). The hexane extract (H.E) and the ethyl acetate extract (EAc.E) which show the

least %FCC also show interesting correlation between their %FCC at certain concentrations. Generally, the %FCC increases with increase in the concentration of the extracts. This can be explained by the fact that and increase in the concentration of extract is equally an increase in the concentration of the chelating agent leading to the formation of greater amount of the Fe^{2+} complex. Some correlations are observed. At the concentration of 175 mg/mL, the E.E and Acq.E have the %FCC, but before this concentration value E.E shows greater %FCC than Acq.E while Acq.E shows greater % FCC than E.E beyond 175 mg/mL. At 200 mg/mL the Acq.E and the M.E have very close values of %FCC. The highest correlation is observed between H.E and EAc.E at 125 mg/mL and also at concentration mid-way between 75 mg/mL-100 mg/mL. EAc.E shows greater %FCC than H.E at concentrations 50 mg/mL and 75 mg/mL while H.E shows greater %FCC than EAc.E at concentrations 100 mg/mL, 150 mg/mL, 175 mg/mL and 200 mg/mL. Generally, extracts of solvents of low polarity show lower %FCC while those of higher polarity show higher %FCC. Chelating capacity is attributed to flavonoids and phenolic compounds which use their redox properties to chelate transition metals (**Gulcin *et al.*, 2010**). Their oxygen atoms and hydroxyl groups can chelate the Fe^{2+} ion. There is a relatively greater amount of phenolic and flavonoids in polar extract than in less polar or non-polar extracts. The entire extracts show %FCC therefore the tested propolis extracts can be considered as potential iron-chelators.

II-6-6: Some regional variations amongst propolis samples

Extracts of propolis from the Northern region were richer in triterpenes and void of alkenyl phenols and resorcinols. They equally contained insignificant amounts of fatty acids and their esters. These same samples showed generally higher antimicrobial activities. Their rich triterpene and triterpene derivatives account for this antimicrobial activity. Samples of propolis from the Western and North-Western regions were rich in alkenyl phenols and alkenyl resorcinols with little amounts of phenolic acids and significant amounts of fatty acid esters. These samples showed higher antiradical activities which could be attributed to the alkenyl phenols, alkenyl resorcinols and phenolic acids that they contain. The presence of significant fatty acids and esters which are constituents of wax in the propolis from Nkambe, North-West region could be justified by the fact that this area is very cold. The wax helps to insulate the hive against heat loss and its production is a highly energetic metabolic process which generates heat that is necessary to keep the hive warm (**Blanc, 2010; Bogdanov, 2015**). Propolis sample from Ndian, South-West region was found to contain a good number of tetracyclic triterpenoids. This information is based on the GC-

MS profiles which also indicated that mangifera indica is a major plant source of propolis in Cameroon. Some alkenyl phenols (cardanols) which are known components of were also present in samples from Nkambe (North-West) and Foumban (West) regions. Cardanols are found worldwide in tropical plants of the Anacardiaceae family, both in their native and cultivated culture. Economic cultivated plants in this family include cashew nut, mango and ginkgo (**Boonsai *et al.*, 2014**).

GENERAL CONCLUSION AND PERSPECTIVES

The results in this thesis have contributed to the ongoing investigation of bioactive metabolites from natural sources and the valorisation of plants and its derived products in traditional medicine. Propolis is a highly medicinal bee product whose use is still yet to be discovered by many Cameroonians. Chromatographic separation of the acetone extract of Nkambe propolis, ethanol extract of Foumban propolis, hexane extract and ethyl acetate extract of Ndian propolis led to the isolation and elucidation of some compounds distributed as follows:

- 16 triterpenes, two of which are novel derivatives namely: 3β,27-dihydroxycycloart-24-en-26-oic acid methyl ester and 3-oxo-6,22-dihydroxy-4,4,14-trimethylspinast-7,23(24)-dien-29-al. The 11 known triterpenoids are: lupenone, oleanolic acid, betulinic acid, mangiferonic acid, lupeol acetate, 27-hydroxymangiferonic acid, betulin, 3β-hydroxylanostan-9,24-dien-21-oic acid, 3β-hydroxycycloart-12,25(26)-diene, 3β-O-acetylbetulin, lup-12,20(29)-dien-3-ol, and a mixture of lupeol and stigmasterol, 24-methylanecycloartenol and stigmasterol, lupeol and β–amyrine.
- 1 new mono-ether of glycerol: 1'-O-eicosanyl glycerol
- 2 fatty acid esters: heptadecyl butanoate and eicosanyl butanoate
- 1 fatty alcohol: n-heptatriacontanol
- Six esters were synthesized, three of lupeol and three of β-amyrine each with 3,4,5-trimethoxybenzoyl chloride, 4-methoxybenzoyl chloride and benzoyl chloride. These reactions showed that the yield is directly proportional to the inductive effect of the methoxyl substituents of the ring.

A triterpene fraction analysed by GC-MS as a mixture of lanosterol, α-amyrine, 28-norolean-12-en-3-ol, Cycloartenol, 3-epi-α-amyrine, lupeol and 24-methylenecycloartenol.

GC-MS profiles of different samples of propolis were established and revealed the presence of over 50 compounds and characterised by alkenyl phenols and resorcinols, fatty acids, triterpenes (as would be expected of tropical and subtropical propolis) and sugars and *mangifera indica* was found to be the major plant source. Samples from Northern part of Cameroon were richer in triterpenes, void of alkenyl phenols and resorcinols and had averagely higher antimicrobial activity while alkenyl phenols and resorcinols and some phenolic compounds were found in samples of North-West and Western regions which showed averagely higher antiradical activity on DPPH. This difference is explained by the variation in vegetation and plants foraged by bees.

The methanol, acetone and hexane extracts (200-600 mg/kg) dose-dependently prevented the formation of ethanol-induced gastric lesions (% inhibition, 61, 54 and 55 %, respectively, at the dose of 600 mg/kg). Increasing doses of the extracts inhibited pylorus ligation–induced lesions by 64.5, 73.1 and 16.2 %, respectively, for the highest dose but none of them showed antisecretory activity compared with controls. The most active further significantly ($P<0.01$) reduced HCl/ethanol-induced ulcer indices from 4.33 ± 0.32 in cytoprotective (acetone) extract (56.6-73.1 % inhibition under highly acidic gastric environments), the controls to 1.25 ± 0.53 and 0.6 ± 0.04 at the dose of 400 and 600 mg/kg, respectively (% inhibition: 71-86%). Furthermore, upon pretreatment of the rats with indomethacin prior to HCl/ethanol, the acetone extract significantly ($P<0.001$) decreased ulcer index from 5.55 ± 0.73 in the controls to 1.89 ± 0.15 at the dose of 600 mg/kg. Although pretreatment with indomethacin reduced the protective effect of the acetone extract by 23 to 27%, cytoprotection remained high (62-66% inhibition). The cytoprotective action of the most active (acetone) extract may involve the mediation of endogenous prostaglandins.

The radical scavenging activity varied from Hexane extract of Foumban propolis (IC_{50} = 5.6 mg/mL) to Methanol extract of Foumban propolis (IC_{50} = 1.07 mg/mL) for the extracts and from 3β-hydroxylanostan-9,24-dien-21-oic acid (IC_{50} = 1.22 mg/mL) to 1'-O-eicosanyl glycerol (IC_{50} = 0.93 mg/mL) for the compounds. Activities of samples were moderate as they remained closer to those of the standard antioxidants Gallic acid (IC_{50} = 0.30 mg/mL) and vitamin C (IC_{50} = 0.80 mg/mL) especially 1'-O-eicosanyl glycerol, the most active compound. The propolis sample from Adamawa Region Djerem 5 shows lowest radical scavenging activity while those of West Region Mbouda 8 and North-West Region Boyo 3 show highest antiradical activity. Also, this high radical activity of Boyo 3 and Mbouda 8 might also be reflected by the presence of aromatic acids and other components revealed in their GC-MS profiles. The presence of Caffeic acid, a metabolite of caffeoylquinic acid, in the two extracts Boyo 3 and Mbouda 8 is undoubtedly responsible for its high radical scavenging activity. In addition to caffeic acid, there is the presence of hydroxybenzoic, chlorogenic and gallic acids in Mbouda 3 and trihydroxymonomethoxy flavone in Mbouda 8 extracts which can also foster the antiradical activity of the extracts.

The antibacterial activity of the ethyl acetate and hexane extracts of Ndian propolis showed that the extracts have a low inhibiting activity on all the tested strains, presenting MICs varying between 6000-12000 µg/mL and consequently did not have any bactericidal effect. The *pseudomonas aeruginisa* proved to be resistant to the ethyl acetate extract. The anti-inflammatory

effect of the hexane and ethyl acetate extracts of Ndian propolis showed that the hexane and ethyl acetate extracts of our propolis reduced meaningfully ($P<0,05$) the edema in relation to the negative control group with percentage of inhibition of 57.14% and 42.86% at a dose of 200 mg/Kg and 71.42% and 57.14% at the dose of 400 mg/Kg of body weight respectively. Globally, of the active extracts against *staphylococcus aureus*, Tala-Mokolo 1, Boyo 3 Boyo 4, Mbere 7 and Mbouda 8 have relatively low antimicrobial activity with MIC 175-184 mg/mL than that of the propolis samples from Adamawa Djerem 5 and Mbere 6 having MICs 156 mg/mL and 159 mg/mL respectively. However, some extracts Boyo 4, Djerem 5 and Mbere 6 showed antifungal activities against *Candida albicans* with the propolis extract from North-West Region Boyo 4 showing lowest activity while all the Adamawa extracts are active with Djerem 5 showing higher activity MIC 78 mg/mL than Mbere 6 with MIC 80 mg/mL. Of the extracts showing both antibacterial activity against *staphylococcus aureus* and antifungal activity against *Candida albicans*, the propolis from North-West Boyo 4 has lowest MIC while the propolis from Adamawa Mbere 6 shows higher MIC than Djerem 5. None of the samples showed antibacterial activity against *Escherichia coli*.

The results of the radical scavenging and antimicrobial activities show a regional variation and the biological activities reported justify the use of propolis in traditional medicine. The GC-MS profiles give an insight of the relationship between biological activity and chemical composition, relationship between propolis and its plant origin as well as regional variation in composition. As future perspectives, we intend to:

- Do spectroscopic analyses of the remaining compounds
- Purify all the extracts and fractions and characterize the compounds obtained
- Carryout toxicity studies on the extracts and compounds
- Perform hemi-synthesis of our compounds to improving their biological activities
- Extract essential oils from propolis and study their compositions as well as their biological activities
- Further studies on the cytoprotectiveactivity of the triterpene fraction (ANT46)
- Develop a traditional cure for gastric ulcers from propolis
- Establish a list of plant sources of propolis in Cameroon.

CHAPTER III: MATERIALS AND METHODS

III-1: MAJOR EQUIPMENT AND MATERIALS USED IN PHYTOCHEMICAL STUDY

Column chromatographies (CC) were performed over silica gel 0.063-0.2 mm Merck, particle size between 0.043 and 0.063 mm in diameter and porosity 230-400 mesh ASTM. Evaporation was done on a BUCHI type rotary evaporator "ROTA VAPOR" under pressure. The masses of extracts and fractions were measured on a Krups type 875 type electronic mass balance while those of compounds and fractions were weighed on a G&G Gebrauchsanleitung K2-20 electronic balance. Filtration was performed on the Whatman type and Macherey-Nagel, Germany type filter papers of diameter 70, 90, 150 and 300 mm. Thin layer chromatography (TLC), were carried out on prefabricated aluminium sheet (thickness 0.2 mm) plates coated with a layer of silica gel 60Å and $60F_{254}$, Merck 20 x 20 cm. These plates were revealed to the UV lamp spectroline model CM-10 at 254 nm and 365 nm wavelengths and by spraying with 10 % dilute sulphuric acid solution followed by heating on a hot plate or by placing in a chamber containing subliming solid iodine.
The GC–MS analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 μm film thickness HP5-MS capillary column.
The ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC and HMBC were recorded on Bruker AV400, Bruker AV500 and Bruker AV600 spectrometer (400, 500 or 600 MHz for ^{1}H and 100, 125 or 150 MHz for ^{13}C) with TMS as internal standard and chemical shifts expressed in parts per million. ESI-MS spectra (ionization voltage 3.8kV) were measured on a LTQ-FT Thermo Scientific spectrometer and Q-TOF Ultima spectrometer. EI-MS spectra were recorded on an MS-JEOL 600 MSroute spectrometer. The structures of the compounds were established based on their 1D and 2D NMR spectra that is ^{1}H, ^{13}C, ^{1}H-^{1}H COSYqf45, HSQC and HMBC and ESI-MS spectra and by comparison with published data. Melting points were recorded on an Electrothermal 9100 device and are uncorrected.

III-2: EXPERIMENTAL PROCEDURES

The studied propolis samples were mostly harvested by scrapping from the walls of the beehives usually after honey collection. Some voucher specimens of the studied propolis samples were deposited in the laboratory of natural products number III, department of organic chemistry,

University of Yaoundé 1. Propolis is called 'Nlaa-nfuu' or 'Mbihdong' in Limbum language, Nkambe and 'Dhatche-Nyaki' by the 'Bororo' inhabitants of this locality. Other popular local names include 'Kilei' in Oku, Bui division of the North-West region and 'Ndaki-goro' by the 'Gbayas' of the Adamawa region amongst others where the use of propolis is becoming very popular. In Nkambe as well as many other localities in Cameroon, propolis is used by local sculptors for ornamental works and mending of calabashes. It is also exploited for its medicinal uses to treat tooth ache, stomach disorders, gastritis and sore throat by chewing directly. Its aqueous extract is used in treating wounds, skin rashes, boils and burns.

III-2-1: Extraction

1 kg of raw propolis sample was harvested from bee hives of an apiary located within the same area in Njap village, Nkambe town, North-West region of Cameroon during the months of February to March 2013, dried and cooled (20^{o}C) and ground in a mortar using a pestle to obtain a powder. The propolis powder was extracted successively by maceration with 10 volumes fold of hexane, acetone and methanol in a tightly closed glass jar kept in a dark cupboard at ambient temperature for 48 hrs with intermittent stirring. The supernatant was carefully decanted and filtered through a Whatmann No. 1 filter paper. The final filtrates were evaporated to near dryness on a rotary evaporator under reduced pressure to remove the solvent and the extract was collected in a clean vial. The maceration, filtration and evaporation processes were repeated three times for each solvent after which the residual powder was dried before introduction of a new solvent. This yielded the hexane extract (PHEN), the acetone extract (PAEN) and the methanol extract of propolis (PMEN). All the extracts were well conserved for GC-MS analysis and antiulcer tests.

120.0 g of propolis collected from Tala-Mokolo (Far-North Region) was powdered and was extracted using maceration method under mechanical agitation. This yielded a dry hexane extract of propolis from Tala-Mokolo, Tala-Mokolo 1. The residue was dried and extracted in the same way with ethyl acetate to obtain the ethyl acetate extract of propolis from Tala-Mokolo, Tala-Mokolo 2. 1.3 kg of propolis from Boyo north-West region was extracted in the same way as the propolis from Tala-Mokolo using hexane and ethyl acetate as described above to give the hexane extract and ethyl acetate extract of Propolis from Boyo, Boyo 3 and Boyo 4 respectively.2 kg of propolis sample collected from Djerem Ngaoundal (Adamawa Region) was powdered and extracted sequentially using hexane and ethyl acetate by percolation method at room temperature to give a brown viscous hexane extract and a dark viscous ethyl acetate extract Djerem 5. Propolis

collected from Mbere Meiganga was powdered (800 g) and was extracted using maceration method under mechanical agitation with ethyl acetate and subjected to two further solvent-solvent extractions with hexane and methanol/water (80/20, v/v) to obtain the extracts Mbere 6 and Mbere 7 respectively. 523 g Propolis collected from Mbouda (West region) was powdered and extracted with 5 L of hexane by maceration at room temperature and a greenish-yellow crude hexane extract Mbouda 8 was obtained. 1 kg of raw propolis sample from Nkambe were dried and cooled (20^{o}C), grounded in a mortar using a pestle to obtain a powder. The propolis powder was extracted successively by maceration with a 10-volume fold of hexane, acetone and methanol. This yielded 175 g of hexane extract PHEN, 388 g of acetone extract PAEN and 182 g of methanol extract of propolis PMEN.

1 kg of propolis sample was collected from the peripheries of Foumban town, Noun division from west region of during the months of October and November 2013. This sample was dried in a shed after which it was powdered using a mortar and pestle. The powder was extracted successively with hexane, ethyl acetate and 95% ethanol solution. Each extraction was done trice and the solvent was removed using a Rotavapor before introduction of a new solvent. This gave the hexane extract (362 g), ethyl acetate extract (150 g) and the ethanol extract (130 g).

550 g of propolis from Ndian, south-west region which was collected in February 2015 was chilled and ground. The powder was then extracted with a 10 folds volume of 70% ethanol at room temperature for 48 hours. This process was repeated three times to yield a hydro-alcoholic solution of the propolis which was concentrated to near dryness to give the ethanol extract. The ethanol extract was extracted successively by liquid-liquid extraction with hexane (3 times) and ethyl acetate (3 times) to obtain a hexane extract (70.1 g) and ethyl acetate extract (65 g).

III-2-2: The GC–MS analysis

The temperature was programmed from 60 to 300°C at a rate of 5°C/min, and a 10 min hold at 300°C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. The split ratio was 1:10, the injector temperature 280°C, the interface temperature 300°C, and the ionization voltage 70 eV. Every extract was analyzed in duplicate. The identification of individual compounds was performed using computer searches on commercial libraries, comparison with spectra of authentic samples and literature data. If no reference spectra were available, identification was performed based on the mass-spectral fragmentation, in such cases for some compounds only tentative structures were proposed. Some constituents remained unidentified because of the lack of relevant

references and information (none of them major constituent). The quantification of individual constituents is based on internal normalization. The percentage figures in the tables refer to percent of the Total Ion Current, TIC, and are semi-quantitative.

III-2-3: Column chromatographic separation of some extracts

III-2-3-1: Column chromatographic separation of Ndian propolis

This procedure involved a coarse separation of the molecules according to their polarity. 25 g of hexane extract were absorbed on 50 g of silica gel. After having dried it, the extract of propolis fixed on silica gel was introduced into the column. The column was filled with 300 g of silica gel prior to the introduction of the absorbed extract. Elution was carried out on hexane/ ethyl acetate and ethyl acetate /methanol gradient system of increasing polarity of. Fractions of 50 ml were collected and concentrated on the rotary evaporator and later analyzed by TLC. The TLC plates were revealed using UV light (254-265nm) and 50% sulphuric acid.

Table 55: Chromatogram of fractioning of hexane extract of propolis from Ndian

Eluent system	Fraction	TLC	Series	Observation
Hex (100)	1-4	Hex/AcOEt (95/5)	A	strains
	5-17	Hex/AcOEt (95/5)	B	strains
Hex/AcOEt (97.5/2.5)	18-21	Hex/AcOEt (90/10)	C	strains
Hex/AcOEt (97.5/2.5)	22-30	Hex/AcOEt (90/10)	D	02 spots, further seperation. ND22’
	31-42	Hex/AcOEt (90/10)	E	Traces
Hex/AcOEt (95/5)	43-58	Hex/AcOEt (90/10)	F	strains
Hex/AcOEt (90 /10)	59-60	Hex/AcOEt (90/10)	G	01 spot, white crystals ND56
Hex/AcOEt (80/20)	61-65	Hex/AcOEt (85/15)	H	strains
	66-68	Hex/AcOEt (50/50)	I	01 spot, light yellow oil ND68
Hex/AcOEt (75/25)	69-79	Hex/AcOEt (75/25)	J	strains
	80-84	Hex/AcOEt (75/25)	K	01 spot, white crystals ND84
	85-90	Hex/AcOEt (75/25)	L	strains
	91-104	Hex/AcOEt (60/40)	M	strains

Table 55: continuation

Hex/AcOEt (70/30)	105-108	Hex/AcOEt (60/40)	N	01 spot, white powder ND106
	109-119	Hex/AcOEt (60/40)	O	strains
Hex/AcOEt (60/40)	120-121	Hex/AcOEt (50/50)	P	strains
	122-126	Hex/AcOEt (50/50)	Q	01 spot, whitish powder ND122
	127-141	Hex/AcOEt(50/30)	R	longstrains
Hex/AcOEt (50 /50)	142-166	Hex/AcOEt (40/60)	S	02 spots +strains
Hex/AcOEt (40/60)	167-184	Hex/AcOEt(30/70)	T	Longue trainées
Hex/AcOEt (30/70)	185-196	Hex/AcOEt (25/75)	U	strains
Hex/AcOEt (20/80)	197-202	Hex/AcOEt (10/90)	V	strains
Hex/AcOEt (20/80)	203-211	Hex/AcOEt(10/90)	W	01 spot, white crystals ND206
	212-216	Hex/AcOEt(10/90)	X	strains
Hex/AcOEt (10/90)	217-225	AcOEt/MeOH (5/95)	Y	02 spots +strains
AcOEt(100)	226-234	AcOEt (100)	Z	03 spots +strains
AcOEt/MeOH (90/10)	335-262	AcOEt/MeOH (85/15)	A1	strains
AcOEt/MeOH (80/20)	363-266	AcOEt/MeOH (75/25)	A2	strains
AcOEt/MeOH (60/40)	267-268	AcOEt/MeOH (55/45)	A3	Long strains
AcOEt/MeOH (40/60)	269-385	AcOEt/MeOH (35/65)	A4	Long strains
AcOEt/MeOH (20/80)	286-296	AcOEt/MeOH (15/85)	A5	strains
MeOH (100)	297-302	MeOH (100)	A6	strains

35 g of ethyl acetate extract of propolis from Ndian were absorbed on 55 g of silica gel and then subjected to column chromatographic separation with silica gel as absorbent using hexane-ethyl acetate (0-100%) and ethyl acetate-MeOH (0-100%) using a column which was filled with 500 g of silica gel prior to the introduction of the absorbed extract. Fractions of 50 mL were collected and concentrated on the rotary evaporator and later analyzed by TLC. The TLC plates were revealed using UV light (254-265 nm) and 50% sulphuric acid.

Table 56: Chromatogram of fractioning of ethyl acetate extract of propolis from Ndian

Eluent system	Fraction	TLC	Series	Observation
Hex (100)	1-14	Hex/AcOEt (90/10)	A’	strain
Hex /AcOEt (97.2/2.5)	15-52	Hex/AcOEt (80/20)	B’	02 spots +strain
	53-57	Hex/AcOEt (80/20)	C’	01 spot, white crystals. PN53
	58-79	Hex/AcOEt (90/10)	D’	strain
Hex/AcOEt (95/5)	80-92	Hex/AcOEt (80/20)	E’	01 spot, white crystals, PN85
	93-143	Hex/AcOEt (80/20)	F’’	strain
Hex/AcOEt (92.5/7.5)	144-176	Hex/AcOEt (80/20)	G’	Traces
Hex/AcOEt (90/10)	177-190	Hex/AcOEt (80/20)	H’	01 spot, white crystals. PN190
	191-207	Hex/AcOEt (80/20)	I’’	Long strain
Hex/AcOEt (87.5/12.5)	208-216	Hex/AcOEt (80/20)	J’	04 spots+ strain
Hex/AcOEt (85/15)	217-227	Hex/AcOEt (80/20)	K’	03 spots+ strain
Hex/AcOEt (82.5/17.5)	228-248	Hex/AcOEt (25/75)	L’	Long strain
Hex/AcOEt (80/20)	249-270	Hex/AcOEt (20/80)	M’	strain
Hex/AcOEt (75/25)	271-276	Hex/AcOEt (70/30)	N’	02 spots
	177-283	Hex/AcOEt (70/30)	O’	01 spot, white crystals. PN277
	284-297	Hex/AcOEt (70/30)	P’	Longue trainées
Hex/AcOEt (70/30)	198-304	Hex/AcOEt (50/50)	Q’	strain
	305-318	Hex/AcOEt (50/50)	R’	strain
Hex/AcOEt (65/35)	319-347	Hex/AcOEt (50/50)	S’	03 spots
Hex/AcOEt (60/49)	349-373	Hex/AcOEt (50/50)	T’	02 spots + strain
Hex/AcOEt (55/45)	374-384	Hex/AcOEt (40/60)	U’	01 spot +strain PN38
Hex/AcOEt (50/50)	385-401	Hex/AcOEt (40/60)	V’	Long strain
Hex/AcOEt (40/60)	402-426	Hex/AcOEt (35/65)	W’	01 spot, white solid. PN306

Table 56: continuation

Hex/AcOEt (30/70)	427-443	Hex/AcOEt (20/80)	X’	02 spots + strain
Hex/AcOEt (20/80)	444-450	Hex/AcOEt (10/90)	Y’	03 taches + trainées
Hex/AcOEt (10/90)	451-500	Hex/AcOEt (5/95)	Z’	strains
AcOEt (100)	501-523	AcOEt (100)	A1	Oxidising brown powder
AcOEt/MeOH (90/10)	524-530	Hex/AcOEt (85/15)	B1	Oxidising brown powder
AcOEt/MeOH (80/20)	531-542	AcOEt/MeOH (75/25)	C1	Oxidising brown powder
AcOEt/MeOH (60/40)	543-550	AcOEt/MeOH (50/50)	D1	Oxidising brown powder
AcOEt/MeOH (40/60)	551-560	AcOEt/MeOH (35/65)	E1	04 spots
AcOEt/MeOH (20/80)	561-569	AcOEt/MeOH (15/95)	F1	strain
MeOH (100)	670-578	MeOH (100)	G1	strain

III-2-3-2: Column chromatographic separation of Nkambe propolis

A sintered glass column of diameter 5 cm and height 80 cm was used. 75 g of the acetone extract was dissolved in MeOH and mixed with 75 g of silica gel, then evaporated to dryness using a rotary evaporator. The column was filled with slurry made up of 375 g silica gel 2 L of hexane. The extract fixed on silica was introduced into the column and eluted with hexane/AcOEt then, AcOEt/MeOH gradient system with increasing polarity and equal volumes of 100 mL fractions were collected each at a time. The fractions were then concentrated on the rotary evaporator, and later analyzed by TLC. The TLC plates were revealed using UV light (254-265 nm) and 50% sulphuric acid.

Table 57: Chromatogram of fractioning of ethyl acetate extract of propolis from Nkambe

Eluent	Fractions	TLC	Series	Observation
Hex/AcOEt 0-2.5%	1-10	Hex 100 %	ANT1-10	White waxy solid (CFB3) + strains
Hex/AcOEt 0-5%	11-44	Hex/AcOEt 5%	ANT11-44	4 spots purified on Hex/CH_2Cl_2 gradient to afford TA1 and TA2
Hex/AcOEt 5-20%	45-82	Hex/AcOEt 15%	ANT45-82	Non-separable spots Large amount of white powder
Hex/AcOEt 20-30%	83-115	Hex/AcOEt 20%	ANT83-115	2 spots + strains. Yielded TAN14 and TA42 upon purification on Hex/AcOEt gradient
30-40%	116-130	Hex/AcOEt 30%	ANT116-130	Brown precipitate of CFB2 + 6 spots Further purified on Hex/CH_2Cl_2 gradient to yield TA5, TA20, TA23, TA33 and TA68
Hex/AcOEt 40-60%	131-161	Hex/ AcOEt 40%	ANT131-156	4 spots
			ANT157-161	1 spot Rf=0.5, brown precipitate (CFB4)
Hex/AcOEt 60-70%	162-170	Hex/ AcOEt 50%	ANT162-170	3 spots, 1 being yellowish
	171-192	Hex/ AcOEt 60%	171-192 F12	5 complex spots
	193-204		ANT193-204	4 complex spots
Hex/AcOEt 70-75%	205-223	Hex/ AcOEt 70%	ANT205-209	strain
			ANT210-223	1 violet spot of brown crystals (CFB5)
Hex/AcOEt 75-80%	224-249	Hex/ AcOEt 70%	ANT224-251	3 spots
Hex/AcOEt 80-90%	250-259	Hex/ AcOEt 80%	ANT252	1 spot. Dirty white crystals (CFB252)
			ANT253-259	2 spots

Table 57: continuation

Hex/AcOEt 80%	260-270	Hex/ AcOEt 80%	ANT260-270	1 spot + strain
Hex/AcOEt 90%	271-281	Hex/ AcOEt 80%	ANT271-281	Oily strain
Hex/AcOEt 100%	282-298	Hex/ AcOEt 92.5%	ANT282-310	Strain
AcOEt/MeOH 5%	299-306			
AcOEt/MeOH 10%	307-310			
AcOEt/MeOH 20%	311-320	AcOEt/MeOH 10%	ANT311-320	1 spot. Brown powder (CFB7)
AcOEt/MeOH 30%	321-333	AcOEt/MeOH 10%	321-333 F29	1 spot + strain. brownish oxidizing solid
AcOEt/MeOH 40%	334-338	AcOEt/MeOH 30%	334-348	Strain
AcOEt/MeOH 60%	339-345	AcOEt/MeOH 40%		Strain
AcOEt/MeOH 80%	346-348	AcOEt/MeOH 60%		Strain
MeOH 100%	349-352	Rinsing of column		

III-2-3-3: Column chromatographic separation of Foumban propolis

The ethanol extract of Foumban propolis was first subjected to Flash chromatography. This enabled us to make an accelerated separation with the aid of a pressure pump compared to that of a system under atmospheric pressure only. Thus, 70 g of ethanol extract were fixed on 150 g of silica. After having dried it at ambient temperature, the absorbed extract was introduced into the column. The column was filled with 800 g of silica prior to the introduction of the absorbed extract. Elution was carried on hexane, hexane- ethyl acetate and ethyl acetate-methanol gradient of increasing polarity and 110 fractions were collected then regrouped on the basis of their TLC profiles.

Table 58: Flash chromatographic fractioning of ethanol extract of propolis from Foumban

Eluent	Fractions	Series	TLC (Hex/AcOEt)	Observation
Hex 100%	1 – 14	A	90/10	4 spots + strain
Hex/AcOEt 90/10	15 – 27	B		5 spots + strain
80/20	28 – 38	C	85/15	3 spots + strain
70/30	39 – 56	D	70/30	3 spots + strain
60/40	57 – 60			
50/50	61 – 65	E		1 spots + strain
40/60	66 – 71	F	60/40	4 spots + strain
30/70	72 – 75			
20/80	76 – 79			
AcOET 100%	80 – 83			
AcOEt/MeOH 90/10	84 – 89			
80/20	90 – 95			
70/30	96 – 99	G	50/50	2 spots + strain
60/40	100 - 102			
40/60	103– 106			
MeOH 100%	107 - 110	-	-	Washing of column

Table 59: Chromatogram showing purification of fraction A and B combined (25 g)

Eluent	**Fractions**	**Series**	**TLC (Hex/AcOEt)**	**Observation**
Hex 100%	1 – 21	A’	95/5	1 spot + strain PF2
Hex/AcOEt 97.5/2.5	22 – 32	B’	97.5/7.5	2 spots + strain Further purified to PF2 and PF3
95/5	33 – 39	C’		
92.5/7.5	40 – 49			
90/10	50 – 57			
87.5/12.5	58 – 64	D’	90/10	3 spots + strain Purified to PF4
85/15	65 – 75	E’	85/15	01 spot + strain PF8
82.5/17.5	76 – 89			
80/20	90 – 101			
77,5/22,5	102 – 107			
75/25	108 – 111			
72.5/27.5	112 - 117			

Table 59: continuation

70/30	118 – 121	F’	80/20	2 spots + strain
67.5/32.5	122 - 125			
65/35	126 – 129			
60/40	130 – 133	G’	50/50	01 spot + Light strain PF10
55/45	134 – 137			
50/50	138 – 140			
40/60	141 – 143			3 spots + Light strain PFIV
20/80	144 – 145			
AcOEt 100%	146 – 149			
AcOEt/MeOH 90/10	150 – 151			
MeOH 100%	152 – 153	-	-	Rinsing of column

Table 60: Chromatogram showing purification of fraction C, D and E combined (15 g)

Eluent	**Fraction**	**Series**	**TLC** (Hex/AcOEt)	**Observation**
Hex 100%	1 – 25	A’’	(95/5)	3 spots + strain ISGI
Hex/AcOEt	26 – 70	B’’		2 spots + strain
95/5				
90/10	71 – 103	C’’		3 spots + strain
80/20	104 - 109	D’’		2 spots + strain
70/30	110 - 125	E’’		
60/40	126– 131	F’’	85/15	2spots+strain
50/50	132 - 138			ISGII
40/60	139 - 142	G’’	60/40	1spot + long strain ISGIII
30/70	143 - 148	H’’	60/40	ISGIV
20/80	149– 151	I’’	50/50	1 spot + strain
10/90	152 - 154			ISGV

Table 60: continuation

10/90	155	J''	20/80	3 spots
AcOEt 100%	156 - 157	K''		2 spots
AcOEt/MeOH	158– 159	L''		strain
95/5				
85/15	160 – 161			
MeOH100%	162 - 165	-	-	Rinsing of column

III-2-4: Chemical transformations

Esterification of lupeol and β-amyrine with benzoyl chlorides

3.0 mg of lupeol (RBI) or β-amyrine (RB1), was dissolved in 5 mL of chloroform and added to a suitable amount (1.12 mmol) of an appropriate acyl chloride previously dissolved in 15 mL of dicholoromethane in a 50 mL round-bottomed flask. Catalytic amount of DMAP (1 mg in 5 mL of DCM) and DIC were added drop wise. The reaction mixture was stirred on a magnetic stirrer for 24 hours, monitored by TLC at 3 hours interval. TLC showed a less polar spot for the product which became intense with time. The mixture was purified using liquid-liquid extraction (DCM-H_2O) followed by preparative TLC. The yields of individual compounds were calculated.

Oxidation of β-amyrine to amyrenone

5 mg of β-amyrine was mixed with 15 mg of PCC in 20 mL of methylene chloride and the mixture was agitated using a magnetic stirrer at room temperature for 48 hours. The product was obtained as a dirty white solid after liquid-liquid separation three times using a mixture of water-CH_2Cl_2 and evaporation on Rotavapor. The product was obtained from the methylene chloride fraction.

III-2-5: Qualitative tests for triterpenes, sterols and saponins

Test for sterols and triterpenes. The Liebermann-Burchard reaction

Five grams of the sample was dissolved in 20 mL of ether. The solution was evaporated leaving behind the residue. To a test tube containing 3 mL of acetic anhydride and 7 mL of $CHCl_3$ together with 2 drops of concentrated H_2SO_4, 1 mg of the residue was added.

Awaited results: sterols gave a dark greenish coloration while triterpenes gave a reddish-violet coloration.

Test for saponins

In a test tube, 0.5 g of the sample was dissolved in 5 mL of distilled water and the mixture was homogenized. It was vigorously heated till boiling point.

Awaited results: The formation of a layer of foam of about 1 cm thick above the boiling mixture persistent for about 15 minutes indicates the presence of saponosides.

III-2-6: Determination of Total Flavonoids content

The total content in flavonoids was determined by a method making use of the Neu reagent (2-aminodiethyl diphenylborinate 1% in pure methanol) with slight modifications. 1 g of the extract was dissolved in 100 mL of 80% methanol. After agitation and sonication, 2 mL were collected unto which 100 µL of Neu reagent was added and mixed. The absorptions were read at 404 nm in a spectrophotometer (Rayleigh Vis-723N) and the values obtained were compared with those of quercetine standard (0.05 mg/mL) treated in the same way with the same reagent. The percentages of total flavonoid contents were calculated in equivalents of quercetine according to the formula below

$F = (0.05 \times Aext./ Aq.) \times 100 / Cext.$

III-2-7: Determination of total phenolic content

The amount of total phenolic compounds in the extracts was determined with Folin–Ciocalteu reagent using gallic acid (0.2 g/L) as a standard. Briefly, 20 µL of extract solution (10 g/mL) was added to a mixture of 200 µL of Folin–Ciocalteu reagent and 1.380 µL of distilled water followed by thorough mixing. After 3 min, 400 µL Na_2CO_3 (20%) was added. The mixture was allowed to stand for 20 min at 40 °C with intermittent shaking. The absorbance was measured at 760 nm using a spectrophotometer (RAYLEIGH VIS-723N). The determination of the total phenolic compounds was carried out after standardization with gallic acid (0.2 g/L) using a straight-line equation obtained from the standard gallic acid calibration graph obtained by plotting optical densities (absorbances) against concentration of gallic acid. The total phenolic content was measured as grams of gallic acid equivalent per 100 g of raw matter.

III-2-8: DPPH radical scavenging activity

Anti-radical is based on the decrease in the absorbance when the diphenyl-picrylhydrazyl (DPPH•) radical is reduced at 517 nm. A series of 8 successive dilutions were prepared from sample stock solutions 10 mg/ml in methanol. For each concentration, 1 mL of DPPH• (20 mg/L in methanol)

was added to 0.5 mL of sample or extract. After 15 minutes of incubation, the absorbance of the mixtures was taken at 517 nm against a blank or control experiment (0.5 mL extract or sample solution in 1 mL of methanol) using a spectrophotometer (Rayleigh VIS-723N). The control experiment with a solution composed of 0.5 mL of pure methanol and 1 mL of DPPH• was used. Butylhydroxytoluene (BHT) and vitamin C were used as references and their absorbances were used in comparing those of the extracts. The antiradical activity of each sample was expressed in percentage of DPPH• reduced as shown by the formula below.

Percentage Radical Scavenging Activity = (1-Abs. sample/Abs. control) ×100

- *Abs. control* = absorbance of the DPPH solution without sample
- *Abs. sample* = absorbance of the tested sample.

III-2-9: Evaluation of Ferrous Ion Chelating Capacity (Binds Fe2+)

The method of FCC (Ferrous ion chelating capacity) is based on the formation of complexes with Fe2+ ion. The reaction solution containing 100 µL (2 mM) ferrous chloride and 400 µL (5 mM) potassium ferricyanide as reagent was prepared. 200 µL test sample of the extract at various concentrations ranging from 50 to 200 mg/mL were prepared in different test tubes. Double distilled water was added to each test tube to 1 mL level and mixed. The above reagent was then added and the reaction mixture was incubated at 20 °C for 10 min. Formation of the potassium hexacyanoferrate complex was measured at 700 nm using a spectrophotometer (Rayleigh VIS-723N). The assay was carried out at 20 °C to prevent Fe2+ oxidation. Lower absorbance indicated a higher iron chelating capacity. The negative control was without any chelating compound or test sample of extract. EDTA was prepared in same way as the test samples and treated with same reagent. Its values (absorbances) were used for comparison. The percent ferrous ion chelating capacity was calculated accordingly by comparing the absorbance of the test samples with that of the negative control.

$$\text{Ferrous ion chelating capacity} = \frac{A_{\text{control}} - A_{extract}}{A_{\text{control}}} \times 100$$

III-2-10: Antimicrobial tests

The MIC of propolis extracts were determined by micro-dilution in liquid medium using 96-well micro-plates with test strains. Stock solutions of samples were prepared as follows: 4–5 mg (exact weight) dry extract were dissolved in 1 mL 70% ethanol. This stock solution was used for serial

dilution in a 96-wells micro-titre micro-plate from 400 – 500 µg/mL to 200–250, 100–125, 50–62.5, 25–31.25, 12.25 -15.62, 6.12 - 7.81 µg/mL. For the broth micro-dilution test, 50 µL of bacterial suspension in exponential phase of the growth was added to the wells of a sterile 96-well micro-titre plate already containing 50 µL of twofold serially diluted propolis extracts in growth medium. Control wells were prepared with culture medium and bacterial suspension only. Three wells of the micro-titre plate were used for each concentration of tested propolis extracts as well as for control sample. Incubation of the micro-plate was done for 24 hours in the cultivation conditions described above. The MIC is the concentration in the last well in the row where no development of the microorganism is detected.

III-2-11: Cytoprotective, Antisecretory tests and evaluation of ulcers

III-2-11-1: HCl/ethanol-induced gastric lesions in rats

The rats were deprived of food for 36 hours prior to experimentation but all the animals had free access to tap water. The HCl/ethanol solution was used to induce ulcers in the gastric mucosa according to the method described by Tan and co-workers (**Tan *et al*., 1996**). The animals received the propolis extract by oral route, 1 h before they were given the necrotizing solution. Positive control rats received sucralfate in place of the extract. They were killed using ether, the abdomen of each opened and the stomachs removed. The ulcers produced in the glandular region of each stomach were measured and scored as earlier described (**Tan *et al*., 1996**) and the ulcer index (UI), percentage of inhibition (% I) and percentage of ulcerated surface (%US) were calculated.

III-2-11-2: HCl/ethanol-induced lesions in rats pre-treated with indomethacin

Indomethacin (Allphamed PHARBIL Arzneimittel GmbH Hildebrandstrasse 12 D-37081 Gottingen, Germany) was given to the rats (20 mg/kg) by intra peritoneal route at the end of the 24 hours fast. This was followed 1 hour later by the HCl/ethanol ulcer procedure as described above. Blood and gastric tissue samples were taken and prepared for the measurement of oxidative stress parameters.

III-2-11-3: Absolute ethanol-induced gastric lesions

The method described previously for the HCl/ethanol method was used, the only difference being that 1 mL of absolute ethanol was used as the necrotizing solution.

III-2-11-4: Pylorus ligated gastric secretion and ulceration in rats

The method of Shay *et al*. (1945) was used to study the ability of the extract to reduce gastric acid secretion as well as prevent gastric ulceration resulting from auto digestion by stomach secretions. The test rats received the extract, while the controls received distilled water (1 mL) or Cimetidine. One hour later, laparotomy was performed under ether anesthesia, the pylorus of each rat was ligatured, and the abdominal incisions stitched up. The gastric juice produced during six subsequent hours was collected from each rat, the volume measured and 1 mL aliquots kept for gastric acid measurement (**Shay *et al*., 1945**). The ulcers produced in the glandular region of the stomachs were measured and ulcer indexes, % of inhibition, % of ulcerated surface were determined.

III-2-11-5: Method of evaluation of ulcers

III-2-11-5-1: Measurement of scores:

The scores were evaluated according to the method of scores attributed to ulcers by Tan and co-workers (Tan *et al.,* 1996).

Table 61: Scores attributed to ulcers by Tan *et al*., (1996).

Range of ulcerated surface in mm^2	**Score**
Ulcerated surface equal to 0.0	0.0
Ulcerated surface greater than 0.0 but less than or equal to 0.5	1.0
Ulcerated surface greater than 0.5 but less than or equal to 2.5	2.0
Ulcerated surface greater than 2.5 but less than or equal to 5.0	3.0
Ulcerated surface greater than 5.0 but less than or equal to 10.0	4.0
Ulcerated surface greater than 10.0 but less than or equal to 15.0	5.0
Ulcerated surface greater than 15.0 but less than or equal to 20.0	6.0
Ulcerated surface greater than 20.0 but less than or equal to 25.0	7.0
Ulcerated surface greater than 25.0 but less than or equal to 30.0	8.0
Ulcerated surface greater than 30.0 but less than or equal to 35.0	9.0
Ulcerated surface greater than 35.0	10.0

III-2-11-5-2: Ulcer index (UI)

UI = Average score ± standard error of average score for each treatment

III-2-11-5-3: Percentage of ulcerated surface (% US)

$$\%US = \frac{Average\ ulcerated\ surface\ (mm)\ \times 100}{675mm2}$$

III-2-11-5-4: Percentage inhibition (%I)

$$\%I = \frac{UI\ of\ negative\ control\ group - UI\ of\ experimental\ group\ \times 100}{UI\ of\ positive\ control\ group}$$

III-2-11-5-5: Measurement of mucus production

The mucus covering of each stomach was gently scraped using a glass slide and the mucus weighed carefully using a sensitive digital electronic balance of mark DHAUSS.

III-2-11-5-6: Measurement of gastric acidity

One ml of centrifuged gastric contents from each rat was assayed for hydrogen ion concentration by pH-metric titration against 0.1 N NaOH using a digital pH meter. Gastric acidity was expressed as mEq/L.

III-2-11-6: Measurement of in vivo antioxidant capacity

Blood and gastric tissue samples were assayed for oxidative stress parameters as follows: Cellular glutathione (GSH) was measured based on the reaction between 2,2-dithio-5,5-dibenzoic acid and the thiol (SH) groups of glutathione to yield a complex whose absorbance was read at 412 nm (**Ellman, 1959**). The glutathione concentration was calculated using the molar extinction coefficient ε= 1.36 104 M^{-1} cm^{-1}. Lipid peroxidation was assessed by measuring the levels of malondialdehyde (**Wilbur *et al.*, 1949**). Quantification of MDA was done using an extinction coefficient of ε = 1.56 105 M^{-1} cm^{-1}.

III-2-12: Evaluation of the anti-inflammatory activity

The anti-inflammatory activity was evaluated as follows. Edema was induced by applying xylene to the external and internal faces of the right ear of each rat. Eighteen (18) rats divided into six (06) groups of 03 rats each were used for the study. The rats were treated by oral route in the following ways:

Group I: (Negative Control) Distilled water (10 mL/kg);

Group II: (positive control) dexamethasone (1 mg/kg);

Group III: hexane extract (200 mg/kg);

Group IV: hexane extract (400 mg/kg);

Group V: ethyl acetate extract (200 mg/kg);

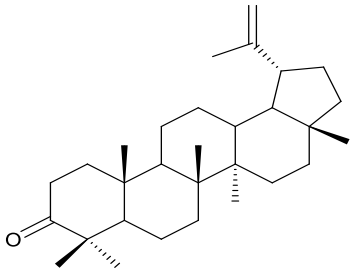
Group VI: ethyl acetate extract (400 mg/kg).

30 minutes later, edema was locally and topically induced by injection of 1 mL of xylene on the internal face of the right ear of each rat of the six groups using a micropipette. After 30 minutes, the animals were sacrificed by under mild anesthesia (ethyl ether) and 0.5 mm sections of each ear were taken and weighed while the left ear was being used as control.

The anti-inflammatory activity is expressed as a percentage inhibition of edema of the ear formation in the rats of the treated groups in comparison with rats of the control groups. The difference between the weight of the right ear and left ear was taken and used in the calculation.

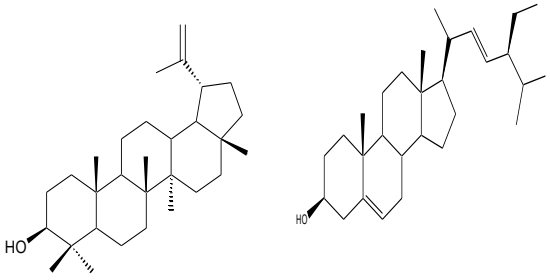
PHYSICO-CHEMICAL CHARACTERISTICS OF SOME OF THE ISOLATED COMPOUNDS

TA1: lupenone (81)



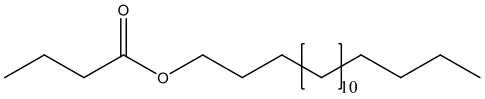
- White crystals
- Liebermann-Burchard test: positive
- Melting point: 170-172ºC
- ITMS-ESI Full MS: $[M+Na]^+$ m/z = 447.8
- Molecular formula: $C_{30}H_{48}O$
- ^1H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 21

TA2: Lupeol and stigmasterol (88 and 134)



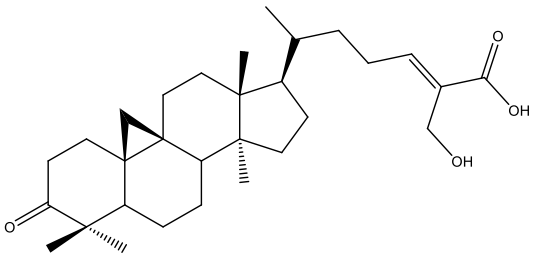
- Dirty white crystals
- Liebermann-Burchard test: positive
- Melting point: 208-214ºC
- ITMS-ESI Full MS: $[2M+Na]^+$ m/z = 875.4 for lupeol
- and $[M+H]^+$ m/z = 413.5 for stigmasterol
- Molecular formulae: $C_{30}H_{50}O$ and $C_{29}H_{48}O$

TA5: Heptadecyl butanoate (146)



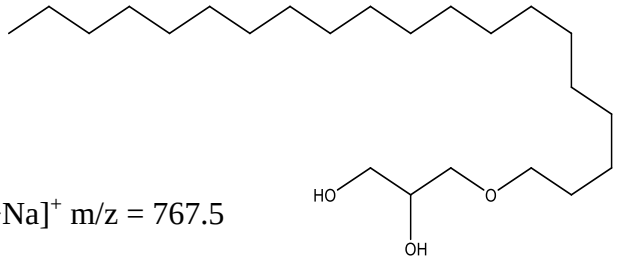
- White powder
- Melting point: 98-102ºC
- ESI-TOF MS: $[M+5H]^+$ m/z = 331.3
- Molecular formula: $C_{21}H_{42}O_2$
- ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 35

CFB252: 27-hydroxymangiferonic acid (135)



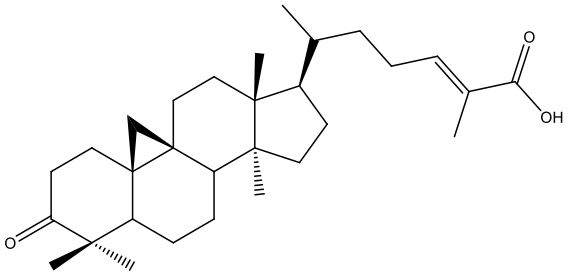
- Pale orange crystals
- Liebermann-Burchard test: positive
- Melting point: 186-188ºC
- ESI-TOF MS: $[M+Na]^+$ m/z = 493.3
- and $[M+2Na]^+$ m/z = 515.3
- Molecular formula: $C_{30}H_{46}O_4$
- ^1H NMR ($CDCl_3$, 600.13 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 22

ISGV: 1'-O-eicosanyl glycerol (145)



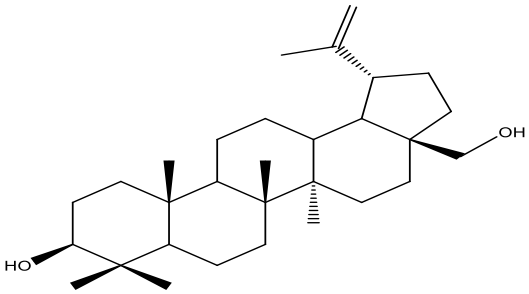
- White crystals
- Melting point: 68-69.2ºC
- ESI-TOF MS: $[M+Na]^+$ m/z = 395.4 and $[2M+Na]^+$ m/z = 767.5
- Molecular formula: $C_{23}H_{48}O_3$
- ^1H NMR ($CDCl_3$, 600.13 MHz) and ^{13}C NMR ($CDCl_3$, 150.92 MHz) data: table 34

ISGII: mangiferonic acid (78)



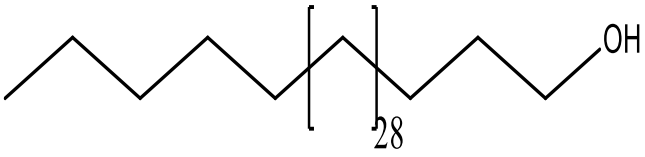
- white solid
- Liebermann-Burchard test: positive
- Melting point: 188-192ºC
- ESI-TOF MS: $[M+Na]^+$ m/z = 477.3
- Molecular formula: $C_{30}H_{46}O_3$
- ^1H NMR ($CDCl_3$, 600.13 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 23

PF2: Betulin (136)



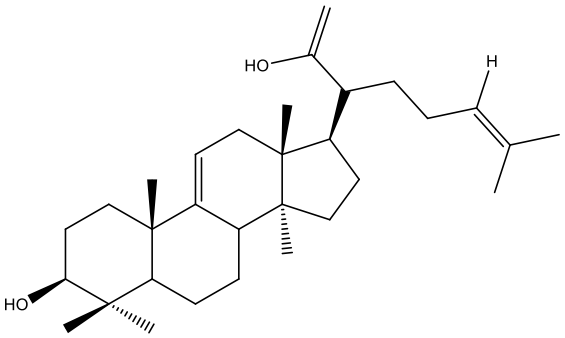
- white crystals
- Liebermann-Burchard test: positive
- Melting point: 254-257ºC
- ESI-TOF MS: $[M+H]^+$ m/z = 442.3
- Molecular formula: $C_{30}H_{46}O_3$
- ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 24

TA14: Heptatriacontanol (147)



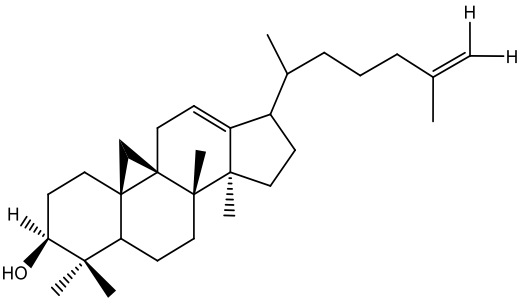
- whitish platelets
- Melting point: 90-91ºC
- ESI-TOF MS: $[M+H]^+$ m/z = 537.3
- Molecular formula: $C_{37}H_{76}O$
- ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 36

PF8: 3β-hydroxylanostan-9,24-dien-21-oic acid (137)



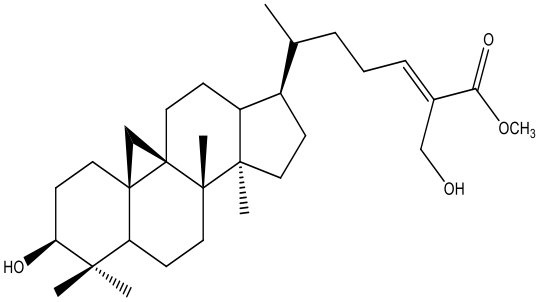
- white flakes
- Liebermann-Burchard test: positive
- Melting point: 134-136^{o}C
- ESI-TOF MS: $[2M+Na]^{+}$ m/z = 935.6
- Molecular formula: $C_{30}H_{48}O_3$
- ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 25

PN53: 3β-hydroxycycloart-12,25(26)-diene (86)



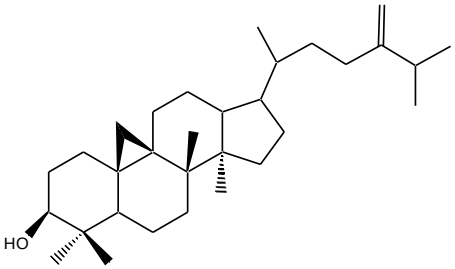
- white crystals
- Liebermann-Burchard test: positive
- Melting point: 181-184^{o}C
- Molecular formula: $C_{30}H_{48}O$
- ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 26

PN306: 27-hydroxymangiferolic acid methyl ester (138)



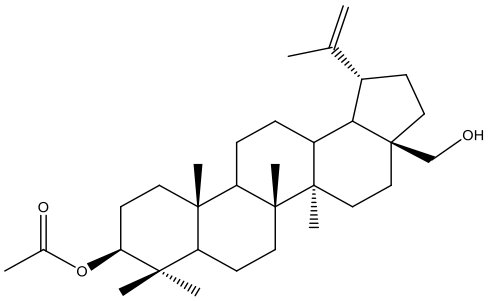
- white crystals
- Liebermann-Burchard test: positive
- Melting point: 167-169.8^{o}C
- ESI-TOF MS: $[M+2Na]^{+}$ m/z = 532.3
- Molecular formula: $C_{31}H_{50}O_4$
- ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data: table 27

TA23: 24-methylenecycloartenol and stigmasterol (141 and 134)



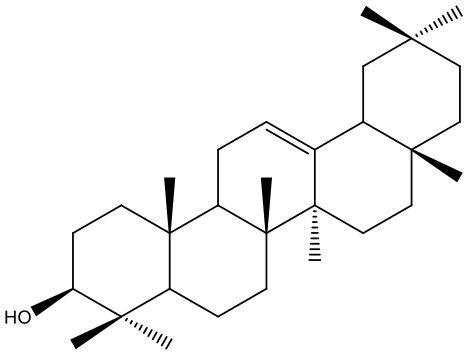
- Dirty white crystals
- Liebermann-Burchard test: positive
- Melting point: 192-195ºC
- ITMS-ESI Full MS negative mode:
- $[M-H]^+$ m/z = 437.6 for 24-methylenecycloartenol and $[M+H]^+$ m/z = 413.5 for stigmasterol
- Molecular formulae: $C_{31}H_{48}O$ and $C_{29}H_{48}O$
- ^1H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 31

PN6: 3-O-acetylbetulin (144)



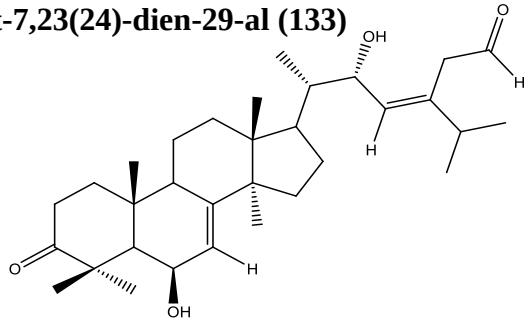
- white needles
- Liebermann-Burchard test: positive
- Melting point: 209-211ºC
- ESI-TOF MS: $[2M+H]^+$ m/z = 969.6
- Molecular formula: $C_{32}H_{52}O_3$
- ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 33

ND22': β-amyrine and lupeol (143 and 88)



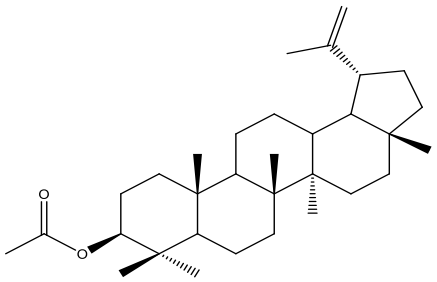
- white crystals
- Liebermann-Burchard test: positive
- Melting point: 215-217ºC
- ESI-TOF MS: $[M+Na]^+$ m/z = 449.3
- Molecular formula: $C_{30}H_{50}O$
- ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 32

PN38: 3-oxo-6,22-dihydroxy-4,4,14-trimethylspinast-7,23(24)-dien-29-al (133)



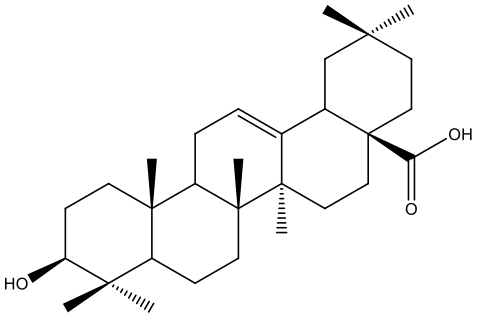
- white crystals
- Liebermann-Burchard test: positive
- Melting point: 167-170ºC
- ESI-TOF MS: $[M+K]^+$ m/z = 537.3
- Molecular formula: $C_{32}H_{50}O4$
- ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data: table 20

TA20: Lupeol acetate (82)



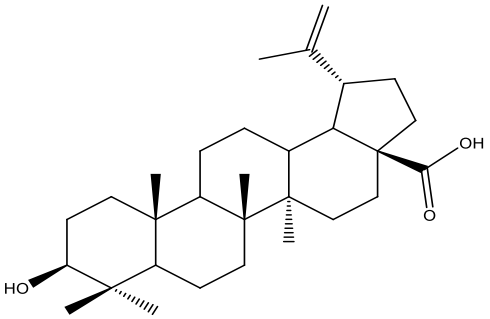
- white crystals
- Liebermann-Burchard test: positive
- Melting point: 216-217ºC
- EIMS: M^+ m/z = 468.3
- Molecular formula: $C_{33}H_{52}O_3$
- ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data: table 28

PF4: Oleanolic acid (139)



- white crystals
- Liebermann-Burchard test: positive
- Melting point: 304-305.5ºC
- EIMS: M^+ m/z = 456.3
- Molecular formula: $C_{30}H_{48}O_3$
- ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data: table 29

PF10: Betulinic acid (140)



- white crystals
- Liebermann-Burchard test: positive
- Melting point: 297-299ºC
- EIMS: M^+ m/z = 456.4
- Molecular formula: $C_{30}H_{48}O_3$
- ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data: table 30

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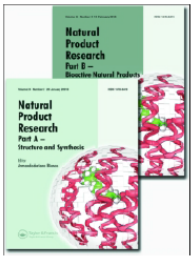

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New mono-ether of glycerol and triterpenes with DPPH radical scavenging activity from Cameroonian propolis

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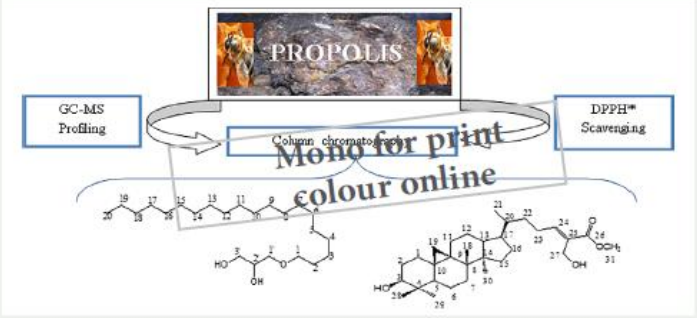
ABSTRACT

The extracts of some propolis samples were analysed by GC-MS and then purified by column chromatography. The latter led to the isolation of a new mono-ether of glycerol, 1′-O-eicosanyl glycerol and a new triterpene, methyl-3β,27-dihydroxycycloart-24-en-26-oate together with known triterpenoids namely betulin, 3β-hydroxylanostan-9,24-dien-21-oic acid, mangiferonic acid, a mixture of ambolic acid and β-sitosterol, 3β-hydroxycycloartan-12,24(25)-diene and 27-hydroxymangiferonic acid. The DPPH radical scavenging potential of some extracts and compounds were measured. The radical scavenging activity varied from Hexane extract of Foumban propolis (IC_{50} = 5.6 mg/mL) to Methanol extract of Foumban propolis (IC_{50} = 1.07 mg/mL) for the extracts and from 3β-hydroxylanostan-9,24-dien-21-oic acid (IC_{50} = 1.22 mg/mL) to 1′-O-eicosanyl glycerol (IC_{50} = 0.93 mg/mL) for the compounds. Activities of samples were moderate as they remained closer to those of the standard antioxidants Gallic acid (IC_{50} = 0.30 mg/mL) and vitamin C (IC_{50} = 0.80 mg/mL), especially 1′-O-eicosanyl glycerol, the most active compound.



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1. Introduction

Propolis is a resinous, gummy and balsamic substance collected by bees from plants and used to close cracks in their hives, reduce the size of the hive entry, or to embalm dead animals that have entered into the hive. Propolis have been shown to possess many biological activities such as antimicrobial (Popova et al. 2013), anti-diabetic (Oršolić et al. 2012), anti-cancerous (Li et al. 2008) antioxidant and anti-ulcer (Socha et al. 2015; Tamfu et al. 2016). It is due to these important biological properties of propolis that it has been used by man for a wide range of purposes and finds applications in cosmetics, agriculture, food technology, human and veterinary medicine. Reports by several authors support the fact that the chemical composition and biological activities of propolis depends on many different factors such as the geographical region, collecting time, and plant source (Meneses et al. 2009). From different botanical and geographical origins of world, more than 300 compounds including volatile organic compounds, flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones, sesquiterpenes, quinones, coumarins, steroids, amino acids, pterocarpans, fatty acid esters and triterpenoids were reported to have been isolated from propolis (Li et al. 2008; Oršolić et al. 2012; Tamfu et al. 2016) and propolis types from unexplored regions of the world have the potential to provide valuable leads to secondary metabolites with important bioactivities (Popova et al. 2013). In this work, extracts were prepared from propolis samples and their GC-MS profiles established. The DPPH radical scavenging activity of the extracts was evaluated and the extracts purified to obtain pure compounds of which some were also tested for DPPH radical scavenging activity.

2. Results and discussion

The unprecedented resolving power of capillary GC (gas chromatography) and the valuable structural information provided by EIMS have proved to be still useful and GC–MS (gas chromatography/mass spectrometry) makes recently a remarkable comeback (Sforcin & Bankova 2011). The GC-MS investigation of different extracts of Foumban propolis revealed the presence of over 30 compounds belonging to a variety of classes of natural products and reported in Tables 1–3. Summarily, the extracts are rich principally in triterpenes, fatty

Table 1. GC-MS profile of ethyl acetate extract of Foumban propolis (silylated sample).

rT	Compound	% of TIC	rT	Compound	% of TIC
23.33	Glycerol	1.9	54.57	Heptadecatrienyl resorcinol	0.7
35.73	Vanillinic acid	0.1	54.73	Heptadecyl resorcinol	5.7
32.63	Hydroxybenzoic acid	0.4	56.63	Anacardic acid (C17:2)	0.7
36.96	Dihydroxybenzoic acid	0.3	56.92	Anacardic acid (C17:1)	1.6
41.16	Hexadecanoic acid	0.9	57.34	Nonadecyl resorcinol	1.8
44.31	Oleic acid	0.6	61.92	Triterpenic ketone	1.8
44.72	Octadecanoic acid	0.2	62.35	Lanosterol	1.8
48.88	Pentadecyl phenol	0.3	62.65	Amyrenone	8.9
51.03	Docosanoic acid	0.2	63.01	β-amyrin	3.0
51.54	Heptadecenyl phenol	0.4	63.18	Triterpenic ketone	2.6
51.86	Heptadecyl phenol	1.3	63.60	Amyrenone + Triterpene [496 (100%), 481 (21), 253 (18), 223 (48), 170 (70)]	13.6
51.96	Pentadecenyl resorcinol	1.0	63.87	Cycloartenol	12.0
52.03	Pentadecyl resorcinol	0.6	66.14	Amyrin acetate	2.3
53.85	Fatty acid 440	0.8		Monosaccharides (sum)	0.6
54.43	Heptadecadienyl resorcinol	3.5			

Table 2. GC-MS profile of methanol extract of Foumban propolis (silylated sample).

rT	Compound	% of TIC
38.65	Triterpenic ketone	0.7
40.19	Triterpenic ketone	1.1
40.73	Triterpenic ketone	3.3
41.14	Triterpenic ketone	1.9
41.96	β-Amyrenone	17.3
42.95	α-Amyrenone	9.1
43.67	Triterpenic ketone (lanostane type)	31.2
44.01	Cycloartenol	5.4
44.52	Amyrine	10.0
46.21	β-Amyrine acetate	1.5
48.09	α-Amyrine acetate	3.5
51.60	Triterpenic aldehyde	1.1

Table 3. GC-MS profile of hexane extract of Foumban propolis (silylated sample).

rT	Compound	% of TIC
23.34	Glycerol	13.3
30.67	Cinnamic acid	0.4
32.61	Hydroxybenzoic acid	0.6
37.27	Pinitol	3.7
41.16	Hexadecanoic acid (palmitic)	2.6
44.30	Octadecenoic acid	0.7
44.71	Octadecanoic acid (stearic)	0.6
47.98	Eicosanoic acid (arachidic)	2.3
51.03	Docosanoic acid (behenic)	2.2
54.38	Heptadecadienyl resorcinol	0.3
54.69	Heptadecenyl resorcinol	0.7
62.58	Triterpene [496 (100%), 481 (21), 253 (18), 223 (48), 170 (70)]	3.8
62.93	β-amyrine	1.8
63.73	α-amyrine	2.6
63.79	Cycloartenol	3.7
	Monosaccharides (sum)	21.3
	Disaccharides (sum)	1.4

Table 4. IC_{50} of $DPPH^{\cdot}$ scavenging activity of some extracts and compounds.

Sample	IC_{50} mg/mL
Hexane extract of Foumban propolis	5.60
Ethyl acetate extract of Foumban propolis	1.40
Methanol extract of Foumban propolis	1.07
Ethyl acetate extract of Ndian propolis	1.65
Hexane extract of Ndian propolis	4.00
1′-O-eicosanyl glycerol	0.93
3β-hydroxylanostan-9,24-dien-21-oic acid	1.22
Mangiferonic acid	1.09
Methyl-3β,27-dihydroxycycloart-24-en-26-oate	0.98
Vitamin C	0.80
Gallic acid	0.30

acids, alkenyl phenols and resorcinols. Cameroonian propolis just like propolis from tropical areas, have shown rich triterpene profile unlike propolis from temperate regions such as France containing mainly aromatic acids and their esters, flavonoids and Sugars with only traces of triterpenes and acids (Popova et al. 2014). Quantitative analyses on propolis samples from Brazil and Poland indicate that it contains mainly phenolic compounds (Inui et al. 2014;

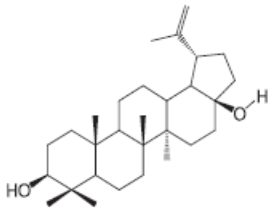

Figure 1. Betulin.

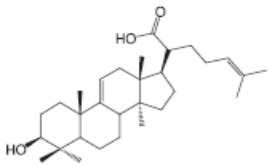

Figure 2. 3β-hydroxylanostan-9,24-dien-21-oic acid.

Socha et al. 2015). The chemical composition of propolis varies from one region to another depending on the local flora. In addition to the triterpenes, Cameroonian propolis is equally rich in fatty acids, alkenyl phenols and alkenyl resorcinols (Kardar et al. 2014; Zhang et al. 2014; Tamfu et al. 2016) which can this far be considered as characteristic in the GC-MS chemical profile of Cameroonian propolis.

The column chromatographic separation of two samples of propolis from two geographical region of Cameroon led to the isolation and characterisation of eight compounds. The structures of the compounds were established based on their spectroscopic data and by comparison with some data existing in literature. The structures of the known compounds were elucidated as; betulin, Figure 1 (Zheng-Fei et al. 2014), 3β-hydroxylanostan-9,24-dien-21-oic acid, Figure 2 (Mosa et al. 2011), mangiferonic acid, **3** (Kardar et al. 2014), mixture of ambolic acid (Kardar et al. 2014) and stigmasterol (Chaturvedula & Indra 2012) Figures 5(a) and 5(b) respectively, 3β-hydroxycycloart-12,25(26)-diene, Figure 6 (Sakava et al. 2014) and 27-hydroxymangiferonic acid, Figure 7 (Tamfu et al. 2016).

Compound **4,** whose structure is given as Figure 4 was isolated as a white powder and melted between 68 and 69.2 °C. It showed a pseudo-molecular ion peak $[M + Na]^+$ at *m/z* 395.4 on its ESI-TOF MS spectra (positive mode) and another diagnostic peak $[2\ M + Na]^+$ at *m/z* 767.5 from which the molecular mass of compound 4 was deduced as 372.4 g/mol for $C_{23}H_{48}O_3$ with a zero double bond equivalence. The ^{1}H NMR (600.13 MHz) spectrum showed chemical shifts of oxymethine protons at δ_H 3.88, 3.55, 3.53 and 3.70 ppm. Thus, at $\delta_H = 3.55$ ppm (2H, t) and $\delta_H = 3.88$ ppm (2H, d) corresponding to the methylene protons of the aliphatic (H-1) and glycerol (H-1′) moieties of the ether function, respectively. The peaks at $\delta_H = 3.53$ ppm (1H, m) and $\delta_H = 3.67$ ppm (2H, dd) are attributable to the oxymethine protons H-2′ and H-3′of the glycerol moiety. The ^{13}C NMR spectra, the three C-atoms of glycerol appear at δ_C 72.57 ppm, 71.88 ppm and 70.37 ppm attributable to C-1′, C-2′ and C-3′, respectively. An aliphatic carbon atom C-1 linked to the oxygen atom of the ether function appears at δ_C 64.35 ppm while a terminal methyl of a long chain appears at δ_C 14.14 ppm. Summarily, the ^{13}C NMR (150.92 MHz) spectra on reveals carbon atoms of the long aliphatic chain linked through an ether function to a molecule of glycerol as follows:

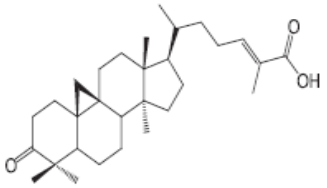

Figure 3. Mangiferonic acid.

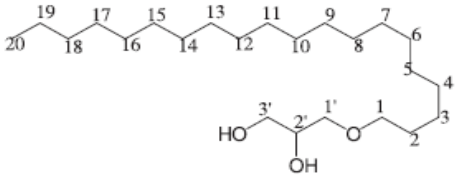

Figure 4. 1′-O-eicosanyl glycerol.

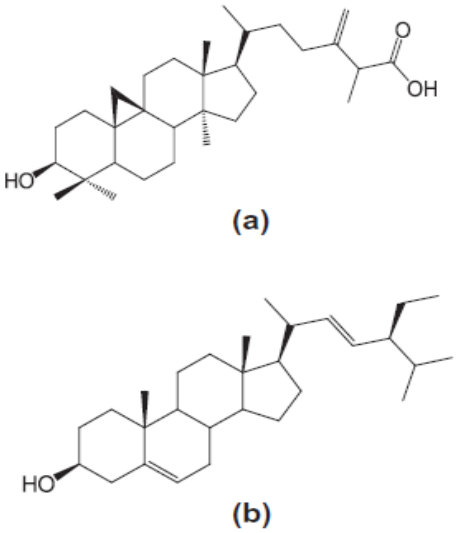

Figure 5. Ambolic acid (a) and stigmasterol (b).

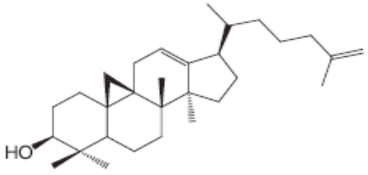

Figure 6. 3β-hydroxycycloart-12,25(26)-diene.

δ_C 71.88 ppm (C-1′), 72.57 ppm (C-2′), 70.37 ppm (C-3′), 64.35 ppm (C-1), 31.95 ppm (C-2), between 29.72 and 29.77 ppm (C-3 to C-19), 14.14 ppm (C-20). The connectivities of aliphatic and glycerol moieties were elucidated as follows: HSQC ($^1J_{H\text{-}C}$) showed correlation between

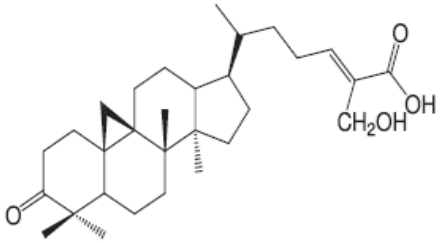

Figure 7. 27-hydroxymangiferonic acid.

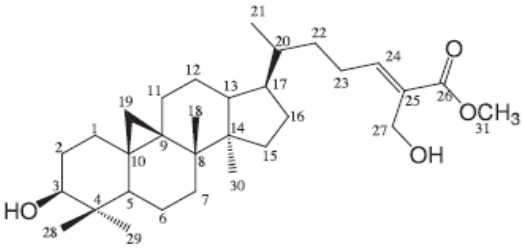

Figure 8. Methyl-3β,27-dihydroxycycloart-24-en-26-oate.

δ_C 14.14 ppm and δ_H 0.80 ppm; δ_C 29.72 ppm δ_H 1.20; δ_C 71.88 ppm and δ_H 3.88; δ_C 64.35 ppm and δ_H 3.55. HMBC showed correlations between C-19 and H-20, C-2 and H-3. Important COSY (^{1}H–^{1}H) correlations were also observed between the following pairs of protons: H-19 and H-20; H-1 and H-2; H-2 and H-3; H-1′and H-2′; H-2′ and H-3′ just to mention the major correlations.

Compound **8**, whose structure is given as Figure 8 was isolated as an amorphous white powder and melted between 167 and 169.8 °C. The ESI-TOF MS spectra of compound **8** showed a pseudo-molecular ion peak $[M + 2Na]^+$ at *m/z* 532.3 from where the molecular formula of the compound was deduced as $C_{31}H_{50}O_4$. The ^{1}H NMR (500 MHz) spectra of compound **8** showed characteristic peaks some of which are similar to those of mangiferolic acid. That is, at δ_H 6.80 ppm (1H, t) characteristic of a conjugated olefin corresponding to the methylene proton H-24, δ_H 4.20 ppm (2H, brs) attributable to the protons of the allylic carbon atom bonded to an oxygen atom (H-27), at δ_H 3.05 ppm a triplet (1H, t) attributable to the oxymethine proton in a α-position with the hydroxyl in β-position H-3. A set of AB doublets at δ_H 0.15 ppm (1H, d) and 0.35 ppm(1H, d) characteristic of a cyclopropane methylene protons Hα-19 and Hβ-19, respectively, allylic methylene protons at δ_H 2.10 ppm (1H, m) and 2.25 ppm (1H, m) corresponding to H_α-23 and H_β-23 and finally signals of five tertiary methyls δ_H 0.67 ppm (3H, s) H-29, 0.70 ppm (3H, s) H-30, 0.75 ppm (3H, d) H-21, 0.80 ppm (3H, s) H-28 and 0.82 ppm (3H, s) H-18 corresponding to the five angular methyl groups. In addition to these, was a broad singlet of 3H at 3.24 ppm attributable to methoxyl protons H-31. ^{13}C NMR (125 MHz) spectrum compound 8 exhibited the following characteristic signal; five methyls δ_C 14.73 ppm (C-30), 18.65 ppm (C-18), 19.84 ppm (C-21), 21.14 ppm (C-29), 26.08 ppm (C-28), a cyclopropane methylene δ_C 29.19 ppm (C-19), two olefinic carbons δ_C 132.70 ppm (C-25) and 148.21 ppm (C-24), an αβ-unsaturated carbonyl ester carbon δ_C 170.17 ppm (C-26), a hydroxyl methylene at δ_C 79.56 ppm (C-3), an alkoxyl carbon at 48.84 ppm (C-31) confirming the presence of a methyl ester seen on the ^{1}H NMR and finally

the oxymethylene at δ_C 56.67 ppm (C-27). These ^{1}H NMR and ^{13}C NMR data closely resembled those of 28-hydroxymangiferolic acid except for the appearance of the signals due to the allylic oxymethylene (δ_H 4.20 ppm, 2H, brs, H-27; δ_C 56.67 ppm, C-27) and the methoxyl group of methylester (δ_H 3.24 ppm, 3H, brs, H-31; δ_C 48.84 ppm, C-31). The hydroxyl group bonded to the allylic methylene C-27 was confirmed on the basis of HMBC correlations between H and 27 (δ_H 4.20 ppm, 2H) with the two olefinic carbons at δ_C 148.21 ppm (C-24) and 132.70 ppm (C-25) and the methyl esterified carbonyl carbon at δ_C 170.17 ppm (C-26). Also, HSQC revealed a correlation between the broad singlet at δ_H 3.24 ppm H-31 and the carbon atom at 48.88 ppm C-31. Other HMBC correlations appeared between δ_C 79.56 ppm C-3 and the methyl protons at δ_H 0.67 ppm (3H, s) H-29 and δ_H 0.80 ppm (3H, s) H-28. Important COSY (^{1}H–^{1}H) correlations were also observed between the following pairs of protons: H-3 with H-2; H-21 with H-20; H-22 with H_α-23 and H_β-23; H_α-19 and H_β-19 amongst others.

It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability. Hence, DPPH radical was scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H (Kumazawa et al. 2004). A comprehensive means of interpreting radical scavenging activity of each extract was to determine the IC_{50} of each extract which is the concentration at which the extract will have a percentage radical scavenging activity of 50. The IC_{50} is inversely proportional to % radical scavenging activity. Therefore, the order of decreasing radical scavenging activity is: hexane extract of Foumban propolis (IC_{50} = 5.6 mg/mL), hexane extract of Ndian propolis (IC_{50} = 4.00 mg/mL), ethyl acetate extract of Ndian propolis (IC_{50} = 1.65 mg/mL), ethyl acetate extract of Foumban propolis (IC_{50} = 1.40 mg/mL), 3β-hydroxylanostan-9,24-dien-21-oic acid (IC_{50} = 1.22 mg/mL) methanol extract of Foumban propolis (IC_{50} = 1.07 mg/mL), mangiferonic acid (IC_{50} = 1.09 mg/mL) methyl-3β,27-dihydroxycycloart-24-en-26-oate (0.98 mg/mL) 1′-O-eicosanyl glycerol (IC_{50} = 0.93 mg/mL), vitamin C (IC_{50} = 0.80 mg/mL) and gallic acid (IC_{50} = 0.30 mg/mL) as shown in Table 4. Although none of the samples showed antiradical activity greater than that of the standards, their activities remained nevertheless closer to those of the standard antioxidants gallic acid and vitamin C. It is observed that the pure compounds showed higher $DPPH^{\cdot}$ radical scavenging activity than the extracts except for methanol extract of Foumban propolis that was more active than mangiferonic acid and 3β-hydroxylanostan-9,24-dien-21-oic acid. Socha and co-workers investigated the antioxidant activities of propolis samples from Poland and found the samples to possess antiradical activity which was highest in samples with higher phenolic and flavonoid contents. The antiradical activity measured towards DPPH radical varied from 1.92 to 2.69 mM TE/g and showed significant correlation with total phenolic and flavonoid contents (Socha et al. 2015). This difference with our results can be attributed to the difference in chemical compositions of the propolis samples. Although our extracts are void of phenolic compounds which are known antiradical agents, their activity might be attributed to triterpenes and alkenyl phenols and resorcinols they contain.

3. Experimental

3.1. Extraction

About 550 g of propolis from Ndian, south-west region of Cameroon which was collected in February 2015 was chilled and ground. The powder was then extracted with a 10-folds

volume of 70% ethanol at room temperature for 48 h. This process was repeated three times to yield a hydro-alcoholic solution of the propolis which was concentrated to near dryness to give the ethanol extract. The ethanol extract was extracted successively by liquid–liquid extraction with hexane (3 times) and ethyl acetate (3 times) to obtain a hexane extract of Ndian propolis (70.1 g) and ethyl acetate extract of Ndian propolis (65 g), respectively. This process was repeated for 1 kg of propolis of Foumban, west region of Cameroon and gave hexane extract of Foumban propolis (362 g), ethyl acetate extract of Foumban propolis (150 g) and methanol extract of Foumban propolis (130 g).

3.2. GC-MS analysis

The GC–MS analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d. and 0.5-µm film thickness HP5-MS capillary column. The temperature was programmed from 60 to 300 °C at a rate of 5 °C/min, and a 10 min hold at 300 °C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. The split ratio was 1:10, the injector temperature 280 °C, the interface temperature 300 °C and the ionisation voltage 70 eV, as described elsewhere (Popova et al. 2014; Tamfu et al. 2016).

3.3. Evaluation of antiradical activity on DPPH·

Anti-radical is based on the decrease in the absorbance when the 2,2′-diphenyl-1-picrylhydrazyl (DPPH·) radical is reduced at 517 nm. This was done according the method described by Talla and co-workers in 2014 (Talla et al. 2014).

3.4. Isolation of secondary metabolites

About 25 g of the ethanol extract of propolis from Foumban was subjected to column chromatography using silica gel using hexane-ethyl acetate (0–100%) then ethyl acetate-MeOH (0–60%) gradient system to afford 110 fractions which were later grouped into 7 pooled fractions (A–G) based on their TLC profiles. Fraction A (2 g) was purified on a Chromatographic column using hexane-ethyl acetate (5–10%) gradient system to betulin (Figure 1, 8 mg, $R_f = 0.7$). Fraction B (400 mg) crystallised into a whitish powder and was filtered out as 3β-hydroxylanostan-9,24-dien-21-oic acid (Figure 2, 65 mg). Fraction C (5 g) was purified by column chromatography using hexane-ethyl acetate (80/20, v/v) gradient system and afforded a mixture of a fatty acid ester and fatty alcohol. Further column chromatography of fraction D (0.5 g) using hexane-ethyl acetate (40/60, v/v) gave 3-oxo-cycloart-24-en-26-oic acid (Figure 3, 63 mg, $R_f = 0.5$) and finally, Fraction E (1.5 g) was purified by column chromatography using hexane-ethyl acetate (20/80, v/v) gradient system and afforded 1-O-eicosanyl glycerol (Figure 4, 30 mg, $R_f = 0.2$).

About 35 g of hexane extract of propolis from Ndian were subjected to column chromatographic separation with silica gel as absorbent using hexane-ethyl acetate (0–100%) and ethyl acetate-MeOH (0–20%) to give 302 fractions which were regrouped into 5 major fractions (A–E) based on their TLC profiles. Successive column Chromatographies using silica gel gave the following compounds from some of the fractions with their respective eluent systems as follows: fraction A (8.2 g) on a gradient system of hexane-ethyl acetate (95/5, v/v)

yielded a mixture of 3β-hydroxy-24-methylenecycloartan-26-oic acid and β-sitosterol (Figures 5(a) and 5(b), 17 mg). Fraction C (5.3 g) upon purification using a gradient system of hexane-ethyl acetate (40/60, v/v) gave 3-oxo-cycloart-24-en-26-oic acid (Figure 3, 13.8 mg).

About 35 g of ethyl acetate extract of propolis from Ndian were subjected to column chromatographic separation with silica gel as absorbent using hexane-ethyl acetate (20–100%) and ethyl acetate-MeOH (0–100%) to give 531 fractions. Fractions 1–318 were regrouped into 7 major fractions (I–VII) based on their TLC profiles. The fractions were each submitted to successive column chromatographies using silica gel and gave the following compounds from some of the fractions with their respective eluent systems as follows: Fraction I afforded 3β-hydroxycycloartan-12,24(25)-diene (Figure 6, 104 mg) on a gradient system of hexane-ethyl acetate (50/50, v/v). Fraction IV (12.3 g) yielded 3-oxo-27-hydroxycycloart-24-en-26-oic acid (Figure 7, 169 mg) on a gradient system of hexane-ethyl acetate (30/70, v/v). Finally, fraction V (2.5 g) on a gradient system of hexane-ethyl acetate (20/80, v/v) led to the isolation of 3β,27-dihydroxycycloart-24-en-26-oic acid methyl ester (Figure 8, 28.6 mg). The structures of the compounds were established based on their 1D and 2D NMR spectra that is ^{1}H, ^{13}C, ^{1}H-^{1}H COSYqf45, HSQC and HMBC which were recorded on a Bruker AV500 and AV600 spectrometer (500 or 600 MHz for ^{1}H and 125 or 150 MHz for ^{13}C) with TMS as internal standard and chemical shifts expressed in parts per million. ESI-MS spectra were measured on a Q-TOF Ultima spectrometer with an ionisation voltage of 3Kv. Their melting points were recorded on an Electrothermal 9100 device and are uncorrected.

3.5. NMR data of compounds 4 and 8 (see spectra in supplementary material)

3.5.1. Methyl-3β,27-dihydroxycycloart-24-en-26-oate (8)

^{13}C NMR (125 MHz, CD_3OD): δ_C 31.05 (C-1), 30.79 (C-2), 79.56 (C-3), 40.64 (C-4), 48.50 (C-5), 21.14 (C-6), 27.24 (C-7), 50.03 (C-8), 19.84 (C-9), 26.41 (C-10), 27.41 (C-11), 33.26 (C-12), 46.55 (C-13), 50.11 (C-14), 38.47 (C-15), 29.59 (C-16), 53.54 (C-17), 18.65 (C-18), 29.19 (C-19), 36.68 (C-20), 19.84 (C-21), 34.17 (C-22), 26.08 (C-23), 148.21 (C-24), 132.70 (C-25), 170.17 (C-26), 56.67 (C-27), 26.08 (C-28), 21.14 (C-29), 14.73 (C-30), 48.84 (C-31). ^{1}H NMR (500 MHz, CD_3OD): δ_H 1.57 (m, H-1), 1.75 (m, H-2), 3.05 (t, H-3), 1.30 (m, H-5), 1.75 (m, H-6), 1.33 (m, H-7), 1.45 (m, H-8), 1.85 (m, H-11), 1.62 (m, H-12), 1.25 (m, H-15), 1.88 (m, H-16), 1.65 (m, H-17), 0.82 (s, H-18), 0.15 & 0.35 (d, H-19), 1.42 (m, H-20), 0.75 (d, H-21), 1.67 (C-22), 2.10 & 2.25 (m, H-23), 6.80 (t, H-24), 4.20 (brs, H-27), 0.80 (s, H-28), 0.67 (s, H-29), 0.70 (s, H-30), 3.24 (brs, H-31).

3.5.2. 1′-O-eicosanyl glycerol (4)

^{13}C NMR (150 MHz, $CDCl_3$): δ_C 64.35 (C-1), 31.95 (C-2), 29.77 (C-3), 29.76 – 29.72 (C-4 to C-19), 14.14 (C-20), 72.57 (C-1′), 71.88 (C-2′), 70.37 (C-3′). ^{1}H NMR (600.13 MHz, $CDCl_3$): δ_H 3.55 (t, 2H, H-1), 1.55 (m, 1H, H-2), 1.25–1.30 (brm, H-3 to H-18), 1.20 (m, 2H, H-19), 0.80 (t, 3H, H-20), 3.88 (m, H-1′), 3.55 (m, H-2′), 3.67 (dd, 2H, H-3′).

4. Conclusion

The GC-MS chemical profiles of some propolis samples showed that they are rich in triterpenes, fatty acids, alkenyl phenols and resorcinols. Some triterpenes were isolated by column chromatography and their antiradical activity on DPPH tested alongside that of the extracts.

Some tested samples showed moderate anti-radical activity against DPPH radical acting as free radical terminators, hence can cause reduction in risk of several chronic diseases thereby increasing their importance. Further purification of the propolis extracts will be done with the aim of obtaining more compounds and testing their antioxidant activities using various methods. Hopefully, this work will attract the attention of scientists to further research on propolis.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Research Article

Chemical Constituents and Anti-ulcer Activity of Propolis from the North-West Region of Cameroon

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Abstract

Three extracts of propolis harvested from Nkambe, North-West region of Cameroon were characterized by GC-MS analysis and their gastric cytoprotective, antisecretory and antioxidant properties evaluated using experimentally-induced gastric ulcers in rats. The propolis extracts were rich in phytoconstituents occurring as sugars, triterpenes and a mixture (fatty acids+triterpenes+alkenyl resorcinols) in the methanol, hexane and acetone extracts, respectively with *Mangifera indica* as major plant source. Three triterpenes, lupeol, lupenone, 27-hydroxymangiferonic acid and an ester of fatty acid heptadecyl butanoate were isolated and characterized. The methanol, acetone and hexane extracts (200-600 mg kg^{-1}) dose-dependently prevented the formation of ethanol-induced gastric lesions (percentage of inhibition, 61, 54 and 55%, respectively, at the dose of 600 mg kg^{-1}). Increasing doses of the extracts inhibited pylorus ligation-induced lesions by 64.5, 73.1 and 16.2%, respectively for the highest dose but none of them showed antisecretory activity compared with controls. The most further significantly ($p<0.01$) reduced HCl/ethanol-induced ulcer indices from 4.33+0.32 in cytoprotective (acetone) extract (56.6-73.1% inhibition under highly acidic gastric environments), the controls to 1.25+0.53 and 0.6+0.04 at the dose of 400 and 600 mg kg^{-1}, respectively (percentage of inhibition: 71-86%). Furthermore, upon pretreatment of the rats with indomethacin prior to HCl/ethanol, the acetone extract significantly ($p<0.001$) decreased ulcer index from 5.55+0.73 in the controls to 1.89+0.15 at the dose of 600 mg kg^{-1}. Although pretreatment with indomethacin reduced the protective effect of the acetone extract by 23-27% and cytoprotection remained high (62-66% inhibition). The cytoprotective action of the most active (acetone) extract may involve the mediation of endogenous prostaglandins.

Key words: Propolis, GC-MS analysis, triterpenes, cytoprotection, antioxidant activity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Peptic ulcers are a deep gastrointestinal erosion disorder that involves the entire mucosal thickness, penetrating the muscular mucosa. An estimated 15.000 deaths occur each year as a consequence of peptic ulcer diseases[1] and as the prevalence of this disease increases over time, one would expect peptic ulcers to continue to have a significant global impact in the basic health and economic systems and in patient's life quality[2]. For decades it was believed that gastrointestinal ulcerations were caused by the excessive secretion of gastric acid, but many patients presenting such ulcerations had normal acid secretion rates[3]. Then some researchers reported that peptic ulcers are caused when the balance between aggressive factors (such as acid and pepsin) and defense mechanisms (such as mucus, bicarbonate, blood flow and mucosal turnover) are shifted in favour of the former[4]. Exogenous aggressive factors such as cigarette smoke, non-steroidal anti-inflammatory drugs (NSAIDs), alcohol, stress, fatty foods and *Helicobacter pylori* infections trigger tissue necrosis through mucosal ischemia, free radical generation and cessation of nutrient delivery. Hydrochloric acid together with pepsin, pancreatic enzymes and bile decrease the defense mechanisms of gastrointestinal mucosa, such as the intercellular junctions, local blood flow, mucus/bicarbonate secretion and cellular growth[5-7]. Although, histamine H_2-receptor blockers (for example ranitidine and famotidine), proton-pump inhibitors (for example omeprazole and lansoprazole), antibiotics (for example metronidazole, amoxicillin, clarithromycin and tetracycline) and other drugs are extensively used in the management of peptic ulcers, there are reports of adverse effects and relapse within one year[8] and also a number of side effects. For example, proton pump inhibitors (omeprazole and lansoprazole) may cause nausea, abdominal pain, constipation, diarrhea and H_2-receptor antagonists (cimetidine) may cause gynaecomastia and loss of libido. Due to the occurrence of many side effects triggered by use of synthetic drugs for many diseases, medicinal plants are considered as the main source of new drugs as they are believed to have less or no side effects. Herbal medicines are considered as safe for the treatment of ulcers with less adverse effects. Drugs for the treatment of gastric ulcers might be very expensive and unaffordable by many. Also in poor countries not everyone can have access to conventional and modern drugs and so they tend to recourse to medicinal plants and other natural products for treatment of various ailments. In addition to being economical, plant sources are effective and relatively less toxic and extensive study is presently being carried out in the study for potent antiulcer agents of plant origin[9-11]. Traditionally plants have not only provided food and shelter for mankind, but have also been used to cure many different ailments[12].

Propolis is an apicultural product that has been used for its various biological properties, particularly as a source of alternative medicines for disease treatment and prevention in different parts of the world. Bees use propolis to narrow the nest entrances, seal cracks and embalm dead organisms inside the hive and the antibiotic properties of propolis provide a healthy hive environment for the honeybee colony[13]. Recently, it has been reported to possess various biological activities such as antinociceptive[14], antimicrobial[15,16], antiviral[17,18], anti-inflammatory[19,20], anticancerous[21,22], antifungal[17,23], antitumoral[21,24], antioxidant[14,25,26], hepatoprotective[27], antiulcer[28,29], antiaging[30], antidiabetes[31,32], immunemodulating[33] and antibacterial[13] properties. The action of propolis against microorganisms represents the most essential pharmaceutical characteristic, for which it has been used by human beings since ancient times[34]. A number of chemical constituents are responsible for these pharmacological activities of propolis. Some of these compounds belong to flavonoids, prenylated p-coumaric acids and acetophenones, lignans, phenolic compounds, di- and triterpenes, caffeoylquinic acids, sugars, sugar alcohols, hydrocarbons and mineral elements[34] and these chemical compositions of propolis depend on the collection site, available plant sources and bee species.

In recent years, a remarkable number of studies have reported many advances made in the chemical and pharmacological studies of medicinal plants and other natural products as well as therapeutically active compounds obtained from propolis. Since incorrect use of the natural products offers can be dangerous to society, it is important to identify the active compounds, linking its structure with the biological activity and to report the correct manner, in which to use them with regard to dose, route of administration and frequency of use. In general, the metabolic profile of an extract gives an insight into its plant origin and allows the identification of its major constituents and also of a number of minor constituents, depending on the technique. It reveals the types of compounds present and gives an idea about the possible activities to be expected.

In the present study, three extracts (methanol, acetone and hexane extracts) were prepared from propolis harvested from Nkambe in the North-West region of Cameroon and the

chemical profiles were characterized by GC-MS analysis. The gastric cytoprotective activity, antisecretory and antioxidant properties of the extracts were then evaluated using experimentally-induced gastric ulcers in rats.

MATERIALS AND METHODS

Collection and significance of propolis in the locality of collection: The propolis was harvested from bee hives of an apiary located within the same area in Njap village, Nkambe town, North-West region of Cameroon during the months of February-March, 2013. A voucher specimen of this sample was deposited in the laboratory of natural products number III, Department of Organic Chemistry, University of Yaoundé 1. Propolis is called 'Nlaa-nfuu' or 'Mbihdong' in Limbum language and 'Dhatche-Nyaki' by the 'Bororo' inhabitants of this locality. Other popular local names include 'Kilei' in Oku, Bui division of the North-West region and 'Ndaki-goro' by the 'Gbayas' of the Adamawa region amongst others, where the use of propolis is becoming very popular. In Nkambe as well as many other localities in Cameroon, propolis is used by local sculptors for ornamental works and mending of calabashes. It is also exploited for its medicinal uses to treat tooth ache, stomach disorders, gastritis and sore throat by chewing directly. Its aqueous extract is used in treating wounds, skin rashes, boils and burns.

Extraction: One gram of raw propolis sample was dried and cooled (20°C) and ground in a mortar using a pestle to obtain a powder. The propolis powder was extracted successively by maceration with 10 V fold of hexane, acetone and methanol in a tightly closed glass jar kept in a dark cupboard at ambient temperature for 48 h with intermittent stirring. The supernatant was carefully decanted and filtered through a Whatmann No. 1 filter paper. The final filtrates were evaporated to near dryness on a rotary evaporator under reduced pressure to remove the solvent and the extract was collected in a clean vial. The maceration, filtration and evaporation process was repeated three times for each solvent after which the residual powder was dried before introduction of a new solvent. This yielded the hexane extract (PHEN), the acetone extract (PAEN) and the methanol extract of propolis (PMEN). All the extracts were well conserved for GC-MS analysis and antiulcer tests.

GC-MS analysis

Preparation of the analyte sample: About 5 mg of each extract were mixed with 50 L of dry (water-free) pyridine and 75 L of bis (trimethylsilyl)-trifluoroacetamide (BSTFA) and heated at 80°C for 20 min. The silylated extracts were analyzed by GC-MS.

GC-MS analysis: The GC–MS analysis was performed with a Hewlett-Packard gas chromatograph 5890 series II Plus linked to a Hewlett-Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d. and 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 60-300°C at a rate of 5°C min^{-1} and a 10 min hold at 300°C. Helium was used as a carrier gas at a flow rate of 0.8 mL min^{-1}. The split ratio was 1:10, the injector temperature 280°C, the interface temperature 300°C and the ionization voltage 70 eV. Every extract was analyzed in duplicate.

Identification and quantification of compounds: The identification of individual compounds were performed using computer searches on commercial libraries, comparison with spectra of authentic samples and literature data. If no reference spectra were available, identification was performed based on the mass-spectral fragmentation and in such cases for some compounds only tentative structures were proposed. Some constituents remained unidentified because of the lack of relevant references and information (none of them major constituents). The quantification of individual constituents is based on internal normalization. The percentage figures in the tables refer to percent of the Total Ion Current (TIC) and are semi-quantitative.

Isolation and characterization of pure compounds: Seventy five grams of the acetone extract were subjected to column chromatography with silica gel on a gradient of hexane-EtOAc (0-100%) then EtOAc-MeOH (0-40%) with increasing polarity to yield 352 fractions indexed ANT 1-352. Based on their TLC profiles, some of the fractions were regrouped into 12 pooled major fractions A-L, while others (ANT10, ANT37, ANT39, ANT46, ANT55, ANT103, ANT252 and ANT315) were left alone. Fraction D (ANT40-ANT45, 205 mg) was purified by column chromatography on silica gel using hexane-CH_2Cl_2 gradient (5-20%) to yield TA1 (48 mg) and TA2 (68 mg). Fraction G (ANT56-ANT84, 600 mg) was purified similarly by column chromatography on silica gel with mobile phase hexane-EtOAc (Hex/AcOEt 40%) to afford 30 mg of TA5. Fraction H (ANT85-ANT100, 2 g) was

purified by column chromatography on silica gel on a hexane-EtOAc40-50% to yield 8 mg of TA20 (hexane-EtOAc, 40% eluate) and 3 mg of TA33 (hexane-EtOAc, 45% eluate). Lastly, fraction I (ANT105-ANT170, 4.5 g) was purified on column chromatography on silica gel on a hexane-EtOAc (40-80%) to yield 24 mg of TA68 (hexane-EtOAc 65% eluate).

The ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC and HMBC were recorded on a Bruker AV500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). The ESI-MS spectra (ionization voltage 3.8 kV) were measured on a LTQ-FT Thermo scientific spectrometer. The structures of the compounds indexed, TA1, TA2, TA5 and ANT252 were elucidated based on their respective spectroscopic data and by comparison with some data reported in literature.

Anti-ulcer tests

HCl/ethanol-induced gastric lesions in rats: The rats were deprived of food for 36 h prior to experimentation but all the animals had free access to tap water. The HCl/ethanol solution was used to induce ulcers in the gastric mucosa according to the method of Hara and Okabe[35]. The animals received the plant extract by oral route, 1 h before they were given the necrotizing solution. Positive control rats received sucralfate in place of the extract. They were killed using ether, the abdomen of each opened and the stomachs removed. The ulcers produced in the glandular region of each stomach were measured and scored as earlier described[36] and the Ulcer Index (UI), percentage of Inhibition (I%) and percentage of Ulcerated Surface (US%) were calculated.

HCl/ethanol-induced lesions in rats pre-treated with indomethacin: Indomethacin (Allphamed PHARBIL Arzneimittel GmbH Hildebrandstrasse 12 D-37081 Gottingen, Germany) was given to the rats (20 mg kg^{-1}) by intra peritoneal route at the end of the 24 h fast. This was followed 1 h later by the HCl/ethanol ulcer procedure as described above. Blood and gastric tissue samples were taken and prepared for the measurement of oxidative stress parameters.

Absolute ethanol-induced gastric lesions: The method described previously for the HCl/ethanol method was used, the only difference being that 1 mL of absolute ethanol was used as the necrotizing solution.

Pylorus ligated gastric secretion and ulceration in rats: The method of Shay *et al.*[37] was used to study the ability of the extract to reduce gastric acid secretion as well as prevent gastric ulceration resulting from auto digestion by stomach secretions. The test rats received the extract, while the controls received distilled water (1 mL) or cimetidine. One hour later, laparotomy was performed under ether anesthesia, the pylorus of each rat was ligatured and the abdominal incisions stitched up. The gastric juice produced during six subsequent hours was collected from each rat, the volume measured and 1 mL aliquots kept for gastric acid measurement. The ulcers produced in the glandular region of the stomachs were measured and ulcer index, percentage of inhibition, percentage of ulcerated surface were determined.

Measurement of mucus production: The mucus covering of each stomach was gently scraped using a glass slide and the mucus weighed carefully using a sensitive digital electronic balance.

Measurement of gastric acidity: One milliliter of centrifuged gastric contents from each rat was assayed for hydrogen ion concentration by pH-metric titration against 0.1 N NaOH using a digital pH meter. Gastric acidity was expressed as meq L^{-1}.

Measurement of *in vivo* antioxidant capacity: Blood and gastric tissue samples were assayed for oxidative stress parameters as follows: Cellular glutathione (GSH) was measured based on the reaction between 2,2-dithio-5,5-dibenzoic acid and the thiol (SH) groups of glutathione to yield a complex, whose absorbence[38] was read at 412 nm. The glutathione concentration was calculated using the molar extinction coefficient $\varepsilon = 1.36\ 104\ M^{-1}\ cm^{-1}$. Lipid peroxidation was assessed by measuring the levels of malondialdehyde[39]. Quantification of MDA was done using an extinction coefficient of $\varepsilon = 1.56\ 105\ M^{-1}\ cm^{-1}$.

Statistical analysis: Pharmacological data were subjected to the one way analysis of variance (ANOVA) followed by the Turkey-Kramer post test. The p-values less than 0.05 were considered significant. Values in tables are given as arithmetic Mean ± Standard Error of the mean (SEM).

RESULTS AND DISCUSSION

Different techniques are appropriate for the purpose of chemical profiling of propolis as demonstrated by numerous

Table 1: GC-MS profile of the methanol (PMEN) extract (silylated sample)

Compounds	TIC (%)
Glycerol	4.0
Glucose	10.2
Fructose	8.2
Pinitol	7.0
Quinic acid	6.3
Pentose	4.0
Manose	4.9
Glucitol	2.3
Inositol	1.2
Hexose	4.2
Pallatinose	1.2
Sucrose	2.8

Table 2: GC-MS profile of the acetone (PAEN) extract (silylated sample)

Compounds	TIC (%)	Compounds	TIC (%)
Glycerol	0.4	Anacardic acid (C17:2)	0.2
Hexadecanoic acid	0.6	Anacardic acid (C17:1)	1.5
Octadecenoic acid	0.8	Nonadecenyl resorcinol	0.4
Octadecanoic acid	0.2	Anacardic acid (C19:1)	0.3
Pentadecyl phenol	0.2	α-amyrenone	7.1
Eicosanoic acid	0.2	Cycloartenol	8.2
Pentadeceny resorcinol	0.9	α-amyrine	12.3
Heptadecyl resorcinol	0.8	Lupenone	10.4
Tetracosanoic acid	0.6	24-methylenecycloartenol	2.7
Anacardic acid (C15:1)	1.0	α-amyrine acetate	1.6
Heptadecadienyl resorcinol	0.6	Lupeol	1.2
Heptadecatrienyl resorcinol	0.6	β-amyrine acetate	5.6
Heptadecenyl resorcinol	2.4	3-hydroxydammarene	1.9

Table 3: GC-MS profile of the hexane (PHEN) extract (silylated sample)

Compound	TIC (%)
α-amyrenone	16.0
Germanicone	2.0
β-amyrine	3.4
Lupenone	24.7
Lupeol	7.1
α-amyrine	3.2
β-amyrine acetate	1.9
α-amyrine acetate	7.3

papers dealing with propolis analysis and hyphenated techniques are the most appropriate ones: HPLC-DAD, LC-MS, LC-MS-MS and GC-MS, etc[32]. The major compounds present in the different extracts PMEN, PAEN and PHEN identified by GC-MS analysis are listed in Table 1-3, respectively. Their percentages are given in the tables and refer to percent of the Total Ion Current (TIC), which are semi-quantitative since the ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.

The GC-MS techniques have been proven to be suitable for chemical profiling of propolis[32]. Even though these techniques provide a sufficient profile and identification of the compounds analyzed[40], the propolis has to be derivatised in order to increase the concentration of volatile compounds for detection. However, not all compounds comprising propolis are able to be derivatised or become volatile after derivatisation[40]. The GC-MS analysis of the silylated samples of the three extracts of propolis (PHEN, PAEN and PMEN) led to the identification of over 40 compounds belonging to various classes of natural products such as triterpenoids, alkenyl phenols and alkenyl resorcinols, fatty acids, sugars and anarcadic acids. The most abundant compounds in the acetone and hexane extracts are triterpenes and triterpene derivatives. The α-amyrenone, α-amyrine, 24-methylenecycloartenol, cycloartenol, α-amyrine acetate and lupenone were the most abundant in the PAEN, while lupenone, α-amyrenone, α-amyrine acetate and lupeol were the most abundant in the PHEN, based on the TIC% values. The hexane and acetone extracts (PHEN and PAEN) are similar in that they all contain α-amyrenone, β-amyrine, lupenone, lupeol, α-amyrine, α-amyrine acetate and β-amyrine acetate, but the PHEN contains germanicone and β-amyrine exclusively. Propolis samples from tropical and subtropical regions such as Cameroon have been proven to be rich in triterpenes and almost deprived of or contain only traces of other constituents[41].

Many studies with African propolis from different regions, like Kenya, Cameroon, Congo and Ethiopia showed that triterpenoids are major chemical components[42-44] and phytochemical studies of Cameroonian and some African propolis samples led to the isolation of or identification of a significant number of triterpenes[14,16,43,45-47]. Triterpenoids including β-amyrin, β-amyrone, lupeol and lupenone and polyprenyl benzophenones such as 7-epi-nemorosone, 7-epi-clusianone, xanthochymol and gambogenone have been detected in propolis samples from the Brazilian Amazon[48] and triterpenoids with major diterpenoids together with caffeate esters were reported in the propolis samples from Egypt but no aromatic acids and flavonoids[49,50]. The major constituents of the PHEN and PAEN are triterpenoids and triterpenes were found to be predominant in the hexanic and EtOAc phases of some Cameroonian propolis samples[45]. Therefore, the major source of triterpenoids is terrestrial vegetation[51]. Generally, the main constituents of propolis are resins and volatiles, which are substances obtained from a variety of botanical processes in different parts of plants found in the site of collection of the propolis samples and beeswax secreted by the bees. Besides triterpenes, a number of fatty acids, hexadecanoic acid, octadecenoic acid, octadecanoic acid, eicosanoic acid and tetracosanoic acid were also identified in the PAEN. A good number of fatty acids and organic acids have been identified in Turkish propolis[52,53] and also fatty acids have been revealed in Omani propolis[54]. To

the best of this knowledge, this is the first time that fatty acids are reported in important amounts in Cameroonian propolis and tropical propolis although methyl esters of these acids have been reported as major constituents of Ethiopian propolis[42]. An alkenyl phenol, pentadecyl phenol together with alkenyl resorcinols, pentadecenyl resorcinol, heptadecyl resorcinol, heptadecadienyl resorcinol, heptadecatrienyl resorcinol, heptadecenyl resorcinol and nonadecenyl resorcinol were also identified in the PAEN.

These compounds were reported previously in Cameroonian propolis[45] with the exception of heptadecadienyl resorcinol identified in Brazilian geopropolis[55] and heptadecatrienyl resorcinol. An inseparable mixture of four alk(en)ylresorcinols, (5-pentadecyl resorcinol, 5-(8′Z,11′Z-heptadecadienyl)-resorcinol, 5-(11′Z-heptadecenyl)-resorcinol and 5-heptadecyl resorcinol) were isolated and characterized from Indonesian propolis together with three cycloartane type triterpenes, mangiferolic acid, isomangiferolic acid and 27-hydroxy isomangiferolic acid[56]. Kardar *et al.*[45] attributed some characterized triterpenes with cycloartenol inclusive, mangiferonic acid, mangiferolic acid and isomangiferolic acid inclusive and some alk(en)ylresorcinols, 5-pentadecylresorcinol, 5-heptadecylresorcinol, 5-(11′Z-heptadecenyl)-resorcinol and 5-(12′Z-heptadecenyl)-resorcinol in Cameroonian propolis as known constituents of mango (*Mangifera indica*, Anacardiaceae) a resin-producing plant widely used in honey production in Cameroon and throughout tropical Africa[57,58]. Therefore mango could be a possible plant source of resin used by bees for the manufacture of propolis from the site of collection in Njap-Nkambe, a hypothesis that might require further verification. This fact is supported by the presence of anacardic acids, anacardic acid, (C15:1), anacardic acid (C17:2), anacardic acid (C17:1) and anacardic acid (C19:1) in the PAEN. Popova *et al.*[54] identified alkylphenol(cardanol), alk(en)ylresorcinols(cardols) and anacardic acids in Omani propolis[54] and documented that these three related compound types, which have been found in propolis samples from Brazil and cardols, which have been detected in propolis from Thailand and Indonesia[56,59,60], most probably originate from *Mangifera indica* fruit bark and are known antifungal substances[56,54]. Glycerol was identified in both PAEN and PMEN. Glycerol has been detected in a good number of propolis samples from different regions around the world for example in Turkish propolis, Canadian propolis and Brazilian geopropolis[52,55,61]. The PMEN is exclusively rich in sugars with glucose TIC% = 10.2, fructose TIC% = 8.2, pinitol TIC% = 7.0 and quinic acid TIC% = 6.3 as predominant constituents. Monosaccharides such as glucose, fructose, ribose, rhamnose, talose, gulose and saccharose are commonly present in propolis[62]. Sugars were also found in Turkish propolis and Omani propolis[52,54] and geopropolis from Northeast Brazil[55]. Some of the polyols alcohols identified in the PMEN such as pinitol, glucitol, inositol and quinic acid are known to possess good biological activities.

The structures of the compounds TA1, TA2, ANT252 and TA5 isolated were elucidated as (1) Lupenone, (2) Lupeol, (3) 27-hydroxymangiferonic and (4) Heptadecyl butanoate, respectively (Fig. 1). For the triterpenes, lupeol and lupenone have been described previously in Cameroonian propolis[14], while 27-hydroxymangiferonic acid was isolated from propolis of Myanmar[63]. Although, as a minor constituent, the presence of mangiferonic acid in the propolis substantiates the fact that *Mangifera indica* could be a major plant source of propolis in Cameroon.

The possible therapeutic usefulness of the rich chemical profiles of the three propolis extracts were tested using well known experimental methods of gastric ulcer, namely, absolute ethanol, HCl/ethanol, HCl/ethanol pretreated with indomethacin-and pylorus ligation-induced gastric ulcer. When the extracts were screened for cytoprotective activity against the highly corrosive absolute ethanol solution, control rats developed hemorrhagic lesions in the glandular portions of their stomachs 1 h after induction of the lesions. The methanol, acetone and hexane extracts (200-600 mg kg^{-1}) dose-dependently prevented the formation of gastric lesions, percentage inhibition attaining 61, 54 and 55%, respectively, at the dose of 600 mg kg^{-1}. Sucralfate (100 mg kg^{-1}) prevented lesion formation by 30.5%. Mucus production increased from 74.6 mg in the controls to 288.8, 375.8 and 375.2 mg, respectively, for the methanol, acetone and hexane extracts compared with 77.4 for sucralfate (Table 4). The highly corrosive nature of absolute ethanol to the gastric mucosa is well known. Absolute ethanol causes gastric mucosal lesions through the release of tissue-derived mediators, such as histamine and leucotriene C_4 as well as by superficial aggressive cellular necrosis. The action of these mediators on gastric microvasculature results in both mucosal and sub mucosal gastric tissue destruction[64]. The significant cytoprotection offered by the propolis extracts against absolute ethanol (54-61% inhibition) was accompanied by highly significant increases in mucus production, suggesting important inhibitory effects by extracts on the generation of the destructive tissue-derived mediators or inhibition of their action on the gastric microvasculature[65,66].

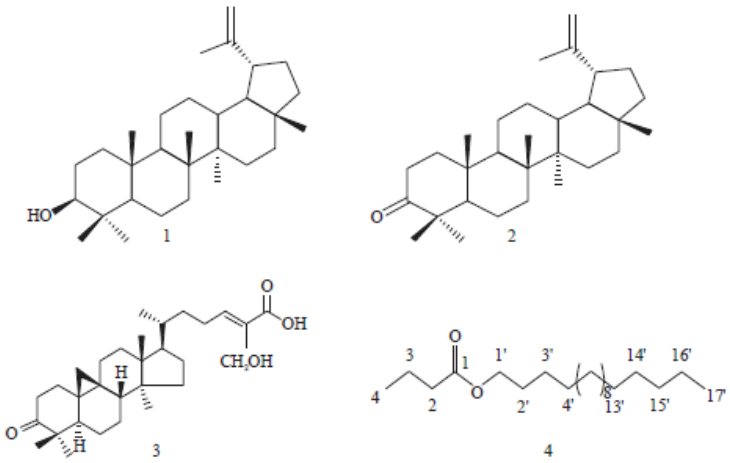

Fig. 1: Structures of the compounds TA1, TA2, ANT252 and TA5 isolated were elucidated as (1) Lupeol, (2) Lupenone, (3) 27-hydroxymangiferonic acid and (4) Heptadecyl butanoate

Table 4: Effects of propolis extracts on gastric ulcers induced by absolute alcohol in rats

Treatment	Dose (mg kg^{-1})	No. of rats	Ulcerated surface (%)	Ulcer index	Mucus production (mg)	Inhibition (%)
Control		5	14.4	5.22±0.40	74.63±6.31	-
Methanol extract	200	5	11.1	3.37±1.40	138.46±19.65*	35.4
	400	5	1 .9	2.32±0.60*	228.77±11.01***	54.9
	600	5	0.4	2.00±0.55*	230.34±17.11***	61.7
Acetone extract	200	5	11.3	4.16±1.20	128.13±19.53*	20.1
	400	5	3.9	3.02±0.18	371.81±52.13***	40.6
	600	5	0.7	2.36±0.64*	375.84±17.56***	54.8
Hexane extract	200	5	15.9	4.76±0.70*	215.60±32.64***	32.6
	400	5	4.9	2.66±0.44*	271.49±50.86***	47.8
	600	5	2.7	2.34±0.62*	275.16±8.95***	55.2
Sucralfate	100	5	1.4	2.04±0.47*	77.44±10.32	60.9

Statistically different relative to control, *p<0.05, ***p<0.001. The values are expressed as Mean±SEM

Since gastric acid and pepsin secretion are very important precursors for the creation of ulcers, the extracts were further screened for their ability to prevent gastric acid secretion using the pylorus ligation technique. In the control rats subjected to pyloric ligature alone, gastric lesion indices were 3.46+0.34. Increasing doses of the methanol, acetone and hexane propolis extracts inhibited lesion formation by 64.5, 73.1 and 16.2%, respectively for the highest dose of extracts compared with 61.8% for cimetidine. Cytoprotection was highest for the acetone extract and lowest for the hexane extract. Although, the cytoprotection was accompanied by significant increases in mucus production (Table 5), none of the extracts showed antisecretory activity compared with the controls (Table 6). Even though gastric acidity for the methanol and acetone extracts (55.8 and 65.6 meq L^{-1}, respectively) were statistically low compared with the controls, previous studies show that gastric acid levels of this magnitude are known to be highly ulcerogenic[36,67,68]. Unlike the methanol and acetone extracts which had slight tendencies to reduce gastric acidity at 400 mg kg^{-1} (45.6 and 34.0% reduction), the hexane extract (400-600 mg kg^{-1}), increased gastric acid levels by 8.1 and 16% compared with the controls (Table 6). Acid substances like hydrochloric acid, acetyl salicylic acid (aspirin) and glacial acetic acid are well known for their ulcerogenic effects on the gastric mucosa. The chemical profiles of the propolis extracts revealed the presence of 11 sugars and 1 acid in the methanol extract, 10 acids, 6 alkenyl resorcinols and phenols, 9 triterpenes and 1 sugar in the acetone extract and 8 triterpenes in the hexane extract. The presence of organic acids in the extracts (especially the methanol and acetone extracts) would have been expected to provide an additive ulcerogenic effect to the pylorus ligation-induced hyperacidity.

Table 5: Effects of propolis extracts on gastric ulcers induced by pylorus ligation in rats

Treatment	Dose (mg kg^{-1})	No. of rats	Ulcerated surface (%)	Ulcer index	Mucus production (mg)	Inhibition (%)
Control	-	5	7.48	3.46±0.34	38.00±5.19	-
Methanol extract	400	5	3.44	2.33±0.66	63.00±5.20*	32.66
	600	5	2.86	1.23±0.53*	63.20±4.39*	64.45
Acetone extract	400	5	1.73	1.50±0.38*	63.80±4.20*	56.65
	600	5	0.57	0.93±0.41**	71.00±8.73**	73.12
Hexane extract	400	5	5.26	3.20±0.33	15.60±1.40**	7.51
	600	5	5.60	2.90±0.60	81.00±6.81***	16.18
Cimetidine	50	5	0.31	1.32±0.61*	88.81±0.13***	61.80

Statistically different relative to control, *p<0.05, **p<0.01, ***p<0.001. The values are expressed as Mean±SEM

Table 6: Effect propolis extracts on gastric secretion in pylorus-ligated rats

Treatment	Dose (mg kg^{-1})	No. of rats	Gastric contents (mL)	Gastric pH	Gastricacidity (meq L^{-1})	Reduction of gastricacidity (%)
Control		5	6.40±0.70	1.93±0.09	84.20±7.07	-
Methanol extract	400	5	4.28±1.32	2.11±0.05	55.80±12.09*	45.6
	600	5	5.54±0.73	2.23±0.07	78.00±4.05	7.4
Acetone extract	400	5	4.28±0.92	2.07±0.10	65.60±12.16*	34.0
	600	5	2.88±0.83**	3.14±0.44**	75.80±8.90	10.0
Hexane extract	400	5	5.96±0.68	2.31±0.15	91.00±4.49	-8.1
	600	5	4.80±0.38	2.24±0.11	97.80±2.15	-16.2
Cimetidine	50	5		4.30±0.34	35.75±0.58**	57.5

Statistically different relative to control, *p<0.05, **p<0.01. The values are expressed as Mean±SEM

This was apparently not the case since the methanol and acetone extracts significantly ($p< 0.01$) prevented the formation of gastric lesions (64.5 and 73.1% inhibition, respectively) in spite of the highly acidic gastric environments (78.0 and 75.8 meq L^{-1}, respectively). The quantification of individual organic constituents of the extracts was done based on internal normalization and gave percentage values of Total Ion Current (TIC%) for each compound. Although, TIC% values are semi-quantitative, they may be useful in explaining the cytoprotective actions observed. The cocktail of 11 sugar molecules in the methanol extract represent 43.2% TIC compared with 6.3% TIC for quinic acid. Intragastric administration of a mannitol, glucose-fructose-sucrose-maltose mixture to pylorus ligated rats prevented the formation of mucosal lesions in an osmolality-dependent manner. The effect occurs by luminal dilution of the necrotising agent and acid without affecting acid content[69]. When Gharzouli *et al.*[70] obtained significant cytoprotection (87-100%) by a glucose-fructose-sucrose-maltose mixture against ethanol, indomethacin-and acidified aspirin-induced lesions in the rat, they concluded that the sugar-rich solutions may prevent gastric damage by a mechanism involving the release of some protective agents. Carbohydrates at high concentrations behave as mild irritants that can induce adaptive cytoprotection[71]. Hexoses, which are present in the methanol extract are major structural components of mucins, which in turn are the major components of the protective gastric mucus.

Six alkenyl resorcinol and alkenyl phenol compounds and 9 triterpenes in the acetone extract together account for 56.9% TIC compared with 5.6% TIC for the 10 acids. The possible ulcerogenic actions of the acid compounds may therefore be masked by the quantitative superiority of the triterpenes, sugars and phenolic compounds. Moreover, the quinic acid present in the methanol extract may well have cytoprotective effects since caffeoylquinic acids from Ligularia species possess peroynitrite-scavenging activity and showed antiulcer activity against HCl/ethanol-and indomethacin/bethanechol and reduced the volume of gastric juice[72]. In addition, the cytoprotective actions of phenolic compounds and triterpenes are well known. A study of antiulcer drugs of plant origin shows that triterpenes because of their ability to strengthen defencive factors such as stimulation of mucous synthesis or maintenance of the prostaglandins content of gastric mucosa at high levels are potentially the compounds with antiulcer activity[73]. These compounds exert cytoprotective actions through increased mucosal blood flow; increased mucus, bicarbonate and prostaglandin secretion and enhancement of the *in vivo* antioxidant status[74-77]. Polyphenolic compounds possess antioxidant activity often attributed to their redox properties which enable them to act like reducing agents and metals chelators and they scavenge free radicals[78]. Most effective medicinal plants are rich in polyphenols and possess high antioxidant potentials[79].

Although, all the three propolis extracts had no antisecretory activity, the acetone extract had the most

Table 7: Effects of propolis extracts on gastric ulcers induced by HCl/ethanol solution in rats

Treatment	Dose (mg kg^{-1})	No. of rats	Ulcerated surface (%)	Ulcer index	Mucus production (mg)	Inhibition (%)
Control	-	5	5.61	4.33±0.32	104.00±8.38	-
Acetone extract	400	5	0.49	1.25±0.53***	109.20±3.01	71.1
Acetone extract	600	5	0.07	0.60±0.40***	152.00±11.85*	86.1
Sucralfate	100	5	1.13	2.80±0.97*	105.90±12.17*	35.3

Statistically different relative to control, *p<0.05, ***p<0.001. The values are expressed as Mean±SEM

Table 8: Effects of propolis extracts on HCl/ethanol-induced gastric lesions in rats pre-treated with indomethacin

Treatment	Dose (mg kg^{-1})	No. of rats	Ulcerated surface (%)	Ulcer Index	Mucus production (mg)	Inhibition (%)
Control	-	5	25.5	5.55±0.73	50.60±4.63	-
Acetone extract	400	5	3.2	2.15±0.20***	120.80±9.46**	62.3
Acetone extract	600	5	2.2	1.89±0.15***	153.60±17.93***	65.9
Sucralfate	100	5	3.1	2.80±0.97*	59.40±6.81	49.5

Statistically different relative to control, **p<0.05, ***p<0.001. The values are expressed as Mean±SEM

Table 9: Effect of propolis extract on oxidative stress parameters in stomach tissues of rats subjected to HCL/Ethanol/Indomethacin-induced gastric lesions

Treatment	Dose (mg kg^{-1})	No. of rats	GSH (µmol g^{-1} of tissue)	MDA (µmol g^{-1} of tissue)
Normal rats	-	5	6.99±0.12	2.26±0.19
Control	-	5	4.09±0.45	4.70±0.49
Acetone extract	400	5	3.70±0.06	6.11±0.95
Acetone extract	600	5	3.65±0.05	6.35±1.07

significant cytoprotection (56.6-73.1% inhibition under highly acidic gastric environments). In addition, 5 out of the 8 triterpenes (α-amyrenone, α-amyrine, lupenone, lupeol and α-amyrine acetate) were present in both the hexane and acetone extracts. The acetone extract was therefore judged to be the most active and further tests were carried out to elucidate its possible mode of action. Table 7 shows the antiulcer actions of the acetone extract against HCl/ethanol solution. The extract significantly ($p<0.01$) reduced ulcer index scores from 4.33+0.32 in the controls to 1.25+0.53 and 0.6+0.04 at the dose of 400 and 600 mg kg^{-1}, respectively (%inhibition: 71-86%). Furthermore, pretreatment of the rats with indomethacin prior to HCl/ethanol raised ulcer index scores to 5.55+0.73 compared with 4.33+0.32 for the HCl/ethanol controls. In response, the acetone extract significantly ($p<0.001$) decreased ulcer index from 5.55+0.73 to 1.89+0.15 at the dose of 600 mg kg^{-1} (Table 8). Inhibition of ulcer formation was accompanied by highly significant ($p<0.001$) increase in mucus production. Although, pretreatment with indomethacin reduced protective effect of the acetone extract by 23-27%, cytoprotection remained high (62-66% inhibition) (Table 8). Indomethacin is a prostaglandin inhibitor, which suppresses gastro-duodenal bicarbonate secretion, disrupts the mucosal barrier, reduces endogenous prostaglandin synthesis as well as gastric mucosa blood flow in animals[80-83]. On the other hand, prostaglandins synthesized in large quantities by the gastrointestinal mucosa are known to prevent experimentally-induced ulcers caused by various ulcerogens. The role of prostaglandins in cytoprotection has been well discussed by Robert[84], Konturek *et al.*[85] and Robert *et al.*[86]. When the cytoprotective action of an antiulcer agent is significantly decreased by pre-treatment with indomethacin, it can be interpreted that the cytoprotection is occurring through the mediation of endogenous prostaglandins[87]. This may well be the case for the acetone extract of propolis used in our experiment. Table 9 shows that subjection of the rats to the HCl/ethanol/indomethacin treatment significantly decreased antioxidant enzyme concentration (GSH) and increased the MDA concentration compared with controls. Treatment with acetone extract did not prevent the drop in the concentration of GSH. The high MDA concentrations (4.70±0.49 mmol g^{-1}) created by the ulceration procedure were not reversed in the extract-treated groups. These results suggest that antioxidant effects may not be involved the mode of antiulcer activity of the propolis extract.

CONCLUSION

Herbal medicines are considered as safe for the treatment of ulcers and propolis, an apicultural by-product of bee farming (often with heterogeneous location-specific chemical composition) has been used for its various biological properties, particularly as a source of alternative medicines for disease treatment and prevention in different parts of the world. In conclusion, the results show that propolis from the Nkambe area of the North West region of Cameroon is rich in phytoconstituents occurring mainly as sugars in the methanol extract, as triterpenes in the hexane extract and as a mixture of acids, triterpenes and alkenyl resorcinols compounds in the

acetone extract. The propolis extracts do not possess antisecretory activity, but show cytoprotective actions that are linked to their phytochemical compositions. The cytoprotective action of the most active (acetone) extract may involve the mediation of endogenous prostaglandins. The propolis showed significant antiulcer activity and provide a justification for the therapeutic use of propolis extracts in the treatment of ulcers and other infectious diseases. These pharmacological activities of Cameroonian propolis are attributable to the presence of diverse chemical compounds including alkenyl resorcinols, fatty acids and triterpenes revealed by the GC-MS profiles of the extracts. *Mangifera indica* could be a major plant source of propolis in Cameroon. In particular, the discovery of propolis plant sources in different geographic regions could be of great importance in addition to the chemistry and biological action of propolis. Hopefully, this study could attract the attention of beekeepers and scientists to further study on propolis and explore its numerous therapeutic effects.

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ABSTRACT

As part of an ongoing investigation of bioactive metabolites from natural sources, extracts of propolis samples from different regions of Cameroon were prepared and screened for their anti-ulcer, antioxidant and antimicrobial properties. Some secondary metabolites were isolated and their structures elucidated.

Thirty-two natural products were isolated: ten compounds and a triterpene fraction from the acetone extract of Nkambe propolis; ten from the ethanol extract of Foumban propolis; six from the hexane extract of Ndian propolis and six from the ethyl acetate extract of Ndian propolis using routine separation and chromatographic techniques. The structures of twenty-four of these compounds were elucidated by interpretation of their physical and spectroscopic data (IR, MS, melting point, 1D & 2D NMR) and by comparison of the latter with similar data reported in literature and due to similarity between some compounds, the number reduced to 20 distributed as follows:

- 16 triterpenes, two of which are novel derivatives namely: 3β,27-dihydroxycycloart-24-en-26-oic acid methyl ester (138) and 3-oxo-6,22-dihydroxy-4,4,14-trimethylspinast-7,23-dien-29-al (133). The 14 known triterpenoids are: lupeol acetate (82), betulinic acid (140), oleanolic acid (139), lupenone (81), 27-hydroxymangiferonic acid (135), mangiferonic acid (78), betulin (136), 3β-hydroxylanostan-9,24-dien-21-oic acid (137), 3β-hydroxycycloart-12,25-diene (86), 3β-O-acetylbetulin (144), lup-12,20(29)-dien-3-ol (142), and a mixture of lupeol and stigmasterol (88 and 134), 24-methylenecycloartenol and stigmasterol (141 and 134), lupeol and β–amyrine (88 and 143).
- 1 new mono-ether of glycerol: 1'-O-eicosanyl glycerol (145)
- 2 fatty acid esters: heptadecyl butanoate (148) and eicosanyl butanoate (146)
- 1 fatty alcohol: n-heptatriacontanol (147)

It is observed that, lupane and oleanane type triterpenoids could be possible chemical markers of Cameroonian propolis.

Also, six benzylic esters (three of lupeol and three of β-amyrine) were synthesized. It resulted from these syntheses that the yield depended on the activating effect of the methoxyl substituents on the benzene ring of the benzoylchloride used. The greater the number of methoxyl groups, the greater the inductive effect and the greater the yield. β-amyrine was also oxidized to amyrenone.

A triterpene fraction was isolated and analysed by GC-MS as a mixture of lanosterol, α-amyrine, 28-norolean-12-en-3-ol, Cycloartenol, 3-epi-α-amyrine, lupeol and 24-methylenecycloartenol.

GC-MS profiles of 13 extracts from different samples of propolis were established and revealed the presence of over 50 compounds belonging to different structural groups but containing mainly triterpenes as would be expected of propolis from tropical and subtropical zones. Characteristically, GC-MS profiles of Cameroonian propolis revealed alkenyl phenols and resorcinols, fatty acids, triterpenes and sugars. The presence of some of these compounds indicated that *Mangifera indica* is the major plant from which bees collect propolis. Samples from Northern part of Cameroon were richer in triterpenes, void of alkenyl phenols and resorcinols and had averagely higher antimicrobial activity while alkenyl phenols and resorcinols and some phenolic compounds were found in samples of North-West and Western regions which showed averagely higher antiradical activity on DPPH. This difference is explained by the variation in vegetation and plants foraged by bees.

The methanol, acetone and hexane extracts of Nkambe propolis (200-600 mg/kg) dose-dependently prevented the formation of ethanol-induced gastric lesions (% inhibition, 61, 54 and 55%, respectively, at the dose of 600 mg/kg). Increasing doses of the extracts inhibited pylorus ligation–induced lesions by 64.5, 73.1 and 16.2 %, respectively, for the highest dose but none of them showed antisecretory activity as compared with controls. The most further significantly ($P<0.01$) reduced HCl/ethanol-induced ulcer indices from $4.33^{\pm}$ 0.32 in cytoprotective (acetone) extract (56.6-73.1 % inhibition under highly acidic gastric environments) to 1.25 $^{\pm}$0.53 and 0.6 $^{\pm}$ 0.04 at the dose of 400 and 600 mg/kg, respectively (% inhibition: 71-86%). Furthermore, upon pretreatment of the rats with indomethacin prior to HCl/ethanol, the acetone extract significantly ($P<0.001$) decreased ulcer index from 5.55 $^{\pm}$ 0.73 in the controls to 1.89 $^{\pm}$ 0.15 at the dose of 600 mg/kg. Although pretreatment with indomethacin reduced the protective effect of the acetone extract by 23 to 27%, cytoprotection remained high (62-66% inhibition). The cytoprotective action of the most active (acetone) extract may involve the mediation of endogenous prostaglandins.

The order of decreasing radical scavenging activity was Hexane extract of Foumban propolis (IC_{50} = 5.6 mg/mL), Hexane extract of Ndian propolis (IC_{50} = 4.00 mg/mL), Ethyl acetate extract of Ndian propolis (IC_{50} = 1.65 mg/mL), Ethyl acetate extract of Foumban propolis (IC_{50} = 1.40 mg/mL), 3β-hydroxylanostan-9,24-dien-21-oic acid (IC_{50} = 1.22 mg/mL), mangiferonic acid (IC_{50} = 1.09 mg/mL), Methanol extract of Foumban propolis (IC_{50} = 1.07 mg/mL),methyl-3β,27-dihydroxycycloart-24-en-26-oate (IC_{50} = 0.98 mg/mL), 1'-O-eicosanyl

glycerol (IC_{50} = 0.93 mg/mL), Vitamin C (IC_{50} = 0.80 mg/mL) and Gallic acid (IC_{50} = 0.30 mg/mL). Although none of the samples showed antiradical activity greater than that of the standards, their activities remained nevertheless closer to those of the standard antioxidants Gallic acid and vitamin C. It is observed that, all the pure compounds showed higher $DPPH^{\bullet}$ radical scavenging activity than the extracts except for methanol extract of Foumban propolis that was more active than mangiferonic acid and 3β-hydroxylanostan-9,24-dien-21-oic acid.

Keywords: Propolis, GC-MS, Triterpenes, 1'-O-eicosanyl glycerol, esterification, DPPH scavenging, anti-ulcer activity, antimicrobial activity.

PUBLICATIONS RESULTING FROM THIS THESIS

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