ISOLATION, CHARACTERISATION AND/OR EVALUATION OF PLANT EXTRACTS FOR ANTICANCER POTENTIAL

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Summary

Chapter One provides a brief introduction to natural products and their applications. Several anticancer compounds from plants, marine animals and microorganisms and several toxins are listed. Screening for bioactive metabolites, MTT assay and Median Lethal Dosage (LD_{50}) are also briefly explained.

Chapter Two is dedicated to the evaluation of *Polygonum bistorta* (Polygonaceae) for anticancer potential using selected cancer cell lines and phytochemical investigations. Hexane and chloroform fractions and a few of their sub-fractions showed moderate to very good activity against LL2 (Lewis lung carcinoma), P388 (Murine lymphocytic leukaemia) and HL60 (Human leukaemia) cancer cell lines. Both active and non-active fractions were investigated for their phytochemical constituents. A total of ten compounds were isolated including three new compounds, *viz*. 24(E)-ethylidene-cycloartanone, 24(E)-ethylidenecycloartan-3α-ol and 24,31-epoxy-24-ethylcycloartan-3α-ol. One rarely encountered compound, cycloartane-3,24-dione was also isolated. The new compound, 24(E)-ethylidenecycloartanone, was screened for its cytotoxicity against P388, HL60, LL2 and WEHI164 cancer cell lines. The Median Lethal Dosage (LD_{50}) of the crude extract, hexane and chloroform fractions was evaluated using Swiss albino mice of both sexes.

Chapter Three deals with the phytochemical investigations of *Eugenia grandis* Myrtaceae). A total of nine compounds were isolated, including one new compound, 2α,3β-dihydroxylup-12-en-28-oic acid, a rarely encountered compound, 3β-hydroxylup-

12-en-28-oic acid and a mixture containing two known compounds *viz*. arjunolic acid and asiatic acid.

Chapter Four is dedicated to the evaluation of sub-fractions of the hexane extract of *Lasiosphaera nipponica* (Basidiomycota) for anticancer potential and identification of the chemical constituents. They showed moderate to good activity against P388 and HL60 cancer cell lines; chemical constituents were identified using GC-MS analysis. One known compound, ergosterol peroxide, was isolated in a pure state and was evaluated against a four cancer cell lines.

Chapter Five is concerned with the evaluation of *Juglans regia*, *Houttuynia cordata* and a mixture containing six herbs for anticancer potential using selected cancer cell lines and the chemical constituents from *Fagraea fragrans*. The sub-fractions of the extract obtained from the seeds of *Juglans regia* (Juglandaceae) were evaluated for their anticancer potential using P388, HL60, LL2, MCF7 and HepG2 cancer cell lines. One known compound, β-sitosterol, was also isolated. Fractions of *Houttuynia cordata* (Saururaceae) and an alcoholic extract of a mixture of six herbs were evaluated for anticancer potential using selected cancer cell lines. *Fagraea fragrans* (Loganiaceae) was investigated for chemical constituents. Several known compounds were identified using GC-MS analysis. However, only two compounds were isolated in a pure state.

Chapter Six is concerned with the conclusion and future studies on *Polygonum bistorta*, *Eugenia grandis*, *Lasiosphaera nipponica* and *Fagraea fragrans*. Both *P. bistorta* and *L.*

nipponica can be further investigated for *in vivo* and/or other *in vitro* pharmacological effects. Similarly, other parts of both *E. grandis* and *F. fragrans* can be investigated both phytochemically and pharmacologically.

List of Tables

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List of Some Common Abbreviations

P338	Murine lymphocytic leukaemia cancer cell lines
HL60	Human leukaemia cancer cell lines
MCF7	Human breast cancer cancer cell lines
LL2	Lewis lung carcinoma cancer cell lines
HepG2	Hepatocellular carcinoma cancer cell lines
J82	Bladder transitional carcinoma cancer cell lines
WEHI164	Murine fibrosarcoma cancer cell lines
NMR	Nuclear magnetic resonance
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
COSY	^{1}H-^{1}H Correlational spectroscopy
DEPT	Distortionless enhancement by electron polarisation transfer
ppm	parts per million
MS	Mass spectrum
EIMS	Electron impact ionisation mass spectrum
FABMS	Fast atom bombardment mass spectrum
ESIMS	Electrospray ionisation mass spectrum
m/z	mass to charge ratio
IR	Infrared
m.p.	melting point
TLC	Thin layer chromatography

Chapter 1

1.1.0 General Introduction

Despite the availability of different approaches for the discovery of therapeutics, natural products still remain as one of the best reservoirs of new structural types. Statistics show that about 25% of prescription drugs sold in the United States are from natural products, while another 25% are from structural modifications of natural products [1]. It was also claimed that about 120 prescription drugs are from higher plants. Of the several hundred thousand plant species around the world, only a small proportion has so far been investigated both phytochemically and pharmacologically. As a single plant may contain thousands of constituents, the possibilities of making new discoveries become evident. The selection of plant material is thus a crucial factor for the ultimate success of the identification of bioactive plant constituents [2].

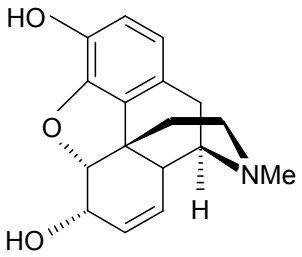
Today, the research facilities have enabled researchers to carry out separation of complex mixtures into single molecular entities and with the help of modern tools *viz*. ultraviolet, infrared, nuclear magnetic resonance and mass spectrometry, a large number of individual compounds from any one plant can be identified unambiguously in a very short period of time. These facilities together with the advancements in molecular biology provide significant improvements in natural product research which is becoming increasingly interconnected with biology, biochemistry, pharmacology and medicine [3].

1.1.1 Natural products and their applications

The term natural products refers to secondary metabolites, small molecules (mol.wt. < 2000 *amu*) produced by an organism and these metabolites are not strictly necessary for the survival of the organism. Natural products include: (1) an entire organism, (2) part of an organism, (3) an extract of an organism or part of an organism and exudates and (4) pure compounds (e.g. alkaloids, coumarins, flavonoids, glycosides, xanthones, lignans, phenylpropanoids, isoprenoids, sugars, etc.) isolated from plants, animals or microorganisms [4]. Many of these compounds show a variety of biological and pharmacological activities and some of these compounds are essential for everyday life, both for humans and animals.

Alkaloids

The term 'alkaloid', is generally limited to organic bases formed in plants. On the whole, alkaloids are very poisonous, but are used medicinally in very small quantity. Morphine **(1)** is an alkaloid found in opium. It is one of the most widely used naturally occurring narcotics in medical treatment and has been prescribed for the relief of moderate to severe pain. Codeine **(2)** is used as an antitussive and is generally accepted as the standard antitussive against which new antitussive medications are compared [5]. Quinine **(3)** is another alkaloid isolated from the bark of *Cinchona* species. It has been used for the treatment of malarial fever.



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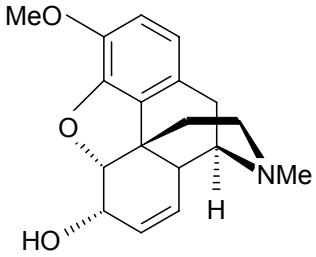

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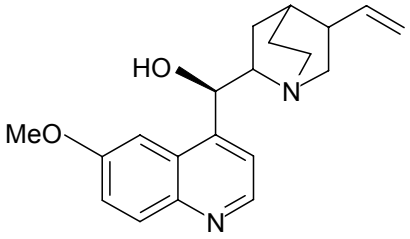

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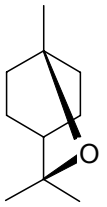
Terpenoids

The diverse, widespread and exceedingly numerous family of natural products constructed from five carbon building-units and so comprising compounds with C_5, C_{10}, C_{15}, C_{20}, etc. skeletons are synonymously termed terpenoids or isoprenoids, with the important subgroup of steroids sometimes singled out as a class in its own right [6]. These compounds are typically found in all parts (i.e. seed, flowers, foliage, roots and wood) of higher plants and also occur in mosses, liverworts, algae and lichens. Some are of insect or microbial origin.

Monoterpenoids play an important role in chemical ecology. For example, 1,8-cineol **(4)** and camphor **(5)** are emitted by assorted plants like eucalyptus and sagebrush and inhibit germination and development of seeds of competing species [7]. Yet others act as attractants to insect pollinators or repellants of insect or larger predators.



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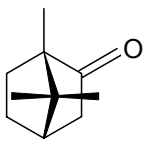

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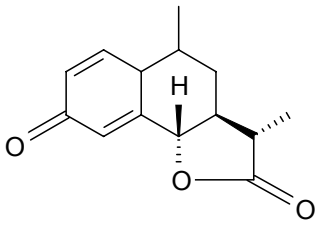
Sesquiterpenoids exhibit a rich variety of biological properties. Their pharmacological properties have also ensured their frequent use in folk medicine. The most famous is α-santonin **(6)** which is widely used to combat intestinal worms [8]. Hernandulcin **(7)** isolated from a Mexican plant is about 1000 times sweeter than sucrose [8].



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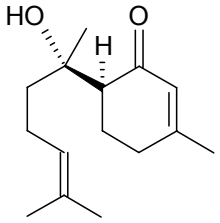

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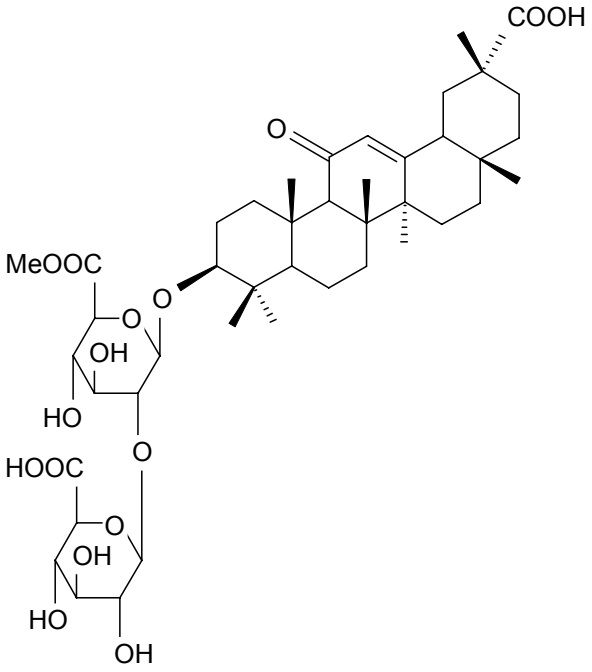
Abrusoside sweeteners, which are based on a cycloartane skeleton, are of approximately equal sweetness potency to that of the widely used natural product sweetener glycyrrhizin **(8)**, an oleanane-type triterpenoid [9]. Abrusoside B **(9)** isolated from *Abrus precatorius* is rated at about 100 times sweeter than a 2 % w/v aqueous sucrose solution [9].



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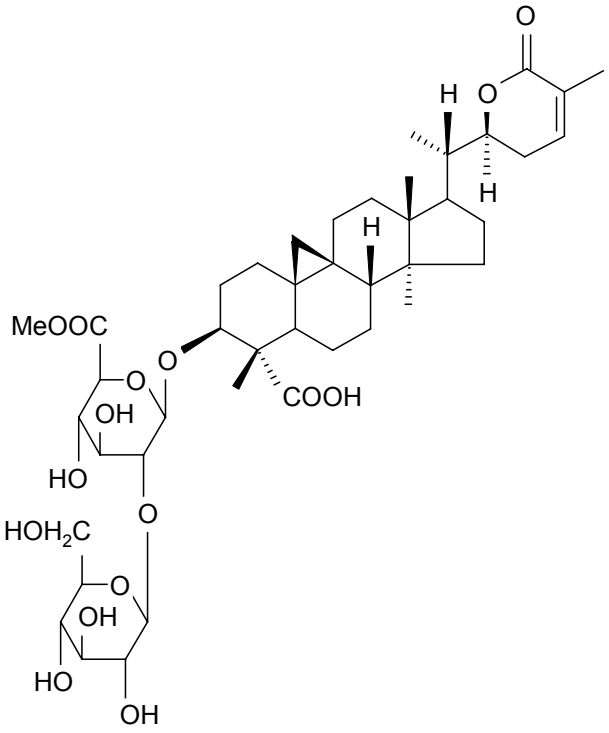

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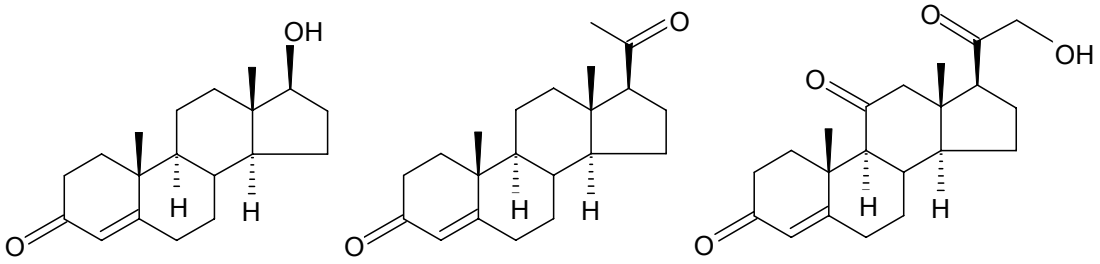
Steroids

Steroids are mostly familiar for their role as hormones, e.g. testosterone **(10)** and progesterone **(11)**. Steroids, such as cortisone **(12)**, are most often used as anti-inflammatory agents, but many have other uses such as in birth control.

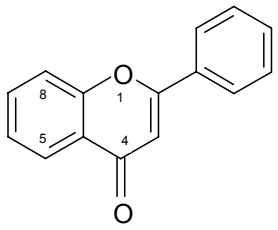


(10) **(11)** **(12)**

A large number of commercially and medicinally valuable steroids available today are made by semi-synthetic preparation.

Flavonoids

Flavonoids are another major class of compounds, whose structure is based on flavone **(13)**. They are also widely distributed in the plant kingdom and exert a variety of biological and pharmacological activities, like other classes of compounds.



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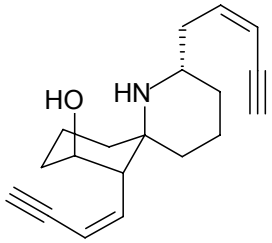
Microorganisms are a prolific source of structurally diverse bioactive metabolites and have yielded some of the most important products of the pharmaceutical industry. These include: antibacterial agents, such as the penicillins (from *Penicillium* species), cephalosporins (from *Cephalosporium acremonium*), aminoglycosides, tetracyclins and polyketides (all from *Streptomyces* species) [10]; cholesterol lowering agents, such as mevastatin and lovastatin (from *Penicillium* species) [10]; immunosuppressive agents, such as cyclosporins and rapamycin (from *Streptomyces* species) [10]. Manoalide, isolated from the marine sponge, *Luffarriella variabilis*, shows potent anti-inflammatory activity. The pseudopterosins, isolated from *Pseudopterogorgia elisabethae*, possess significant analgesic and anti-infammatory activities.

1.1.2 Pharmacology

The interaction of chemical agents with biological systems, how they affect the various tissues and their mechanism of actions are studied in pharmacology. Many natural products have pharmacological significance.

Toxins

As mentioned earlier alkaloids (plant origins) are the most familiar toxic compounds. However, some animals also serve as a potent source of toxic compounds. For example, histrionicotoxin **(14)**, a spirocyclic piperidine, has been extracted from small brightly coloured frogs, *Dendrobates histrionicus*. The spirocyclic core is unique in the world of natural products and the *cis*-enyne moiety is also a very unusual feature in the natural product kingdom.



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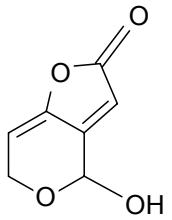
When the frogs are kept in captivity, the level of the toxins that they produce is severely diminished and in most species not produced at all. This has led to the assertion that the toxins are somehow introduced into the frog via diet or by some other outside influence. Batrachotoxin, homobatrachotoxin, conatoxins, tetrodotoxin and epibatidine are some other examples of toxins derived from animals. Patulin **(15)**, zearalenone **(16)**, aflatoxin B1 **(17)**, atropine **(18)**, psoralene **(19)**, vomitoxin **(20),** 3,4-dihydroxyphenylalanine **(21)** and sporodesmin **(22)** are some examples of plant-or fungi-derived toxins [11-16]. Median Lethal Dosages (LD_{50}) of some biotoxins, are listed in Table 1.



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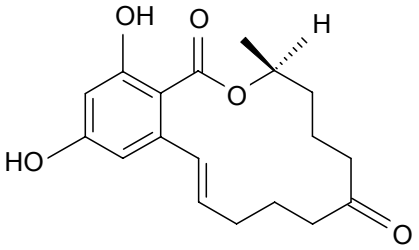

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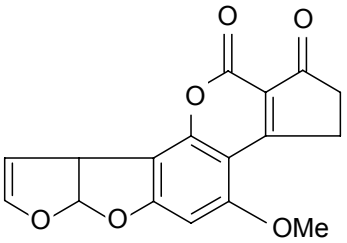

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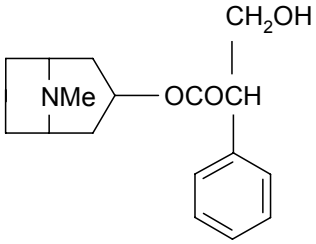

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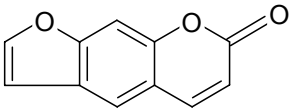

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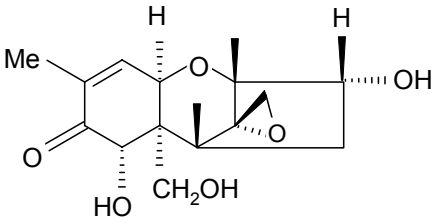

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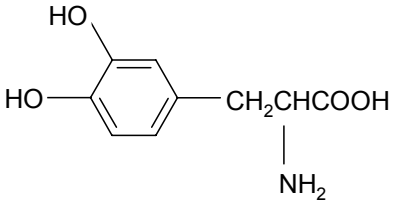

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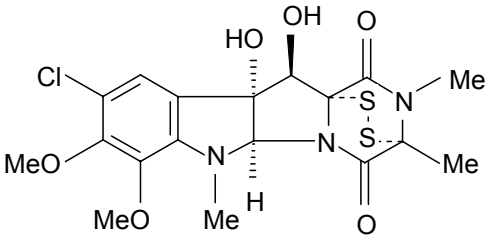

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Table 1
Median Lethal Dosages (LD_{50}) of some biotoxins.

Toxin	Organism	Common name	Lethal Dose (LD_{50})
Botulinum toxin-a	*Clostridium botulinum*	Bacterium	0.00003
Tetanus toxin	*Clostridium tetani*	Bacterium	0.0001
Ricin	*Ricinus communis*	Castor Bean Plant	0.02
Diphtheria toxin	*Corynebacterium diphtheriae*	Bacterium	0.3
Koki toxin	*Phyllobates bicolor*	Poison Arrow Frog	2.7
Tetrodotoxin	*Sphaeroides rubripes*	Puffer Fish	8
Saxitoxin	*Aphanizomenon flos-aquae*	Cyanobacterium	9
Cobra toxin	*Naja naja*	Cobra	20
Nodularin	*Nodularia spumigena*	Cyanobacterium	30-50
Microcystin-LR	*Microcystis aeruginosa*	Cyanobacterium	50
Anatoxin-a	*Anabaena flos-aquae*	Cyanobacterium	200
Microcystin-PR	*Microcystis aeruginosa*	Cyanobacterium	300-600
Curare	*Chrondodendron tomentosum*	Brazilian poison arrow Plant	500
Strychnine	*Strychnos nux-vomica*	Plant	500
Amatoxin	*Amanita phalloides*	Fungus	600
Muscarin	*Amanita muscaria*	Fungus	1100
Phallotoxin	*Amanita phalloides*	Fungus	1800
Glenodin	*Peridinium polonicum*	Dinoflagellate Alga	2500

LD_{50} is in µg per kg bodyweight, based on intraperitoneal injection of mice or rats.

Cancer

Cancer in its many diverse forms is a most insidious disease and represents the second major cause of death [17]. The word cancer actually refers to over 100 different diseases, but in all cases, certain body cells multiply in an abnormal and unregulated manner. If normal cells begin to grow abnormally and reproduce too rapidly, a mass of abnormal cells eventually develops. This is called a tumour. The tumour cells localise at the site of the origin in the body and if they multiply slowly the tumour is called benign. Benign tumours such as cysts, warts, moles and polyps do not spread to other parts of the body and are not life threatening. Benign tumours can be removed by surgery and will not re-grow after surgical excision; thus the victims will recover fully from the tumours. On the other hand malignant tumours (called cancers) are composed of cells that grow rapidly and invade other normal tissues.

Cancers are medically classified according to the organ or kind of tissue in which they originate. Four major categories of cancers are carcinomas, sarcomas, leukaemias and lymphomas [18]. Within these major categories are numerous subgroups that generally describe the organ in which the cancer originates such as adenocarcinoma of the stomach or oat cell carcinoma of the lung.

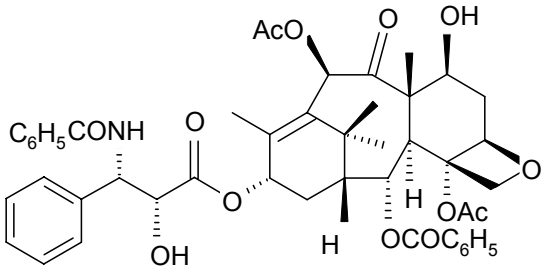
Recently, pharmacological manipulations on inhibiting tumour cell growth and the antiproliferation of malignant cells through induction of apoptosis (or suicidal cell death) have been recognised as a novel strategy for the identification and screening of potential chemotherapeutic agents.

1.1.3 Natural products in the treatment of cancer

Plant sources

The anticancer principles, vinblastine **(23)** and vincristine **(24)**, are alkaloids obtained from the periwinkle plant, *Catharanthus roseus*. This is one of the most successful higher plant materials used in cancer chemotherapy. The above mentioned anticancer principles have now been extracted commercially for the treatment of leukaemia [19-27].

Taxol® **(25)** is a complex polyoxygenated diterpenoid. It was isolated from the bark of the Pacific yew tree (*Taxus brevifolia*). The US Food and Drug Administration (FDA) approved taxol for the treatment of ovarian and breast cancers. The natural source, the Pacific yew tree, is an environmentally protected species, which is also one of the slowest growing trees in the world. Isolation of the compound, which is contained in the bark, involves killing the trees and the quantities available are small. It would take six 100-year old trees to provide enough taxol to treat just one patient.



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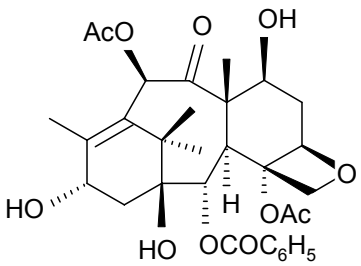


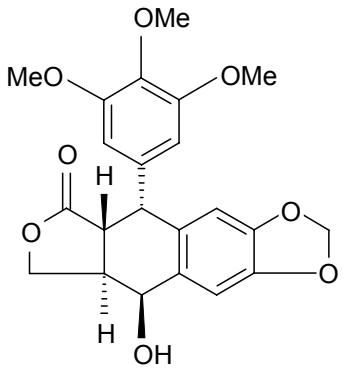
(26)

However, a closely related analogue of taxol, called baccatin III **(26)** was discovered in the needles and twigs of the European yew tree (*Taxus baccata)* and other different types of yews. As the needles are quickly replenishable by the trees, harvesting large quantities

has little effect on the population of yew trees. Baccatin III **(26)** was chemically modified to form taxol. Methods of total synthesis of taxol take more than 30 steps with less than 0.05% yield [28-30]. This is not considered commercially viable.

Camptothecin^{TM} **(30)** is a quinoline alkaloid, isolated from the Chinese ornamental tree, *Camptotheca acuminate*. Topotecan (TPT), irinotecan (CPT-11), 9-aminocamptothecin and 9-nitrocamptothecin, were semi-synthetically derived from camptothecin [31]. Camptothecin and its derivatives are active against carcinomas of stomach, head, neck and bladder. Both TPT and CPT-11 have been approved for treating colorectal and ovarian cancers.

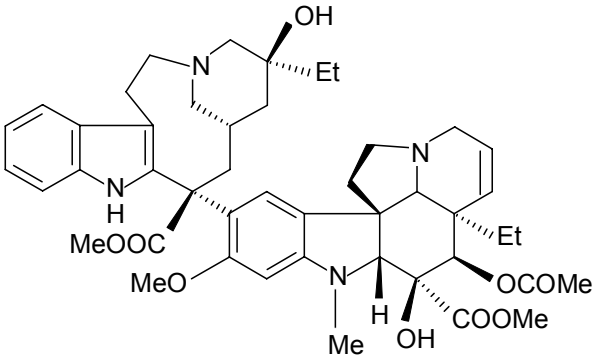
Epipodophyllotoxin **(30a)** was isolated as the active antitumour agent from the roots of the genus *Podophyllum*. These plants possess a long history of medicinal use by early American and Asian cultures [32]. Etoposide and teniposide are two clinically active agents, which are semi-synthetic derivatives of epipodophyllotoxin. Some cytotoxic compounds obtained from plant sources are given in Table 2.



(30a)

Table 2
Some cytotoxic compounds obtained from plants [33].

Name	Source	Class
Vinblastine **(23)**/ Vincristine **(24)**	*Catharanthus roseus*	Indole alkaloid
Taxol® **(25)**	*Taxus brevifolia*	Diterpene alkaloid
Emetine	*Cephaelis acuminata*	Isoquinoline alkaloid
Maquiroside A	*Maquira calophylla*	Cardiac glycoside
Kamebanin	*Rabdosia umbrosa*	Diterpenoid
Lariciresinol	*Wilkstroemia elliptica*	Lignan
3',4'-Deoxypsorospermin	*Psorospermum febrifugum*	Xanthone
Larreantin	*Larrea tridentata*	Quinone
Allamandin **(27)**	*Allamanda cathartica*	Monoterpenoid
Cudraisoflavone	*Cudrania cochinchinensis*	Flavonoid
Helenalin **(28)**	*Heliotropium indicum*	Sesquiterpenoid
Cucurcubitacin	*Marah oreganus*	Triterpenoid
Colchicine	*Colchicum speciosum*	Alkaloid
Indicine-N-Oxide **(29)**	*Heliotropium indicum*	Pyrrolizidine alkaloid
Camptothecin™ **(30)**	*Camptotheca acuminata*	Quinoline alkaloid
Umbelliferone **(31)**	*Wilkstroemia elliptica*	Coumarin



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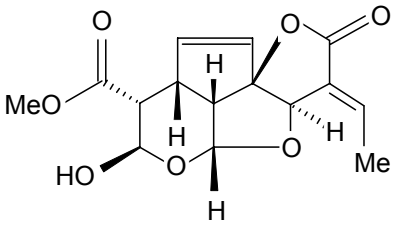

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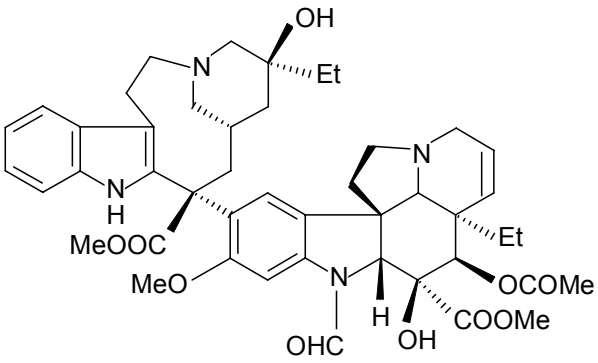

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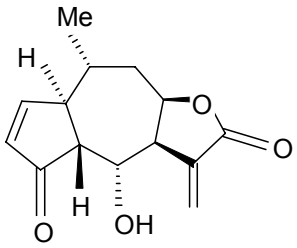

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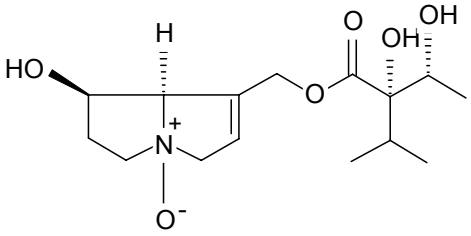

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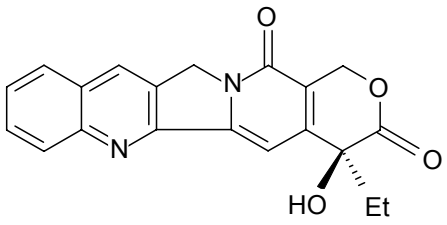

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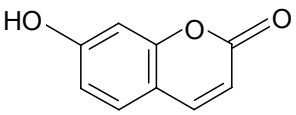
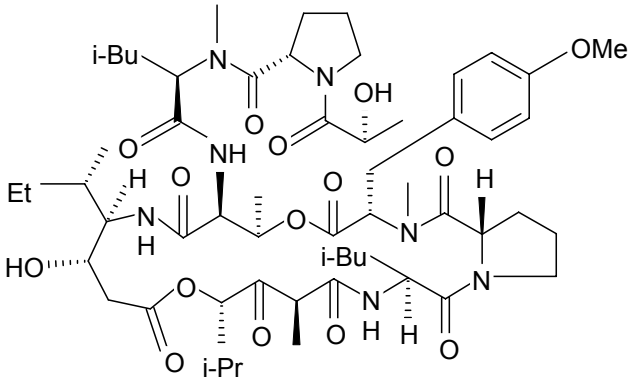


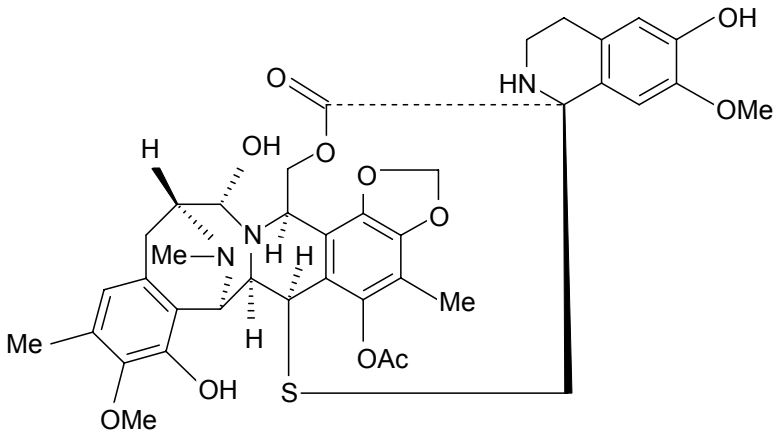
(31)

Marine sources

The marine environment is a rich source of bioactive compounds, many of which belong to totally novel classes not found in terrestrial sources [34]. The first marine-derived compound to enter clinical trials is didemnin B **(31a)**, isolated from the tunicate, *Tridemnum silidum* [35]. Ecteinascidin 743 **(31b)**, a metabolite produced by another tunicate *Ecteinascidia turbinata*, has significant *in vivo* activity against the murine B16 melanoma and human MX-1 breast carcinoma models.

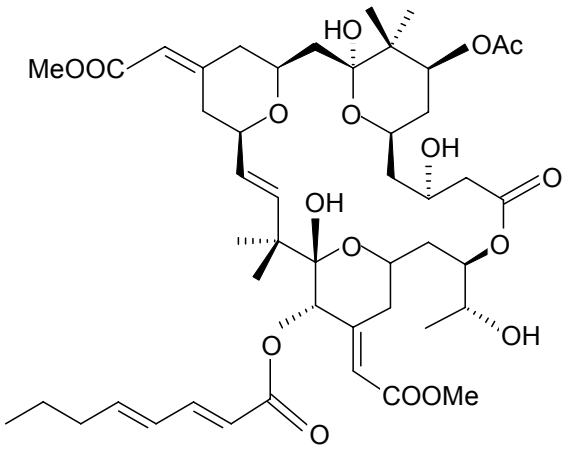


(31a)



(31b)

Bryostatins are a group of novel macrocyclic lactones derived from *Bugula neritina*, a marine invertebrate. More than 13 different but structurally closely related compounds have been isolated [36,37] and a variety of synthetic analogs have been prepared [38-41].



(32)

Studies showed that bryostatin 1 **(32)** has significant antineoplastic activity against lymphomas, leukaemia, melanoma and breast cancer cell lines. Preliminary results of phase I studies had also supported these observations [42]. It has subsequently been investigated extensively in phase II clinical trials as a single agent. The status of some cytotoxic natural products obtained from marine sources in clinical and preclinical trials are listed in Table 3.

Table 3
Status of some cytotoxic natural products obtained from marine sources in clinical and preclinical trials [43-71].

Name	Source	Status
Bryostatin 1 **(32)**	*Bugula neritina*	Phase II
Dolastatin 10	*Dolabella auricularia*	Phase I/II
Thiocoraline	*Micromonospora Marina*	Preclinical
Kahalalide F	*Eylsia rufescens/Bryopsis sp*.	Phase II
Bengamide derivative	*Jaspis sp*.	PhaseI
Squalamine	*Squalus acanthias*	Phase II
KRN-7000	*Agelas mauritianus*	Phase I
Cryptophycins	*Nostoc sp*.	Phase I
E-7389	*Lissodendoryx sp*	Phase I
Salicylihalimides A	*Haliclona sp*.	Preclinical
Aplidine	*Aplidium albicans*	Phase II
Ecteinascidin 743	*Ecteinascidia turbinate*	Phase II/III
ES-285 (spisulosine)	*Spisula polynyma*	Phase I
Dictyodendrins	*Dictyodendrilla verongiformis*	Preclinical
Variolins	*Kirpatrickia variolosa*	Preclinical
Arnastarin	*Dysidea arenaria*	Phase I
Peloruside A	*Mycale hentscheli*	Preclinical

Microbial sources

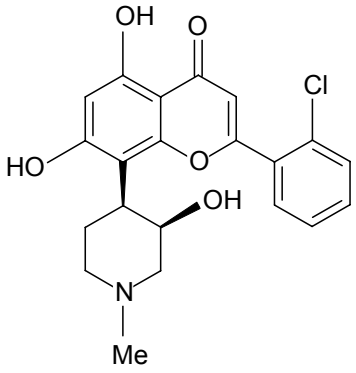
Clinically useful anticancer microbial agents include daunomycin-related agents, daunomycin itself, doxorubicin (Adriamycin®), idarubicin, epirubicin, the glycopeptidic bleomycins A_2 and B_2, mitomycin C, mithramycin etc. All were isolated from various *Streptomyces* species. Other clinically active agents isolated from *Sreptomyces* include streptozocin and deoxycoformycin.

Microbial metabolites in past and present clinical trials include acivicin, aclacinomycin, deoxyspergualin, echinomycin, fostriecin, 17-hydroxystaurosporine (UCN-01), menogaril, porfiromycin, quinocarmycin and rhizoxin. Microbial products predominate amongst the agents under development by the Division of Cancer Treatment and Diagnosis of the NCI. These include FR-901228, a novel bicyclic depsipeptide isolated from a *Chromobacterium violaceum* strain and derivatives of quinocarmycin (DX-52-1), spicamycin (KRN-500), tetracycline (COL-3), rapamycin and rebeccamycin. Recent exciting discoveries are the epothilones isolated from myxobacteria [72]. This class of compounds has been shown to act by a similar mechanism of action as paclitaxel and could complement the taxanes as chemotherapeutic agents.

Natural products for the inhibition of protein kinases

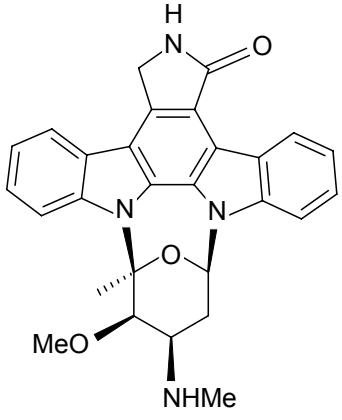
Recently, the emphasis in cancer drug discovery has focused on novel compounds that target protein kinases. Compounds that target the inhibition of Cyclin Dependent Kinases (CDKs) are particularly attractive from the perspective of anticancer drug design.

Flavopiridol **(33)** is a semi-synthetic flavone derived from rohitukine, an alkaloid isolated from a plant indigenous to India, *Dysoxylum binectarieferum*. The National Cancer Institute (NCI) identified flavopiridol as a potent antiproliferative compound against all 60 human cell lines [73,74]. It inhibits CDK1, 2 and 4.



(33)

Staurosporine **(34)** is a natural product originally isolated from the bacterium *Streptomyces staurosporeus* and found to be a nonspecific inhibitor of many kinases causing cell cycle arrest in G_1 and G_2 phases in different cell types. It has significant toxicities, which precluded its clinical development.



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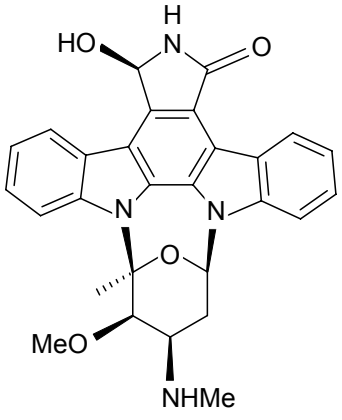


(35)

UCN-01 (17-hydroxystaurosporine), **(35)** isolated from a *Streptomyces* species, is a naturally occurring derivative of staurosporine. It is relatively more selective for CDKs (CDK1, 2 and 4) and PKC (Protein Kinase C), although many kinases are also affected. It possesses significant antiproliferative activity in several human tumour cell lines and tolerable toxicity profile.

1.1.4 Screening for bioactive metabolites

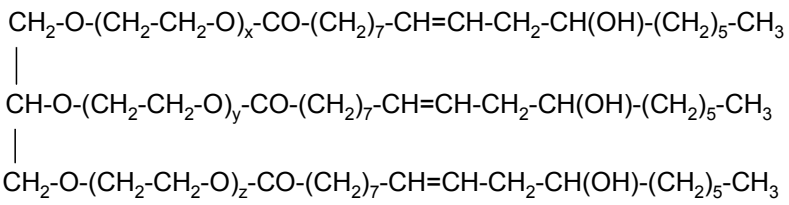
The screening of organisms for bioactive metabolites requires a multidisciplinary approach**,** involving chemists, pharmacologists, biochemists, etc. The rapid progress in the field of bioactive metabolites is due in large part to the utilisation of bioassay-guided fractionation techniques. Many factors can complicate matters when using bioassay-guided fractionation. For example, in the isolation of leurosine, a dimeric indole alkaloid related to vinblastine, the crude alkaloid fraction showed no *in vitro* activity against the P1534 leukaemia system, but the pure alkaloid showed pronounced cytotoxicity in the same test. On the other hand, fractionation of the extracts from *Combretum caffrum* was complicated by loss of *in vivo* activity. Modification of the extraction of a new batch of the plant eventually led to the isolation of the antineoplastic combretastatin A-1.

Cytotoxic assay (MTT Assay)

Cytotoxic assays are widely used particularly in the field of new drug development. Use of a tetrazolium dye [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (MTT)] to measure the cell number was first reported in the early 1980's [75]. National Cancer Institute (NCI) evaluated MTT-dye reduction as a possible endpoint in a rapid screening assay [76]. Cells in exponential phase of growth are exposed to a cytotoxic

drug. Surviving cell numbers are then determined indirectly by MTT dye reduction. MTT is a yellow water soluble tetrazolium dye that is reduced, by live but not dead cells, to a purple formazan product that is insoluble in aqueous solutions [77]. The amount of MTT-formazan produced can be determined spectrophotometrically once solubilised in a suitable solvent.

The compound obtained from either natural sources or entirely synthetic routes is screened for cytotoxic activity *in vitro* and *in vivo*. Once the screening process has been completed, the compound should be properly pharmaceutically-formulated and subsequently subjected to human phase I, II and III studies [78,79]. For initial screening, dimethylsulfoxide (DMSO) has been used as a suitable solvent with the restriction that it is applicable only to hydrophilic compounds. After the identification of paclitaxel (Trade name: Taxol®) as the active ingredient in the crude extract of the bark of the Pacific Yew tree, *Taxus brevifolia,* against several murine tumours [80], further development was suspended for more than a decade due to problems associated with the solubilisation of the compound. Paclitaxel is highly hydrophobic in nature and is insoluble in DMSO. Cremophor EL (CrEL) **(36)** has been suggested as a solvent for hydrophobic compounds. CrEL is produced by the reaction of castor oil with ethylene oxide at a molar ratio of 1:35 [81]. Castor oil is a colourless or pale yellow oil obtained from the seeds of *Ricinus communis*, with an extremely high viscosity and consists of highly variable compositions, with the major component identified as oxylated triglycerides of ricinoleic acid (i.e.polyoxyethyleneglycerol triricinoleate).



(x+y+z ~ 35)

(36)

CrEL is now being used as a vehicle for the solubilisation of a wide variety of hydrophobic drugs, including anaesthetics, photosensitisers, sedatives, immuno-suppressive agents and anticancer drugs [82]. In contrast to earlier reports, CrEL is not an inert vehicle, but exerts a range of biological effects, some of which have important clinical implications. CrEL alone has shown cytotoxic effect at high concentrations *in vitro* [83,84].

1.1.5 Median Lethal Dosage (LD_{50})

Investigation of the acute toxicity is the first step in the toxicological investigations of an unknown substance. The index of the acute toxicity is the Median Lethal Dosage (LD_{50}). LD_{50} is a dosage that will cause a death of fifty percent of the experimental animals. However, LD_{50} should not be regarded as a biological constant, since different results are obtained on repetition or when the determinations are carried out in different laboratories [83,85]. To determine whether a chemical substance or an extract was very toxic, toxic, and less toxic or whether the toxic effects are of no significance, it is desirable to have a test. Thus, a means of determining the likelihood of death, as exactly as possible, was sought [86]. Moreover, even if LD_{50} could be measured exactly and reproducibly, the knowledge of its precise numerical value would barely be of practical importance,

because an extrapolation from the experimental animals to humans is hardly possible. The acute toxicity should be tested in two steps.

1. Different doses are administered to animals to establish the toxic effects.
2. Based on the result, further specific doses are administered to calculate LD_{50}.

The use of single animal in each group can lead to false assessment when, by chance, the animal in the non-toxic range dies or in the toxic range survives. For this reason it is proposed that at least three animals in each group should be used to determine the toxic range. The result of this test is used as a basis for selecting the subsequent doses. The following assumptions are made with respect to the subsequent dosage schedules.

1. Substances with LD_{50} values less than 1mg/kg (amount of substance/body weight) are very toxic; it is not so important to calculate the LD_{50} exactly.
2. LD_{50} values greater than 5,000 mg/kg are of no practical interest.
3. An approximate figure for the LD_{50} is usually adequate to estimate the risk of acute intoxication.

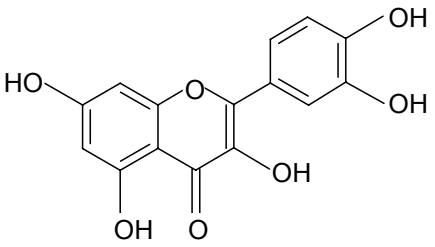
Chapter 2

Isolation, characterisation and evaluation of *Polygonum bistorta* (Polygonaceae) for anticancer potential using selected cancer cell lines

2.1.0 Introduction to Polygonaceae Family

The Polygonaceae are herbs, shrubs or trees comprising about 30 genera and 1,000 species. The leaves are simple, alternate or rarely opposite and usually stipulate. Many species have ocreas at their stem's nodes. Ocrea is a sheath developed from stipules at the attaching leave's bases. Some ocreas have mildly hairy fringes. The infloresences (flower heads) are conspicuous, but the actual flowers are usually tiny and with no petals. The flowers are actinomorphic, usually small and are bisexual or occasionally unisexual.

Many compounds have been reported from Polygonaceae family. Quercetin **(37)**, rhamnetin **(38)** and quercetin-3-O-D-glucopyranoside **(39)** were reported from *Antigonon leptopus* (Mexican creeper) [87,88].



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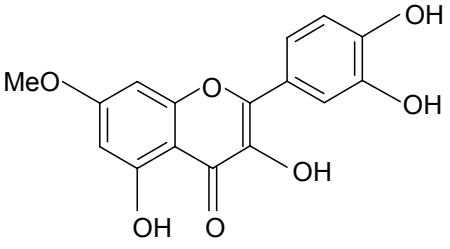

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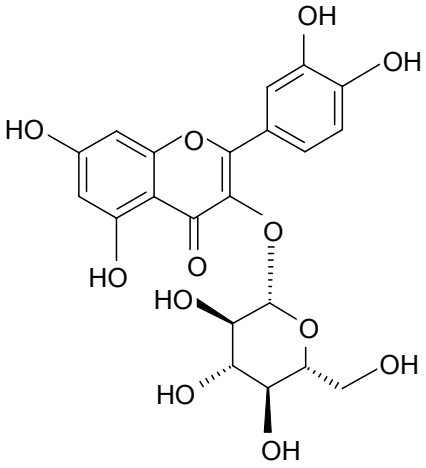
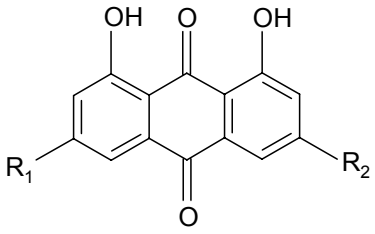


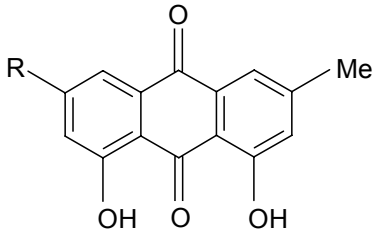
(39)

Emodin **(40)**, rhein **(41)**, chrysophanol **(42)** and physcion **(43)** were isolated from *Coccoloba uvifera* (sea grape) [89].



(40) R_1 = OH; R_2 = Me

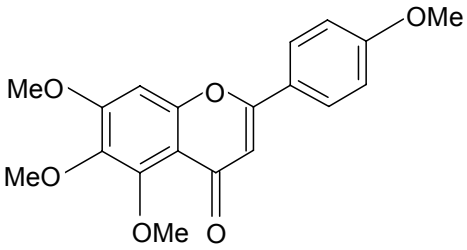
(41) R_1 = H; R_2 = COOH



(42) R = H

(43) R = MeO

5,6,7,4'-Tetramethoxyflavanone **(44)**, 6-methoxyplumbagin **(45)**, oleanolic acid **(46)** and β-sitosterol **(47)** were reported from *Polygonum aviculare* [90]. Danilol **(48)** was reported from *Polygonum newberryi* [91].



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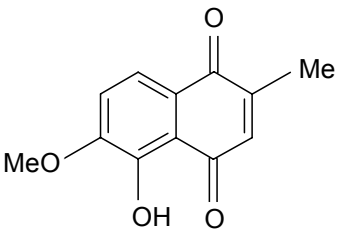

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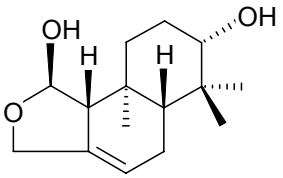

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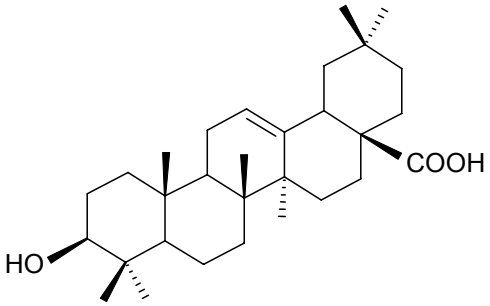

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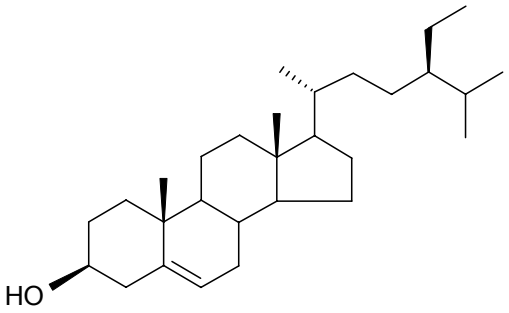
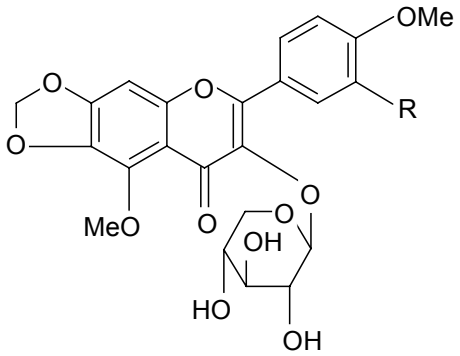


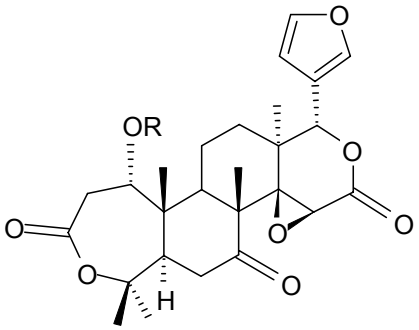
(47)

5,4'-Dimethoxy-6,7-methylenedioxyflavone-3-O-D-xylopyranoside (viviparum A) **(49)** and 3'-hydroxy-5,4'-dimethoxy-6,7-methylenedioxyflavone-3-O-D-xylopyranoside (viviparum B) **(50)** were reported from *Polygonum viviparum* [92].



(49) R = H; **(50)** R = OH

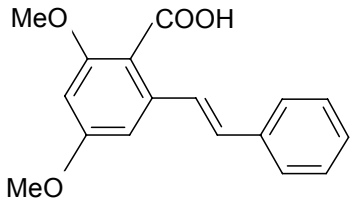
Flavonoid compounds and their glycosides were isolated from the dried leaves of *Polygonum hydropiper* (Laksa leaves) [93]. Limonoids, polygonumin A **(51)** and polygonumin B **(52)**, together with four other limonoids, deacetylnomilin, nomilin, rutaevin and rutaevin acetate were isolated from the whole plant of *Polygonum orientale* [94].



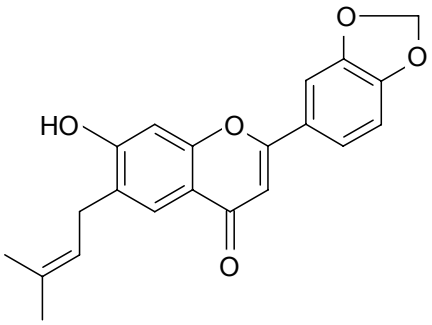
(51) R = (E) - feruloyl

(52) R = (Z) - feruloyl

A stilbene compound, 2-carboxy-3,5-dimethoxy-E-stilbene (persilben) **(53)** was isolated from *Polygonum persicaria* [95]. A flavonoid compound **(54)** was isolated from *Fagopyrum cymosum* [96].

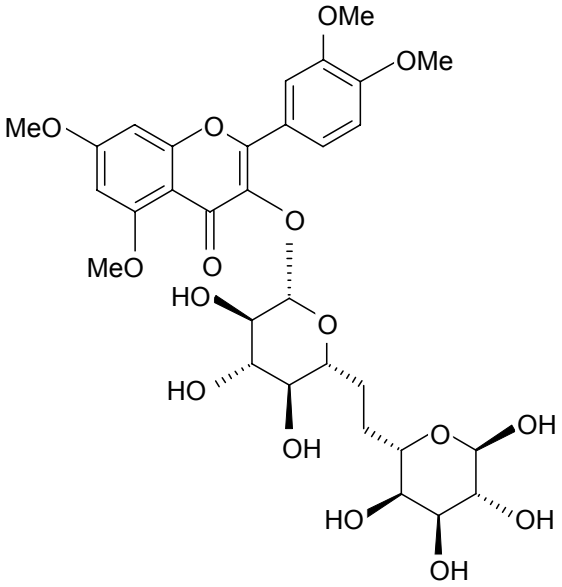


(53)



(54)

A flavonoid compound **(55)** was isolated from *Fagopyrum tataricum* [97]. Diesters of 2, 3-dihydroxyisodrimeninol **(56-59)** and flavonoid compounds were reported from *Polygonum glabrum* [98,99].



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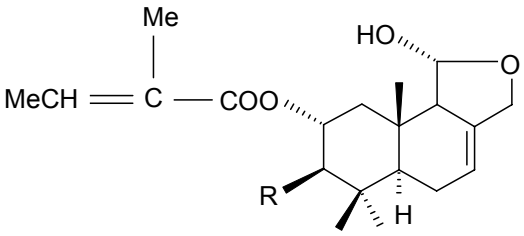
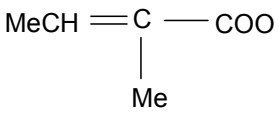
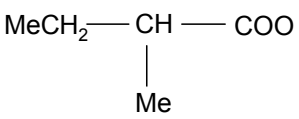


R =



(56)

R =



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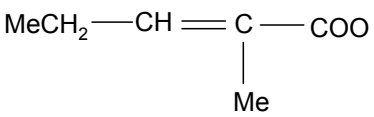

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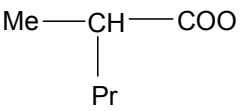
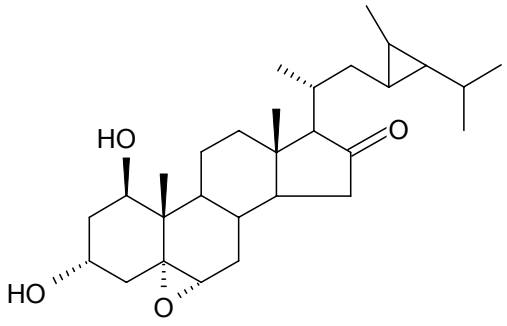


(59)

Steroids, zhonghualiaoine I **(60)**, zhonghualiaoine II **(61)**, cyclobutane compounds **(62, 63)** and a flavanonol **(64)**, were reported together with some known compounds from *Polygonum nodosum* (Chinese name: *Zhonghualiao*) [100,101].



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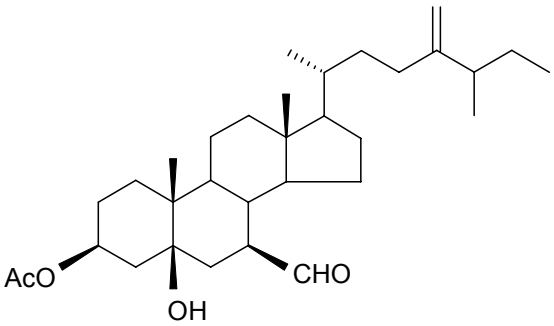

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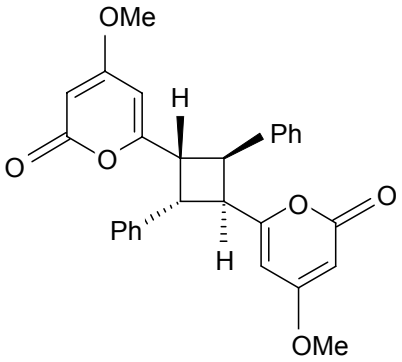

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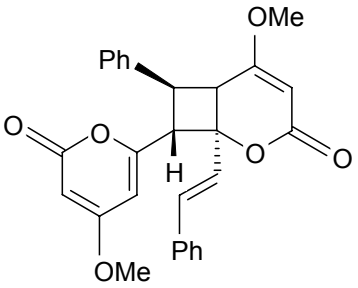

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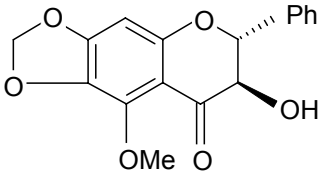
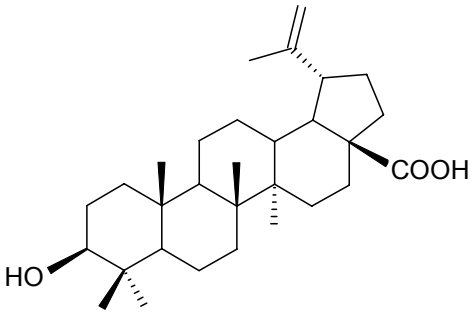


(64)

The flowers of *Polygonum plebejum* contained oleanolic acid **(46)**, betulinic acid **(65)**, 3β-friedelinol **(66)** and β-sitosterol **(47)** [102].



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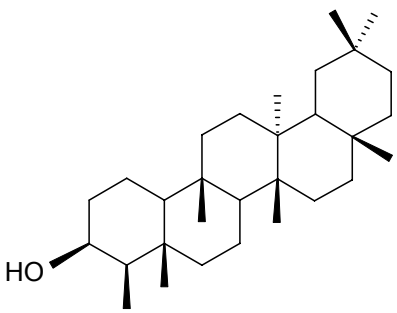
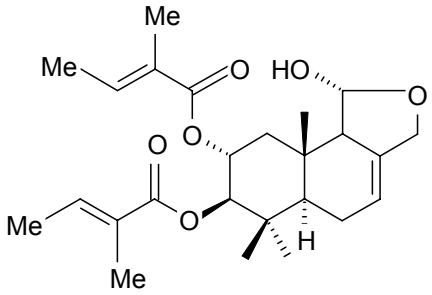


(66)

The sesquiterpenoid, stagninol **(67)** was reported from *Polygonum stagnina* [103].



(67)

Polygonum vaccinifolium, *Rheum palmatum*, *Rheum sp*., *Rumex alpinus*, *Rheum rhaponicum*, *Eriogonum inflatum* (Desert trumpets), *Eriogonum umbellatum* (Sulfur flower), *Pterogonum alatum* (Winged buckwheat), *Stenogonum salsuginosum*, *Polygonum heterophyllum*, *Coccoloba pubescens*, *Polygonum amphibium*, *Lapathum alpinum*, *Lapathum aquaticum*, *Lapathum sylvestris*, *Polygonum divaricatum*, *Polygonum convolvulus* (Black bindweed), *Rumex albescens*, *Fagopyrum esculentum*, *Polygonum aubertii*, *Polygonum cuspidatum*, *Polygonum maritimum*, *Polygonum thumbergii*, *Eriogonum compositum* (Northern buckwheat), *Eriogonum sp*., (Wild buckwheat), *Homalocladium platycladum* (Ribbon bush), *Muehlenbeckia axillaries* etc. are some other species that belong to the Polygonaceae family.

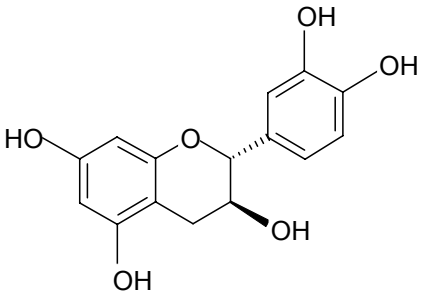
2.1.1 Introduction to *Polygonum bistorta*

Polygonum bistorta, commonly known as Bistort or Snakeroot, belongs to the Polygonaceae family. It is one of the strongest herb astringents. It is excellent for the treatment of cholera, diarrhea and dysentery. When applied to a wound, it can stop bleeding. It has been used in traditional Chinese medicine as a remedy for smallpox, measles, pimples, jaundice, insect stings, snake bites and expelling worms. It is also taken

for the treatment of a wide range of complaints including cystitis, irritable bowel syndrome, peptic ulcers and ulcerative colitis.

2.1.2 Previously reported chemical constituents from *P. bistorta*

Catechin **(68)**, epicatechin **(69)**, flavonoids **(37, 38, 70-75)**, aromatic compounds **(31, 76-85)**, flavonoid glycosides **(39, 86-90)**, sitosterols and a few pentacyclic triterpenoids **(91, 92)** were reported from this plant.



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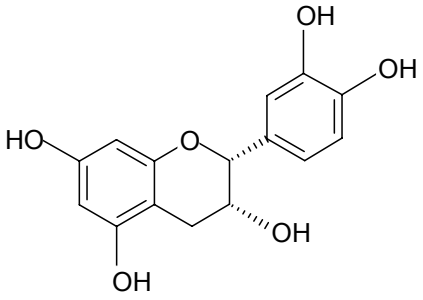

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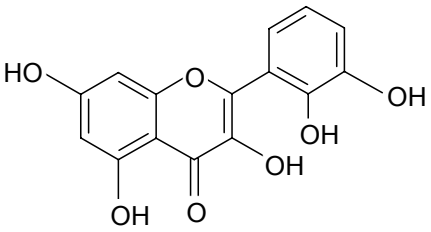

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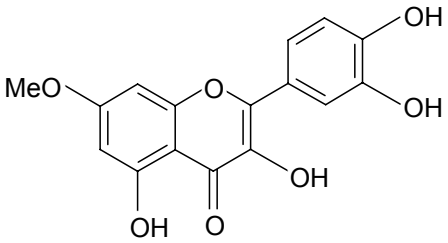

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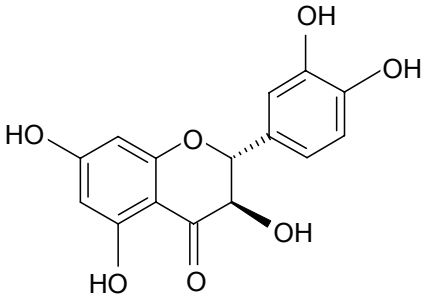

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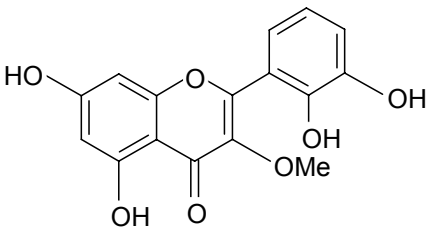

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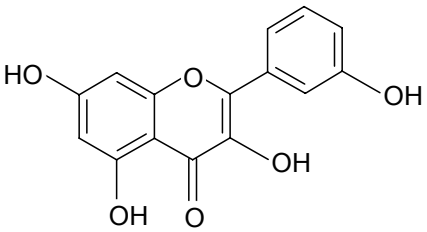

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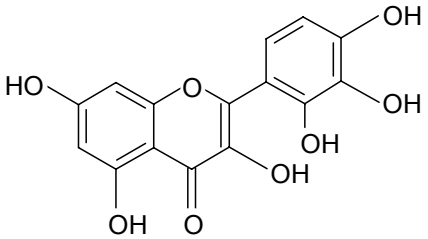

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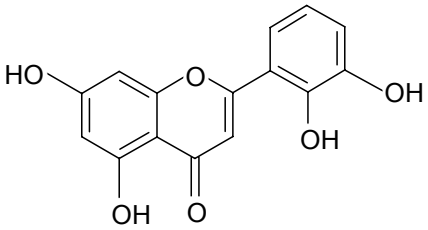

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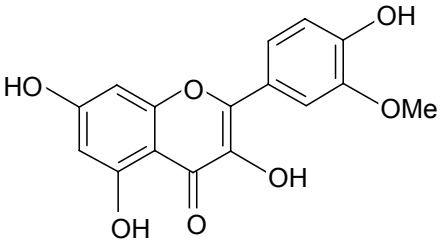

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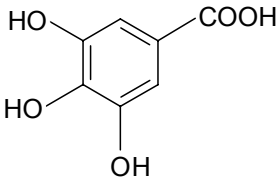

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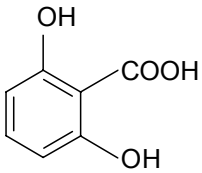

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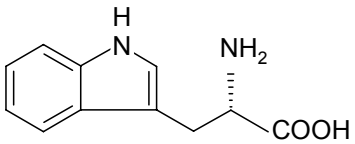

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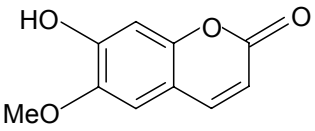

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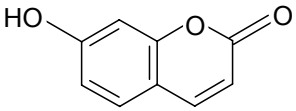

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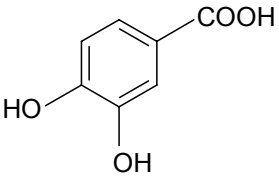

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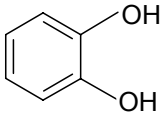

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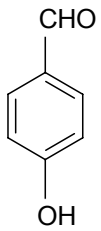

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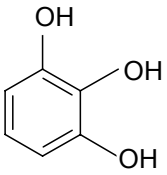

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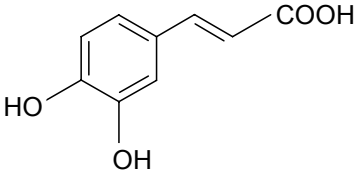

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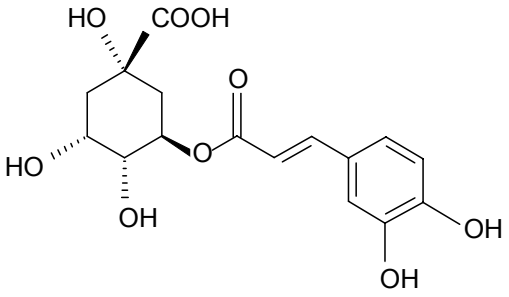

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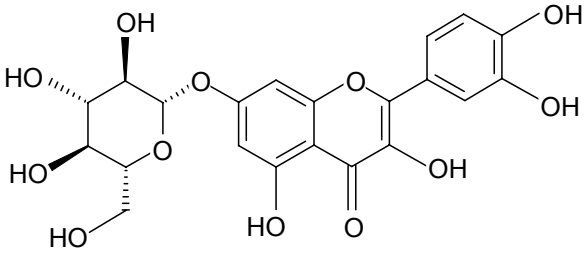

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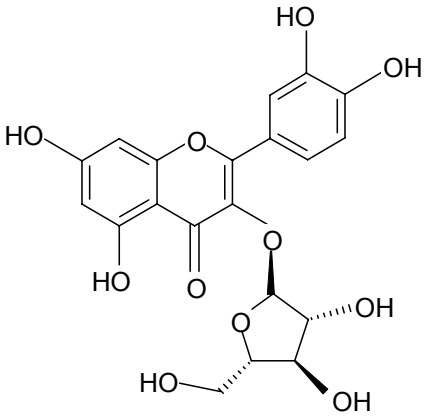

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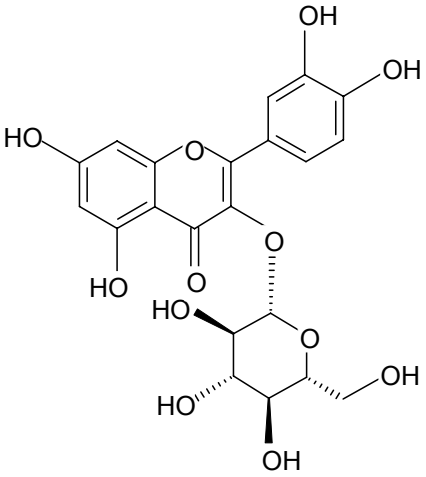

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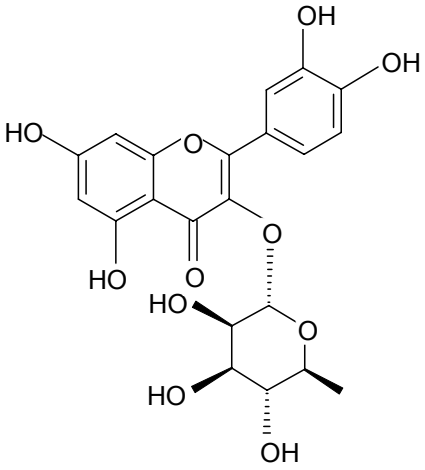

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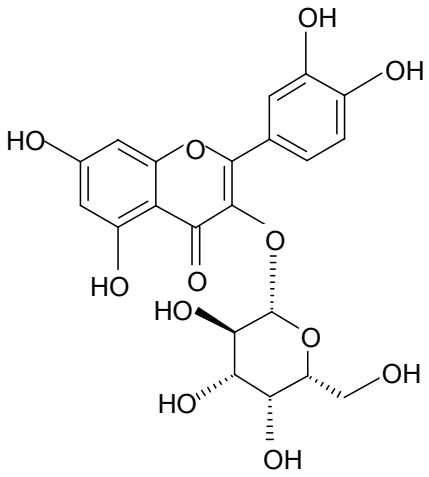

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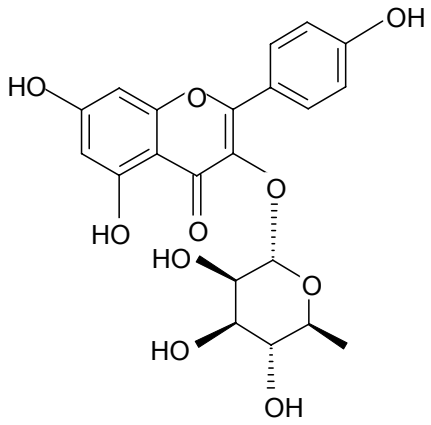

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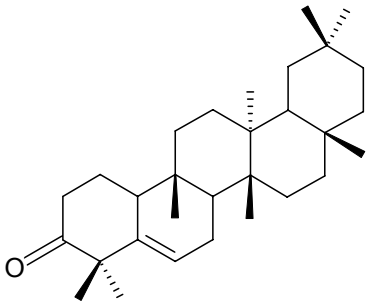

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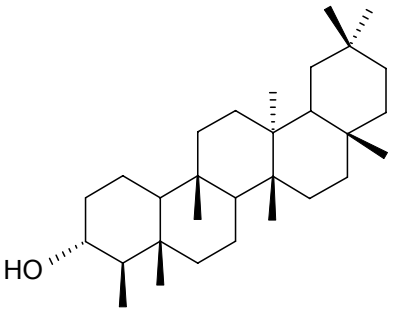


(92)

As far as the biological acitivities of this plant were concerned, it was reported that the ethanolic extract caused strong anti-inflammatory effect [104]; 5-glutinen-3-one (alnusenone) **(91)** and friedelinol **(92)** were identified as active constituents for such effect [105]. It was also reported that the aqueous extract strongly inhibits the mutagenicity of Trp-P-1 [106].

2.2.0 Results and Discussion

2.2.1 Evaluation of *P. bistorta* for anticancer potential using selected cancer cell lines

We have evaluated the chloroform and hexane fractions and their sub-fractions of rhizomes of *P. bistorta* for their anticancer potential using selected cancer cell lines in culture. Both the chloroform and hexane fractions and a few of their sub-fractions showed moderate to very good activity against P388, HL60 and LL2 cancer cell lines. The results are summarised in Tables 4 and 5.

Table 4
Cytotoxicity (IC_{50} values) of hexane fraction and its sub-fractions (A-E) against murine and human cancer cell lines in culture.

	Cancer cell lines and IC_{50} values					
Fractions	P388	HepG2	J82	HL60	MCF7	LL2
Hexane fraction	<10	80.6	>100	17.8	72.0	62.4
A	60.8	>100	80.8	72.0	>100	>100
B	40.8	74.3	64.8	91.2	65.6	>100
C	16.2	63.6	82.1	18.6	64.8	<10
D	50.2	52.3	>100	78.8	40.8	75.0
E	27.6	70.5	>100	63.6	92.6	19.4

P338: Murine lymphocytic leukaemia; HL60: Human leukaemia; MCF7: Human breast cancer; LL2: Lewis lung carcinoma; HepG2: Hepatocellular carcinoma; J82: Bladder transitional carcinoma; IC_{50} values are expressed in µg/mL; IC_{50} values less than 30 µg/mL are considered active; NT-Not tested; No. of replicates, n = 8 for each concentration; Hexane fraction was obtained from the first batch of plant materials (600g); Sub-fractions A-E, were obtained from the second batch of plant materials (12kg); 6-Mercaptopurine (6MP) was used as positive control for P388 cancer cell lines; Doxorubicin was used as positive control for MCF7 cancer cell lines. Positive controls were not maintained throughout experiments. The experiments were conducted over a period of time and the availability of cell line concentrations varied at each time; positive controls were omitted when there was shortage. The 50% inhibitory concentrations (IC_{50}) were measured by MTT assay after 3-days culture.

Table 5
Cytotoxicity (IC_{50} values) of chloroform fraction and its sub-fractions against murine and human cancer cell lines in culture.

	Cancer cell lines and IC_{50} values						
Fractions	P388	HepG2	J82	HL60	MCF7	LL2	WEHI164
Chloroform fraction	<10	78.2	>100	<10	66.6	52.3	NT
F	65.4	>100	70.4	76.0	>100	90.8	NT
G	51.8	90.4	>100	19.4	>100	<10	NT
H	65.4	80.2	92.0	80.8	35.8	70.0	NT
*Pure compound	>221	NT	NT	>221	NT	>221	>221

P338: Murine lymphocytic leukaemia; HL60: Human leukaemia; MCF7: Human breast cancer; LL2: Lewis lung carcinoma; HepG2: Hepatocellular carcinoma; J82: Bladder transitional carcinoma; WEHI164: Murine fibrosarcoma; IC_{50} values are expressed in µg/mL; IC_{50} values less than 30 µg/mL are considered active; *Pure compound = 24(E)-ethylidenecycloartanone **(93)**; For the pure compound, 24(E)-ethylidenecycloartanone **(93)**, the IC_{50} values are expressed in µM; NT-Not tested; No. of replicates, n = 8 for each concentration; Chlorofrom fraction was obtained from the first batch of plant materials (600g); Sub-fractions F-H, were obtained from the second batch of plant materials (12kg); 6-Mercaptopurine (6MP) was used as positive control for P388 cancer cell lines; Doxorubicin was used as positive control for MCF7 cancer cell lines. Positive controls were not maintained throughout experiments. The experiments were conducted over a period of time and the availability of cell line concentrations varied at each time; positive controls were omitted when there was shortage. The 50% inhibitory concentrations (IC_{50}) were measured by MTT assay after 3-days culture.

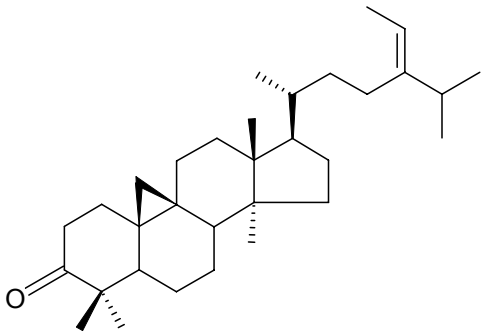
The hexane fraction of *P. bistorta* (obtained from the first batch of plant materials, 600g) was screened against LL2 (Murine Lewis lung carcinoma), HL60 (Human leukaemia), P388 (Murine lymphocytic leukeamia), MCF7 (Human breast cancer), HepG2 (Hepatocellular carcinoma) and J82 (Bladder transitional carcinoma) cancer cell lines. The IC_{50} values are given in Table 4. The hexane fraction showed very good activity against P388 cancer cell lines with IC_{50} value <10 µg/mL, the lowest concentration tested

and good activity against HL60 with IC_{50} value of 17.8 μg/mL but was inactive against all other cell lines screened; the IC_{50} values were 62.4, 72.0, 80.6 and >100 μg/mL, respectively against LL2, MCF7, HepG2 and J82 cancer cell lines. Owing to the promising activity of the hexane extract against P388 and HL60 cancer cell lines, we repeated the experiments with 12 kg of plant materials. This time the hexane fraction was not screened again for its cytotoxicity but chromatographed over a silica gel column using hexane and eluted with solvents of increasing polarity. Five major sub-fractions, (A-E), three minor fractions and one more fraction (A1) of about 90 % purity were obtained. The five major fractions (A-E) were screened against the above mentioned cancer cell lines and the results are given in Table 4.

Fraction A showed no promising activity against all the cancer cell lines tested. It was not active even at a concentration of 100 μg/mL against HepG2, MCF7 and LL2 cancer cell lines and the IC_{50} values were 60.8, 72.0 and 80.8 μg/mL respectively, against P388, HL60 and J82 cancer cell lines. Fraction B showed weak activity against P388 with IC_{50} value of 40.8 μg/mL and was inactive against all other cancer cell lines tested with IC_{50} values of 65.6, 74.3, 64.8, 91.2, and > 100 μg/mL, respectively against MCF7, HepG2, J82, HL60 and LL2 cancer cell lines. Fraction C showed very good activity against LL2 with IC_{50} value < 10 μg/mL, the lowest concentration tested and good activity against P388 and HL60 with IC_{50} values of 16.2 and 18.6 μg /mL, respectively. But it was inactive against three other cell lines with the IC_{50} values of 82.1, 63.6 and 64.8 μg/mL, respectively against J82, HepG2 and MCF7 cancer cell lines. Fraction D showed no activity against all the cell lines tested but the IC_{50} values were 40.8, 50.2 and 52.3 μg/

mL, respectively against MCF7, P388 and HepG2 cancer cell lines; the IC_{50} value was >75 μg/mL against both HL60 and LL2, while it was >100 μg/mL against J82 cancer cell lines. Fraction E showed good activity against LL2 with IC_{50} value of 9.4 μg/mL and moderate activity against P388 with IC_{50} value 27.6 μg/mL; the IC_{50} values were 63.6, 70.5 and 92.6 μg/mL respectively against HL60, HepG2 and MCF7 cancer cell lines; while it was >100 μg/mL against J82 cancer cell lines.

Although Fraction A showed no activity against all the cancer cell lines tested, purification of this fraction led to isolation of a new triterpenoid compound of the cycloartane-type, 24(E)-ethylidenecycloartanone **(93)**. Purification of Fractions B, C and D gave the known compounds friedelin **(94)**, 3β-friedelinol **(66)** and β-sitosterol **(47),** respectively. Fraction E gave cycloartane-3,24-dione **(95)**, a rarely encountered compound. Fraction (A1), obtained in about 90 % purity, was not screened for its cytotoxicity and on recrystalisation with acetone yielded the pure compound, 24(E)-ethylidenecycloartanone **(93)**. The minor fractions were also not screened for their cytotoxicity due to their small quantities but preparative TLC led to isolation of the known compounds, β-sitosterol **(47)** and γ-sitosterol **(96)** and again the new compound 24(E)-ethylidenecycloartanone **(93)**, one compound from each fraction.



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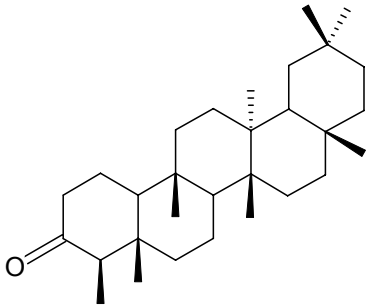

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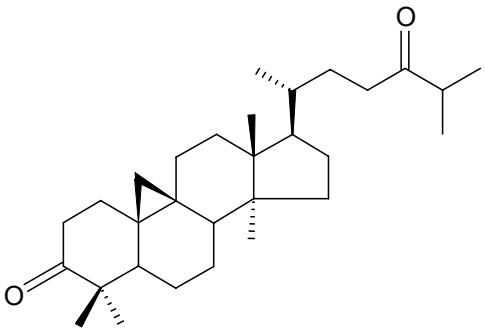

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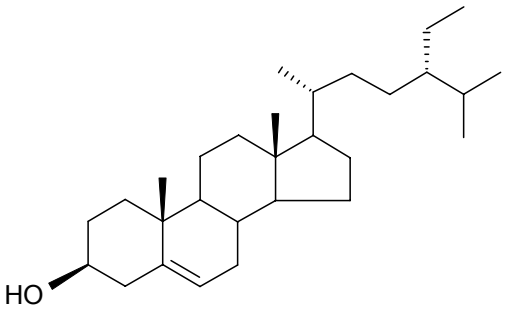

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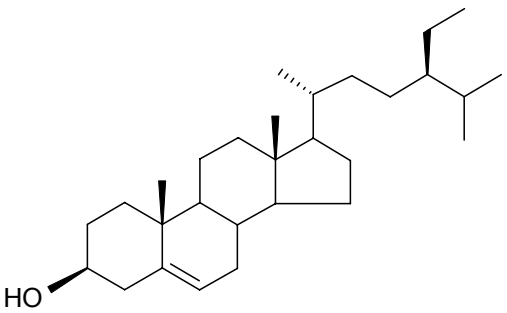

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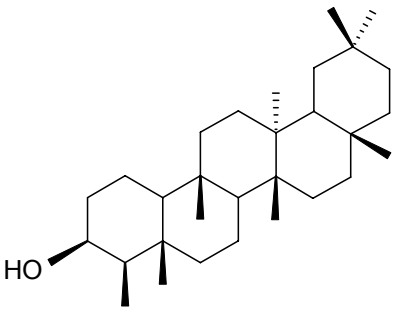


(66)

Cycloartane-3,24-dione **(95)** was not screened for its cytotoxicity since it was isolated in a small quantity. The known compounds friedelin **(94)**, 3β-friedelinol **(66)**, β-sitosterol **(47)** and γ-sitosterol **(96)** were also not tested for their cytotoxicity since these compounds are very common natural products and there have been several reports citing

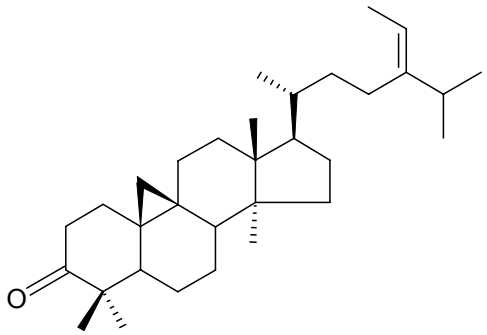
their cytotoxicity in the literature. Of the isolated pure compounds, only the new compound, 24(E)-ethylidenecycloartanone **(93)**, was screened for its cytotoxicity against P388, HL60, LL2 and WEHI164 cancer cell lines and the results are given in Table 5.

The chloroform fraction (obtained from 600g of plant material) was screened against the above mentioned cancer cell lines. It showed very good activity against both P388 and HL60 cancer cell lines with IC_{50} value < 10 μg/mL, the lowest concentration tested, but was inactive against all other cancer cell lines tested. It showed the IC_{50} values of 52.3, 66.6 and 78.2 μg/mL, respectively, against LL2, MCF7 and HepG2 cancer cell lines; while it was > 100 μg/mL against J82 cancer cell lines. This chloroform fraction (obtained from 600g plant material) was chromatographed over silica gel using hexane and eluted with solvents of increasing polarity. Further purification of a major fraction by HPLC (hexane/chloroform, 1:9) followed by preparative TLC (chloroform/methanol, 9:1) afforded 24-methylenecycloartanone **(97)** (7 mg) [107,108].

The chloroform fraction obtained from the second batch of plant material (12 kg), was not screened again for its cytotoxicity, but was chromatographed. Three major fractions F, G and H and four minor fractions were obtained. The major fractions, F-H were screened for their cytotoxicity against the above mentioned cancer cell lines. Fraction F showed no activity against MCF7, HepG2, P388, J82, HL60 and LL2 cancer cell lines. The IC_{50} value was found to be >100 μg/mL on both MCF7 and HepG2 cancer cell lines; while it was 65.4, 70.4, 76.0 and 90.8 μg/mL, respectively against P388, J82, HL60 and LL2 cancer cell lines. Fraction G showed very good activity against LL2 with IC_{50} value < 10

μg/mL and good activity against HL60 with IC_{50} value of 19.4 μg/mL, but was inactive against all other cell lines; the IC_{50} values were 51.8 and 90.4 μg/mL against P388 and HepG2 cancer cell lines and it was > 100 μg/mL against both MCF7 and J82 cancer cell lines. Fraction H showed borderline activity against MCF7 with IC_{50} value of 35.8 μg/mL but was inactive against all other cancer cell lines tested; the IC_{50} values were 65.4, 70.0, 80.2, 80.8 and 92.0 μg/mL, respectively against P388, LL2, HepG2, HL60 and J82 cancer cell lines. The minor fractions were not screened for their cytotoxicity since they were obtained in small quantities.

The new compound, 24(E)-ethylidenecycloartanone **(93)**, was isolated from Fraction F which was already isolated from the hexane sub-fractions. Another new compound, 24(E)-ethylidenecycloartan-3α-ol **(98)** was isolated from Fraction G. It was already discussed that Fraction G, showed promising activity against LL2 and HL60 cancer cell lines with IC_{50} values of <10 μg/mL and 19.4 μg/mL, respectively. However, this compound **(98)** was not tested for cytotoxicity since it was obtained in small quantity. A known compound, β-sitosterol **(47)** was isolated from Fraction H. The known compound, β-sitosterol **(47)** and 24(E)-ethylidenecycloartanone **(93)** were again isolated from two minor fractions, one compound from each fraction. From the two other minor fractions, a new compound, 24,31-epoxy-24-ethylcycloartan-3α-ol **(99)** and the known compound β-sitosterone **(100)**, were isolated, one compound from each fraction. These compounds could not be tested for their cytotoxicity due to their poor yield.



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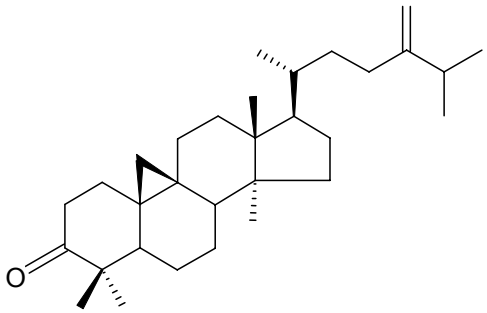

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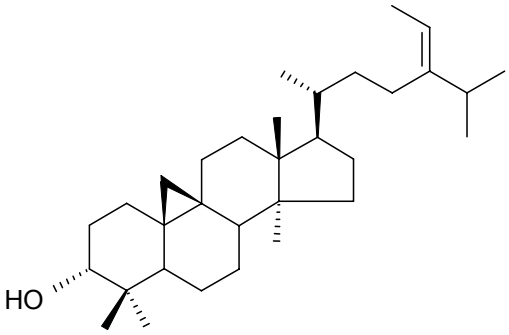

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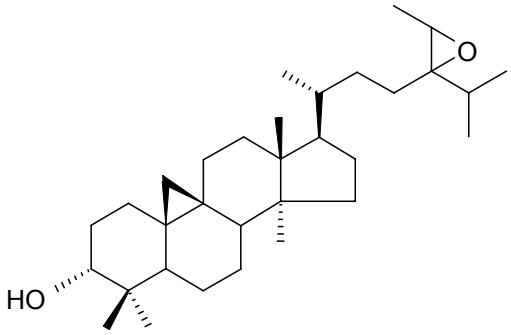

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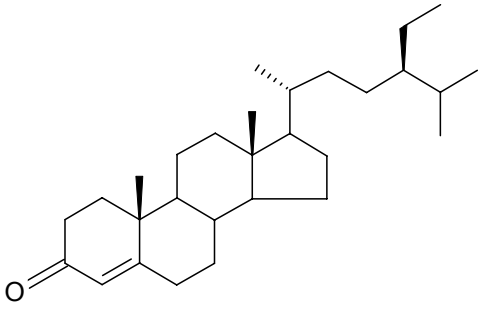

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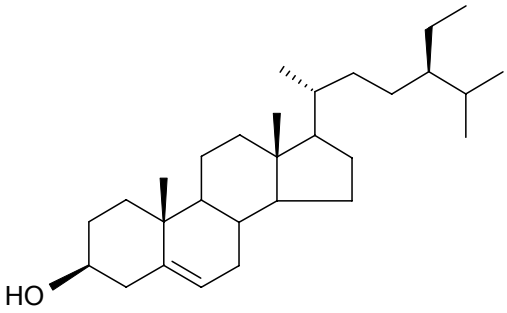


(47)

Although 3β-friedelinol **(66)** was isolated from the active fraction C, our literature search revealed that it was not only inactive against P388 but also A549, MCF7, HT29 and KB cancer cell lines [109]. Friedelin **(94)** also showed no activity against P388, A549, MCF7, HT29 and KB cancer cell lines [109]. It was reported that friedelin **(94)** was cytotoxic against PC3 and U251 cancer cell lines [110]. At a concentration of 31μM, the

percentage of inhibition caused by friedelin, were 61.9, 25.8 and zero, respectively on PC3, U251 and K562 cancer cell lines [110]. Friedelin **(94)** is inactive against MCF7 (breast cancer), TK10 (renal) UACC62 (melanoma), NCI-H460 (lung cancer) and SF-268 (CNS) cancer cell lines [111].

β-Sitosterol **(47)** is the major phytosterol in higher plants, including fruits and vegetables. This compound has shown potential for prevention and therapy for human cancer. β-Sitosterol **(47)** treatment resulted in the inhibition of HCT116 human colon cancer cell proliferation in a concentration-dependent manner [112]. Breast cancer cell growth was inhibited by 66% after 3 days and by 80% after 5 days with 16 μM β-sitosterol. Supplementation of β-sitosterol **(47)** for 3 days at 16 μM resulted increase in apoptosis in cells. It was reported that β-sitosterol **(47)**, by a still-unknown mechanism, protects from breast cancer by inhibiting growth and stimulating apoptosis [113-115]. This compound effectively inhibits invasion of tumour cells and metastasis [116]. β-Sitosterol **(47)**-treated premalignant and malignant cells, accumulated in the G0/G1 and G2/M phases, respectively [117]. β-Sitosterol **(47)** exhibits a growth suppressing effect against two human cancer cell lines, MCF7 and BT20 [118,119]. β-Sitosterol **(47)** caused a high degree of growth inhibition on Hep-2 and McCoy cells [120]. It showed cytotoxic activity against human nasopharynx epidermoid cells [121].

The methanol-water fraction was not tested for its cytotoxicity. However, purification of one of the eluted fractions afforded β-sitosterol **(47)**.

2.2.2 Characterization of the isolated pure compounds from *P. bistorta*

Our investigations on the chemical constituents of this plant, revealed the presence of a three new and two known cycloartane-type triterpenoids, as mentioned earlier. Other known compounds, β-sitosterol **(47)**, friedelin **(94)**, γ-sitosterol **(96)**, 3β-friedelinol **(66)** and β-sitosterone **(100)** were also isolated. All the cycloartane-type compounds, friedelin **(94)** and β-sitosterone **(100)** were reported for the first time from this plant. The characterisation and/or identification of all these compounds are discussed below.

24(E)-Ethylidenecycloartanone (93)

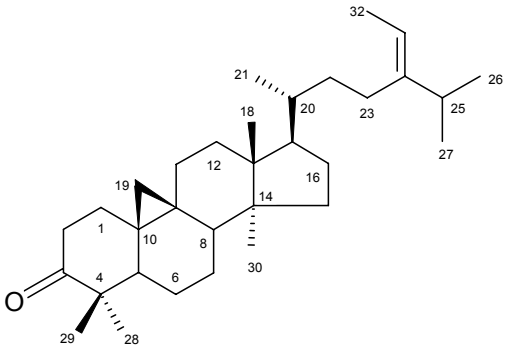
The molecular formula for the compound, 24(E)-ethylidenecycloartanone **(93)** was deduced as $C_{32}H_{52}O$ by the molecular ion peak at *m/z* 452.4019 in the HREIMS and ^{13}C NMR analysis. The IR spectrum showed absorption peak for ketone functionality (ν_{max} 1712 cm^{-1}). Inspection of its ^{13}C and HSQC-DEPT spectra, revealed the presence 32 signals; eight methyl, eleven methylene, six methine and seven quaternary carbons. The ^{1}H NMR spectrum of the compound revealed the presence of a cycloartane type skeleton with typical high-field AB doublets due to the non-equivalent hydrogens [δ 0.80 (d, J = 4.5 Hz, Hα) and 0.59 (d, J = 4.3 Hz, Hβ)] at C-19 in the cyclopropane ring [122]. The presence of a cycloartane skeleton was supported by a fragmentation peak at *m/z* 313 (M-$C_{10}H_{19}$) in the MS, which was due to loss of the side chain [123]. The major MS fragmentation pattern of compound **(93)** is given in Scheme 1.

The ^{1}H-^{1}H COSY spectrum together with HMQC data revealed that the compound **(93)** has seven distinct ^{1}H-^{1}H spin systems: (a) [-CH_2-CH_2-], (b) [>CH-CH_2-CH_2-CH<], (c)

[-CH_2-CH_2-], (d) [-CH_2-CH_2-CH<], (e) [-CH (CH_3)-CH_2-CH_2-], (f) [CH_3-CH-] and (g) [-CH-(CH_3)$_2$]. The ^{1}H-^{1}H spin system (a) [-CH_2-CH_2-] was assigned for C1 and C-2; since in the HMBC spectrum, C-1 showed correlation with H-2 and C-2 showed correlation with H-1. H-1 protons resonated at δ 1.87 (ddd, *J* = 13.8, 4.4, 1.5 Hz, Hα) and δ 1.56 (ddd, *J* = 13.5, 6.4, 2.7 Hz, Hβ). Similarly, H-2 protons resonated at δ 2.72 (ddd, *J* = 13.8, 4.4, 2.7 Hz, Hα) and δ 2.32 (ddd, *J* = 13.5, 6.4, 4.5Hz, Hβ). The multiplicity, ddd, indicated that each proton showed ^3J couplings with vicinal protons (both Hα and Hβ) as well as ^2J coupling with a geminal proton. The large coupling constant value, *J* >13 Hz was due to ^3J *trans* coupling of each proton with its vicinal protons. The C-3 (δ 216.4) showed correlations with methyl protons at H-28 and H-29; similarly C-4 also showed correlations with methyl protons at H-28 and H-29. These correlations as well as the chemical shift value of δ 216.4 (C-3) allowed us to place the carbonyl carbon at C-3 position and the attachment two methyl groups (C-28 and C-29) to the same carbon at C-4. The H-1 and H-11 protons showed correlation with carbon at C-19 (δ 29.4) and C-5 showed correlation with H-19. These correlations allowed us to place a methylene group (belongs to the cyclopropane ring) between C-9 and C-10. Further, H-19 protons are resonated at δ 0.80 (d, *J* = 4.5 Hz, Hα,) and 0.59 (d, *J* = 4.3 Hz, Hβ)]; they are non-equivalent and each proton split in to a doublets due to ^2J geminal couplings with each other. The spin system (b) [>CH-CH_2-CH_2-CH<] was assigned for the positions C-5 to C-8. C-5 showed correlation with H-19 and C-6 showed correlation with H-5. The single proton H-5 resonated at δ 1.73 (dd, *J* = 12.5, 4.5 Hz); the multiplicity, dd and the coupling constant values, *J* = 12.5 and 4.5 Hz were respectively, due the ^3J *trans* and *cis* couplings with H-6 protons. Similarly, C-7 showed correlation with H-6 and C-8 showed

correlation with H-30. H-8 resonated at δ1.61 (dd, J = 12.5, 4.9 Hz) and the multiplicity, dd and the coupling constant values were due to ^{3}J vicinal couplings with H-7 protons. All these correlations are in good agreement with the above spin system. The spin system (c) [-CH_2-CH_2-] was assigned for the positions C-11 and C-12; since C-11 showed correlations with H-8 and H-9 and C-12 showed correlation with H-18. The spin system (d) [-CH_2-CH_2-CH<] was assigned for positions C-15 to C-17. C-15 showed correlations with H-16 and H-30. C-17 showed correlation with H-21. Each proton in this spin system i.e. H-15, H-16 and H-17, exhibited a multiplet due to an availablity of several protons for both ^{3}J and ^{2}J couplings. The spin system (e) [-CH (CH_3)-CH_2-CH_2-] was assigned for the positions C-20 to C-23. C-20 showed correlation with methyl protons at H-21 and this methyl protons (δ 0.92, d, J = 6.5 Hz) split in to a doublets due to ^{3}J coupling with H-20. C-21 showed correlation with H-17 and C-22 showed correlation with H-21. The spin system (f) [CH_3-CH-] was assigned for an ethylidene moiety at C-24. C-31 showed correlations with H-23, H-25 and H-32. Similarly, C-32 showed correlation with H-31. Further, C-24 (δ 145.8) showed correlations with H-31 and H-32. In the 1D proton NMR spectrum, the protons on methyl group at C-32 gave a clearly defined doublets at δ1.60 (d, J = 6.0 Hz), due to ^{3}J coupling with H-32, expected for an ethylidene group. The single proton on C-31 was also clearly evident showing the expected quartet at δ 5.13 (q, J = 6.5 Hz). All these correlations supported the presence of an ethylidene moiety at C-24. Finally, the spin system (g) [-CH-(CH_3)$_2$] was assigned for an isopropyl moiety at C-24. In the HMBC spectrum, C-25 showed correlations with H-26 and H-27 and C-24 showed correlations with H-25, H-26 and H-27. The methyl protons at H-26 and H-27, each split in to a doublets due to ^{3}J coupling with H-25. All these correlations indicated the

presence of an isopropyl moiety also at C-24. Other significant HMBC and ^{1}H-^{1}H COSY correlations are listed in Table 6.



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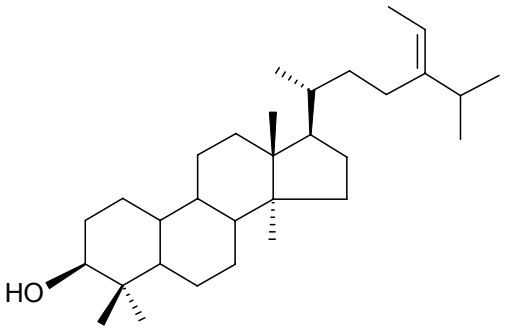
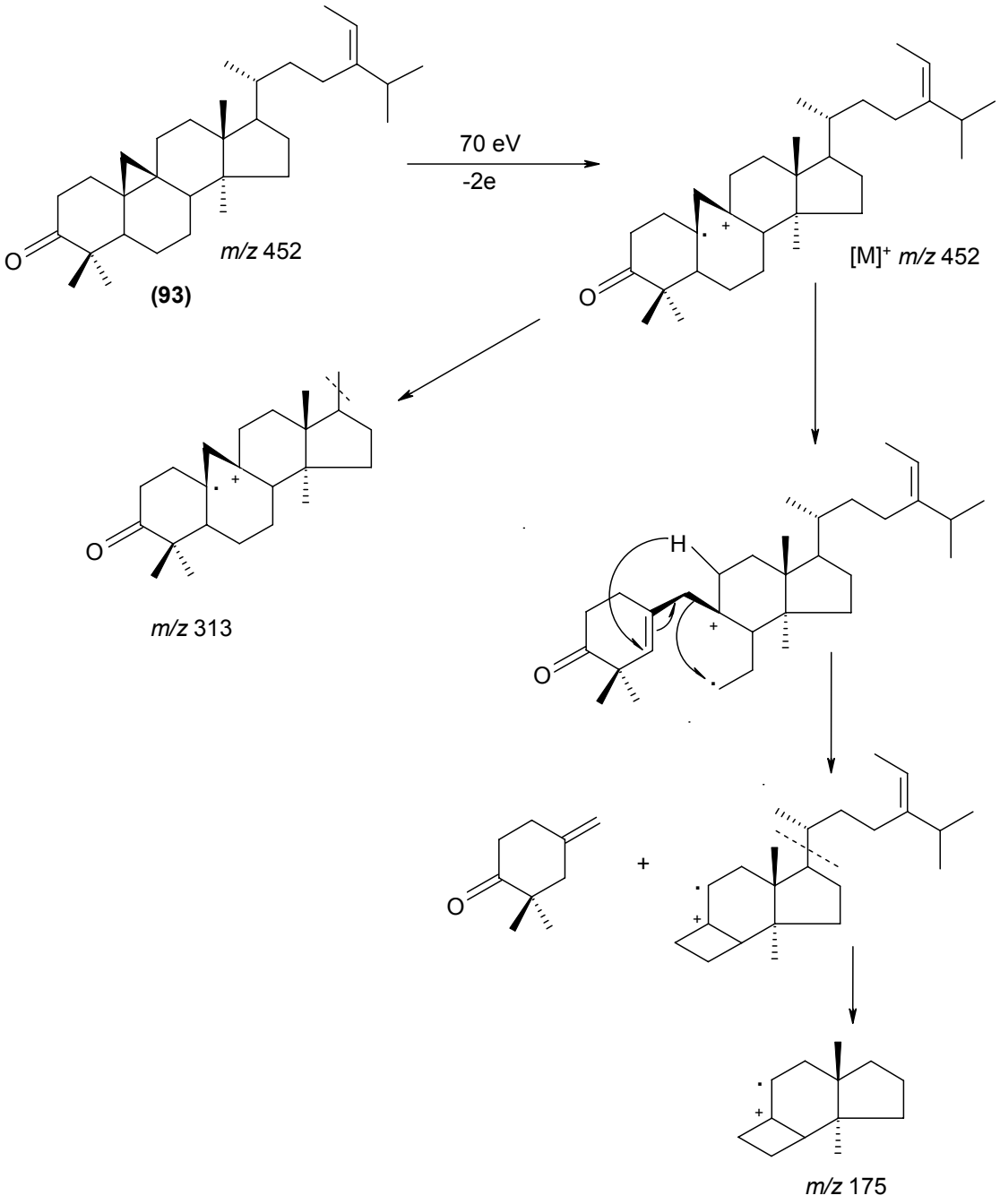


(101)

Both 24(E)-ethylidenecycloartanone **(93)** and fucosterol **(101)** have an identical side chain structure. The ^{1}H NMR chemical shift values for the latter compound at H-32 and H-31 were δ1.57 and 5.17, respectively [124]. Such a correlation also supported the proposed structure as well as used to assign the stereochemistry of 24(E)-ethylidene-cycloartanone **(93)** at C-24, which is similar to that of fucosterol **(101)**. In other words, the double bond has E stereochemistry.



Scheme 1
Major MS fragmentation pattern of 24(E)-ethylidenecycloartanone **(93)**

Table 6
NMR data of 24E-ethylidenecycloartanone **(93)** in $CDCl_3$

Position	^{13}C / DEPT	δ_C (ppm)	δ_H (ppm) *J* in Hz	HMBC (C → H)	^1H-^1H COSY
1	CH_2	33.3	α: 1.87 ddd (13.8, 4.4, 1.5)	2	2
			β: 1.56 ddd (13.5, 6.4, 2.7)		2
2	CH_2	37.4	α: 2.72 ddd (13.5, 4.4, 2.7)	1	1
			β: 2.32 ddd (13.5, 6.4, 4.5)		1
3	C	216.4		2, 28, 29	
4	C	50.2		28, 29	
5	CH	48.4	1.73 dd (12.5, 4.5)	19	6
6	CH_2	21.5	α: 1.57 m; β : 0.96 m	5	5, 7
7	CH_2	25.8	α: 1.39 m; β : 1.16 m	6	6, 8
8	CH	47.8	1.61 dd (12.5, 4.9)	30	7
9	C	21.0		1	
10	C	25.9		6	
11	CH_2	26.7	α: 2.06 m; β: 1.19 m	8, 9	12
12	CH_2	32.7	1.68 m	18	11
13	C	45.3		8, 18	
14	C	48.7		18	
15	CH_2	35.5	1.33 m	16, 30	16
16	CH_2	28.1	α: 1.95 m; β: 1.33 m		15, 17
17	CH	52.2	1.63 m	21	16, 20
18	CH_3	18.0	1.01 s	12, 17	
19	CH_2	29.4	α: 0.80 d (4.5)	1, 11	
			β: 0.59 d (4.3)		
20	CH	36.4	1.41 m	21	17, 22
21	CH_3	18.3	0.92 d (6.5)	17	20
22	CH_2	36.2	α: 1.55 m; β: 1.12 m	21	23
23	CH_2	28.2	α: 2.03 m; β: 1.78 m		22
24	C	145.8		23, 25, 26, 27, 31, 32	
25	CH	28.5	2.84 m	26, 27	26, 27
26	CH_3	21.0	1.00 d (6.4)	25, 27	25
27	CH_3	21.0	1.00 d (6.4)	25, 26	25
28	CH_3	22.1	1.06 s	5, 29	
29	CH_3	20.7	1.11 s	5, 28	
30	CH_3	19.2	1.79 s	8, 15	
31	CH	116.4	5.13 q (6.5)	23, 25, 32	32
32	CH_3	12.7	1.60 d (6.0)	31	31

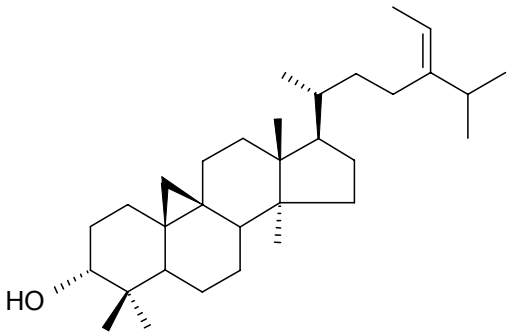
24(E)-Ethylidenecycloartan-3α-ol (98)

24(E)-Ethylidenecycloartan-3α-ol **(98)** was obtained as colourless crystals. It gave a molecular ion peak at *m/z* 454.4175 in the HREIMS, corresponding to the molecular formula $C_{32}H_{54}O$. Inspection of its ^{13}C and HSQC-DEPT spectra, revealed the presence 32 signals; eight methyl, eleven methylene, seven methine and six quaternary carbons. The ^{1}H NMR spectrum indicated the presence of cycloartane-type skeleton with typical high-field AB doublets due to the non-equivalent hydrogens [δ 0.49 (d, J = 4.0 Hz, Hα) and 0.33 (d, J = 4.0 Hz, Hβ)] at C-19 in the cyclopropane ring (Table 7). In the MS spectrum, a fragment peak at *m/z* 315 (M- $C_{10}H_{19}$) was observed, which was an indicative of the presence of a cycloartane skeleton with hydroxyl group in the nucleus and C_{10} side chain. Most of our spectral correlations were similar to 24E-ethylidenecycloartanone **(93)** but a mass unit difference of 2 *amu* was observed in the MS spectrum.

The ^{1}H-^{1}H COSY spectrum together with HMQC data revealed that the compound **(98)** has seven distinct ^{1}H-^{1}H spin systems: (a) [-CH_2-CH_2-CH(O)], (b) [>CH-CH_2-CH_2-CH<], (c) [-CH_2-CH_2-], (d) [-CH_2-CH_2-CH<], (e) [-CH-(CH_3)-CH_2-CH_2-], (f) [CH_3-CH-] and (g) [-CH-(CH_3)$_2$]. The ^{1}H-^{1}H spin system (a) [-CH_2-CH_2-CH (O)-] was assigned for C1, C-2 and C-3. In the HMBC spectrum, C-1 showed correlation with H-2; C-2 showed correlation with H-1 and C-3 (δ 77.0) showed correlations with H-2, H-28 and H-29. Thus, the oxymethine proton was placed in this spin system at C-3. Similarly, C-4 also showed correlations with methyl protons at H-28 and H-29. These correlations allowed us to place the two methyl groups (C-28 and C-29) to the same carbon at C-4. The H-1 and H-11 protons showed correlation with carbon at C-19 (δ 29.7) and C-5 showed

correlation with H-19. These correlations allowed us to place a methylene group (belongs to the cyclopropane ring) between C-9 and C-10. Further, H-19 protons are resonated at δ 0.49 (d, J = 4.0 Hz, Hα,) and 0.33 (d, J = 4.0 Hz, Hβ)]; they are non-equivalent and each proton split in to doublets due to ^{2}J geminal couplings with each other. The spin system (b) [>CH-CH_2-CH_2-CH<] was assigned for the positions C-5 to C-8. C-5 showed correlation with H-19 and C-6 showed correlation with H-5. Similarly, C-7 showed correlation with H-6 and C-8 showed correlations with H-19 and H-30. H-8 resonated at δ1.50 (m) and this multiplet was due to ^{3}J vicinal couplings with H-7 and ^{2}J geminal coupling with H-8. All these correlations are in good agreement with the above spin system. The spin system (c) [-CH_2-CH_2-] was assigned for the positions C-11 and C-12; since C-11 showed correlations with H-8 and H-9 and C-12 showed correlation with H-18. The spin system (d) [-CH_2-CH_2-CH<] was assigned for positions C-15 to C-17. C-17 showed correlation with H-21. Each proton in this spin system i.e. H-15, H-16 and H-17, exhibited a multiplet due to an availablity of several protons for both ^{3}J and ^{2}J couplings. The spin system (e) [-CH-(CH_3)-CH_2-CH_2-] was assigned for the positions C-20 to C-23. C-20 showed correlation with methyl protons at H-21 and this methyl protons (δ 0.92, d, J = 5.2 Hz) split in to a doublets due to ^{3}J coupling with H-20. C-21 showed correlation with H-17 and C-22 showed correlation with H-21. The spin system (f) [CH_3-CH-] was assigned for an ethylidene moiety at C-24. C-31 showed correlations with H-23, H-25 and H-32. Similarly, C-32 showed correlation with H-31. Further, C-24 (δ 149.5) showed correlations with H-31 and H-32. In the 1D proton NMR spectrum, the protons on methyl group at C-32 gave a doublets at δ1.58 (d, J = 6.1 Hz), due to ^{3}J coupling with H-32, expected for an ethylidene group. The single proton on C-31 was also clearly evident

showing the expected quartet at δ 5.17 (q, J = 6.7 Hz). All these correlations supported the presence of an ethylidene moiety at C-24. Finally, the spin system (g) [-CH-$(CH_3)_2$] was assigned for an isopropyl moiety at C-24. In the HMBC spectrum, C-25 showed correlations with H-26 and H-27 and C-24 showed correlations with H-23, H-25, H-26 and H-27. The methyl protons at H-26 and H-27, each split in to a doublets due to ^3J coupling with H-25. All these correlations indicated the presence of an isopropyl moiety also at C-24.



(98)

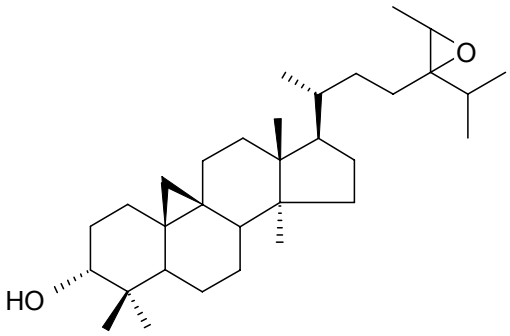
Overall, the HMBC and ^{1}H-^{1}H COSY spectral correlations of compound **(98)** were comparable to 24(E)-ethylidenecycloartanone **(93)**, but in the ^{1}H-^{1}H COSY spectrum, H-3 also showed correlation with H-2. The ^{1}H and ^{13}C chemical shift values at C-3 position were observed as δ 3.45 (br, s) and 77.0 respectively, indicated the presence of a hydroxyl functionality at C-3. Further, the stereochemical orientation of this hydroxyl functionality was observed as α, which was confirmed by comparing the above chemical shift values and multiplicity at this position with a related compound [125]. For a β-orientation, these chemical shift values would be around δ 3.28 (m) and 78.8, respectively [126,127]. The spectral correlations regarding its side chain structure and NMR chemical shift values for other positions are also comparable to 24(E)-ethylidenecycloartanone **(93)**. The ^{1}H-^{1}H COSY and significant HMBC correlations are listed in Table 7.

Table 7
NMR data of 24(E)-ethylidenecycloartan-3α-ol **(98)** in $CDCl_3$

Position	^{13}C / DEPT	δ_C (ppm)	δ_H (ppm) *J* in Hz	HMBC (C → H)	^1H-^1H COSY
1	CH_2	27.5	α: 1.56 m; β: 1.24 m	2	2
2	CH_2	28.5	α: 2.72 m; β: 1.56 m	1	1, 3
3	CH	77.0	3.45 br *s*	2, 28, 29	2
4	C	39.5		28, 29	
5	CH	41.0	1.28 m	19	6
6	CH_2	21.1	α: 1.58 m; β: 0.80 m	5	5, 7
7	CH_2	28.2	α: 1.87 m; β: 1.28 m	6	6, 8
8	CH	47.8	1.50 m	19, 30	7
9	C	19.8		1	
10	C	26.4		6	
11	CH_2	26.2	α: 1.96 m; β:1.10 m	8, 9	12
12	CH_2	32.8	1.68 m	18	11
13	C	45.3		8, 18	
14	C	48.9		18	
15	CH_2	35.4	1.28 m		16
16	CH_2	25.9	α: 1.33 m; β: 1.10 m		15, 17
17	CH	52.0	1.56 m	21	16, 20
18	CH_3	19.3	0.94 s	12, 17	
19	CH_2	29.7	α: 0.49 d (4.0) β: 0.33 d (4.0)	1, 11	
20	CH	35.9	1.36 m	21	17, 22
21	CH_3	18.1	0.92 d (5.2)	17	20
22	CH_2	34.7	α: 1.34 m; β: 0.92 m	21	23
23	CH_2	26.8	α: 1.33 m; β: 1.10 m		22
24	C	149.5		23, 25, 26, 27, 31, 32	
25	CH	29.5	2.80 m	26, 27	26, 27
26	CH_3	21.9	0.92 d (6.0)	25, 27	25
27	CH_3	21.9	0.81 d (6.0)	25, 26	25
28	CH_3	22.2	0.88 s	5, 29	
29	CH_3	20.8	0.92 s	5, 28	
30	CH_3	19.3	0.85 s	8, 15	
31	CH	116.4	5.17 q (6.7)	23, 25, 32	32
32	CH_3	12.8	1.58 d (6.1)	31	31

24,31-Epoxy-24-ethylcycloartan-3α-ol (99)

24,31-Epoxy-24-ethylcycloartan-3α-ol **(99)** was obtained as colourless amorphous powder and gave a molecular ion peak 470.4123 in the HREIMS, corresponding to the molecular formula $C_{32}H_{54}O_2$. Inspection of its ^{13}C and HSQC-DEPT spectra, revealed the presence 32 signals; eight methyl, eleven methylene, seven methine and six quaternary carbons. The ^{1}H NMR spectrum of compound indicated the presence of a cycloartane skeleton with typical high-field AB doublets due to non-equivalent protons at C-19 of the cyclopropane ring as reported in the other two new compounds, **(93)** and **(98)**. The fragment ion at *m/z* 315(M-$C_{10}H_{19}O$) was also an evidence of the presence of a cycloartane skeleton with a hydroxyl functionality in the nucleus and C_{10} side chain.



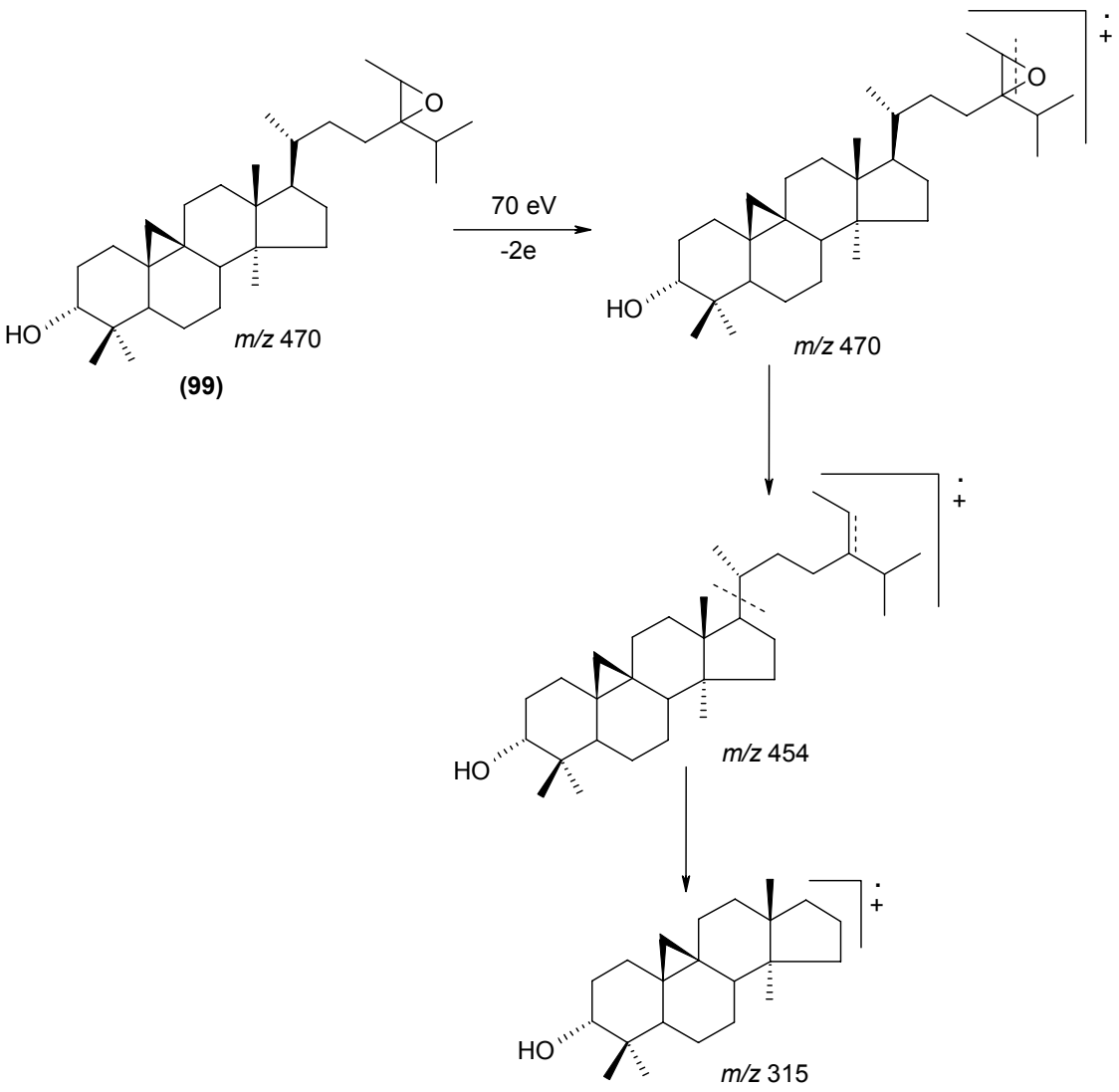
(99)

In the HMBC spectrum, C-1 showed correlation with H-2; C-2 showed correlation with H-1 and C-3 (δ 77.2) showed correlations with H-2, H-28 and H-29. Similarly, C-4 also showed correlations with methyl protons at H-28 and H-29. These correlations together with ^{13}C NMR (δ 77.2) and ^{1}H NMR (δ 3.50) chemical shift values, allowed us to place one of the oxymethine protons at C-3 and the two methyl groups (C-28 and C-29) to the same carbon at C-4. The H-1 and H-11 protons showed correlation with carbon at C-19 (δ 29.7) and C-5 and C-8 showed correlation with H-19. These correlations allowed us to

place a methylene group (belongs to the cyclopropane ring) between C-9 and C-10. Further, H-19 protons are resonated at δ 0.51 (d, J = 4.2 Hz, Hα) and 0.36 (d, J = 4.2 Hz, Hβ); they are non-equivalent and each proton split in to a doublets due to ^{2}J geminal coupling with each other. C-5 showed correlation with H-19 and C-6 showed correlation with H-5. Similarly, C-7 showed correlation with H-6 and C-8 showed correlations with H-19 and H-30. H-8 resonated at δ1.47 (m) and this multiplet was due to ^{3}J vicinal coupling with H-7 and ^{2}J geminal coupling with H-8. These correlations allowed us to form a skeleton of C-5 to C-8. C-15 showed correlations with H-16 and H-30. These correlations together with the correlation of C-8 with H-30 allowed us to place a methyl group (C-30) at C-14 position. Similarly, C-11 showed correlations with H-8 and H-9 and C-12 showed correlation with H-18. C-13 showed correlations with H-8 and H-18. C-14 showed correlation with H-18. All these correlations allowed us to place another methyl group (C-18) at C-13 position. C-11 showed correlations with H-8 and H-9 and C-12 showed correlation with H-18. These correlations allowed us to form a skeleton of C-11 and C-12. C-15 showed correlations with H-16 and H-30 and C-17 showed correlation with H-21. These correlations allowed us to form a skeleton of C-15 to C-17. Further, each proton at H-15, H-16 and H-17 exhibited a multiplet due to an availablity of several protons for both ^{3}J and ^{2}J couplings. C-20 showed correlation with methyl protons at H-21 and this methyl protons (δ 0.95, d, J = 5.0 Hz) split in to a doublets due to ^{3}J coupling with H-20. C-21 showed correlation with H-17 and C-22 showed correlation with H-21. C-31 showed correlations with H-23, H-25 and H-32. Similarly, C-32 showed correlation with H-31. Further, C-24 (δ 149.5) showed correlations with H-31 and H-32. In the 1d proton NMR spectrum, the protons on methyl group at C-32 gave a doublets at δ1.58 (d,

J = 6.2 Hz), due to ^{3}J coupling with H-32. The single proton on C-31 gave a quartet at δ 2.87 (q, J = 6.5 Hz). All these correlations were indicated the presence of a [CH_3-CH-] moiety at C-24. In the HMBC spectrum, C-25 showed correlations with H-26 and H-27 and C-24 showed correlations with H-23, H-25, H-26 and H-27. The methyl protons at H-26 and H-27, each split in to a doublets due to ^{3}J coupling with H-25. All these correlations indicated the presence of an isopropyl moiety also at C-24.

Most of the spectral correlations have close similarity to 24(E)-ethylidenecycloartan-3α-ol **(98)** including the stereochemistry at C-3 position. However, the ^{13}C spectrum exhibited three oxygenated carbons, one of them was assigned to C-3 (δ 77.2) and two other were assigned to respectively, C-24 (δ 60.2) and C-31 (δ 65.2) (Table 8). Further, HSQC-DEPT spectrum revealed that C-24 and C-31 were respectively quaternary and methine carbons. Attaching hydroxyl group on both or any one of the carbons at C-24 or C-31 was ruled out, since doing so will cause the molecular weight to be exceeded than actual. Based on these observations, we placed the oxygen atom as an epoxy function between C-24 and C-31 positions, unambiguously. A fragment ion in the mass spectrum at *m/z* 454 (M-O) was a further evidence that one of the oxygen atoms was present in the form of epoxy function. The major fragment ions were depicted in Scheme 2. However, the stereochemistry at these positions remained unassigned.



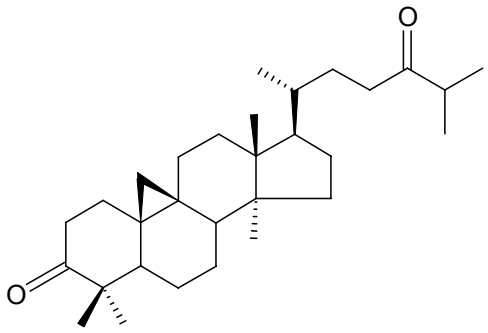
Scheme 2
Major fragmentation pattern of 24,31-epoxy-24-ethylcycloartan-3α-ol **(99)**.

Table 8
NMR data of 24,31-epoxy-24-ethylcycloartan-3α-ol **(99)** in $CDCl_3$.

Position	^{13}C / DEPT	δ_C (ppm)	δ_H (ppm) *J* in Hz	HMBC (C → H)
1	CH_2	27.3	α: 1.57 m; β: 1.25 m	2
2	CH_2	28.4	α: 1.75 m; β: 1.56 m	1
3	CH	77.2	3.50 br s	2, 28, 29
4	C	39.7		28, 29
5	CH	41.0	1.28 m	19
6	CH_2	21.2	α: 1.60 m; β: 0.83 m	5
7	CH_2	28.2	α: 1.89 m; β: 1.28 m	6
8	CH	48.0	1.47 m	19, 30
9	C	19.8		1
10	C	26.4		6
11	CH_2	26.2	α: 1.90 m; β: 1.12 m	8, 9
12	CH_2	32.8	1.60 m	18
13	C	45.5		8, 18
14	C	49.2		18
15	CH_2	35.6	1.31 m	16, 30
16	CH_2	26.1	α: 1.27 m; β: 1.12 m	
17	CH	52.2	1.60 m	21
18	CH_3	19.5	0.98 s	12, 17
19	CH_2	29.7	α: 0.51 d (4.2) β: 0.36 d (4.2)	1, 11
20	CH	36.1	1.40 m	21
21	CH_3	18.3	0.95 d (5.0)	17
22	CH_2	34.8	α: 1.36 m; β: 0.95 m	21
23	CH_2	26.8	α: 1.29 m; β: 1.12 m	
24	C	65.2		23, 25, 26, 27, 31, 32
25	CH	29.8	2.82 m	26, 27
26	CH_3	21.9	0.95 d (5.9)	25, 27
27	CH_3	21.9	0.82 d (5.9)	25, 26
28	CH_3	22.4	0.90 s	5, 29
29	CH_3	20.8	0.92 s	5, 28
30	CH_3	19.5	0.84 s	8, 15
31	CH	60.2	2.87 q (6.5)	23, 25, 32
32	CH_3	12.8	1.58 d (6.2)	31

Cycloartane-3,24-dione (95)

Cycloartane-3,24-dione **(95)** was obtained as colourless crystals. It gave a molecular ion peak at *m/z* 440.3655 in the HREIMS, corresponding to the molecular formula $C_{30}H_{48}O_2$. Inspection of its ^{13}C and HSQC-DEPT spectra, revealed the presence 30 signals; seven methyl, eleven methylene, five methine and seven quaternary carbons. The ^{13}C NMR chemical shift values of two carbons were observed at δ 215.4 and 216.5 respectively, assigned for two carbonyl groups. No signals were observed for an olefinic bond or hydroxyl group. The ^{1}H NMR spectrum indicated the presence of a methylene group in the form of cyclopropane ring (cycloartane-type) and the two non-equivalent hydrogens resonated at high-field region at δ 0.78 (d, *J* = 4.3 Hz, Hα) and 0.57 (d, *J* = 4.3 Hz, Hβ) respectively (Table 9). The fragment peak at *m/z* 313 (M- $C_8H_{15}O$) in the MS spectrum was supported the presence of a cycloartane skeleton; which also supported the presence of one of the carbonyl groups at the nucleus and a C_8 side chain.



(95)

In the HMBC spectrum, C-1 showed correlation with H-2 and H-19 and C-2 showed correlation with H-1. H-1 protons resonated at δ 1.85 (ddd, *J* = 13.5, 4.0, 1.4 Hz, Hα) and δ 1.56 (ddd, *J* = 13.5, 4.4, 2.7 Hz, Hβ). Similarly, H-2 protons resonated at δ 2.72 (ddd, *J* = 13.5, 4.4, 2.7 Hz, Hα) and δ 2.32 (ddd, *J* = 13.5, 6.5, 4.5 Hz, Hβ). The multiplicity, ddd,

indicated that each proton showed ^{3}J couplings with vicinal protons (Hα and Hβ) as well as ^{2}J coupling with a geminal proton. The C-3 (δ 216.4) carbon showed correlations with methyl protons at H-28 and H-29; similarly C-4 also showed correlations with methyl protons at H-28 and H-29. These correlations as well as the chemical shift value δ 216.4 (C-3) allowed us to place the carbonyl carbon at C-3 position and the attachment two methyl groups (C-28 and C-29) to the same carbon at C-4. The H-1 and H-11 protons showed correlation with carbon at C-19 (δ 29.5) and C-5 showed correlation with H-19. These correlations allowed us to place a methylene group (belongs to the cyclopropane ring) between C-9 and C-10. Further, H-19 protons are resonated at δ 0.78 (d, J = 4.3 Hz, Hα,) and 0.57 (d, J = 4.3 Hz, Hβ); they are non-equivalent and each proton split in to a doublets due to ^{2}J geminal coupling with each other. C-5 showed correlation with H-19 and C-6 showed correlation with H-5. The single proton H-5 resonated at δ 1.70 (dd, J = 12.5, 4.5 Hz); the multiplicity, dd and the coupling constant values J = 12.5 and 4.5 Hz were, respectively, due to ^{3}J *trans* and *cis* couplings with H-6 protons. Similarly, C-7 showed correlation with H-6 and C-8 showed correlation with H-30. H-6 protons are resonated at δ 1.37 (dddd, J = 12.5, 12.5, 12.5, 3.0 Hz, Hα,) and 1.12 (dddd, J = 12.5, 4.9, 4.9, 2.5 Hz, Hβ); the multiplicity indicated that each proton showed ^{3}J vicinal couplings with H-7 and H-5 and ^{2}J geminal coupling with H-6. H-8 resonated at δ1.58 (dd, 12.5, 4.9 Hz) and the multiplicity, dd and the coupling constant values were due to ^{3}J vicinal couplings with H-7 protons. These correlations allowed us to form a skeleton of C-5 to C-8. C-15 showed correlations with H-30. These correlations together with the correlation of C-8 with H-30 allowed us to place a methyl group (C-30) at C-14 position. Similarly, C-11 showed correlations with H-19 and C-12 showed correlation with H-18. C-13

showed correlation with H-18 and H-30. C-14 showed correlation with H-18 and H-30. All these correlations allowed us to place another methyl group (C-18) at C-13 position. C-11 showed correlations with H-9 and C-12 showed correlation with H-18. These correlations allowed us to form a skeleton of C-11 and C-12. C-15 showed correlations with H-30 and C-17 showed correlation with H-18 and H-21. These correlations allowed us to form a skeleton of C-15 to C-17. Further, each proton at H-15, H-16 and H-17 exhibited a multiplet due to an availablity of several protons for both ^{3}J and ^{2}J couplings. C-20 showed correlation with methyl protons at H-21 and this methyl protons (δ 1.09, d, J = 6.5 Hz) split in to a doublets due to ^{3}J coupling with H-20. H-17, H-20 and H-22 showed correlation with methyl carbon at C-21. H-23, H-25, H-26 and H-27 showed correlations with C-24. The methyl protons at H-26 and H-27, each split in to a doublets due to ^{3}J coupling with H-25. All these correlations indicated the presence of an isopropyl moiety also at C-24 (δ 215.4) and the attachment of two methyl groups (C-26 and C-27) to the same carbon at C-25. In addition, the same correlations allowed us to place the second carbonyl groups at C-24 (δ 215.4). The McLafferty rearrangement product at *m/z* 354 supported the presence of a carbonyl group in the side chain at C-24. Other significant HMBC correlations are listed in Table 9.

Although this compound had earlier been reported as an inseparable mixture, the identification was made based on only MS fragmentation pattern of GC-MS analysis [128]. However the same compound was isolated from another species and reported in a pure state [107] but it had inconsistency with our assignment, in particular at the C-3 position. The MS fragmentation pattern obtained for cycloartane-3,24-dione **(95)** was

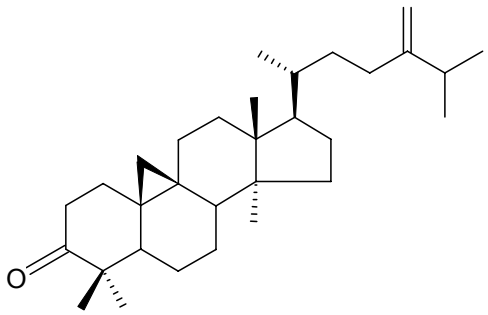
identical as reported in the literature [107]. NMR assignments were made for this compound and are listed in Table 9.

Table 9
NMR data of cycloartane-3,24-dione **(95)** in $CDCl_3$.

Position	^{13}C/ DEPT	δ_C(ppm)	δ_H (ppm) *J* in Hz	HMBC (C → H)
1	CH_2	33.3	α: 1.85 ddd (13.5, 4.0, 1.4) β: 1.56 ddd (13.5, 4.4, 2.7)	2, 19
2	CH_2	37.4	α: 2.72 ddd (13.5, 4.4, 2.7) β: 2.32 ddd (13.5, 6.5, 4.5)	1
3	C	216.4		2, 28, 29
4	C	50.2		28, 29
5	CH	48.4	1.70 dd (12.5, 4.5)	19
6	CH_2	21.5	α: 1.37 dddd (12.5, 12.5, 12.5, 3.0) β: 1.12 dddd (12.5, 4.9, 4.9, 2.5)	5
7	CH_2	25.8	α: 1.39 m; β: 1.16 m	6
8	CH	47.9	1.58 dd (12.5, 4.9)	30
9	C	21.0		8, 11
10	C	25.9		5, 19
11	CH_2	26.7	α: 2.05 m; β: 1.16 m	19
12	CH_2	32.8	1.64 m	18
13	C	45.3		18, 30
14	C	48.7		18, 30
15	CH_2	35.5	1.32 m	30
16	CH_2	28.0	α: 1.94 m; β: 1.32 m	
17	CH	52.2	1.59 m	18, 21
18	CH_3	18.3	1.09 s	
19	CH_2	29.5	α: 0.78 d (4.3); β: 0.57 d (4.3)	1, 11
20	CH	35.6	1.39 m	21
21	CH_3	20.8	1.09 d (6.5)	
22	CH_2	30.0	α: 1.77 m; β: 1.24 m	21
23	CH_2	37.4	α: 2.14 ddd (16.6 ,9.7, 6.0) β: 2.14 ddd (16.6, 10.0, 5.2)	
24	C	215.4		23, 25, 26, 27,
25	CH	40.8	2.61 m	26, 27
26	CH_3	18.6	0.86 d (6.4)	25, 27
27	CH_3	18.0	0.98 d (6.4)	25, 26
28	CH_3	22.1	1.04 s	5, 29
29	CH_3	20.7	1.09 s	5, 28
30	CH_3	19.3	0.90 s	8, 15

24-Methylenecycloartanone (97)

24-Methylenecycloartanone **(97)** was obtained as colourless crystals. The molecular formula of the compound was deduced as $C_{31}H_{50}O$ by the molecular ion peak at *m/z* 438 in the EIMS and ^{13}C NMR analysis. The IR spectrum of this compound showed absorption for ketone functionality (ν_{max} 1712 cm^{-1}). Inspection of its ^{13}C and HSQC-DEPT spectra, revealed the presence 31 signals; seven methyl, twelve methylene, five methine and seven quaternary carbons. There was one carbonyl carbon (δ 216.5) and one double bond (δ156.9 and 105.9) as revealed from its ^{13}C NMR chemical shift values. The ^{1}H NMR spectrum of the compound revealed the presence of cycloartane-type skeleton with typical high-field AB doublets due to the non-equivalent hydrogens at δ 0.52 (d, *J* = 4.2 Hz, Hα) and 0.35 (d, *J* = 4.2 Hz, Hβ) at C-19 in the cyclopropane ring [122]. The fragment peak at *m/z* 313 (M-$C_{10}H_{19}O$) in the MS spectrum was also supported the presence of a cycloartane skeleton with a carbonyl group in the nucleus and C_9 side chain.



(97)

In the HMBC spectrum, C-1 showed correlation with H-2 and C-2 showed correlation with H-1. H-1 protons resonated at δ 1.88 (ddd, *J* = 13.5, 4.0, 1.4 Hz, Hα) and 1.04 (m, Hβ). Similarly, H-2 protons resonated at δ 2.72 m, Hα and 1.96 (ddd, *J* = 13.5, 6.5, 4.5 Hz, Hβ). The multiplicity indicated that each proton showed ^{3}J couplings with vicinal

protons (Hα and Hβ) as well as ^{2}J coupling with a geminal proton. The C-3 (δ 216.4) carbon showed correlations with methyl protons at H-28 and H-29; similarly C-4 also showed correlations with methyl protons at H-28 and H-29. These correlations as well as the chemical shift value δ 216.5 (C-3) allowed us to place the carbonyl carbon at C-3 position and the attachment two methyl groups (C-28 and C-29) to the same carbon at C-4. The H-1 and H-11 protons showed correlation with carbon at C-19 (δ 29.8) and C-5 showed correlation with H-19. These correlations allowed us to place a methylene group (belongs to the cyclopropane ring) between C-9 and C-10. Further, H-19 protons are resonated at δ 0.52 (d, J = 4.2 Hz, Hα,) and 0.35 (d, J = 4.2 Hz, Hβ); they are non-equivalent and each proton split in to a doublets due to ^{2}J geminal coupling with each other. C-5 showed correlation with H-19 and C-6 showed correlation with H-5. The single proton H-5 resonated at δ 1.85 (dd, J = 12.6, 4.5 Hz); the multiplicity, dd and the coupling constant values J = 12.5 and 4.5 Hz were, respectively, due to ^{3}J *trans* and *cis* couplings with H-6 protons. Similarly, C-7 showed correlation with H-6 and C-8 showed correlations with H-19 and H-30. H-6 protons are resonated at δ 1.51 (dddd, J = 12.6, 4.6, 4.5, 2.7 Hz, Hα,) and 0.79 (dddd, J = 12.6, 12.6, 12.6, 2.5 Hz, Hβ); the multiplicity indicated that each proton showed ^{3}J vicinal couplings with H-7 and H-5 and ^{2}J geminal coupling with H-6 proton. Similarly, H-7 protons are resonated at δ 1.15 (dddd, J = 12.6, 12.6, 12.7, 2.5 Hz, Hα,) and 1.33 (dddd, J = 12.6, 4.6, 4.6, 2.6 Hz, Hβ); the multiplicity indicated that each proton showed ^{3}J vicinal couplings with H-6 and H-8 and ^{2}J geminal coupling with H-7 proton. H-8 resonated at δ1.56 (dd, 12.6, 4.6 Hz) and the multiplicity, dd and the coupling constant values were due to ^{3}J vicinal couplings with H-7 protons. These correlations allowed us to form a skeleton of C-5 to C-8. C-15 showed correlations

with H-30. These correlations together with the correlation of C-8 with H-30 allowed us to place a methyl group (C-30) at C-14 position. Similarly, C-11 showed correlations with H-19 and C-12 showed correlation with H-18. C-13 showed correlation with H-18 and H-30. C-14 showed correlation with H-18. All these correlations allowed us to place another methyl group (C-18) at C-13 position. C-11 showed correlations with H-8 and H-9 and the H-11 protons resonated at δ 2.03 (ddd, J = 15.0, 8.0, 7.5 Hz, Hα) and δ 1.16 (ddd, J = 15.0, 7.5, 6.0 Hz, Hβ). The multiplicity indicated that each proton showed ^{3}J couplings with vicinal protons (Hα and Hβ) at H-12 as well as ^{2}J coupling with a geminal proton at H-11. C-12 showed correlation with H-18. These correlations allowed us to form a skeleton of C-11 and C-12. Further, each proton at H-15, H-16 and H-17 exhibited a multiplet due to an availablity of several protons for both ^{3}J and ^{2}J couplings. C-20 showed correlation with methyl protons at H-21 and this methyl protons δ 0.90 (d, J = 7.0 Hz) split in to a doublets due to ^{3}J coupling with H-20. The protons at H-17, H-20 and H-22 showed correlation with methyl carbon at C-21. H-23, H-25, H-26 and H-27 showed correlations with C-24. H-17 showed correlation with methyl carbon at C-21. H-25, H-26 and H-27 showed correlations with C-24; H-26 and H-27 showed correlations with C-25. Similarly, C-31 showed correlation with H-25. All these correlations allowed us to place C-31 methylene group at C-24 (δ 156.9) and the attachment of two methyl groups (C-26 and C-27) to the same carbonyl at C-25. The methylene protons at C-31 were non-equivalent and also olefinic. Since, they exhibited ^{1}H NMR chemical shift values at δ 4.66 (1H, d, J = 1.5 Hz) and 4.71 (1H, br, s). The placement of double bond between C-24 and C-31 was also supported by the ^{13}C NMR chemical shift values of C-31 (δ 105.9) and C-24 (δ 156.9). Compound **(97)** is a very common cycloartane triterpenoid and has

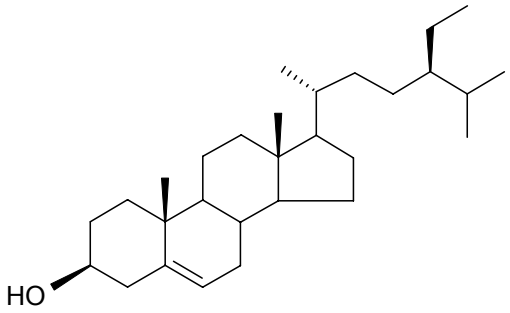
been reported several times from other species. Comparison of spectral data with those reported previously had good agreement with our data [107,108]. Other significant HMBC correlations are listed in Table 10.

Table 10
NMR data of 24-methylenecycloartanone **(97)** in $CDCl_3$

Position	^{13}C/ DEPT	δ_C (ppm)	δ_H (ppm) *J* in Hz	HMBC (C → H)
1	CH_2	27.5	α: 1.88 ddd (13.8, 13.8, 2.7) β: 1.04 m	2
2	CH_2	28.6	α: 1.66 m β: 1.96 ddd (13.8, 13.8, 4.0)	1
3	C	216.5		2, 28, 29
4	C	39.6		28, 29
5	CH	41.1	1.85 dd (12.6, 4.5)	19
6	CH_2	21.1	α: 1.51 dddd (12.6, 4.6, 4.5, 2.7) β: 0.79 dddd (12.6, 12.6, 2.5, 12.6)	5
7	CH_2	25.7	α: 1.15 dddd (12.6, 12.6, 2.5, 12.7) β: 1.33 dddd (12.6, 2.6, 4.6, 4.6)	6
8	CH	48.0	1.56 dd (12.6, 4.6)	19, 30
9	C	19.8		1
10	C	26.4		6
11	CH_2	26.2	α: 2.03 ddd (15.0, 8.0, 7.5) β: 1.16 ddd (15.0, 7.5, 6.0)	8, 9
12	CH_2	32.8	1.65 m	18
13	C	45.3		8, 18
14	C	48.9		18
15	CH_2	35.5	1.32 m	
16	CH_2	28.2	α: 1.94 m; β: 1.32 m	
17	CH	52.3	1.64 m	21
18	CH_3	18.0	0.97 s	12, 17
19	CH_2	29.8	α: 0.52 d (4.2); β: 0.35 d (4.2)	11
20	CH	36.2	1.42 m	21
21	CH_3	18.3	0.90 d (7.0)	17
22	CH_2	35.0	α: 1.19 m; β: 1.60 m	21
23	CH_2	31.3	α: 1.19 m; β: 2.15 m	31
24	C	156.9		25, 26, 27
25	CH	33.8	2.26 m	26, 27
26	CH_3	21.9	1.03 d (6.8)	25, 27
27	CH_3	22.0	1.03 d (6.8)	25, 26
28	CH_3	25.9	0.95 s	5, 29
29	CH_3	21.2	0.88 s	5, 28
30	CH_3	19.3	0.91 s	8, 15
31	CH_2	105.9	4.66, br, d (1.5) (1H); 4.71 br, s (1H)	25

β-Sitosterol (47)

β-Sitosterol **(47)** was obtained as colourless flakes. The IR spectrum demonstrated the presence of hydroxyl group (ν_{max} 3429 cm^{-1}) and double bond (ν_{max} 1662 cm^{-1}) functions in the molecule. The mass spectrum of the compound showed a molecular ion peak at *m/z* 414 and a peak at *m/z* 396 (M-18) due to loss of a water molecule.

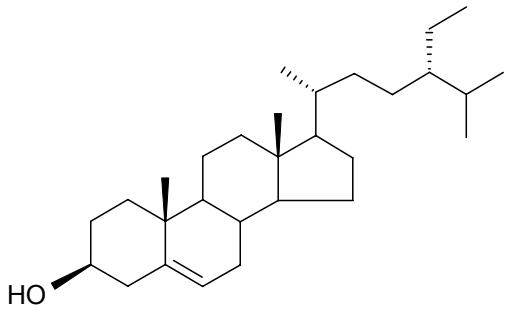


(47)

A characteristic signal at 5.36 (br d, J = 5.4 Hz) indicated the presence of an olefinic proton and a peak at δ 3.52 (m, 1H) showed the presence of a hydroxyl group. The ^{13}C NMR spectrum gave 29 signals; six methyl, eleven methylene, nine methine and three quaternary carbons were sorted by HSQC-DEPT spectrum. The ^{13}C NMR spectrum showed peaks at δ 71.8 and 140.8 & 121.7 that confirmed the presence of a hydroxyl and olefinic carbons, respectively. Melting point range was found to be 136.1-138.0 ˚C which had good agreement with reported literature value [129,130]. With all these information, the compound was confirmed as β-sitosterol **(47)**. Comparison of the spectral data with those reported in the literature also had good agreement [131,132].

γ-Sitosterol (96)

γ-Sitosterol **(96)** was obtained as colourless crystals. Most of the spectral correlations are identical to β-sitosterol **(47)**. The only difference between this compound and β-sitosterol **(47)** is the stereochemistry at C-24.

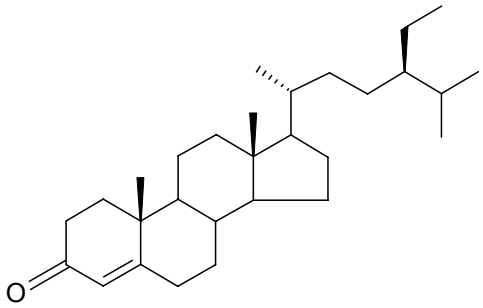


(96)

However this compound can easily be distinguished from β-sitosterol **(47)** by its melting point, 147-148 ˚C [133,134] and relative intensity of the molecular ion and fragment peaks in the MS. The melting point difference between compounds, β-sitosterol **(47)** and γ-sitosterol **(96)** is ~ 10 ˚C.

β-Sitosterone (100)

β-Sitosterone **(100)** was obtained as an amorphous powder. It gave a molecular ion peak at *m/z* 412 and other fragment peaks at *m/z* 397, 370, 289, 229,149, 124, 55 and 43. The fragmentation peak at *m/z* 124 indicates the presence of a keto group in the ring.

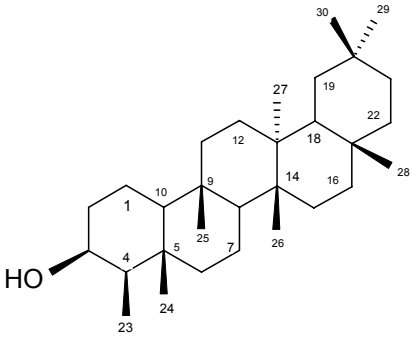


(100)

It gave a peak at δ 5.7 (1H, s) was characteristic for an olefinic proton. Its spectral data compared well with those reported in the literature [135].

3β-Friedelinol (66)

3β-Friedelinol **(66)** was obtained as colourless crystals. The IR spectrum revealed the presence of hydroxyl functionality in the molecule (ν_{max} 3500 cm^{-1}). The mass spectrum gave a molecular ion peak at *m/z* 428. The ^{13}C NMR spectrum gave 30 signals; eight methyl, eleven methylene, five methine and six quaternary carbons were sorted by HSQC-DEPT spectrum. One of the carbons resonated at δ 72.8 and indicated the presence of a hydroxyl group. The ^{1}H NMR spectrum gave a peak at δ 3.73 which corresponds to H-3.



(66)

In the HMBC spectrum, C-1 showed correlations with H-3 and H-10. H-3 resonated at 3.73 (q-like, *J* = 9.5, 3.0 Hz); the HMBC correlations and this chemical shift value allowed us to place the hydroxyl functionality at this position (C-3). H-10 resonated at δ 0.86 (dd, *J* = 12.0, 2.0 Hz); the multiplicity, dd, was due to a coupling of this proton with H-1 protons. C-3 (δ 72.8) showed correlations with H-2 and H-23. These correlations and the chemical shift value supported to place the hydroxyl functionality at C-3 and a methyl group (C-23) at C-4. Further, in the 1D proton NMR spectrum, the methyl group at C-23 split in to a doublets, which was due to the coupling of these methyl protons with H-4 and fits the characteristic of this methyl group (C-23) at C-4. C-4 showed correlation with H-

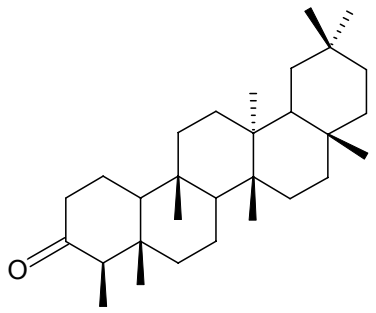
2, H-23 and H-24 and the quaternary carbon C-5 showed correlations with H-1, H-3, H-6 and H-24. These correlations supported the attachment of C-23 methyl group at C-4 and C-24 methyl group at C-5. The methyl protons at H-25 showed correlations with C-8, C-9, C-10 and C-11. These correlations supported the attachment of the C-25 methyl group at C-9 position. Similarly, the methyl protons at H-26 showed correlations with C-8, C-13, C-14 and C-15; the methyl protons at H-27 showed correlations with C-12, C-13, C-14 and C-18. These correlations allowed us to place the C-26 methyl group at C-15 and C-27 methyl group at C-13, respectively. The methyl protons at H-28 showed correlations with C-16, C-17, C-18 and C-22. These correlations allowed us to place the C-28 methyl group at C-17. Finally, C-29 showed correlations with H-19 and H-30; C-30 showed correlations with H-19, H-21 and H-29; C-20 showed correlations with H-19, H-21, H-29 and H-30. C-21 showed correlation with H-22, H-29 and H-30; C-21 showed correlation with H-22, H-29 and H-30. All these correlations supported the attachment of both C-29 and C-30 methyl groups to the same carbon at C-20 and the compound is an oleanane type triterpenoid. Other significant HMBC correlations are listed in Table 11. The β-orientation of the hydroxyl group at C-3 was confirmed by comparing the proton coupling constant values at this position with literature values [142,143]. The 3.0 Hz coupling between H-3 and H-4 and H-2 confirms the stereochemistry. Melting point range was found to be 280.0-282.0 ˚C. These data had good agreements with the reported literature data **(66)** [136-138].

Table 11
NMR data of 3β-friedelinol **(66)** in $CDCl_3$

Position	DEPT	δ_C (ppm)	δ_H (ppm) *J* in Hz	HMBC (C → H)
1	CH_2	15.8	α: 1.52 m; β: 1.41 m	3, 10
2	CH_2	35.2	α: 1.86 qd (9.5, 3.0) β: 1.56 m	
3	CH	72.8	α: 3.73 q-like (3.0)	2, 23
4	CH	49.2	1.22 m	2, 23, 24
5	C	37.1		1, 3, 6, 24,
6	CH_2	41.7	α: 1.69 m; β: 0.96 m	24
7	CH_2	17.6	1.36 m	6, 8,
8	CH	53.2	1.25 m	6, 10, 11, 15, 25, 26
9	C	48.4		8, 25,
10	CH	61.4	0.86 dd (12.0, 2.0)	1, 2, 6, 24, 25
11	CH_2	35.3	α: 1.27 m; β: 1.19 m	25
12	CH_2	30.6	1.28 m	27
13	C	37.8		8, 15, 26, 27
14	C	39.7		18, 26, 27
15	CH_2	32.3	α: 1.46 m; β: 1.27 m	8, 26
16	CH_2	36.1	α: 1.52 m; β: 1.31 m	22, 28
17	C	30.0		15, 18, 19, 28
18	CH	42.8	1.50 dd (11.0, 6.0)	12, 27, 28
19	CH_2	35.6	α: 1.42 m; β: 1.11 m	18, 29, 30
20	C	28.2		19, 21, 29, 30
21	CH_2	32.8	α: 1.45 m; β: 1.25 m	22, 29, 30
22	CH_2	39.3	1.46 dd (12.0, 2.0)	18, 28
23	CH_3	11.6	0.91 d (7.0)	4
24	CH_3	16.4	0.93 s	4, 10
25	CH_3	18.2	0.83 s	8
26	CH_3	18.6	0.98 s	15
27	CH_3	20.1	0.95 s	18
28	CH_3	31.8	0.97 s	18, 22
29	CH_3	35.0	0.92 s	19, 30
30	CH_3	32.1	1.14 s	19, 21, 29

Friedelin (94)

Friedelin **(94)** was obtained as colourless crystals. The IR spectrum demonstrated the presence of ketone functionality (ν_{max} 1719 cm^{-1}) in the molecule. It gave a molecular ion peak at *m/z* 426 in the MS. The ^{1}H NMR spectrum showed signals at high-field region corresponding to eight methyl groups and these were confirmed also through HSQC-DEPT spectrum. The ^{13}C NMR spectrum gave 30 signals; eight methyl, eleven methylene, four methine and seven quaternary carbons were sorted by HSQC-DEPT spectrum. The ^{13}C NMR spectrum showed a peak at δ 213.2, which corresponds to keto group of C-3 position.



(94)

Most of the spectral correlations were similar to 3β-friedelinol **(66)**, but the C-3 position was assigned for a keto group. Melting point range was found to be 263.1-265.0 ˚C. These data had good agreements with reported literature data [139,140].

2.2.3 Evaluation of the new compound, 24(E)-ethylidenecycloartanone (93) for anticancer potential using selected cancer cell lines in culture

The pure compound, 24(E)-ethylidenecycloartanone **(93)**, was tested for its cytotoxicity against four cancer cell lines *viz*. LL2 (Murine Lewis lung carcinoma), HL60 (Human leukaemia), P388 (Murine lymphocytic leukaemia) and WEHI164 (Murine fibrosarcoma). It showed no activity even at a concentration of 100 µg/mL against all the cancer cell lines tested, i.e. the IC_{50} value was >100 µg/mL against all the four cancer cell lines tested (Table 5). The percentage of growth inhibition at each concentration is given in Table 12.

Table 12
Growth inhibition (%) of cancer cell lines at various concentrations of the new compound, 24(E)-ethylidenecycloartanone **(93)**

	Cancer cell lines and % of growth inhibition			
Concentration (µM)	LL2	P388	WEHI164	HL60
221.00	33.3 (±3.9)	36.6 (±6.0)	25.9 (±0.7)	10.8 (±2.5)
110.50	22.9 (±6.6)	29.8 (±1.6)	11.0 (±0.9)	5.6 (±0.4)
55.25	17.4 (±5.5)	12.7 (±1.9)	10.1 (±0.8)	4.1 (±0.1)
22.10	17.0 (±4.5)	12.2 (±2.3)	6.4 (±1.0)	2.3 (±0.3)
2.21	2.1 (±6.1)	8.2 (±5.1)	5.4 (±0.6)	2.0 (±0.3)

P338: Murine lymphocytic leukaemia; HL60: Human leukaemia; LL2: Lewis lung carcinoma; WEHI164: Murine fibrosarcoma; No. of replicates, n = 8 for each concentration; Data show mean ± SD (standard deviation); Inhibitory concentrations were measured by MTT assay after 3-days culture.

It showed only 33.3% of growth inhibition with a standard deviation of ±3.9 % on LL2 cancer cell lines at a concentration of 221.0 μM, the highest concentration tested. The percentage of growth inhibitions at a concentration of 110.5, 55.25, 22.10 and 2.21 μM, were 22.9 (±6.6), 17.4 (±5.5), 17.0 (±4.5) and 2.1 (±6.1) %, respectively, against LL2 cancer cell lines. Similarly, with P388 cancer cell lines, the results were comparable to that of LL2 cancer cell lines, showing 33.6 (±6.0), 29.8 (±1.6), 12.7 (±1.9), 12.2 (±2.3) and 8.2 (±5.1) % growth inhibitions at a concentration of 221.0, 110.5, 55.25, 22.10 and 2.21 μM, respectively. With WEHI164 cancer cell lines, at a concentration of 221.0, 110.5, 55.25, 22.10 and 2.21 μM, the percentage of growth inhibitions were 25.9 (±0.7), 11.0 (±0.9), 10.1 (±0.8), 6.4 (±1.0) and 5.4 (±0.6), respectively. With HL60 cancer cell lines the percentage of growth inhibitions were10.8 (±2.5), 5.6 (±0.4), 4.1 (±0.1), 2.3 (±0.3) and 2.0 (±0.3), respectively, at a concentration of 221.0, 110.5, 55.25, 22.10 and 2.21 μM.

From Table 12, it was observed that the percentage of inhibition gradually increased with increasing concentrations. This indicated that the solvent did not play an adverse effect on the experiments at a proportion of 2 drops of CrEL and 0.5 mL of ethanol solvent mixture, so further dilutions were made with water (see Experimental Section). However, in our earlier attempts to screen the cytotoxicity of the same compound with solvent mixture of CrEL and ethanol (1:1) 1 mL each and further dilutions were made with water, the results obtained were erratic. This indicated that the solvent, CrEL had played a significant role and showed adverse effects at somewhat higher ratio. This is consistent with the report that CrEL showed cytotoxic effect at high concentrations *in vitro* [83].

Some cycloartane-type triterpenoids, mostly in the form of saponins or with several functional groups, showed cytotoxic acitivities against several cancer cell lines, *viz*. HepG2 (Hepatocellular carcinoma cells), HL60 (Human leukaemia cells), KB Cells, A2780 (Ovarian cancer cells), 26L5 (Murine colon cells), HSC2 (Human oral squamous cells), U251 (CNS), PC3 (Prostate cancer cells), HCT15 (Colon carcinoma cells), MCF7 (Human breast cancer cells), K562 (Leukaemia cancer cells) *etc*. [141-150]. These results indicated that some cycloartane-type compounds are cytotoxic against some cancer cell lines. However, the new compound, 24(E)-ethylidenecycloartanone **(93)**, though it is a cycloartane-type compound, did not show any activity against a panel of cancer cell lines. The new compound, 24(E)-ethylidenecycloartanone **(93)**, has only a ketone functional group and one double bond. The lack of functionality would be a probable reason for its inactivity on the cancer cell lines tested or it may not induce any inhibition on the cancer cell lines selected for the testing.

2.2.4 Deriving Median Lethal Dosage (LD_{50}) of crude extract, chloroform and hexane fractions of *P. bistorta*

LD_{50} is a dosage that will cause a death of fifty percent of the experimental animals. We assessed the $LD_{50}$of crude extract, chloroform and hexane fractions of *P. bistorta* using Swiss albino mice and the results are given in Tables 13 and 14.

Table 13
General behaviours of mice after the administration of 200 mg of plant extract/kg of body weight of mice

Days	*P. bistorta* crude extract	Chloroform fraction	Hexane fraction	Control
1	Slow respiration and sedative; locomotion was arrested within a few hours of administration.	Slow respiration and sedative; locomotion was arrested within a few hours of administration.	Slow respiration and sedative; locomotion was arrested within a few hours of administration.	Normal
2	Death of three mice; the live one was dull.	Death of two mice and the live mice were dull.	Less active; all mice were live but were dull.	Normal
3	Recovered and gained weight; locomotive behaviour became normal.	Recovered and gained weight; locomotive behaviour became normal.	Recovered and gained weight; locomotive behaviour became normal.	Normal
4-14	Normal	Normal	Normal	Normal

No. of mice used in each group, n = 4; Swiss albino mice of both sexes, each weighing about 30 g, were used. Extracts/fractions in DMSO (200 mg of extract/kg of body weight of mice) were administered to mice by intraperitoneal injection. Crude extract/fractions were dissolved in 10 mL of 100% DMSO. Control group was received DMSO.

Table 14
General behaviours of mice after the administration of 150 mg of plant extracts/kg of body weight of mice

Days	*P. bistorta* crude extract	Chloroform fraction	Hexane fraction	Control
1	Slow respiration and sedative; locomotion was arrested within a few hours of administration.	Slow respiration and sedative; locomotion was arrested within a few hours of administration.	Slow respiration and sedative; locomotion was arrested within a few hours of administration.	Normal
2	Death of two mice; the live mice were dull.	Death of one mouse and live mice were dull.	Less active, all mice were live but were dull.	Normal
3	Recovered and gained weight; locomotive behaviour became normal.	Recovered and gained weight; locomotive behaviour became normal.	Recovered and gained weight; locomotive behaviour became normal.	Normal
4-14	Normal	Normal	Normal	Normal

No. of mice used in each group, n = 4; Swiss albino mice of both sexes, each weighing about 30 g, were used. Extracts/fractions in DMSO (150 mg of extract/kg of body weight of mice) were administered to mice by intraperitoneal injection. Crude extract/fractions were dissolved in 10 mL of 100% DMSO. Control group was received DMSO.

The experimental animals were administered with crude extract or fractions by the intraperitoneal injection. Those groups treated with extract or fractions showed slow respiration, sedative and locomotor behaviours. These behaviours were observed within a few hours after the administration; while the control experimental animals were normal throughout the experiments. On day two, three animals died in the group in which the animals were treated with crude extract and two died in the group in which the animals were administered with chloroform; while no animals died in the group in which hexane

fraction was treated but they were less active and very dull. On day three the survived animals were recovered and the locomotive behaviour was also normal in all the groups of experimental animals. The same observations were seen on all other days. The experimentation was completed on the fifteenth day and the experimental animals were sacrificed. The animal bodies were dissected; no gross abnormalities were observed. These experiments showed that the LD_{50} value of crude extract was less than 200 mg/kg of body weight and that of chloroform fraction was 200 mg/kg of body weight; while hexane fraction was more than 200 mg/kg of body weight.

The experiment was repeated by preparing the crude extract and fractions concentration as 150 mg/kg body weight. The general behaviours of mice were observed as previously. On day one, day three and the rest of the days, the observations were the same as observed previously. But on day two, two animals died in the group in which the animals were treated with crude extract; one died in the group in which the animals were treated with chloroform fraction, while in the hexane fraction-treated group, no animals died but they were less active and very dull. We did not do any further experimentation with lower concentrations. We concluded that the LD_{50} value of crude extract was 150 mg/kg of body weight and that of chloroform extract was 200 mg/kg of body weight; the hexane extract has an LD_{50} value >200 mg/kg of body weight. This is the first such report in this plant.

2.3.0 Experimental

General experimental procedures

Melting points were determined on a Buchi Melting point B-540 apparatus. IR spectra were recorded on a Bio Rad, Class II Laser product. ^{1}H, ^{13}C NMR and 2D NMR spectra were recorded on Bruker, 300 and/or 500 MHz spectrometers. Standard microprograms supplied by Bruker were used to run 1D and 2D NMR spectroscopy. Chemical shifts were reported in parts per million (ppm) with TMS as a reference standard or with reference to solvent peaks and coupling constants (*J*) expressed in hertz. LREIMS were measured on a Finnigan/MAT MAT 95 XL-T or VG Micromass 7035. HREIMS were measured on Finnigan/MAT MAT 95 XL-T mass spectrometers. HPLC was carried on a Waters associates, μ-Porasil (300 x 5 mm) column with a Shimadzu RID-10A, refractive index detector. Silica gel 60 (Merck, 0.063- 0.200 m) was used for column chromatography. Lichroprep RP-18 (Merck, 40-63 μm) was used for separation and/or purification. Precoated silica gel plates (Merck, Kieselgel 60F 254, 0.25 mm or Baker Si250F, 0.25 mm) were used for preparative TLC and/or analytical TLC. Spots were detected using UV light or staining with iodine or by spraying with 50% H_2SO_4 followed by heating at 110˚C for 5 minutes. The following instruments were used at the Pharmacology laboratory; Biological safety cabinet (NUAIRE, Plymouth, USA), ELX 800 Microplate reader (Bio-Tek Instruments Inc., USA), Hemocytometer (Fortuna, Germany), Leitz Fluovert microscope (Ernst Leitz Wetzlar GMBH, Germany), Water incubator (Everbloom Medical & Scientific Pte. Ltd., Singapore), -86˚C Freezer (Forma Scientific), Beckman Avanti J-251 Cenrtifuge (Fullerton, CA, USA), Beckman Optima L-90K and Ultracentrifuge (Fullerton, CA, USA).

Cell Culture

The following cancer cell lines were obtained from American Type Cell Culture (ATCC) (Manassas, VA, USA).

P338 (Murine lymphocytic leukaemia cells), HL60 (Human leukaemia cells), MCF7 (Human breast cancer cells), LL2 (Lewis lung carcinoma cells), HepG2 (Hepatocellular carcinoma cells), J82 (Bladder transitional carcinoma cells) and WEHI1640 (Murine fibrosarcoma cells)

Cells were cultured in Corning disposable flasks using RPMI-1640 or DEME medium supplemented with 5% fetal bovine serum and streptomycin and incubated at 37˚C in a humidified atmosphere of 5% CO_2. To ensure an exponential growth, cells were resuspended in fresh medium every 36h or 48h. Cell concentration and viability were determined using the trypan blue exclusion test. 20 µL of cell suspension was taken and equal volume of trypan blue solution (0.4%) was added. The number of live cells was counted using a Hemocytometer under a Leitz light microscope. The cultured cell lines were then diluted to required concentration using medium. 90 µL of cultured cells were transferred to each well of a 96 well plate and 10 µL extract of various concentrations was added to the cultures and incubated at 37˚C in the humidified atmosphere of 5% CO_2. On day three, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/mL) was added to the culture medium [151]. After a further 4h of incubation, 100 µL solution of 10% sodium dodecyl sulphate (SDS) in 0.01 N HCl was added to each well and the formazan crystals in each well were dissolved. Optical density (absorbance) measurements were made with a microplate reader at 570 nm. Cytotoxicity was calculated by the following formula [152].

Growth inhibition, (GI) = [(OD_{570} of control – OD_{570} of test sample)/ OD_{570} of control] x 100%.

Preparation of solutions for cell culture study

The extracts or their sub-fractions were first dissolved in a minimum quantity of (0.5 or 1.0 mL) dimethylsulfoxide (DMSO) and then diluted to required quantity by water. A stock solution of DMSO/water solvent mixture of same proportion was prepared and used for subsequent dilutions. DMSO/water solvent mixture of the same proportion without extract or sub-fractions served as negative control. In all cases, the proportion of DMSO was kept constant.

The new compound, 24(E)-ethylidenecycloartanone **(93)** is highly hydrophobic; it is insoluble in DMSO which is the solvent mainly used for cytotoxic screening in the MTT assay. Therefore we used a CrEL/ethanol solvent mixture at the first, followed by water. 10 mg of the new compound **(93)** was first dissolved in a minimum quantity of CrEL (2 drops) and 0.5 mL of ethanol solvent mixture. The solution was stirred with a spatula in order to attain homogeneity and the solution was made up to 10 mL using water and was shaken well. As per calculation, this served as a solution of concentration 221.0 μM (100 μg/mL). A stock solution of CrEL/ethanol solvent mixture and water of the same proportion was prepared and used for subsequent dilutions. A total of five concentrations, 221.0, 110.5, 55.25, 22.1 and 2.21 μM (i.e.100, 50, 25, 10 and 1 μg/mL) were prepared. CrEL/ethanol solvent mixture and water of the above mentioned proportion without compound was served as negative control.

Experimental animals for toxicity study

White Swiss albino mice of both sexes weighing about 30g each were used. The animals were divided into four groups, each consisting of four animals. The first three groups were treated with the crude extract or fractions while the fourth one served as a control group. A solution of crude extract, hexane and chloroform fractions were prepared in 10 mL of 100% DMSO and administered to each mouse by intraperitoneal injection. The solutions were prepared by dissolving them as per the ratio of 200 mg of extracts/kg bodyweight of mice in 10 mL of 100% DMSO. Their general behaviours were assessed after the administration (by intraperitoneal injection) of the extract or fractions as reported in the literature [153,154]. The experimental animals were sacrificed on the fifteenth day. We repeated the experiments by administering the crude extract, hexane and chloroform fractions of 150 mg extract/kg of body weight of mice in 10 mL of 100% DMSO. The general behaviours of experimental mice were monitored as previously; the animals were sacrificed on the fifteenth day.

Plant Material

The plant materials were purchased from a local market and a voucher specimen (KMano PB 2003) is deposited in the Department of Biological Sciences, National University of Singapore, Singapore.

Extraction and Isolation

The rhizomes of *P. bistorta* (600g) were ground into powder and then extracted with chloroform (3L x 4) at room temperature. The residue was dissolved in a water/methanol

mixture (95:5) and was then extracted successively with hexane and chloroform. After the preliminary screening followed by the isolation of 24-methylenecycloartanone **(97)**, we carried out further investigation by purchasing 12 kg of plant material and extracted it as previously. Rota-vapour was used to remove solvent from hexane and chloroform fractions. The hexane extract was chromatographed over silica gel using hexane and eluted in a gradient fashion with increasing polarity. Purification of the eluted fractions afforded friedelin **(94)** (6 mg)**,** β-sitosterol **(47)** (1.2 g), γ-sitosterol **(96)** (4 mg), 24(E)-ethylidenecycloartanone **(93)** (580 mg) and cycloartane-3,24-dione **(95)** (ca.1.5 mg). The chloroform extract was chromatographed over Lichroprep RP-18 and eluted in isocratic fashion with methanol. Purification of eluted fractions afforded β-sitosterol **(47)** (110 mg), β-sitosterone **(100)** (ca. 0.5 mg), 24(E)-ethylidenecycloartanone **(93)** (7.0 mg) and 24(E)-ethylidenecycloartan-3α-ol **(98)** (ca. 1.0 mg). For methanol-water fraction, Rota-vapour was used first to remove as much solvent as possible followed by the use of Freeze dryer/Lyophilizer to remove the remaining solvent. The residue was chromatographed over Lichroprep RP-18 and eluted in isocratic fashion with methanol. Purification of one of the eluted fractions afforded β-sitosterol **(47)**.

List of spectral data and physical properties of compounds

24(E)-Ethylidenecycloartanone (93)

Colourless crystals; mp 135.4-137.1 ˚C; IR (KBr) ν_{max} 3046, 2939, 2864, 1712, 1448, 1376 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 452 $[M]^+$ (72), 437 (42), 354 (86), 340 (48), 313 (72), 271 (44), 216 (78), 175 (51), 95 (100), 55 (54); HREIMS *m/z* 452.4019 (calcd. for $C_{32}H_{52}O$, 452.4018); ^1H and ^{13}C NMR data: see Table 6.

24(E)-Ethylidenecycloarta-3α-ol (98)

Colourless solid; MS (EI, 70eV), *m/z* (rel.int. %): 454 $[M]^+$ (72), 437 (42), 354 (86), 340 (48), 315 (72), 271 (44), 216 (78), 175 (51), 95 (100), 55 (54); HREIMS *m/z* 454.4175 (calcd for $C_{32}H_{54}O$, 454.4174); ^1H and ^{13}C NMR data: see Table 7.

24,31-Epoxy-24-ethylcycloartan-3α-ol (99)

Colourless amorphous powder; MS (EI, 70eV), *m/z* (rel.int. %): 470 $[M]^+$ (8), 454 (48), 439 (64), 356 (33), 315 (45), 271 (44), 201 (78), 175 (92), 95 (100), 55 (60); HREIMS *m/z* 470.4108 (calcd. for $C_{32}H_{52}O$, 470.4123); ^1H and ^{13}C NMR data: see Table 8.

Cycloartane-3,24-dione (95)

Colourless crystals; MS (EI, 70eV), *m/z* (rel.int. %): 440 $[M]^+$ (84), 425 (61), 407 (21), 379 (19), 354 (78), 342 (30), 313 (100), 302 (59), 271 (20), 203 (66), 175 (84), 135 (87), 127 (77); HREIMS *m/z* 440.3655 (calcd for $C_{32}H_{52}O$, 440.3654); ^1H and ^{13}C NMR data: see Table 9.

24-Methylenecycloartanone (97)

Colourless crystals; mp 111-113 ˚C; IR (KBr) v_{max} 3040, 2939, 2862, 1712, 1376, 1442, 814 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 438 $[M]^+$ (100), 423 (47), 395 (31), 355 (28), 340 (40),313 (75), 300 (39), 219 (34),175 (54), 147 (49), 95 (68), 69 (28), 55(15); ^1H and ^{13}C NMR data: see Table10.

β-Sitosterol (47)

Colourless flakes; mp.136.1-138 ˚C; IR (KBr) v_{max} 3429, 2960, 2880, 1662, 1464, 1377, 1330, 1049 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 414 $[M]^+$ (64), 396 (100), 381 (42), 303 (30), 255 (50), 231 (40), 173 (18), 145 (78),121 (46), 107 (56), 81 (40), 55 (38); ^1H-NMR ($CDCl_3$) δ 1.15 m, Hα, 1.89 m, Hβ (H-1), 1.85 m, Hα, 1.56 m, Hβ , (H-2), 3.52 (1H, m,

H-3), 2.28 m (2H, H-4), 5.36 (1H, br d, 5.4 Hz, H-6), 1.53 m (2H, H-7), 1.93 m (1H, H-8), 0.98 m (1H, H-9), 1.51 m (H-11), 2.03 m, Hα, 1.19 m, Hβ (H-12), 1.00 (1H, H-14), 1.61 m, Hα, 1.13 m, Hβ, (H-15), 1.86 m, Hα, 1.35 m, Hβ, (H-16), 1.10 (1H, H-17), 0.68 (3H, s, H-18), 1.01 (3H, s, H-19), 1.37 m (1H, H-20), 0.92 (3H, d, 6.4 Hz, H-21), 1.35 m, Hα, 1.04 m, Hβ (H-22), 1.20 m (2H, H-23), 0.97 m (1H, H-24), 1.70 m (1H, H-25), 0.83 (3H, d, 6.8 Hz, H-26), 0.81(3H, d, 6.4 Hz, H-27),1.27(1H, H-28), 0.845 (3H, t, 6.6 Hz, H-29); ^{13}C-NMR ($CDCl_3$) δ37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.3(C-4), 140.8 (C-5), 121.7 (C-6), 31.8 (C-7), 31.9 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.7 (C-12), 42.3 (C-13), 56.8 (C-14), 24.3 (C-15), 28.2 (C-16), 56.1 (C-17), 11.9 (C-18), 19.4 (C-19), 36.1 (C-20), 18.9 (C-21), 33.9 (C-22), 26.0 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 19.0 (C-27), 23.0 (C-28), 12.0 (C-29).

γ-Sitosterol (96)

Colourless crystals; mp147-148 ˚C; MS (EI, 70eV), *m/z* (rel.int. %): 414 $[M]^+$ (16), 145 (45), 95 (100), 83 (26), 93 (85), 71 (46), 55 (27).

β-Sitosterone (100)

Amorphous solid; MS (EI, 70eV), *m/z* (rel.int. %): 412 $[M]^+$ (90), 397 (32), 370 (60), 327 (28), 289 (70), 271 (42), 229 (96), 187 (30),149 (64), 124 (100), 69 (32), 55 (38) 43 (46); [60].

3β-Friedelinol (66)

Colourless crystals; mp 280-282 ˚C; IR (KBr) ν_{max} 3500, 1380, 1370, 1265, 1175, 1020, 800 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 428 $[M]^+$ (215), 413 (27), 275 (46) 248 (26), 231 (52), 220 (42), 205 (53), 177 (57), 165 (82), 125 (98), 95 (100), 69 (76), 55(34); ^{1}H and ^{13}C NMR data: see Table 11.

Friedelin (94)

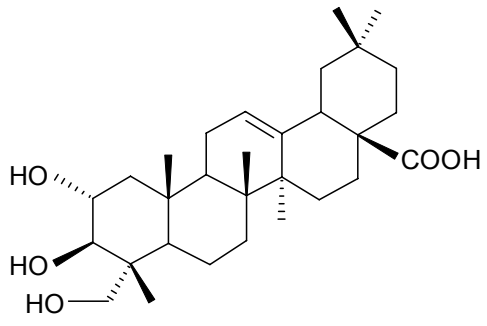
Colourless crystals; mp 263-265 ˚C; IR (KBr) v_{max} 2946, 2882, 2869, 1719, 1464, 1385 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 426 $[M]^+$ (28), 341 (12), 302 (32), 273 (54), 231 (38), 205 (56), 191 (44), 163 (57), 13 (100), 109 (98), 69 (75), 55 (43); ^1H NMR ($CDCl_3$) δ 1.95, 1.71, (2H, ddd, H-1), 2.37, 2.27 (2H, ddd, H-2), 2.25 (1H, q, H-4), 1.73,1.28 (2H, d, H-6), 1.49, 1.36 (2H, m, H-7), 1.38 (1H, dd, H-8), 1.53 (1H, m, H-10), 1.45, 1.26 (2H, m, H-11), 1.33, 1.33 (2H, m, H-12), 1.47, 1.27 (2H, m, H-15), 1.58, 1.35 (2H, m, H-16), 1.56 (1H, m, H-18), 1.37, 1.21 (2H, m, H-19), 1.50, 1.31 (2H, m, H-21), 1.50, 0.94 (2H, m, H-22), 0.88 (3H, d, H-23), 0.71 (3H, s, H-24), 0.86 (3H, s, H-25), 1.00 (3H, s, H-26), 1.04 (3H, s, H-27), 1.17 (3H, s, H-28), 0.99 (3H, s, H-29), 0.94 (3H, s, H-30); ^{13}C NMR ($CDCl_3$) δ22.3 (C-1), 41.5 (C-2), 213.2 (C-3), 58.2 (C-4), 42.1 (C-5), 41.3 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.4 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.4 (C-15), 36.0 (C-16), 30.0 (C-17), 42.8 (C-18), 35.3 (C-19), 28.1 (C-20), 32.7 (C-21), 39.2 (C-22), 7.2 (C-23), 15.0 (C-24), 17.9 (C-25), 20.2 (C-26), 18.6 (C-27), 32.1 (C-28), 35.7 (C-29), 32.8 (C-30).

Chapter 3

Constituents of *Eugenia grandis* (Myrtaceae)

3.1.0 Introduction to Myrtaceae family

The genera *Syzigium* and *Eugenia* both belong to the Myrtaceae family and consist of approximately 700 species confined mainly to the warmer parts of the world [155]. The genus *Eugenia* consists of approximately 600 species in the tropics. These trees or shrubs are often planted for ornaments in warm regions with species yielding edible fruits [155]. A number of species have been reported to contain triterpenes and/or β-sitosterol **(47)** [156]. Examples of triterpenoids isolated were arjunolic acid **(102)**, asiatic acid **(103)**, methyl arjunolate **(104)**, methyl asiatate **(105)**, lupeol **(106)**, betulinic acid **(65)**, α-amyrin **(107)** , β-amyrin **(108)**, methyl 2α-acetoxy-3β-hydroxyolean-12-en-28-oate **(109)**, methyl 2α-acetoxy-3β-hydroxyurs-12-en-28-oate **(110)**, 2α-hydroxyursolic acid **(111)**, methyl maslinate (methyl 2α,3β-dihydroxyolean-12-en-28-oate) **(112)**, methyl 2α,3β-dihydroxyurs-12-en-28-oate **(113)**, oleanolic acid **(46)**, epioleanolic acid **(114)**, ursolic acid **(115)**, epiursolic acid **(116)**, crategolic acid **(117)**, erythrodiol **(118)**, friedelin **(94)** and 3β-friedelinol **(66)** [156-159]. The only steroid isolated was β-sitosterol **(47)**.



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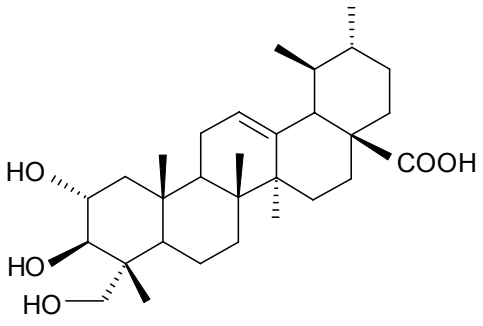

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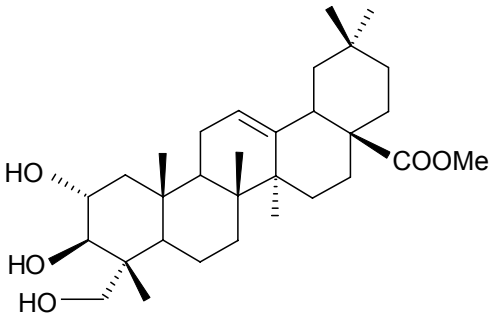

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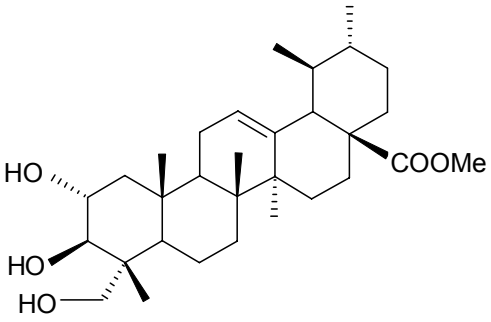

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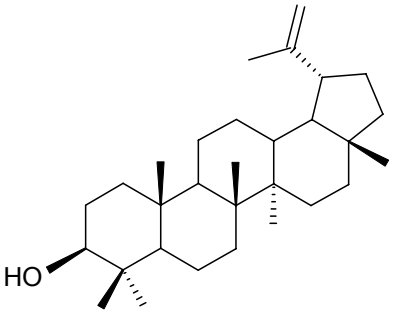

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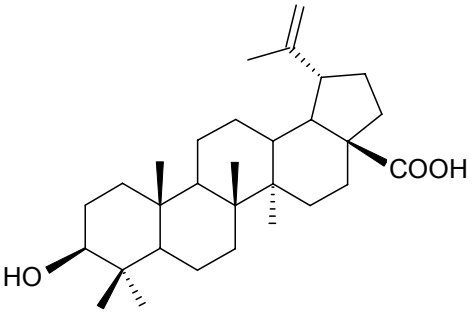

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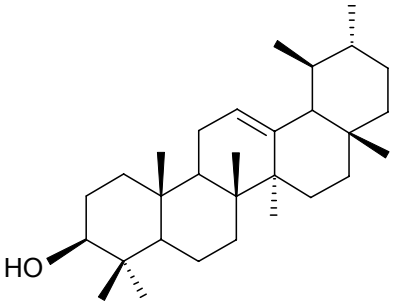

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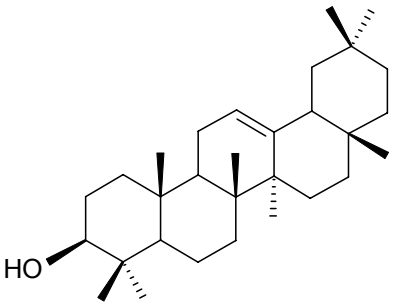

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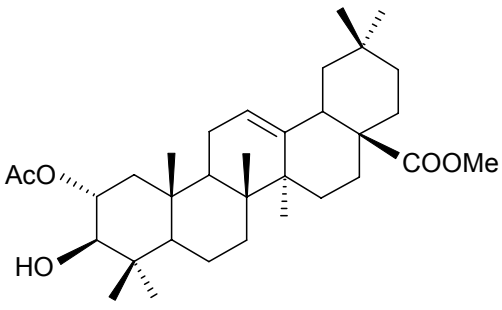

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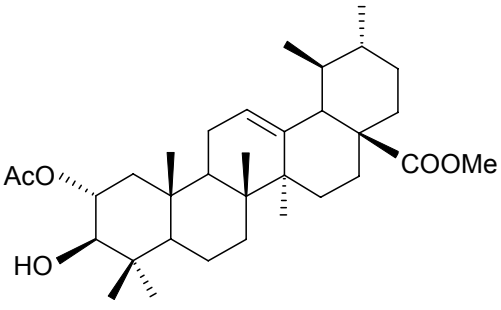

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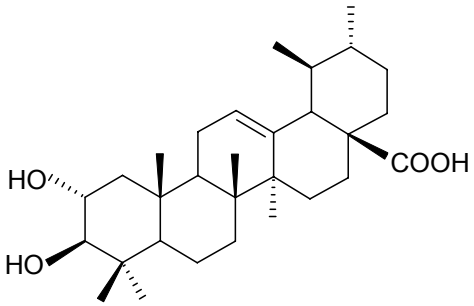

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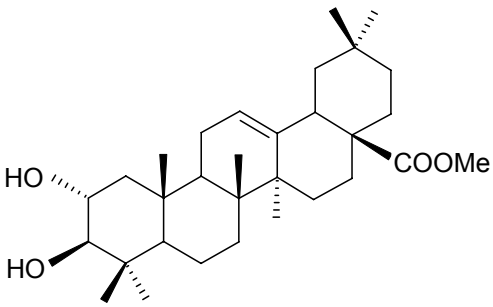

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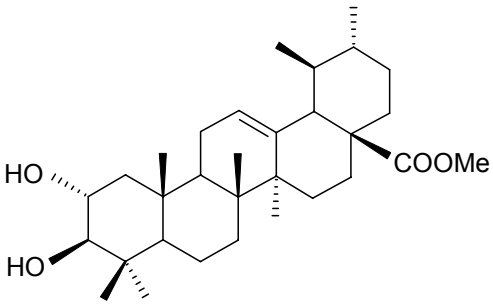

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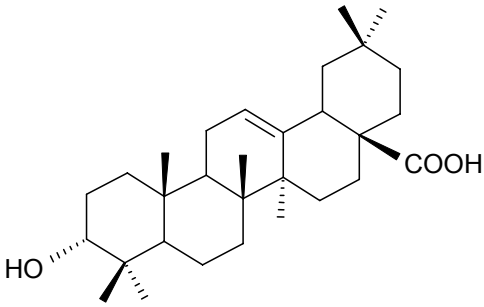

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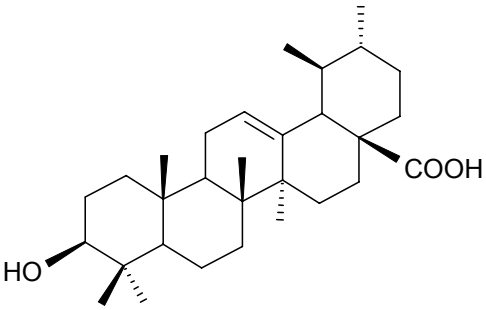

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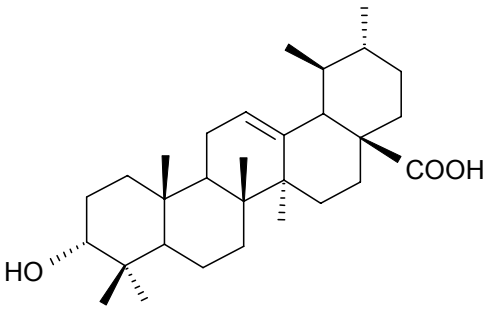

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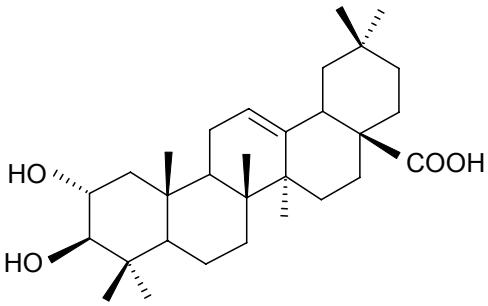

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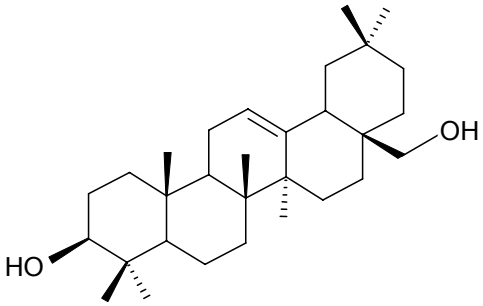


(118)

Eugenia aromatica (syn: *Syzigium aromaticum*, *Eugenia caryophyllata*, *Caryophyllus aromaticus*), *Jambosa caryophyllus*, *Eugenia jambolana* (syn: *Syzigium cumini*), *Eugenia maire*, *Eugenia fructicosa*, *Eugenia wallichii*, *Syzigium cordatum*, *Eugenia javanica* (syn: *Syzigium samarangense*, *Eugenia formosa*), *Eugenia mooniana*, *Eugenia biflora* etc. are some examples of species belong to the Myrtaceae family. Among the *Eugenia* species, *Eugenia caryophyllata* has been most extensively studied due to its medicinal properties and economic values. Extracts from the plant have been used in treating tooth ache and gum diseases, dandruff control, antibaldness, scalp-treatment, transdermal and antitumour pharmaceuticals, food flavourings and as antioxidants for fats.

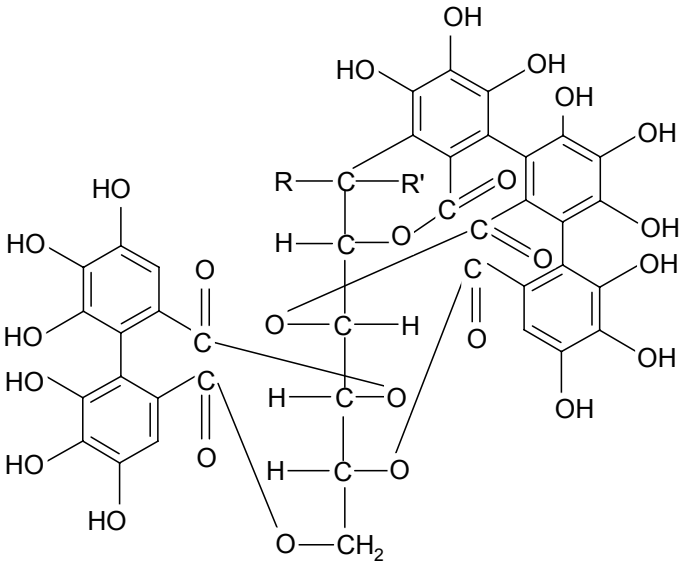
3.1.1 Introduction to *Eugenia grandis*

*Eugenia grandis (*syn. *Syzygium grande*) (Myrtaceae) also known locally as Sea apple or Jambu Laut, is a common seashore tree. They are often planted along the roadsides to give shade. The trees are tall, growing to 30 m and have an irregular crown. The leaves are large, shiny, dark green, elliptic in shape and have a distinct down-turned tip. The flowers are oblong, large, white and fluffy.

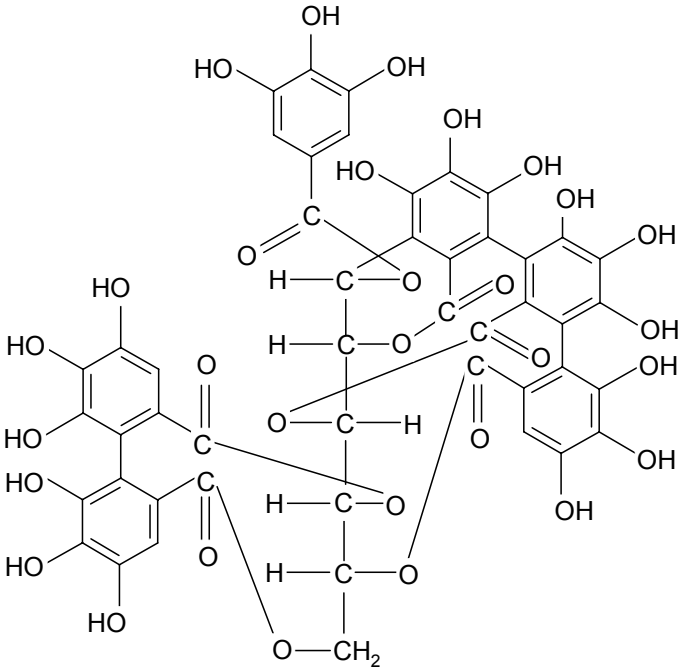
3.1.2 Previously reported chemical constituents from *E. grandis*

A thorough literature search indicated that there was only one report on the chemical constituents of *E. grandis*. The isolation of castalagin **(119)**, vescalagin **(120)** and

ellagitannin (1-O-galloyl castalagin) **(121)** have earlier been reported from the leaves of *E. grandis* [160].



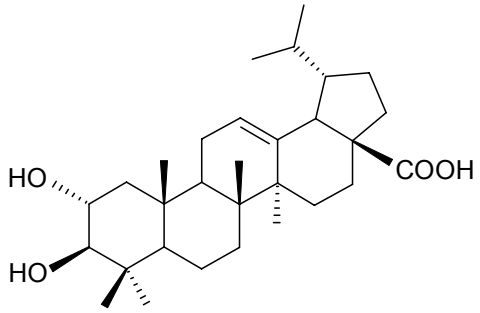
(119) R = H, R’ = OH; **(120)** R = OH, R’ = H



(121)

3.2.0 Results and Discussion

We investigated the chloroform extract of stem-bark of *E. grandis* for their chemical constituents. A new compound, 2α,3β-dihydroxylup-12-en-28-oic acid **(122)**, a rarely encountered compound, 3β-hydroxylup-12-en-28-oic acid **(123)**, a mixture containing two known compounds *viz*. arjunolic acid **(102)** and asiatic acid **(103)** together with other five known compounds, fridelin **(94)**, 3β-friedelinol **(66)**, β-sitosterol **(47)**, oleanolic acid **(46)** and betulinic acid **(65)** were isolated



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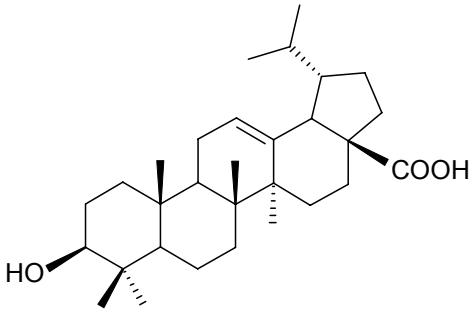

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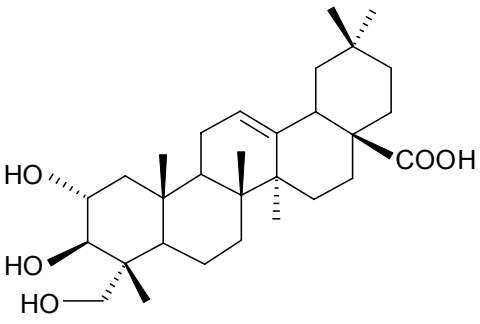
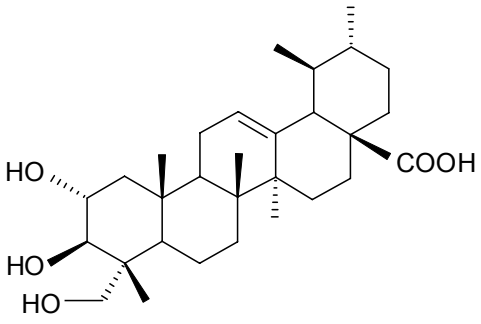
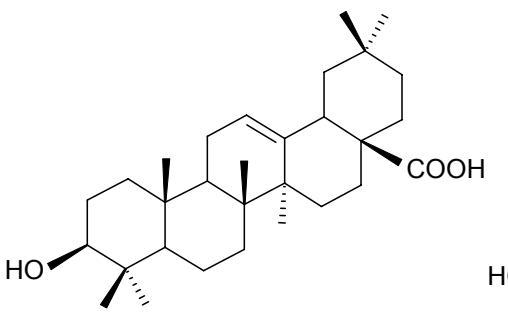


(102)



(103)



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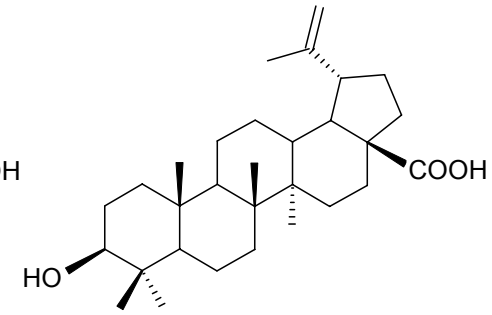

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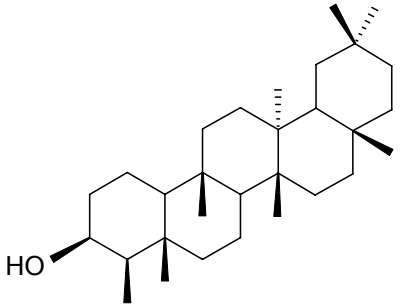
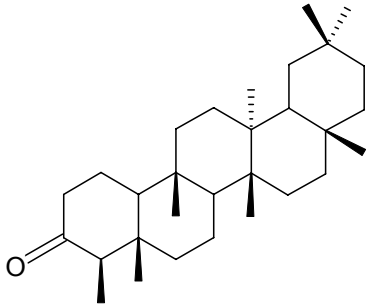
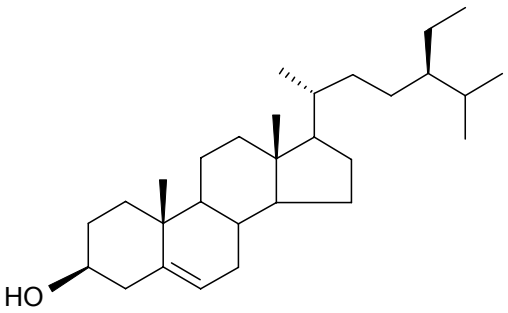


(66)



(94)



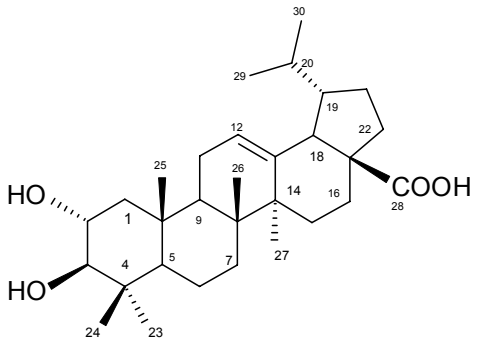
(47)

3.2.1 Characterisation of the isolated chemical constituents from *E. grandis*

2α,3β-Dihydroxylup-12-en-28-oic acid (122)

2α,3β-Dihydroxylup-12-en-28-oic acid **(122)** was obtained as colourless crystals. The molecular formula was determined to be $C_{30}H_{48}O_4$ by high-resolution EIMS (*m/z* 472.3549). The IR spectrum gave absorption peaks at ν_{max} 3437, 1692 and 1642 cm^{-1}; these are characteristic of hydroxyl, carboxyl and double bond respectively. The ^{13}C NMR gave thirty signals. Seven methyl, eight methylene, eight methine and seven quaternary carbons were sorted by HSQC-DEPT spectrum. Two of the methine carbon signals were observed at δ 68.6 and 83.6 indicating the presence of two hydroxyl groups. Further, the chemical shift values of another methine and a quaternary carbon were observed at δ 125.8 and 138.7, respectively, indicated the presence of a double bond. One of the quaternary carbons showed a chemical shift value at δ 180.9 indicated the presence of a carboxylic group. The ring olefinic double bond and the fact that the compound has a lup-12-ene skeleton was indicated by resonances of the sp^2 carbons C-12 (methine) at δ 125.8 and C-13 (quaternary carbon) at δ 138.7 and by the analysis of the methine and methylene resonances. In the HMBC spectrum, it is evident that the signal at δ 5.26 (t, 3.6, Hz) attributed to hydrogen H-12, which is also a typical feature of ring olefinic proton [161], showed correlations with carbon signals at C-9, C-11, C-14 and C-18. Similarly, C-13 showed correlations with H-11, H-15, H-18 and H-27. Therefore, the olefinic double bond was placed between C-12 and C-13 based on these correlations. Further, the carboxyl group was attached at C-17, since C-28 (δ 180.9) assigned for the carboxyl group showed correlations with H-16 and H-18. The position of the double bond and the carboxyl group was collectively supported by an MS fragmentation peak at *m/z*

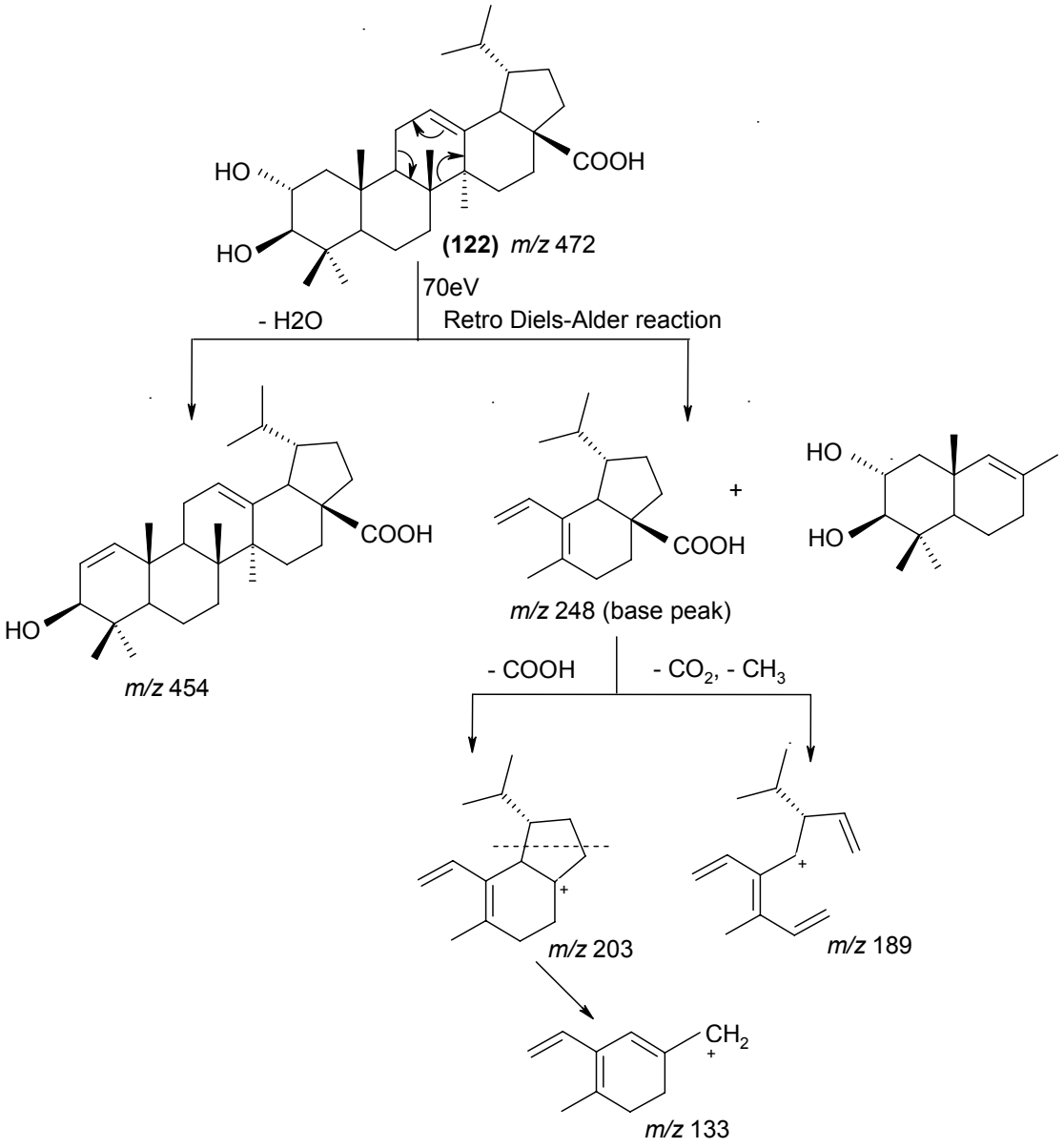
248 (base peak), which was due to retro Diels-Alder cleavage of the molecule as shown in Scheme 3. Other fragmentation peaks were observed at *m/z* 203, 189 and 133 (Scheme 3). These are characteristics of a lupane or oleanane or ursane-type triterpenoids [161-163]. The fragment peak at *m/z* 203 was due to loss of a carboxyl group from *m/z* 248.



(122)

The ^{1}H NMR spectrum showed signals at δ 3.64 (ddd, J = 11.3, 9.9, 4.6 Hz, 1H) and 2.93 (d, J = 9.9 Hz, 1H) ascribable to two hydroxymethine protons. Through analysing HSQC and HMBC spectra, these two hydroxymethine protons were respectively assigned as H-2 and H-3. In the HMBC spectrum, it is evident that the H-2 proton (δ 3.64) showed correlations with C-10 and H-3 proton (δ 2.93) showed correlations with C-23 and C-24. Further, H-2 has a β-orientation while H-3 has an α-orientation. In other words the hydroxyl group at C-2 position has the α-orientation while it has the β-orientation at C-3 position. These orientations were confirmed through NOESY and comparing their coupling constant values and/or multiplicity with related compounds [164-166]. The NOESY correlations of H-2 with H-24 and H-25; H-3 with H-5 and H-23, indicated the relative configurations at C-2 and C-3. Other significant correlations observed were H-9 with H-5 and H-27; H-11 with H-12 and H-25; H-24 with H-25 and H-25 with H-11 and

H-24. With all these information, the structure of this new compound was derived as 2α,3β-dihydroxylup-12-en-28-oic acid **(122)**.



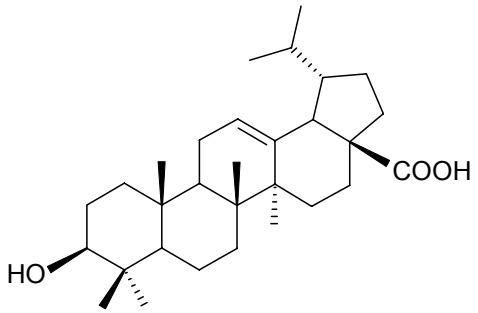
Scheme 3
Major MS fragmentation pattern of 2α,3β-dihydroxylup-12-en-28-oic acid **(122)**.

Table 15
NMR data of 2α,3β-dihydroxylup-12-en-28-oic acid **(122)** in CD_3OD.

Position	^{13}C/ DEPT	δ_C (ppm)	δ_H (ppm) *J* in Hz	HMBC (C → H)	NOESY
1	CH_2	47.4	α: 1.98 m β: 0.92 m	9, 25	
2	CH	68.6	β: 3.64 ddd (11.3, 9.9, 4.6)	3	24, 25
3	CH	83.6	α: 2.93 d (9.9)	2, 23, 24	5, 23
4	C	39.5		23, 24, 25	
5	CH	55.8	0.87 m	1, 23, 24	
6	CH_2	18.6	α: 1.58 m β: 1.45 m	5, 7	
7	CH_2	33.3	α: 1.38 m β: 1.58 m	5, 26	
8	C	39.8		6, 7, 9, 11, 26	
9	CH	48.0	1.63 m	5, 11, 12, 25, 26	5, 27
10	C	38.2		2, 5, 11, 25	
11	CH_2	23.6	α: 1.99 m β: 1.99 m	9, 12	12, 25
12	CH	125.8	5.26 t (3.6)	11, 18	
13	C	138.7		11, 15, 18, 27	
14	C	42.2		9, 12, 15, 18, 26	
15	CH_2	28.3	α: 1.95 m β: 1.11 m	16, 27	
16	CH_2	24.4	α: 2.06 m β: 1.67 m	15, 18, 22	
17	C	48.2		15, 18, 19	
18	CH	53.5	2.30 d (11.6)	12, 16, 19	
19	CH	39.5	1.41 m	18, 29, 30	
20	CH	39.6	1.00 m	18, 19, 29, 30	
21	CH_2	30.9	α: 1.52 m β: 1.37 m	22	
22	CH_2	37.2	α: 1.72 m β: 1.66 m		
23	CH_3	28.5	0.83 s	3, 24	
24	CH_3	16.6	0.83 s	3, 23	25
25	CH_3	16.3	1.04 s	1, 9	11, 24
26	CH_3	16.9	0.87 s	9	
27	CH_3	23.2	1.15 s	15	
28	C	180.9		16, 18, 22	
29	CH_3	20.7	0.99 d (6.5)	19, 30	
30	CH_3	16.8	0.91 d (6.5)	19, 29	

3β-Hydroxylup-12-en-28-oic acid (123)

3β-Hydroxylup-12-en-28-oic acid **(123)** was obtained as colourless crystals. The molecular formula was determined to be $C_{30}H_{48}O_3$ by high-resolution EIMS (*m/z* 456.3588). The IR spectrum gave absorption bands at ν_{max} 3406, 1689 and 1639 cm^{-1}; these are characteristics of hyrdroxyl, carboxyl and double bond, respectively. The ^{13}C NMR gave thirty signals; seven methyl, nine methylene, seven methine and seven quaternary carbons were sorted by HSQC-DEPT spectrum. The ^{13}C NMR signal of a methine carbon was observed at δ 79.0 indicated the presence of hydroxyl functionality. The chemical shift values of another methine and a quaternary carbon were observed at δ 126.1 and 138.8, respectively, indicated the presence of a double bond. One of the quaternary carbons showed a chemical shift value at δ 181.1 that indicated the presence of a carboxylic group.



(123)

Its mass spectrum gave a base peak at *m/z* 248 and other fragmentation peaks at *m/z* 235, 203, 189 and 133, which is a characteristic of a lupane or oleanane or ursane-type triterpenoids with C-12 unsaturation and a carboxyl group at C-17 position. Most of the HMBC spectral correlations have close similarity with compound, 2α,3β-dihydroxylup-12-en-28-oic acid **(122)** except at C-2 position in which the hydroxyl group is absent. The

signal observed at δ 3.71 (dd, *J* = 11.6, 4.8 Hz) was diagnostic for H-3, deducing from an HMBC correlations between H-23 (δ 0.98), H-24 (δ 0.77) and C-3 (δ 79.0). The chemical shift value and *J* couplings (diaxial and axial/equatorial interactions) suggested the presence of a β-hydroxyl substitution at C-3 [161]. The same compound **(123)** has been reported twice previously [161,167]. However, complete NMR data were not given in both reports, as well as to the best of our knowledge, assignments were not found from any other source. Thus, we did the complete assignments and list these in Table 16.

Table 16
NMR data of 3β-hydroxylup-12-en-28-oic acid **(123)** in $CDCl_3$ and CD_3OD

Position	DEPT	δ_C (ppm)	δ_H (ppm) *J* in Hz	HMBC (C → H)
1	CH_2	39.2	α: 1.66 m; β: 1.66 m	5, 25
2	CH_2	27.1	α: 1.63 m; β: 1.58 m	
3	CH	79.0	α: 3.71 dd (11.6, 4.8)	1, 23, 24
4	C	39.2		5, 23, 24
5	CH	55.2	0.74 m	1, 6, 23, 24, 25
6	CH_2	18.8	α: 1.54 m; β: 1.38 m	5
7	CH_2	33.5	α: 1.53 m; β: 1.35 m	5, 9, 26
8	C	40.1		6, 7, 9, 11, 26
9	CH	48.1	1.54 m	7, 11, 12, 25, 26
10	C	37.4		2, 5, 6, 9, 11, 25
11	CH_2	23.7	α: 1.92 m; β: 1.92 m	9, 12
12	CH	126.1	5.24 t (3.6)	11, 18
13	C	138.8		11, 18, 27
14	C	42.9		9, 12, 15, 18, 26, 27
15	CH_2	28.4	α: 1.90 m; β: 1.09 m	16, 27
16	CH_2	24.6	α: 2.02 m; β: 1.65 m	15, 18
17	C	48.3		15, 16, 18, 19
18	CH	53.4	2.19 d (11.5)	12, 19
19	CH	39.6	1.36 m	18, 20, 29, 30
20	CH	39.5	0.99 m	19, 29, 30
21	CH_2	31.1	α: 1.50 m; β: 1.33 m	
22	CH_2	36.3	α: 1.70 m; β: 1.64 m	
23	CH_3	28.2	0.98 s	3, 5, 24
24	CH_3	15.8	0.77 s	3, 5, 23
25	CH_3	15.6	0.96 s	1, 5, 9
26	CH_3	17.1	0.84 s	9
27	CH_3	23.7	1.11 s	15
28	C	181.1		16, 18
29	CH_3	21.2	0.95 d (6.5)	30
30	CH_3	17.2	0.88 d (6.5)	29

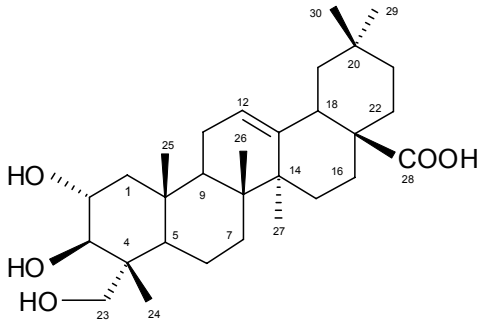
Mixture of two compounds (102) and (103)

The mixture of two compounds, arjunolic acid **(102)** and asiatic acid **(103)**, were obtained as colourless crystals. Both compounds have the same molecular formula, $C_{30}H_{48}O_5$, deduced from HREIMS *m/z* 488.3497. The EIMS gave a single molecular ion peak at *m/z* 488 and a base peak at *m/z* 248 gave information about the presence of a pentacyclic triterpenoids. We have confirmed this molecular ion peak with ESI and FAB mass spectroscopy. Both gave $[M-1]^+$ ion at *m/z* 487. However, there was inconsistency in the observed molecular weight and the number of ^{13}C NMR signals. In the ^{13}C NMR spectrum, we observed more than forty peaks. Some peaks displayed doublets with unequal intensities, which were not due to incomplete ^1H-decoupling. For example, a peak at about δ 181, which indicated the carboxyl carbon, showed two very close signals at δ 180.9 and 181.1. Further, both HMBC and HSQC-DEPT gave peaks with stacking one over another or with very close chemical shift values. Based on these observations, we understood that it was a mixture of two closely related compounds. The MS fragmentation pattern has close similarity to the new compound, 2α,3β-dihydroxylup-12-en-28-oic acid **(122)** except a mass difference of 16 *amu*. This observation allowed us to assume the presence of an extra hydroxyl group in the sample compared to the new compound, 2α,3β-dihydroxylup-12-en-28-oic acid **(122)**. As expected, on inspection of its chemical shift values in ^{13}C and DEPT spectra at the hydroxyl region, three oxygenated carbons were observed, one at δ 75.2 another at δ 66.8, both attached to methine carbons with the third one at δ 63.3 attached to a methylene carbon. However each of the three peaks displayed two peaks with a negligibly small chemical shift difference. We have placed one hydroxyl group at C-2 and another group at C-3 based on

the spectral correlations obtained in the new compound, 2α,3β-dihydroxylup-12-en-28-oic acid **(122)**. According to the number of different types of carbons, the third hydroxyl group, as it attached to methylene carbon, should replace any one of methyl hydrogens, so that it will form $–CH_2OH$ group. It is impossible to use all other positions which will make the carbon either methine or quaternary.

As mentioned earlier that EIMS gave a base peak at *m/z* 248 and other fragment peaks at *m/z* 203, 189 and 133 indicative of lupene or oleanene or ursene type triterpenoids with a double bond between C-12 and C-13 and a carboxyl group at C-17 position. This observation allowed us to exclude the presence of a hydroxyl group in rings C, D and E. Any changes in any one of the position in these rings will not follow this fragmentation pattern. In other words, it is impossible to utilise the methyl group present in these rings for the formation of $–CH_2OH$. The only possibility is to utilise one of the methyl groups present in ring A or B *i.e*. C-23, C-24, C-25 and C-26. Inspection of its HMBC revealed that the single proton at C-3 position correlates with an oxygenated methylene carbon at C-23 position. This allowed us to exclude the possibility of C-24, C-25 and C-26 positions. This is consistent with reports of NMR signals of methyl groups of pentacyclic triterpenoids with oxygen functions at 2, 3 and 23 positions [168].

For this interpretation, three structures are possible, *viz*. 2α,3β,23-trihydroxyolean-12-en-28-oic acid (arjunolic acid) **(102)**, 2α,3β,23-trihydroxyurs-12-en-28-oic acid (asiatic acid) **(103)** and 2α,3β,23-trihydroxylup-12-en-28-oic acid **(124)**.



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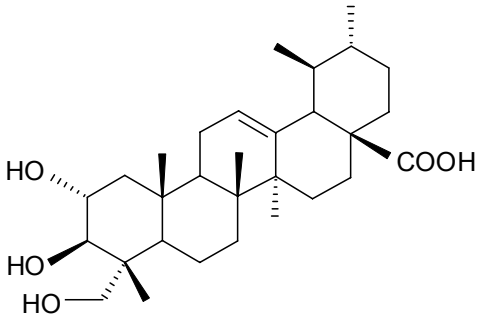
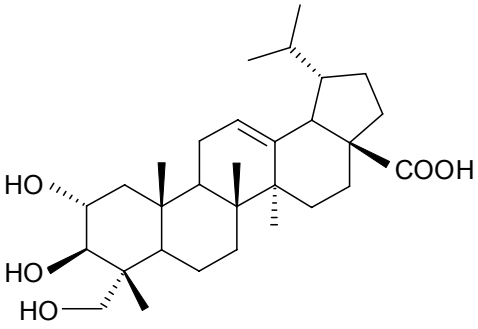


(103)



(124)

We were unable to get the two possible structures based on their 2D NMR spectral correlations, due to their extreme complexity, in particular at the high-field region. However, analysis of its ^{13}C NMR or DEPT spectrum in the olefinic region showed that peaks were not stacked one over another; we observed four independent peaks with reasonably good chemical shift difference. Further, it was also observed that two of them were methine and the other two were quaternary carbons. These peaks were due to C-12 and C-13 positions. The chemical shift values of one of the methine and a quaternary carbon were observed at δ 122.6 and 144.6, respectively. These values were in very good agreement with chemical shift values of arjunolic acid **(102)** at its C-12 and C-13 positions. Similarly the chemical shift values of another methine and a quaternary carbon

were observed at δ 125.9 and δ 139.0, respectively. These values were in very good agreement with chemical shift values of asiatic acid **(103)** at its C-12 and C-13 positions.

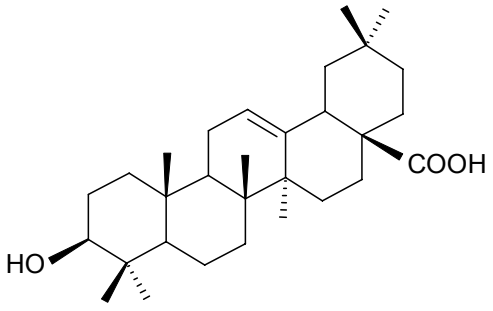
As stated earlier that the ^{13}C NMR and HMBC spectra were very complicated at the high-field region; we were unable to get the chemical shift values for the individual compounds. Thus, we tentatively assigned that the mixture of two compounds were arjunolic acid and asiatic acid. Our literature search indicated that reports of a mixture containing two compounds are very common, in particular the pentacyclic triterpenoids [169,170]. Our literature search also revealed that the existence of the compound, 2α,3β,23-trihydroxylup-12-en-28-oic acid **(124)** has not been reported so far. Further, oleanane- and ursane-type compounds were reported as a mixture rather than lupane & oleanane or lupane & ursane [169,170]. The NMR chemical shift values for the individual compounds and/or their methyl esters are available in the literature [171-175].

Arjunolic acid **(102)** and its semisynthetic derivatives were shown to exhibit inhibitory effects on Epstein-Barr virus (EBV) activation in Raji cells. Arjunolic acid derivatives could be valuable compounds as antitumour-promoters. Their inhibitory effects on skin tumour promoters were greater than those of previously studied natural products [176,177]. The anticancer effect of asiatic acid in two human breast cancer cell lines, MCF7 and MDA-MB-231 was reported earlier. Asiatic acid exhibited effective cell growth inhibition by inducing cancer cells to undergo S-G2/M phase arrest and apoptosis [178]. Asiatic acid **(103)** decreased viability and induced apoptosis in SK-MEL-2 (Human melanoma cells) and HepG2 (Human hepatoma cells) in a time- and dose-

dependent manner [179,180]. Asiatic acid dose-dependently showed cytotoxicity in HT29 (Human colon adenocarcinoma cell lines). The structural relationships of asiatic acid **(103)** and its derivatives to cytotoxicity and antihepatofibrotic activity in HSC-T6 cells were reported. Modification of the carboxylic acid group at C28 also reduced the cytotoxicity in HSC-T6 cancer cell lines [181].

Oleanolic acid (46)

Oleanolic acid **(46)** was obtained as colourless crystals. The mass spectrum of the compound showed molecular ion peak at *m/z* 456. The ^{13}C NMR gave thirty signals; seven methyl, ten methylene, five methine and eight quaternary carbons were sorted by DEPT analysis. A methine carbon showed a chemical shift value at *δ* 78.2, indicated the presence of hydroxyl functionality. The chemical shift values of another methine and a quaternary carbon were observed at δ 122.6 and 144.8 respectively indicated the presence of a double bond. One of the quaternary carbons showed the chemical shift value at δ 180.0 in the ^{13}C NMR, indicated the presence of a carboxylic group.



(46)

The mass spectrum gave a base peak at *m/z* 248 and other fragmentation peaks at *m/z* 235, 203, 189 and 133, which are characteristic of a lupene- or oleanene- or ursene-type triterpenoids with C-12 unsaturation and a carboxyl group at C-17 position. The single

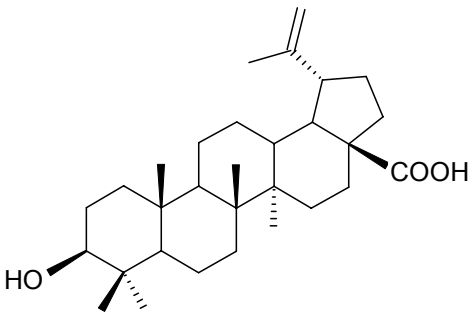
hydrogen at C-12 gave a chemical shift value centered at δ 5.49 (t, J = 3.6 Hz) in the ^{1}H NMR spectrum, which is a typical feature of ring olifinic proton [167]. A peak at δ 3.45 (dd, J = 11.2, 4.6 Hz) is due to H-3. Melting point range was found to be 196-198 ˚C. Comparison of its spectral data with those reported in the literature had good agreement [162,182-184].

Oleanolic acid **(46)** and ursolic acid **(115)** are pentacyclic triterpenoic acids having closely related chemical structure and are the major components of some traditional herbal medicines. There is a growing interest in the evaluation of the biological roles of these triterpenoid compounds. A review paper summarised the biological activities of triterpenoid acids (anti-inflammatory, hepatoprotective, gastroprotective, anti-ulcer, anti-HIV, cardiovascular, hypolipidemic, antiatherosclerotic and immunoregulatory effects) [185]. Oleanolic acid **(46)** is relatively non-toxic and could be used as chemopreventive/ chemoprotective agents in clinical practice [185,186]. The IC_{50} values for oleanolic acid on HCT15 (Human colon carcinoma cell lines) was 60 μmol/L. Proliferation assay showed that proliferation of oleanolic acid-treated cells was slightly increased at 24 h and significantly decreased at 48 h and 60 h. [187]. The IC_{50} values of oleanolic acid on WI 38, VA13 and HepG2 cancer cell lines were 14.5, 123 and 165 μM, respectively [188]. Oleanolic acid has been shown to inhibit mouse skin tumour promotion and showed bioactivity against a panel of 6 human solid tumour cell lines [189].

Betulinic acid (65)

Betulinic acid **(65)** was obtained as colourless crystals. The ^{13}C NMR gave thirty signals; six methyl, eleven methylene, six methine and seven quaternary carbons were sorted by

DEPT analysis. A methine carbon showed the chemical shift value at δ 78.2 indicated the presence of a hydroxyl group. The ^{1}H NMR spectrum showed a peak at δ 3.5 (t, J = 7.2 Hz) was due to H-3.



(65)

The chemical shift values of a methylene and a quaternary carbon were observed at δ 110.0 and 151.0, respectively, indicated the presence of a double bond. One of the quaternary carbons showed a chemical shift value at δ180.0 in the ^{13}C NMR indicated the presence of a carboxylic group. Two peaks as singlet at δ 4.95 (s) and 4.77 (s) in the ^{1}H NMR spectrum are characteristics of end methylene hydrogens. Comparison of its spectral data with those reported in the literature had good agreement [162,190].

Betulinic acid (3-hydroxy-lup-20(29)-en-28-oic acid) **(65)** is a pentacyclic lupane-type triterpenoid that is widely distributed in the plant kingdom. A variety of biological activities has been ascribed to betulinic acid **(65)**. With regard to its anticancer properties, it exhibited selective cytotoxicity against several melanoma-derived cell lines; because of its selective cytotoxicity against tumour cells and favourable therapeutic index, betulinic acid is a very promising new chemotherapeutic agent for the treatment of cancer. To date, dihydrobetulinic acid is a most potent pentacyclic triterpenoid to inhibit eukaryotic

topoisomerase I with IC_{50} value of 0.5 μM and can be exploited as a strong candidate for anti-tumour drug design [191-200].

Betulinic acid **(65)** inhibited the growth of K562 tumour cell lines with IC_{50} values 6.25 μg/mL and induces apoptosis in leukemia cells and further evaluation as a future drug to treat leukemia is on [196, 201-203]. Betulinic markedly inhibited cell growth of A-549, SK-OV-3, SK-MEL-2, XF498 and HCT15 human tumour cells [204]. The IC_{50} values of betulinic acid **(65)** on WI 38, VA13 and HepG2 cancer cell lines were respectively 1.3, 11.6 and 21μM [188].

β-Sitosterol (47), friedelin (94) and 3β-friedelinol (66)

For interpretation, refer to Chapter 2.

3.3.0 Experimental

General experimental procedure was followed elsewhere as described in chapter 2

Plant Material

The plant material was collected in Singapore along Kent Ridge Road and identified by Associate Prof. Hugh Tan Tiang Wah, Dept. of Biological Sciences, NUS and Chua Keng Soon, Senior Laboratory Officer (RMBR), Herbarium, NUS. A voucher specimen (KM20041122) was deposited in the herbarium, Department of Biological Sciences, National University of Singapore, Singapore.

Extraction and Isolation

A whole plant weighing about 20 kg (wet weight) was cut and chopped into small pieces. The air-dried material was then exhaustively extracted with chloroform and the extract was chromatographed over silica gel using hexane and eluted with the solvents of increasing polarity. Further purification of eluted fractions by repeated column chromatography and/or preparative TLC afforded 2α,3β-dihydroxylup-12-en-28-oic acid **(122)**, 3β-hydroxylup-12-en-28-oic acid **(123)**, oleanolic acid **(46)**, betulinic acid **(65)**, mixture of two compounds, arjunolic acid **(102)** and asiatic acid **(103)**, β-sitosterol **(47)**, friedelin **(94)** and 3β-friedelinol **(66)**.

List of spectral data and physical properties of compounds

2α,3β-Dihydroxylup-12-en-28-oic acid (122)

Colourless crystals; mp 258.2 - 260.4 ˚C; IR (KBr) ν_{max} 3437, 2927, 2856, 1692, 1642, 1514, 1449, 1377, 1047 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 472 $[M]^+$ (8), 454 (5), 248 (100), 203 (48), 189 (15), 133 (24), 119 (18), 69 (16), 44 (34); HREIMS *m/z* 472.3549 (calcd. for $C_{30}H_{48}O_3$, 472.3552); ^1H and ^{13}C NMR data: see Table 14.

3β-Hydroxylup-12-en-28-oic acid (123)

Colourless crystals; mp 288.2 - 290.6 ˚C; IR (KBr) ν_{max} 3406, 2922, 2851, 1689, 1639, 1462, 1383, 1314, 1274, 1038 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 456 $[M]^+$ (6), 410 (14), 248 (100), 203 (54), 189 (34), 133 (40), 119 (26), 69 (26), 41 (34); HREIMS *m/z* 456.3588 (calcd. for $C_{30}H_{48}O_4$, 456.3603); ^1H and ^{13}C NMR data: see Table 15.

Mixture of two compounds (102) and **(103)**

Colourless crystals; IR (KBr) ν_{max} 3535, 3372, 2925, 2856, 1694, 1639, 1458, 1389, 1306, 1270, 1047 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 488 $[M]^+$ (4), 470 (3), 452 (10), 248 (100), 203 (88), 189 (34), 133 (54), 119 (24), 69 (26), 41 (16); HREIMS *m/z* 488.3497 (calcd. for $C_{30}H_{48}O_5$, 488.3501)

Oleanolic acid (46)

Colourless crystals; mp 196-198˚C; MS (EI, 70eV), *m/z* (rel.int. %): 456 $[M]^+$ (8), 248 (100), 207 (25), 203 (34), 189 (15), 133 (14), 119 (18), 69 (16), 44 (34); ^1H-NMR δ 0.93 (3H, s), 0.97 (3H, s), 1.02 (3H, s), 1.04 (3H, s), 1.02 (3H, s), 1.24 (3H, s), 1.30 (3H, s), 3.30 (1H, ddd, *J* = 14.6, 4.5 Hz; H-18), 3.44 (1H, dd, *J* = 11.2, 4.6 Hz; H-3), 5.49 (1H, t, *J* = 3.6 Hz; H-18); ^{13}C-NMR δ 39.0 (C-1), 28.1 (C-2), 78.2 (C-3), 39.4 (C-4), 55.9 (C-5), 18.8 (C-6), 33.3 (C-7), 39.8 (C-8), 48.2 (C-9), 37.4 (C-10), 23.8 (C-11), 122.6 (C-12), 144.8 (C-13), 42.2 (C-14), 28.3 (C-15), 23.8 (C-16), 46.7 (C-17), 42.1 (C-18), 46.6 (C-19), 31.0 (C-20), 34.3 (C-21), 33.2 (C-22), 28.8 (C-23), 16.5 (C-24), 15.6 (C-25), 17.5 (C-26), 26.2 (C-27), 180.0 (C-28), 33.4 (C-29), 23.8 (C-30).

Betulinic acid (65)

Colourless crystals; MS (EI, 70eV), *m/z* (rel.int. %): 456 $[M]^+$ (36), 438 (12), 411 (6), 248 (54), 228 (62), 207 (60), 203 (48), 189 (100); ^1H-NMR δ 0.83 (3H, s), 1.00 (3H, s),

1.06 (3H, s), 1.07 (3H, s), 1.23 (3H, s), 1.80 (3H, s), 3.46 (1H, t, *J* = 7.2 Hz; H-3); 3.55 (1H, m; H-19), 4.77 (1H, s; H-30), 4.95 (1H, s; H-30); ^{13}C-NMR δ 39.3 (C-1), 28.3 (C-2), 78.2 (C-3), 39.5 (C-4), 55.9 (C-5), 18.8 (C-6), 34.8 (C-7), 41.1 (C-8), 50.9 (C-9), 37.5 (C-10), 21.2 (C-11), 26.1 (C-12), 38.6 (C-13), 42.8 (C-14), 30.3 (C-15), 32.8 (C-16), 56.6 (C-17), 49.8 (C-18), 47.8 (C-19), 151.3 (C-20), 31.2 (C-21), 37.5 (C-22), 28.6 (C-23), 16.3 (C-24), 16.4 (C-25), 16.4 (C-26), 14.9 (C-27), 178.8 (C-28), 109.9 (C-29), 19.5 (C-30).

β-Sitosterol (47), friedelin (94) and 3β-friedelinol (66)

For mp, IR, MS and NMR data, refer to Chapter 2.

Chapter 4

Evaluation of *Lasiosphaera nipponica* (Basidiomycota) for anticancer potential and identification of the chemical constituents

4.1.0 Introduction to Basidiomycota division

The Basidiomycota Division of the Fungi Kingdom consists of mushrooms, jelly fungi, polypores, tooth fungi, crust fungi, chanterelles, coral fungi and puffballs. These fungi have fleshy bodies, with many of them edible, while others are very poisonous. Mushrooms, puffballs *etc*. are only a part of the fungus. The main body of a fungus is a network of tiny threads, called mycelia, which are usually hidden under tree bark, dead leaves or soil.

Edible mushrooms

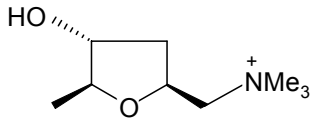
The edible pore mushrooms grow in open deciduous woods. The king boletus and many other species of the same genus are suitable for food. These mushroom species are usually cultivated commercially in dark cellars or specially constructed mushroom houses in which the proper humidity and temperature are maintained. The field or garden mushroom is a common and widespread species in pastures, grassy areas and manured fields. It has the same desirable qualities as the cultivated species. The Chanterelle, a gill fungus with a nutlike flavour, has been popular in Europe since ancient times and most desirable for eating. The oyster mushroom has a pleasant, oysterlike flavour. This mushroom grows in bracketlike clusters on decaying tree trunks. Sulfur mushroom, shaggy-mane, giant puffballs, true morels and related species are excellent edible forms.

Poisonous mushroom

The number of poisonous mushroom species is more than 200. Some mushrooms, especially amanitas, are extremely poisonous and are often fatal if ingested by humans. They contain toxins that destroy cells in the central nervous system, blood vessels, kidneys, liver and musculature. Medically, the most important toxins formed by fungi are ibotenic acid, muscarine, monomethylhydrazine and the amatoxins and phallotoxins.

Muscarine (125), **monomethylhydrazine (126) and ibotenic acid (127)**

Certain mushrooms, particularly *Inocybe* and *Clitocybe* species produced muscurine **(125)**. It was first isolated from *Amanita muscaria*. The symptoms of muscarine poisoning are a dramatic increase in salivation, lacrimation, perspiration accompanied by vomiting, diarrhoea and stomach pains. Death rarely occurs with this poisoning but has been recorded.



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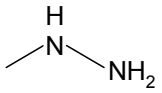

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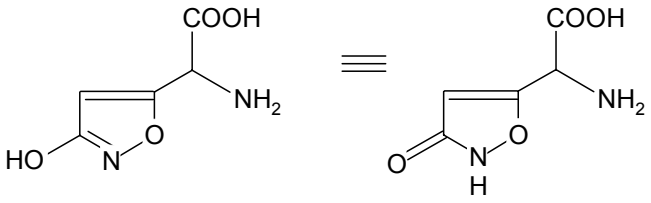

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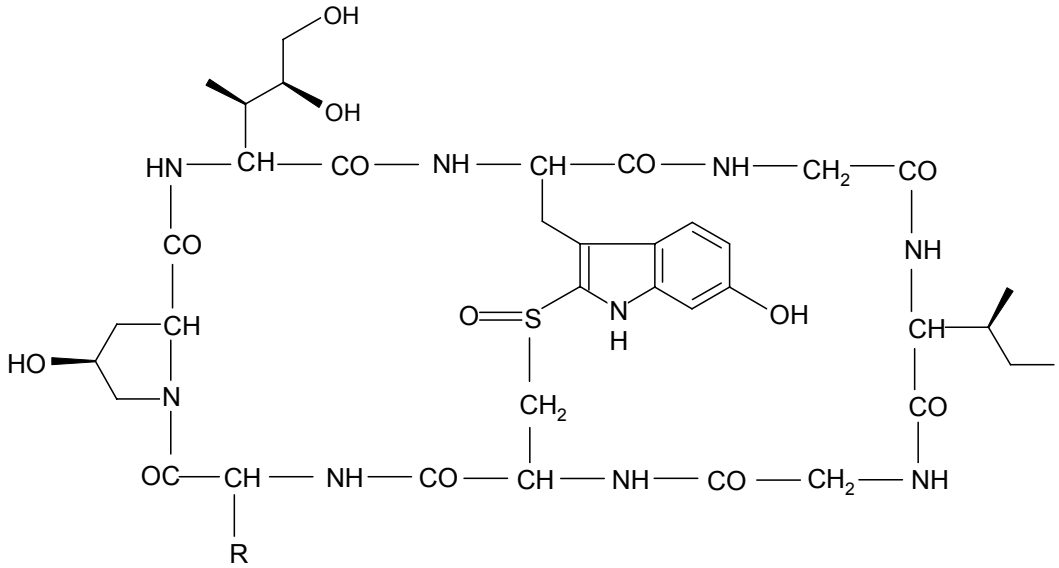
Monomethylhydrazine **(126)** is a toxin produced by the mushroom gyromitrin, *Gyromitra esculenta*. It occurs in the poisonous false morels, which is often confused with true

morels [205]. Ibotenic acid **(127)** is the principal toxin in the fly amanita. It will cause damage to the central nervous system.

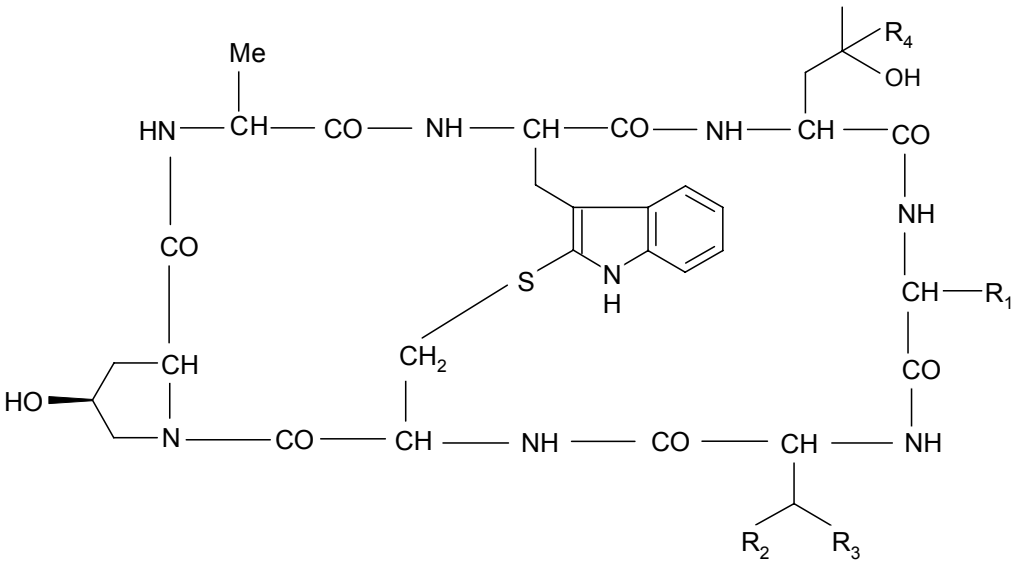
Amatoxins and phallotoxins

Amatoxins **(128, 129)** are bicyclic octapeptides. Phallotoxins **(130, 131)** are bicyclic heptapeptides. These are very toxic compounds. Symptoms usually become apparent 8 to 12 hours or even longer after the mushroom is eaten; death follows in 2 or 3 days. Treatment for poisoning by amatoxins is supportive after the mushrooms have been cleansed from the gastrointestinal tract. Thioctic acid is administered to individuals poisoned by amatoxins, but its effectiveness is uncertain.



(128) α-Amanitin; R = $-CH_2CONH_2$

(129) β-Amanitin; R = $-CH_2COOH$



(130) Phalloin; $R_1 = R_3 = R_4 = Me$; $R_2 = OH$

(131) Phallacidin; $R_1 = CHMe_2$; $R_2 = OH$; $R_3 = COOH$; $R_4 = CH_2OH$

Many other mushrooms are generally avoided because their edibility is doubtful or they have a disagreeable odour. The emetic mushroom and its near relatives should be avoided [206-211].

Puffball fungi

Puffball fungi come in two different sizes. Puffball of size bigger than a (non-baby) potato or a baseball is in the genus *Calvatia*. The largest ones are members of the genus *Calvatia*. Puffballs of golf ball size or smaller are probably of the genera *Lycoperdon* or *Calbovista* (*Bovista*). Puffballs are usually white and round and are attached to the ground with little or no apparent stem. Puffballs are edible when young and when still fleshy-white within and not edible once they have darkened and begun to go to spore. Although many wild mushrooms are delicious and safe to eat, some are not. When cut open, the gleba (interior) must not have any sign of yellow, brown, purple but fleshy whiteness. It should be regarded as toxic if it is of any colour other than white.

4.1.1 Introduction to *Lasiosphaera nipponica*

Lasiosphaera nipponica (syn: *Lasiosphaera fenzlii*, *Lasiosphaera fenzlei*) belongs to Lycoperdaceae family [212]. It is a puffball mushroom commonly known as mabo.

Kingdom : Fungi

Division : Basidiomycota

Class : Gasteromycetes

Order : Lycoperdales

Family : Lycoperdaceae

Genus : Calvatia

Species : *Lasiosphaera nipponica* (syn: *Lasiosphaera fenzlii*, *Lasiosphaera fenzlei*)

Chinese name : Mabo

English name : Puffballs

Pharmaceutical name/ Latin name : *Lasiosphaera seu Calvatia*

It was reported that the water extract of *L. nipponica*, showed complete inhibition of HIV-1 induced CPE in MT-4 cells and the minimum concentration required was about 250 μg /mL [212]. It has been used in Japanese and Chinese folk medicine as a haemostatic [213]. The cytotoxicity of this mushroom extract has not been reported so far. Further, there has been only one publication describing its chemical constituents [214]. The present study was undertaken to evaluate the sub-fractions of the hexane extract of this fungus for cytotoxic activity against P338 (Murine lymphocytic leukaemia), HL60 (Human leukaemia), MCF7 (Human breast cancer), HepG2 (Hepatocellular carcinoma)

and J82 (Bladder transitional carcinoma) cancer cell lines and to identify the chemical constituents of the fractions.

4.2.0 Results and Discussion

The hexane extract was pooled into six fractions. Three pure compounds were obtained from three of the six fractions, one compound from each fraction. The pure compounds isolated are characterised as ergosterol peroxide **(132)**, hexadecanoic acid **(133)** and octadecanoic acid **(134)** by spectroscopic means. Three other Fractions A-C, were screened against murine and human cancer cell lines and the results are given in Table 17.

Table 17
Cytotoxicity (IC_{50} values) of sub-fractions (A-C) of hexane extract of *L. nipponica* and ergosterol peroxide **(132)**

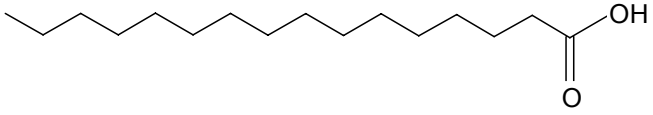
	Cancer cell lines and IC_{50} values				
Fractions	P388	HL60	MCF7	HepG2	J82
A	9.8	18.2	72.6	>100	>100
B	19.4	16.2	>100	64.8	>100
C	25.3	80.6	>100	>100	>100
EP	NT	77.0	NT	>234	>234

P338: Murine lymphocytic leukaemia; MCF7: Human breast cancer; HL60: Human leukaemia; HepG2: Hepatocellular carcinoma; J82: Bladder transitional carcinoma; EP = Ergosterol peroxide **(132)**; NT- Not Tested; No. of replicates, n = 8 for each concentration; 6-Mercaptopurine (6MP) was used as positive control for P388 cancer cell lines; Doxorubicin was used as positive control for MCF7 cancer cell lines; IC_{50} values are in µg/mL; IC_{50} values less than 30 µg/mL are considered active. For the pure compound, ergosterol peroxide **(132)**, the IC_{50} values are expressed in µM. The 50% inhibitory concentrations (IC_{50}) were measured by MTT assay after 3-days culture.

Fraction A showed very good activity against P388 cancer cell lines with IC_{50} value of 9.8 μg/mL and good activity against HL60 with IC_{50} value of 18.2 μg/mL, but was inactive against MCF7, HepG2 and J82 cancer cell lines. It showed IC_{50} value 72.6 μg/mL against MCF cancer cell lines and it was >100 μg/mL against both HepG2 and J82 cancer cell lines, the highest concentration tested. Fraction B showed good activity against P388 and HL60 cancer cell lines with IC_{50} values of 19.4 and 16.2 μg /mL, respectively but was inactive against MCF7, HepG2 and J82 cell lines; it showed IC_{50} value 64.8 μg/mL against HepG2 cancer cell lines and it was >100 μg/mL against both MCF7 and J82 cancer cell lines. Fraction C showed only a moderate activity against P338 with IC_{50} value of 25.3 μg/mL but was inactive against other cell lines; it showed the IC_{50} value 80.6 μg/mL against HL60 cancer cell lines and it was >100 μg/mL against both MCF7, HepG2 and J82 cancer cell lines. All the three fractions were subjected to GC-MS analysis for their chemical constituents. Fatty acids, esters of fatty acids and aromatic compounds were identified. The chemical constituents of the individual fraction are given in Tables 18, 19 and 20. Plasticizers may contaminate solvents, filter papers, plastic apparatus and chromatographic stationary phases stored in plastic containers. Phthalate esters are the plasticizers most likely to be encountered [215]. Thus, the compounds **(137)**, **(142)**, **(143)**, **(147)**, **(148)** and **(149)** listed in these Tables are contaminants.

Table 18
Chemical constituents of Fraction A of hexane extract of *L. nipponica*.

No	Retention time (min)	Compounds
1	9.217	Octanoic acid **(135)**
2	16.911	Tetradecanoic acid **(136)**
3	19.819	Butyl isobutyl phthalate **(137)**
4	20.562	n-Hexadecanoic acid **(133)**
5	22.166	Heptadecanoic acid **(138)**
6	22.818	11(Z)-Hexadecenoic acid **(139)**
7	23.280	Methyl octadecanoate **(140)**
8	24.052	Octadecanoic acid **(134)**
9	25.286	Ethyl linoleate **(141)**
10	29.649	Mono(2-ethylhexyl) phthalate **(142)**
11	29.679	Diisooctyl phthalate **(143)**



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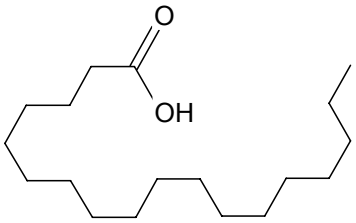

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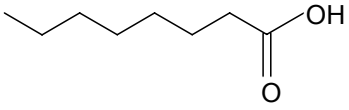

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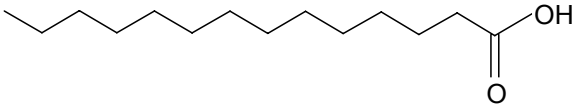

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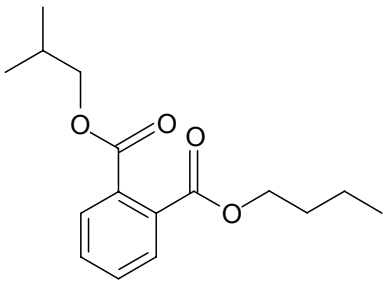

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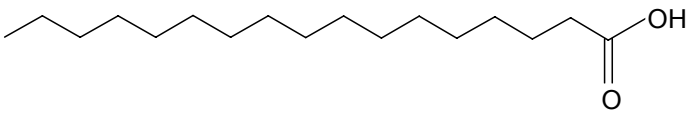

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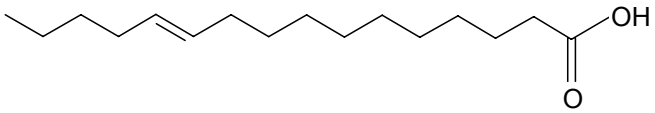

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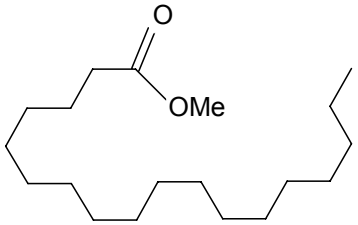

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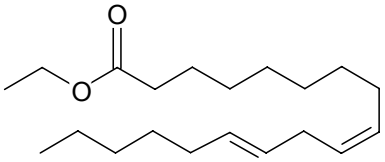

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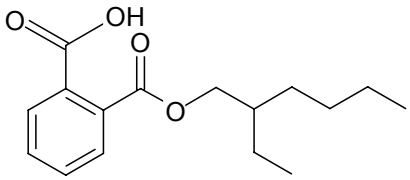

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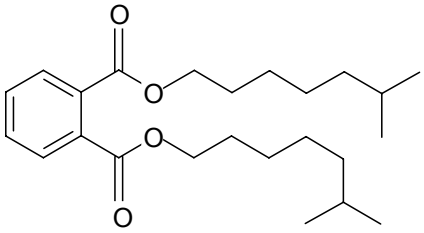
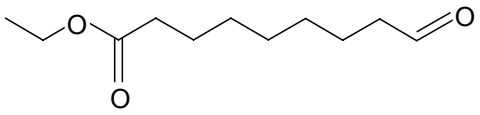


(143)

Table 19
Chemical constituents of Fraction B of hexane extract of *L. nipponica*.

No	Retention time (min)	Compounds
1	13.239	Ethyl 9-oxononanoate**(144)**
2	13.350	Ethyl 11(Z)-hexadecenoate **(145)**
3	14.032	Ethyl hexadecanoate **(146)**
4	19.778	Dibutyl phthalate **(147)**
5	19.784	Butyl isobutyl phthalate **(137)**



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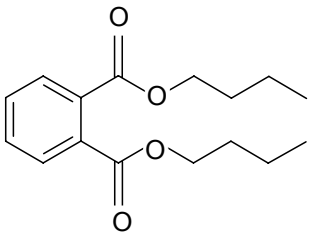

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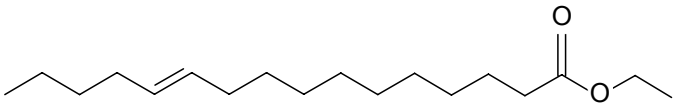

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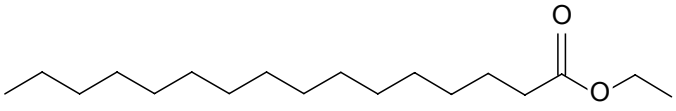
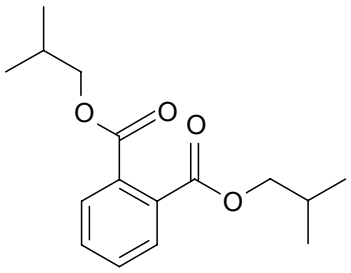


(146)

Table 20
Chemical constituents of Fraction C of hexane extract of *L. nipponica*.

No	Retention time (min)	Compounds
1	18.224	Diisobutyl phthalate **(148)**
2	19.796	Butyl isobutyl phthalate **(137)**
3	33.971	Bis(2-ethylhexyl) phthalate **(149)**



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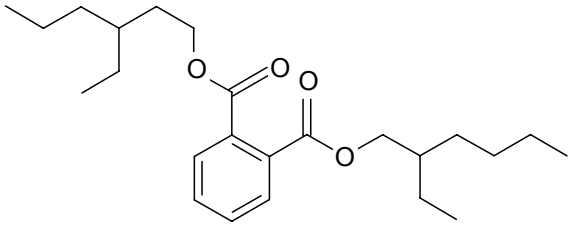


(149)

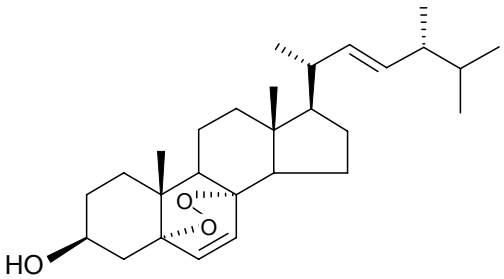
Hexadecanoic acid (133) and octadecanoic acid (134)

Hexadecanoic acid **(133)** and octadecanoic acid **(134)** were mainly identified from their mass spectral data. Both compounds displayed only two peaks at the high-field region in the ^{1}H NMR spectrum; one of them was assigned for methyl group while the other one was found to be very intense due to all the methylene protons. Octadecanoic **(133)** and hexadecanoic acids **(134)** were obtained in small quantities and could not be tested for their cytotoxic activity.

Ergosterol peroxide (132)

Ergosterol peroxide **(132)** was obtained as colourless crystals with molecular ion peak at *m/z* 428 in its EI mass spectrum. The NMR spectrum showed the presence of four

secondary methyls and two tertiary methyls and one methine attached to hydroxyl functionality. The signal at δ 3.96 (1H, m) was due to hydrogen at H-3. The signals at δ 5.18 (2H, m, H-22 and H-23), 6.22 (1H, d, *J* = 8.5 Hz, H-6) and 6.50 (1H, d, *J* = 8.5 Hz, H-7) were assigned to the protons of the double bonds. The ^{13}C NMR gave peak at δ 66.2 which was due to oxygenated carbon at C-3 position. Another two carbons were also oxygenated since they exhibited their chemical values at δ 79.3 and 82.1, respectively. These were due to C-5 and C-8 carbons respectively. The chemical shift values at δ 135.0, 130.5, 135.3 and 132.1 were assigned for C-6, C-7, C-22 and C-23 carbons respectively. The mass spectrum gave major fragmentation peaks at *m/z* 410 [M-H_2O], 396 [M-O_2] and 303 [M-side chain]. The peak at *m/z* 396 was due to loss of two oxygen atoms (peroxide moiety) present in the ring B. Comparison of spectral data of ergosterol peroxide have good agreements with literature data [214,216-218].

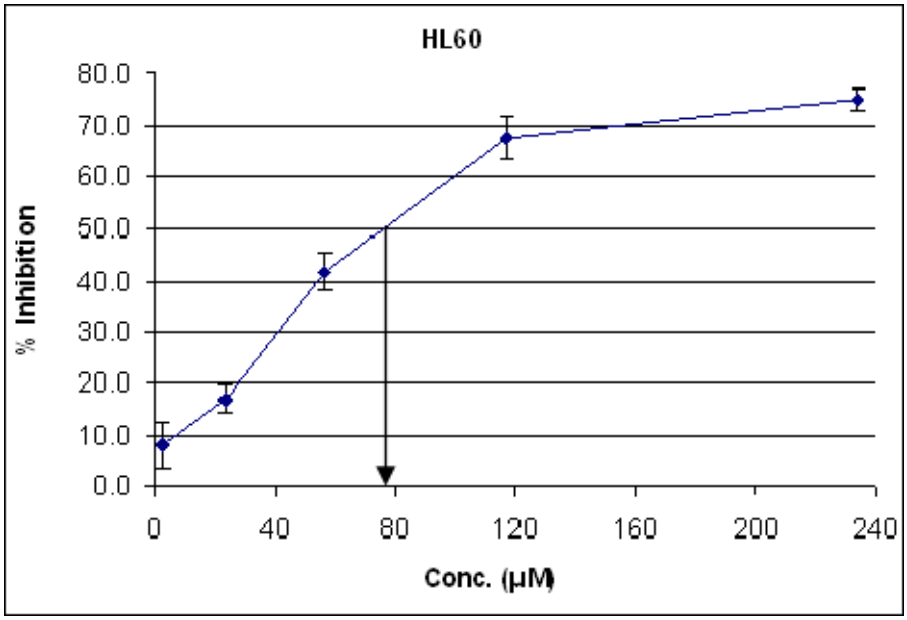


(132)

4.2.1 Evaluation of ergosterol peroxide (132) for anticancer potential

Ergosterol peroxide **(132)** was screened for its cytotoxicity against HL60, HepG2 and J82 cancer cell lines. It showed only a borderline activity against HL60 cancer cell lines with IC_{50} value of ~ 77.0 μM (Figure 1).

Figure 1
Cytotoxicity (IC_{50} values) of the pure compound, ergosterol peroxide **(132)** against HL60 (Human leukaemia cancer cell lines)



It showed 8.1% of growth inhibition at a concentratin of 2.34 µM with a standard deviation of (SD) ± 4.39. Similarly the growth inhibitions at a concentrations of 23.4, 56.5, 117.0 and 234.0 µM were respectively, 17.0 (± 2.57), 41.7 (± 3.70), 67.7 (± 3.98) and 75.3 (± 2.14) %. Ergosterol peroxide **(132)** was inactive towards HepG2 and J82 cancer cell lines with IC_{50} value > 234 µM on both cancer cell lines (Table 18). Its activity against P388 and MCF7 cancer cell lines were not tested. Literature search revealed that ergosterol peroxide **(132)** showed strong anticomple- mentary activity [219]. It showed antitumor activity against MCF7 (Human breast cancer) and Walker 256 (Carcinosarcoma) cancer cell lines [220,221]. It showed activity against SNU-1 (IC_{50} = 8.0 µg/ml), SNU-C4 (IC_{50} = 67.7 µg/ml), SNU-354 (IC_{50} = 36.2 µg/ml), Sarcoma-180 (IC_{50} = 31.7 µg/ml) cancer cell lines [222]. It markedly inhibited the tumour-promoting effect of TPA in 7,12-dimethylbenz[a]anthracene-initiated mice [223].

4.3.0 Experimental

General experimental procedure was followed elsewhere as described in Chapter 2

Plant material

The plant materials were purchased from a local market and a voucher specimen (KMano LN2002) was deposited in the Department of Biological Sciences, National University of Singapore, Singapore.

Extraction and Isolation

The whole plant material of *L. nipponica* (600g) was crushed into powder and then extracted with hexane (3L x 4) at room temperature. Other experimental procedures were the same as described in Chapter 2. The extracts were pooled into six fractions. One pure compound was obtained from each of the three fractions. They were ergosterol peroxide **(132)**, hexadecanoic acid **(133)** and octadecanoic acid **(134)**. The other three fractions (A-C) were subjected to GC-MS analysis for the identification of their chemical constituents.

GC-MS analysis

Using Nist MS database, Fraction A was analysed for its chemical constituents on an Agilent GC-MS instrument with a column having 50 m length x 0.25 μm film thickness. The oven temperature was fixed initially at 50˚C for 2 min and then increased by 10˚C /min until 180˚C, maintained for 2 min at this temperature and then increased 5˚C /min up to 280˚C, which was maintained for a further 3 min. Fractions B and C were analysed for their chemical constituents in the same instrument. The oven temperature was fixed initially at 50˚C for 2 min and then increased by 10˚C /min until 180˚C was attained. This was maintained for 2 min and then increased by 5˚C /min up to 300˚C and maintained for a further 4 min.

Cytotoxic assay

The same procedure was followed as described in Chapter 2.

Ergosterol peroxide (132)

Colourless needles, mp 180-181˚C; IR (KBr) ν_{max} 3360, 1460, 1380, 1277, 1040, 1030, 960, 940 cm^{-1}; MS (EI, 70eV) 428 M^+ (22), 410 (M-H_2O) (23), 396 (M-O_2) (65), 251 (83), 209 (38), 197 (45), 141 (35) 69 (100); ^1H NMR ($CDCl_3$): δ 0.81 (3H, s), 0.82 (3H, d, *J* = 6.8 Hz), 0.83 (3H, d, *J* = 6.8 Hz), 0.88 (3H, s), 0.94 (3H, d, *J* = 6.6 Hz), 1.02 (3H, d, *J* = 6.6 Hz), 3.96 (1H, m, H-3), 5.18 (2H, m, H-22 and H-23), 6.22, 6.50 (each 1H, d, *J* = 8.4 Hz, H-6, H-7); ^{13}C NMR: δ 34.7 (C1), 30.0 (C2), 66.2 (C3), 36.8 (C4), 79.3 (C5) , 135.0 (C6),130.5 (C7), 82.1 (C8), 51.0 (C9), 39.3 (C10), 20.6 (C11), 39.3 (C12), 44.5 (C13), 51.6 (C14), 23.4 (C15), 28.6 (C16), 56.1 (C17), 12.8 (C18), 18.1(C19), 39.6 (C20), 20.8 (C21), 135.3 (C22), 132.1 (C23), 42.7 (C24), 33.0 (C25), 19.6 (C26), 19.9 (C27), 17.5 (C28).

Hexadecanoic acid (133)

Colourless crystals; MS (EI, 70eV) 256 M^+ (95), 227 (24), 213 (70), 185 (38), 171 (35), 143 (15), 129 (74), 97 (48), 73 (100), 55 (90), 43 (86).

Octadecanoic acid (134)

Colourless crystals; MS (EI, 70eV) 284 M^+ (100), 241 (52), 213 (10), 185 (42), 157 (8), 129 (54), 97 (28), 73 (74), 57 (55)

Chapter 5

Evaluation of *Juglans regia*, *Houttuynia cordata* and a mixture containing six herbs for anticancer potential using selected cancer cell lines and the chemical constituents of *Fagraea fragrans*.

5.1.0 Introduction to Juglandaceae family

The species belong to Juglandaceae family are mostly trees, rarely shrubs, deciduous, with gray or brownish bark. Terminal buds are larger than lateral buds. Leaves are alternate (or opposite), aromatic, usually odd, rarely even and pinnately compound. There are about 7 genera, 59 species (2 genera, 17 species in the flora). The fruits in Juglandaceae are usually hard stone like materials, the nutmeat inside is coverd by a soft and fleshy husk [224].

Juglans regia (Common walnut or Persian walnut), *Juglans sigillata* (Iron walnut), *Juglans australis* (syn: *Juglans boliviana*) (Argentine walnut), *Juglans brasiliensis* (Brazilian walnut), *Juglans californica* (California walnut), *Juglans jamaicensis* (syn: *Juglans insularis*) (West Indies walnut), *Juglans major* (Heller Arizona walnut), *Juglans neotropica* (syn: *Juglans honorei*) (Andean walnut), *Juglans nigra* (Black walnut), *Juglans olanchana*, *Juglans peruviana* (Peruvian walnut), *Juglans soratensis*, *Juglans steyermarkii* (Guatemalan walnut), *Juglans venezuelensis* (Venezuela walnut), *Juglans ailantifolia* (Japanese walnut or Heartnut), *Juglans stenocarpa* (Chinese walnut or Manchurian walnut), *Juglans cinerea* (Butternut) etc. are some examples of species belong to the Juglandaceae family.

5.1.1 Introduction to *Juglans regia*

Juglans regia commonly known as walnut (also called English walnut or Persian walnut) belongs to the Juglandaceae family.

Kingdom : Planate

Division : Magnoliphyta

Class : Magnoliopsida

Order : Fagales

Family : Juglandaceae

Genus : Juglans

Species : *Juglans regia*

There are about 15 edible varieties of walnuts, foremost among which is the common walnut. Used since ancient time, the versatile walnut is grown throughout the world. Today, this variety is produced mostly in California. Over 98 % of the total U.S. commercial crop and two-thirds of the world's commercial walnuts are produced in California. Walnuts are available year-round. English walnuts have a thin shell that is easily cracked and the curly nutmeat have a sweet flavour. The wood of English walnuts is used for furniture, panelling and gunstocks.

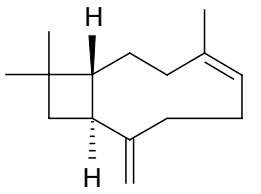
Functions of walnut

The fat in walnut is beneficial to the formation of albumin in the body. In traditional Chinese medicine, it is claimed that walnut is good for nourishing the blood and helping inspiration to relieve asthma. It is often used with other herbs such as *Cortex eucommiae*, *Fructus psoraleae*, *Astragali complanati*, *Fructus corni*, *Radix Ginseng*, *Armeniacae*

amarum, *Fructus schisandrae*, etc. for reinforcing the kidney to keep vital essence. Walnut has been used for lumbago due to kidney-deficiency, dizziness, tinnitus (ringing ear), tired extremities, insomnia, forgetfulness, spermatorrhoea and frequent urination. About 20 g walnut and 20 g black sesame, all fried to a little bit brown, may be eaten at bedtime once daily for habitual constipation. Melatonin is present in walnuts and when eaten, increase blood melatonin concentrations. The increase in blood melatonin levels correlates with an increased antioxidative capacity [225].

5.1.2 Previously reported chemical constituents from *Juglans regia*

The amount of secondary metabolites differs depending on the development stage. Tocopherols were reported as the main constituents [226]. Leaves are rich in mono- and sesquiterpenoids. Caryophyllene **(150)**, eudesmol **(151)** and the phenolic compound, eugenol (eugenic acid) **(152)** have also been reported [227].



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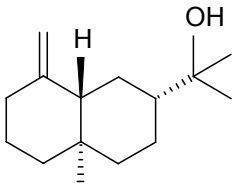

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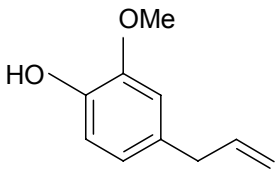
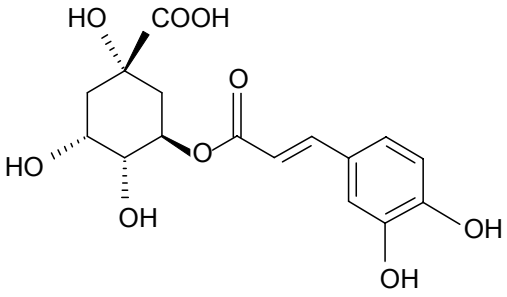


(152)

Juglone (5-hydroxy-1,4-naphthalenedione) **(153)**, syringaldehyde **(154)**, phenolic acids, chlorogenic acid **(85)**, ellagic acid **(155)**, caffeic acid **(156)**, p-coumaric acid **(157)**, syringic acid **(158)**, sinapic acid **(159)** and ferulic acid **(160)** were identified in ripe fruits [228].



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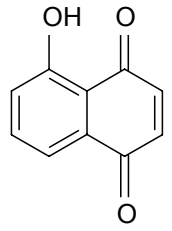

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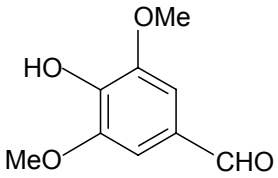

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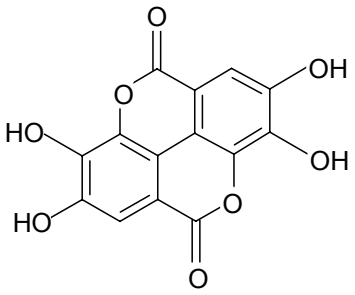

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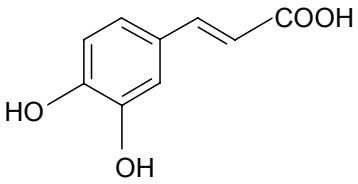

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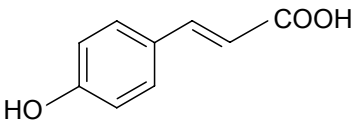

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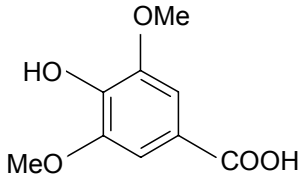

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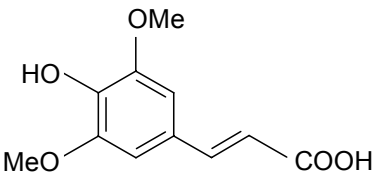

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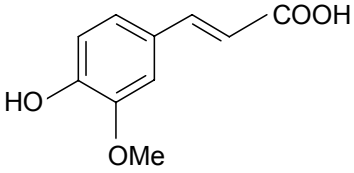
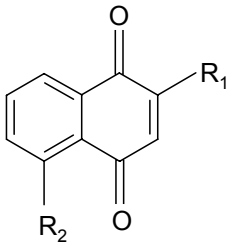


(160)

Flavonoid glycosides, hydrolyzable tannins together with adenosine and adenine were also reported [229,230]. Juglone (5-hydroxy-1,4-naphthoquinone) **(153)**, 2-methyl-1,4-naphthoquinone **(161)**, 1,4-naphthoquinone **(162)** and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) **(163)** occur in walnut husks [231,232].

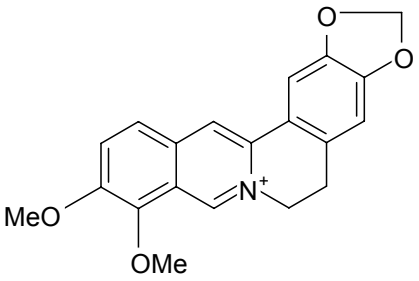


(161) R_1 = Me; R_2 = H

(162) R_1 = H; R_2 = H

(163) R_1 = Me; R_2 = OH

The mature stem bark yielded the yellow coloured alkaloid, berberine **(164)** [233]. The presence of sterols and carbohydrates had been reported [234]. The presence of ascorbic acid **(165)** was also reported [235].



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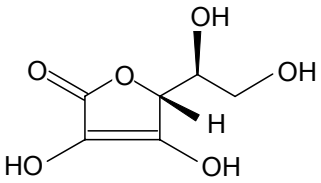
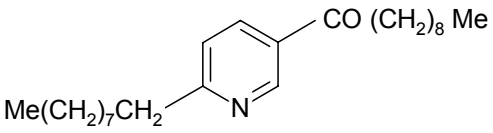


(165)

5.1.3 Introduction to *Houttuynia cordata* (Saururaceae)

Houttuynia cordata (Saururaceae) is a Chinese herbal medicine. Fresh *H. cordata* has the actions of clearing heat, eliminating toxins, reducing swelling and relieving stagnation. However, dry *H. cordata* has traditionally been used in clinical application instead of the fresh counterpart [236]. It was reported that a hot water extract of a mixture of tea leaves, spearmint leaves and *H. cordata* leaves was effective in preventing mucosal inflammation due to influenza, common cold, tonsillitis, etc.

The presence of the flavonol glycosides, rutin, hyperin, afzelin, quercitrin and isoquercitrin was identified through HPLC analysis [237]. 3-Decanoyl-6-nonylpyridine **(166)** together with several 1,4-dihydropyridine alkaloids were isolated [238]. Aristolactam AII **(167)**, cepharanone B **(168)**, piperolactam A **(169)** and norcepharandione B **(170)** have been isolated from *H. cordata*. Some of them exhibited significant cytotoxicity against A549, SKOV3, SKMEL2, XF498 and HCT5 human tumour cell lines [239].



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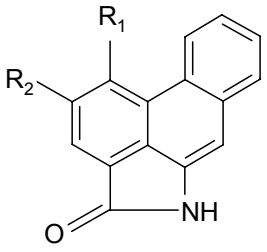
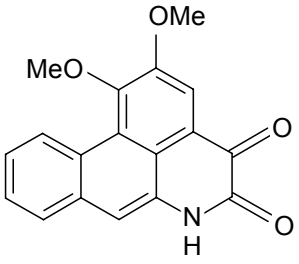


(167) R_1 = OMe; R_2 = OH

(168) R_1 = OMe; R_2 = OMe

(169) R_1 = OH; R_2 = OMe



(170)

The extract of *H. cordata* inhibited the growth of influenza virus in cultures. Methyl n-nonyl ketone **(171)** n-decylaldehyde **(172)** and n-dodecylaldehyde **(173)** were identified as antiviral substances.

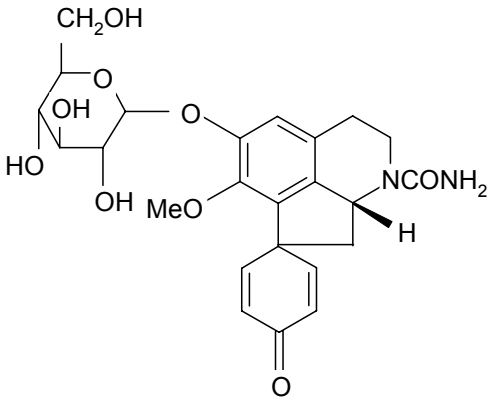
$MeCO(CH_2)_8Me$ **(171)** $Me(CH_2)_8CHO$ **(172)** $Me(CH_2)_{10}CHO$ **(173)**

5.1.4 Introduction to mixture of six herbs

The mixture of six herbs included *Stephania venosa* (Menispermaceae) 10.5 %, *Stephania glabra* (Menispermaceae) 8.5 %, *Stephania suberosa* (Menispermaceae) 3.5 %, *Hedychium coronarium* (Zingiberaceae) 20.0 %, *Zingiber officinale* (Zingiberaceae) 35.5 % and *Curcuma amada* (Zingiberaceae) 22.0 %.

Stephania venosa

Several alkaloids have been reported from *S. venosa* including kamaline **(174)** tetrahydropalmatine (gindarine) **(175)** and N-carboxamidostepharine **(176)** [240,241].



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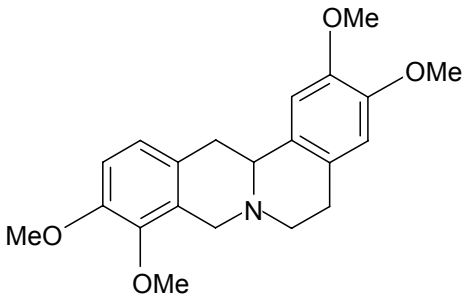

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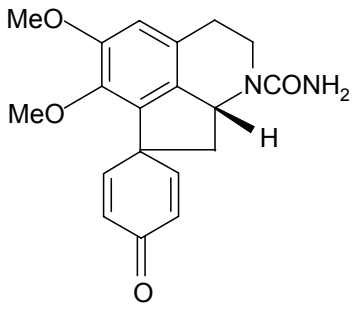
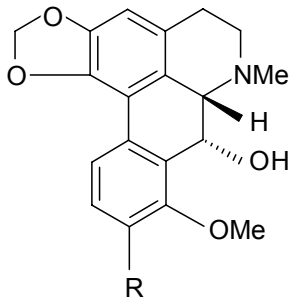


(176)

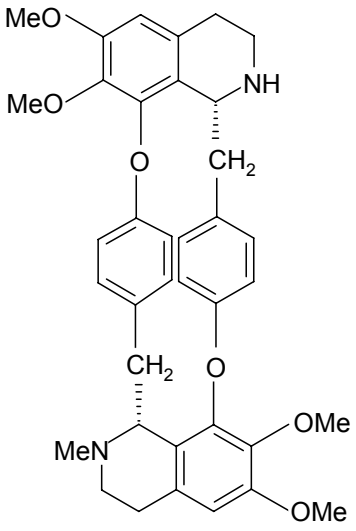
Crebanine, dehydrocrebanine, tetrahydropalmatine, kikemanine, liriodenine, oxocrebanine, acetylsukhodianine, ayuthianine **(177)**, sukhodianine **(178)**, oxostephanosine and ushinsunine were obtained from the leaves of *S. venosa* [242,243].



(177) R = H; **(178)** R = OMe

Stephania glabra

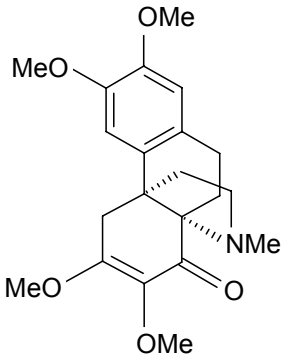
Gindarine, an alkaloid obtained from *S. glabra*, had slight and variable actions on systemic arterial blood pressure, heart rate, cardiac contractility and skeletal muscle. It was devoid of surface anesthetic activity, but produced conduction and infiltration anesthesia. It did not possess antibacterial property [244]. Bisbenzylisoquinoline alkaloid **(179)**, cycleanine, capaurine, corynoxidine and tetrahydroprotoberberine were isolated from the rhizomes of *S. glabra*. Tetrahydropalmatine, corydalmine, stepholidine, the proaporphine alkaloids, pronuciferine, stepharine, the quaternary protoberberine salts, palmatine, dehydrocorydalmine, jatrorrhizine and stepharanine were also reported from this plant [245].



(179)

Stephania suberosa

A promorphinane alkaloid, stephaphylline **(180)**, along with hasubanane, delavaine, nordelavaine and stephanubine **(181)** were reported from *S. suberosa* [246].



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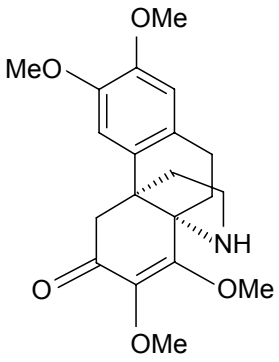
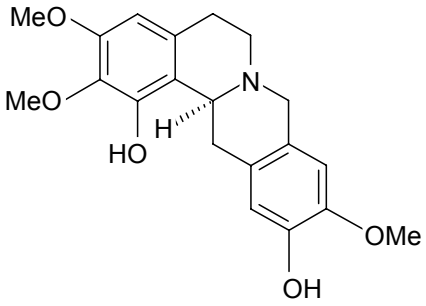


(181)

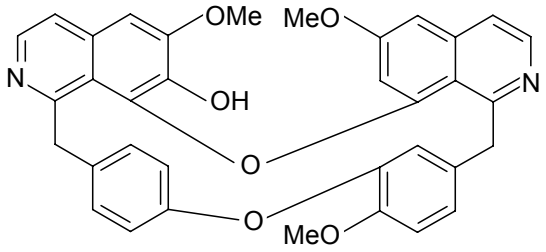
Several protoberberines were reported from *S. suberosa* root extracts. They were tetrahydrostephabine, stephabinamine **(182)**, stephabine, 8-oxypseudopalmatine, trans-xylopinine N-oxide and cis-xylopinine N-oxide. Other alkaloids, tetrahydropalmatine,

tetrahydropalmatrubine, stepholidine, kikemanine, capaurimine, coreximine, corytenchine, discretine, pseudopalmatine and xylopinine were also reported [247].



(182)

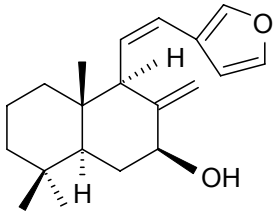
Bisbenzylisoquinoline alkaloids, (+)-2-norcepharanthine **(183)**, cepharanthine, strephasubine, norstrephasubine and stephasubimine were also obtained from *S. suberosa* [248].



(183)

Hedychium coronarium

Farnesane-type sesquiterpenes, labdane-type diterpenes, hedychilactones, hedychiols were isolated from the methanolic extract of the fresh rhizomes of *H. coronarium* [249,250]. Labdane-type diterpenes, coronarin A, B, C and D **(184-187)** were isolated as cytotoxic principles from the rhizomes of *H. coronarium* [251-253].



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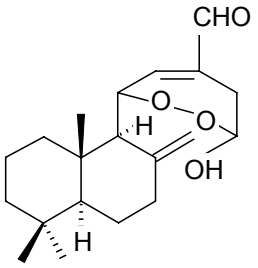

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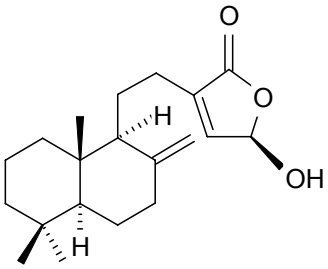

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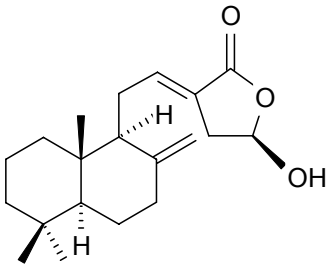
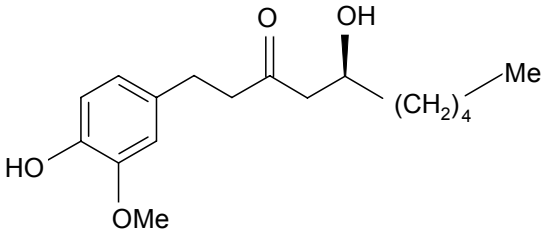


(187)

Zingiber officinale

Zingiber officinale **(**Ginger) has been widely used as a dietary spice as well as in traditional medicine. The rhizome of ginger contains pungent vanillyl ketones, including 6-gingerol **(188)** and 6-paradol **(189)** and has been reported to possess a strong anti-inflammatory activity. It was also reported that the pungent vanilloids found in ginger possess potential chemopreventive activities. These vanilloid structures were found in other chemopreventive phytochemicals including curcumin [254,255].



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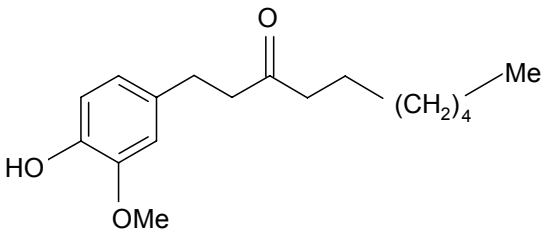
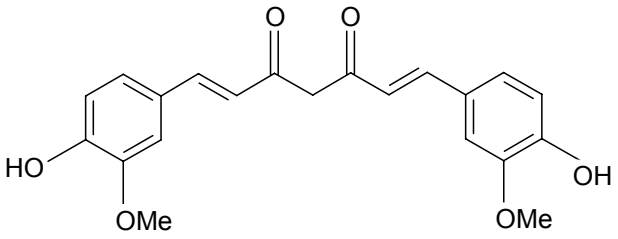


(189)

Ginger is a natural dietary component with antioxidant and anticarcinogenic properties. The effect of ginger on the initiation and post-initiation stages of 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in male Wistar rats was reported. The number of tumors as well as the incidence of cancer was significantly decreased on treatment with ginger [256].

Curcuma amada

Extracts of fresh or dried *Curcuma amada*, preferably the rhizomes have medicinal properties. They can be used as immunosuppressants and for the alleviation of pain. Specifically, they are used for preparing medicaments for the treatment or prevention of hypersensitivity diseases.



(190)

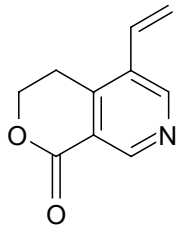
Curcumin **(190)**, an orange-yellow colouring compound present in rhizomes, has long been known to possess antioxidant property. β-Sitosterol and α-pinene were also reported as the main constituents from the rhizomes of *C. amada* [257].

5.1.5 Introduction to *Fagraea fragrans*

Fagraea fragrans belongs to the Loganiaceae family. The vernacular name for this plant is *Tembusu Padang*. The tree is impressive in its 40 m or so height and single trunk with deeply fissured bark. Branches arise from the main trunk and grow upwards. The leaves

are simple, elliptical and narrow towards the tip. Flowers are large in bunches, creamy white, turning yellow. They are very fragrant, especially during the late evenings, for they attract night-flying moths which help their pollination. The tree flowers twice a year, towards the middle and the end of the year. Fruits are small, roundish berries, found in small bunches, turning orange then scarlet with maturity [258].

A thorough literature search indicated that the plant was not extensively studied, both phytochemically and pharmacologically. There was a report which described the isolation of an alkaloid, gentianine **(191)**, from the leaves and fruits of this plant. The alkaloid content of the plant showed marked seasonal variation. The yield of alkaloid from the leaves was highest (0.32%) at the beginning of the flowering season (May-June) and lowest (0.005%) after fruiting had occurred (October-November). The ripe fruits contained approximately 0.10% alkaloids [259].



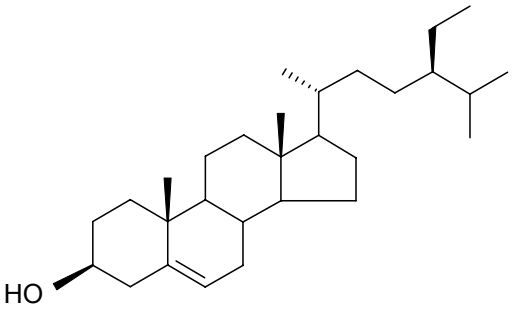
(191)

There was another report which described the pharmacological effects of the gentianine **(191)** on experimental animals. Gentianine **(191)**, when administered orally to rats at doses of 50 and 100 mg/kg, produced 20 and 30%, analgesia, respectively. It had no antipyretic activity or diuretic effect in rats, no hypoglycemic activity in guinea pigs and no cardiovascular or central nervous system effects on anesthetised cats [260].

5.2.0 Results and Discussion

Juglans regia

The seeds of *J. regia* were extracted with chloroform; an oily liquid and residue was obtained. Five sub-fractions were obtained from this residue. Four of them contained a gum like material; we were unable to isolate any pure compounds from these fractions. However, these four sub-fractions and the original residue were screened for their cytotoxicity against P388, HL60, LL2, MCF7 and HepG2 cancer cell lines. They showed no activity against all these five cancer cell lines tested even at a concentration of 100 μg/mL. The fifth fraction was not tested for its cytotoxicity since it was obtained in small quantity. However, a known compound, β-sitosterol **(47)** was isolated from this fraction. This compound had already been isolated several times earlier and the identity was confirmed through a direct comparison with the original spectra.



(47)

The oily liquid was subjected to GC-MS analysis, for the identification of its chemical constituents and they are listed in Table 21.

Table 21
Chemical constituents of the oily liquid obtained from seeds of *Juglans regia*, identified by GC-MS analysis.

Peak	Rt (min)	Area	Library
1	8.29	1.26	n-Hexanoic acid
2	8.75	4.70	Ethyl hexadecanoate
3	10.30	2.77	Methyl l9,12-octadecadienoate
4	10.468	16.55	Methyl 8(E)-octadecenoate
5	11.29	9.30	9,12 (Z,Z)-Octadecadienoic acid
6	11.75	29.93	Ethyl linoleate
7	11.96	6.59	Ethyl oleate
8	21.30	4.98	Unknown
9	26.88	3.46	γ-Tocopherol
10	30.19	16.95	Unknown
11	31.08	3.51	Unknown

Rt = Retention time

Houttuynia cordata

The crude extract, ethyl acetate, butanol and alcohol-water fractions of *H. cordata* were screened for their cytotoxicity against P388, HL60, LL2, MCF7 and HepG2 cancer cell lines. They showed no activity against all these five cancer cell lines tested even at a concentration of 70.0 μg/mL. Though some alkaloids isolated from this plant showed significant activity against A549, SKOV3, SKMEL2, XF498 and HCT5 human tumour cell lines [255], in the present study the crude extract and its fractions showed no activity

against the five cancer cell lines tested. The reason for this may be the chemical constituents of the crude extract or its fractions may not induce cytotoxicity against P388, HL60, LL2, MCF7 and HepG2 cancer cell lines or the active constituents may exist in small amounts and under the experimental condition the active constituents are insufficient to induce cytotoxicity.

Mixture of six herbs

The alcoholic extract of mixture of herbs was screened against HL60, P388, MCF7, J82 and HepG2 cancer cell lines. It showed no activity against all the five cancer cell lines tested even at a concentration 100 μg/mL, the highest concentration tested. It showed growth inhibition of 2.3, 5.0, 7.8 and 10.2 %, respectively at 10, 25, 50 and 100 μg/mL concentrations on P388 cancer cell lines. It showed growth inhibition of 4.5, 7.8, 12.2 and 15.4 %, respectively at 10, 25, 50 and 100 μg/mL concentrations on J82 cancer cell lines. It showed growth inhibition of 1.5, 5.4, 9.8 and 20.0 %, respectively 10, 25, 50 and 100 μg/mL concentrations on HL60 cancer cell lines. It showed growth inhibition of 2.2, 3.5, 4.2 and 9.4 %, respectively at 10, 25, 50 and 100 μg/mL concentrations on HepG2 cancer cell lines. It showed growth inhibition of 7.2, 13.8, 24.6 and 39.2 %, respectively at 10, 25, 50 and 100 μg/mL concentrations on MCF7 cancer cell lines.

Of the six herbs, *Z. officinale* was reported to have a variety of biological activity including anticancer activities. *H. coronarium* also showed anticancer property; some labdane-type diterpenes were identified as cytotoxic principles [269-271]. For the other four herbs, no reports were seen concerning their cytotoxicity. In the present study the

crude extract showed no activity against five cancer cell lines. It was due to the same reason as cited earlier that the chemical constituents of the extract may not induce cytotoxicity against P388, HL60, J82, MCF7 and HepG2 cancer cell lines or the active constituents may exist in small amounts and under the experimental condition these constituents are insufficient to induce cytotoxicity.

Fagraea fragrans

The crude extract of *F. fragrans* was analysed for its chemical constituents using GC-MS analysis. Several aromatic compounds, a few fatty acids and their esters and a few sterols were identified. The results are given in Table 22.

Table 22
GC-MS analysis of the crude extract obtained from stem-bark of the *F. fragrans*.

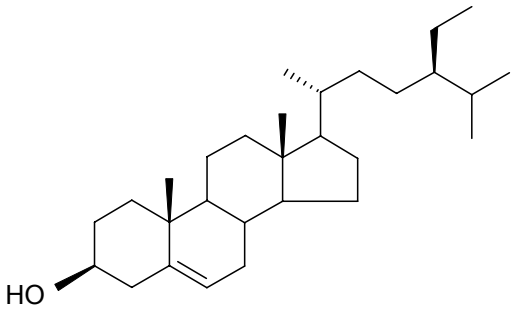
Pk#	Rt (min)	% of Area	Library (Identification)
1	8.71	2.240	Benzoic acid
2	13.72	0.793	E-Stilbene
3	14.63	0.319	4-Hydroxy-3,5-dimethylbenzaldehyde
4	14.67	0.478	Unknown
5	14.87	0.221	4-Hydroxy-3,5-dimethoxybenzaldehyde
6	15.21	0.457	4-Hydroxy-3,5-dimethoxybenzyl alcohol
7	15.48	0.458	4-Hydroxy-2-methoxycinnamaldehyde
8	15.60	9.647	Unknown
9	17.19	0.326	Unknown
10	18.10	0.543	4-Hydroxy-3,5-dimethoxycinnamaldehyde
11	18.29	7.065	Unknown
12	18.46	1.407	n-Hexadecanoic acid
13	18.71	0.531	Unknown
14	20.42	0.310	Methyl 9,12-octadecadienoate
15	21.03	4.595	9,12 (Z,Z)-Octadecadienoic acid
16	21.15	3.244	Unknown
17	21.54	0.784	Octadecanoic acid
18	26.13	0.354	Unknown
19	31.57	3.097	Unknown
20	33.78	1.866	Unknown
21	34.02	0.435	Stigmastan-3,5,22-triene
22	35.15	0.295	Unknown
23	35.42	3.202	Unknown
24	35.67	3.074	Unknown
25	35.92	24.565	Stigmasterol
26	36.20	11.767	Sitosterol
27	36.62	12.143	Unknown
28	37.33	2.468	Unknown
29	37.68	1.376	Unknown
30	38.13	1.211	Unknown
31	38.33	0.726	Unknown

Although GC-MS analysis can give quick and accurate results of the existence of known compounds, it is unsuitable for the identification of less familiar compounds. The chloroform fraction obtained from the crude extract was chromatographed on a silica gel column using hexane solvent and eluted with solvents of increasing polarity. The first

few fractions obtained were either oily liquid or solid materials. However, all those fractions, when analysed by TLC, had showed no significant spots and were not analysed any further. Following this, stigmasterol **(192)** (62 mg) and β-sitosterol **(47)** (ca.6 mg), were isolated from two fractions, one compound from each fraction.

Characterisation of stigmasterol (193) and β-sitosterol (47)

β-Sitosterol was isolated several times earlier and the structure was confirmed by direct comparison with the original spectra. Refer to Chapter 2 for the interpretation.



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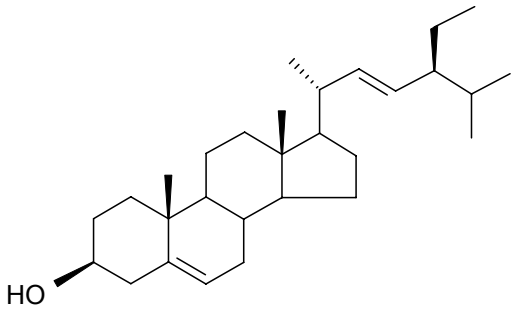

(192)

Stigmasterol **(193)** was obtained as colourless crystals. The mass spectrum gave a molecular ion peak at *m/z* 412, which determined the molecular formula $C_{29}H_{48}O$. The ^{1}H NMR spectrum showed four signals which were well separated and with high shift values. One of the signals resonated at δ 3.52, which corresponds to H-3. Another signal was observed at δ 5.35 (br, d, J = 5.4 Hz), which was due to ring olefinic hydrogen, H-6. Two other peaks resonated at δ 5.13 (dd, J = 8.4, 5.4 Hz) and 5.00 (dd, J = 8.4, 1.5 Hz); these are due to the side chain olefinic protons at H-22 and H-23, respectively. The ^{13}C NMR spectrum gave a peak at δ 72.0 that indicated the presence of a hydroxyl group. Further, two peaks were resonated at δ 140.8 and 121.8, respectively. These values showed the presence of a double bond and were assigned to C-5 and C-6 carbons, respectively. Two

other peaks also resonated at the olefinic region, one at δ 138.3 and another at δ 129.7, respectively. These were due to double bond at the side chain between C-22 and C-23, respectively. Comparison of its spectral data with those reported in the literature showed good agreement [126,245]. Original spectrum of ^{1}H NMR was printed in the literature [261]. Stigmasterol **(192)** is a phytosterol with a wide range of metabolism and therapeutic actions on animals and humans [262].

5.3.0 Experimental

General experimental procedures were followed as described in Chapter 2.

Cytotoxic study

The same procedure was followed as described in Chapter 2.

Plant material, Extraction and Isolation

Juglans regia

The seed material was purchased from a local market and a voucher specimen (KMano SJ 2003) was deposited in the Department of Biological Sciences, National University of Singapore, Singapore.

The seeds were extracted with chloroform and this procedure was repeated several times. The extract was kept in a china-dish and left to stand for a few days. An oily liquid was separated out and removed by simple decantation. The residue thus obtained was chromatographed over a silica gel column, using hexane and eluted with solvents of increasing polarity. Fractions were collected and monitored as per the procedure described in the Chapter 2. After collection of several fractions, β-sitosterol was obtained along with impurities. Further purification followed by recrystallisation with acetone yielded the pure compound (c.a. 3 mg).

Using Nist MS database, the oily liquid was analysed for its chemical constituents on an Agilent GC-MS instrument with a column having 50 m length x 0.25 μm film thickness. The oven temperature was fixed initially at 150˚C for 20 min and then increased by 10˚C

/min until 200˚C, maintained for 5 min at this temperature and then increased by 5˚C/min up to 290˚C, which was maintained for a further 10 min.

β-Sitosterol (47) For mp, IR, MS, ^{1}H NMR and ^{13}C NMR; refer to Chapter 2.

***Houttuynia cordata* and mixture of herbs**

H. cordata crude extract and their fractions and the mixture of six herbs powder weighing about 250 g were provided by Assoc. Prof. Tan, Kwong Huat, Benny, Traditional Medicine & Natural Products Research Laboratory, Department of Pharmacology, NUS. The mixture of herbs powder was extracted with 80% alcohol. Rota-vapour was used to remove solvents as much as possible at the first followed by the use of a freeze dryer/lyophilizer. The crude extract thus obtained was used for cytotoxic screening.

Fagrae fragrans

The stem-bark of the plant material was collected in Singapore and identified by Associate Prof. Hugh Tan Tiang Wah, Dept. of Biological Sciences, NUS and Chua Keng Soon, Senior Laboratory Officer (RMBR), Herbarium, NUS. A voucher specimen (KM20041122) was deposited in the herbarium, Department of Biological Sciences, National University of Singapore, Singapore.

A branch weighing about 25 kg was cut and chopped into small pieces. The air-dried material was extracted with methanol. The methanol extract was chromatographed using hexane solvent but we faced problems for further elutions. Actually, the solvent did not flow through and latex-like material was initially observed. Thus we recovered the sample from the column. The extract was then suspended in methanol/water (8:2) and partitioned between this solvent mixture and chloroform. This partition was repeated

several times and the chloroform fraction was collected separately. The methanol/water fraction was obtained as gum-like material and was discarded. Solvent was removed from the chloroform fraction using a Rota-vapour and the residue thus obtained was chromatogaphed again using hexane and then eluted with solvents of increasing polarity.

β-Sitosterol (47)

For mp, IR, MS, ^{1}H NMR and ^{13}C NMR, refer to Chapter 2.

Stigmasterol (5,22-Stigmastadien-3β-ol) (192)

Colourless flakes; mp.166.2-168.7˚C; MS (EI, 70eV), *m/z* (rel.int. %): 412 $[M]^{+}$ (96), 382 (72), 369 (30), 351 (48), 255 (100), 231 (32), 173 (34), 145 (78),133 (60), 107 (74), 83 (84), 55 (62); ^{1}H NMR ($CDCl_3$): δ 1.08 m, Hα, 1.84 m, Hβ (H-1), 1.83 m, Hα, 1.51 m, Hβ (H-2), 3.51 (1H, m, H-3), 2.30 m, Hα, 2.23 m, Hβ (H-4), 5.34 (1H, m, 5.4 Hz, H-6), 1.50 m, Hα,1.97 m, Hβ (H-7), 1.46 m (1H, H-8), 0.94 m (1H, H-9), 1.50 m, Hα and Hβ (2H, H-11), 1.19 m, Hα, 2.00 m, Hβ (H-12), 1.01 (1H, H-14), 1.56 m, Hα, 1.06 m, Hβ, (H-15), 1.72 m, Hα, 1.28 m, Hβ, (H-16), 1.15 (1H, H-17), 0.70 (3H, s, H-18), 1.01 (3H, s, H-19), 2.06 m (1H, H-20), 1.03 (3H, d, *J* = 6.4 Hz, H-21), 5.17 (1H, dd, *J* = 15.2 Hz, H-22), 5.04 m (1H, dd, *J* = 8.6 Hz, H-23), 1.54 m (1H, H-24), 1.55 m (1H, H-25), 0.85 (3H, d, 6.8 Hz, H-26), 0.80 (3H, d, *J* = 6.4 Hz, H-27),1.43 m (1H, H-28), 0.81 (3H, t, *J* = 6.6 Hz, H-29); ^{13}C NMR ($CDCl_3$): δ 37.6 (C-1), 31.9 (C-2), 72.0 (C-3), 42.5 (C-4), 140.8 (C-5), 121.8 (C-6), 32.1 (C-7), 32.2 (C-8), 50.5 (C-9), 36.5 (C-10), 21.2 (C-11), 40.0 (C-12), 42.2 (C-13), 57.1 (C-14), 24.5 (C-15), 28.9 (C-16), 56.3 (C-17), 12.2 (C-18), 19.5 (C-19), 40.4 (C-20), 21.4 (C-21), 138.3 (C-22), 129.7 (C-23), 51.5 (C-24), 32.2 (C-25), 21.2 (C-26), 19.2 (C-27), 25.4 (C-28), 12.2 (C-29).

Chapter 6

Conclusion and future studies

Polygonum bistorta

P. bistorta was screened for its cytotoxicity against selected cancer cell lines, assessed for its toxicity on experimental animals (Swiss albino mice) and investigated for their chemical constituents. Both chloroform and hexane fractions and a few of their their sub-fractions showed moderate to very good activity against P388, HL60 and LL2 cancer cell lines. We found that the LD_{50} value of crude extract was 150 mg/kg of body weight of mice and that of chloroform extract was 200 mg/kg of body weight of mice; while, the hexane extract has an LD_{50} value >200 mg/kg of body weight of mice. This is the first such report in this plant. Our investigations on the chemical constituents of this plant revealed the presence of three new and two known cycloartane-type triterpenoids, two known pentacyclic triterpenoids and three steroids. Two of the cycloartane triterpenoids, 24-methylenecycloartanone **(97)** and 24(E)-ethylidenecycloartan-3α-ol **(98)**, were isolated from active fractions. These two compounds could not be tested for their pharmacological activity since they were obtained in small quantities. One of the new compounds, 24(E)-ethylidenecycloartanone **(93)**, was screened for its cytotoxicity against a panel of cancer cell lines, but did not show any significant activity.

Some cycloartane-type triterpenoids, mostly in the form of saponins or with several functional groups, showed cytotoxic acitivities against several cancer cell lines, *viz*. HepG2 (Hepatocellular carcinoma cells), HL60 (Human leukaemia cells), KB Cells, A2780 (Ovarian cancer cells), 26-L5 (Murine colon cells), HSC2 (Human oral squamous cell carcininoma), U251 (CNS), PC3 (Prostate cancer cells), HCT15 (Colon carcinoma

cells), MCF7 (Human breast cancer cells), K562 (Leukaemia) *etc*. [135-144]. These results indicated that some cycloartane-type compounds are cytotoxic against some cancer cell lines. However, the new compound, 24(E)-ethylidenecycloartanone **(93)**, though it is a cycloartane-type compound, did not show any activity on a panel of cancer cell lines. The new compound, 24(E)-ethylidenecycloartanone **(93)**, has only a ketone functional group and one double bond. The lack of functionality would be a probable reason for its inactivity on the cancer cell lines tested or it may not induce any inhibition on the cancer cell lines selected for the testing. Synthetic analogues of this new compound, 24(E)-ethylidenecycloartanone **(93)**, can be prepared and could be tested for their cytotoxic effects. The hexane and chloroform factions can also be further investigated in *in vivo* and/or other *in vitro* pharmacological assays eg. annexin V, caspase-3, etc.

Eugenia grandis

The chloroform extract of the stem-bark of *E. grandis* was investigated for its chemical constituents. A new pentacyclic triterpenoid, 2α,3β-dihydroxylup-12-en-28-oic acid **(122)**, a rarely encountered triterpenoid, 3β-hydroxylup-12-en-28-oic acid **(123)** and some known pentacyclic triterpenoids, were isolated. From this result, we understand that *E. grandis* is rich in pentacyclic triterpenoids. Pentacyclic triterpenoids, in general, exerted a variety of biological and pharmacological effects. To the best our knowledge, not only the chemical constituents but also the pharmacology of this plant material has not been extensively studied so far. Thus, other parts of the plant material may be investigated both phytochemically and pharmacologically.

Lasiosphaera nipponica

The present study was a small scale screening of the fungus, *L. nipponica*. The sub-fractions of the hexane extract of *L. nipponica* showed very good to good activity against P388 and HL60 cancer cell lines. This is the first report on cytotoxicity of this fungus material. Therefore, the sub-fractions of the extract may be subjected to further *in-vivo* and/or other *in-vitro* pharmacological assays eg. annexin V, caspase-3, etc. One pure compound, ergosterol peroxide **(132)** was isolated along with fractions that showed borderline activity against HL60 (Human leukaemia cancer cell lines). Ergosterol peroxide **(132)** showed strong anticomplementary activity [223]. It showed antitumour activity against MCF7 (Human breast cancer cell lines) and Walker 256 (Carcinosarcoma cell lines) [224,225]. It also showed activity against SNU-1 (IC_{50} = 8.0 μg/ml), SNU-C4 (IC_{50} = 67.7 μg/ml), SNU-354 (IC_{50} = 36.2 μg/ml) and Sarcoma-180 (IC_{50} = 31.7 μg/ml) cancer cell lines [226]. It markedly inhibited the tumor-promoting effect of TPA in 7, 12-dimethylbenz[a]anthracene-initiated mice [227]. Ergosterol peroxide **(132)** is one of the major constituents in this fungus material and therefore more ergosterol peroxide **(132)** may be obtained for *in vivo* pharmacological studies. As the chemical constituents of this fungus material have not been extensively studied, isolation of new compounds is also possible.

Fagraea fragrans

This plant material is also not extensively studied, both phytochemically and pharmacologically. The stem-bark of the plant material was investigated for its phytochemical constituents. When extracted with methanol, a latex-like material was

obtained. After solvent-solvent partition followed by purification, only two known steroids were isolated. Other parts of plant material may be investigated both phytochemically and pharmacologically.

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Publication

1. Karuppiah Pillai Manoharan., Tan Kwong Huat Benny., Daiwen Yang., (2005) Cycloartane-type triterpenoids from the rhizomes of *Polygonum bistorta* *Phytochemistry* 66, 2304-2308.

Presentations

1. Karuppiah Pillai Manoharan., Tan Kwong Huat Benny., Daiwen Yang., Title: Evaluation of *Polygonum bistorta* for anticancer potential using selected cancer cell lines. Inaugural International Congress on Complementary and Alternative Medicines (ICCAM 2005), at Raffles City Convention Centre, Singapore, on 26-28 February 2005 (Poster presentation).

2. Karuppiah Pillai Manoharan., Tan Kwong Huat Benny., Daiwen Yang., Title: Novel Cycloartane-type compounds from the rhizomes of *Polygonum bistorta*. 1st Singapore Mini-Symposium on Medicinal Chemistry, at National University of Singapore, on 6th July 2005 (Poster presentation).