EGE UNIVERSITY GRADUATE SCHOOL OF
NATURAL AND APPLIED SCIENCES
(M. Sc. THESIS)

ISOLATION, PURIFICATION AND CHARACTERIZATION OF SECONDARY METABOLITES FROM *Nigella arvensis* var. *involucrata*

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Kenan BIÇAK tarafından Yüksek Lisans tezi olarak sunulan "**Isolation Purification and Characterization of Secondary Metabolites from *Nigella arvensis* var. *involucrata* (*Nigella arvensis* var. *involucrata*'dan Sekonder Metabolitlerin İzolasyonu, Saflandırılması ve Karakterizasyonu)**" başlıklı bu çalışma E.Ü. Lisansüstü Eğitim ve Öğretim Yönetmeliği ile E.Ü. Fen Bilimleri Enstitüsü Eğitim ve Öğretim Yönergesi'nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve 11.02.2015 tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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E.Ü. Lisansüstü Eğitim ve Öğretim Yönetmeliğinin ilgili hükümleri uyarınca Yüksek Lisans Tezi olarak sunduğum "**Isolation Purification and Characterization of Secondary Metabolites from *Nigella arvensis* var. *involucrata* (*Nigella arvensis* var. *involucrata*'dan Sekonder Metabolitlerin İzolasyonu, Saflandırılması ve Karakterizasyonu)**" başlıklı bu tezin kendi çalışmam olduğunu, sunduğum tüm sonuç, doküman, bilgi ve belgeleri bizzat ve bu tez çalışması kapsamında elde ettiğimi, bu tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara atıf yaptığımı ve bunları kaynaklar listesinde usulüne uygun olarak verdiğimi, tez çalışması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığını, bu tezin herhangi bir bölümünü bu üniversite veya diğer bir üniversitede başka bir tez çalışması içinde sunmadığımı, bu tezin planlanmasından yazımına kadar bütün safhalarda bilimsel etik kurallarına uygun olarak davrandığımı ve aksinin ortaya çıkması durumunda her türlü yasal sonucu kabul edeceğimi beyan ederim.

11.02.2015

Kenan BIÇAK

ÖZET

Nigella arvensis var. *involucrata*'DAN SEKONDER METABOLİTLERİN İZOLASYONU, SAFLANDIRILMASI VE KARAKTERİZASYONU

Kenan BIÇAK

Yüksek Lisans Tezi, Kimya Anabilim Dalı

Tez Danışmanı: Prof. Dr. Özgen ÇALIŞKAN

Şubat 2015, 62 sayfa

Bu çalışmada *Nigella arvensis* türünden 13 tane glikozid izole edilerek, izole edilen bileşiklerden iki tanesinin yapısı spektral teknikler kullanılarak 3-O-β-D glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-ribopyranoside ve 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-ribopyranosyl-28-O-[α-L rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] olarak belirlenmiştir.

Anahtar Kelimeler: *Nigella arvensis,* triterpenoid saponin, oleanan

ABSTRACT

ISOLATION PURIFICATION AND CHARACTERIZATION OF SECONDARY METABOLITES FROM *Nigella arvensis* var. *involucrata*

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In this thesis, 13 glycosides were isolated from *Nigella arvensis* species and structures of the two of the isolated compounds determined as 3-O-β-D glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-ribopyranoside. and 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-ribopyranosyl-28-O-[α-L rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] by using spectroscopic techniques.

Keywords: *Nigella arvensis,* triterpenoid saponin, oleanane

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ABBREVIATIONS

UV	Ultraviolet
NMR	Nuclear Magnetic Resonance
CC	Column Chromatography
CTLC	Centrifugal Thin Layer Chromatography
LPLC	Low-pressure Liquid Chromatography
MPLC	Medium-pressure Liquid Chromatography
HPLC	High Performance Liquid Chromatography
RP-VLC	Reverse Phase Vacuum Liquid Chromatography
SPE	Solid Phase Extraction
s	Singlet
d	Doublet
t	Triplet
q	Quartet
m	Multiplet

1 INTRODUCTION

1.1 Natural Products

Plants synthesize a vast array of intricate and fascinating organic molecules. Natural Products Chemistry is the study of these molecules. It involves the determination of structures by chemical and spectral methods, the study of the relationships between structures and functions, biosyntheses and syntheses, and the uses mankind has made of the host plants through the ages.

The use of natural products, especially plants, for healing is as ancient and universal as medicine itself. The therapeutic use of plants certainly goes back to the Sumerian civilization, and 400 years before the Common Era, it has been recorded that Hippocrates used approximately 400 different plant species for medicinal purposes. Natural products played a prominent role in ancient traditional medicine systems, such as Chinese, Ayurveda, and Egyptian, which are still in common use today. According to the World Health Organization (WHO), 75% of people still rely on plant-based traditional medicines for primary health care globally (Sarker *et al.,* 2006).

Naturally occuring compounds may be divided into three broad categories. Firstly, there are those compounds which occur in all cells and play a central role in the metabolism and reproduction of those cells. These compounds include the nucleic acids and the common amino acids and sugars. They are known as primary metabolites. Secondly, there are the high-molecular-weight polymeric materials such as cellulose, the lignins and the proteins which form the cellula structures. Finally, there are those compounds that are characteristic of a limited range of species. These are the secondary metabolites. Most primary metabolites exert their biological effect within the cell or organism that is responsible for their production. Secondary metabolites, on the other hand, have often attracted interest becouse of their biological effect on other organisms (Hanson, 2003).

The biologically active constituents of medicinal, commercial and poisonous plants have been studied throughout the devolopment of organic chemistry. Many of these compounds are secondary metabolites. It has been estimated that over 40% of medicines have their origins in these natural products. A number of screening programmes for bioactive compounds exist and have led to new drugs, for example taxol, which is used for the treatment of various

cancers (Figure 1.1). Natural products often have an ecological role in regulating the interactions between plants, microorganisms, insects and animals. They can be defensive substances, antifeedants, attractants and pheromones. Chemotaxonomy provides another reason for examming the constituents of plants. Phytochemical surveys can reveal natural products that are "markers" for botanical and evolutionary relationships (Hanson, 2003).

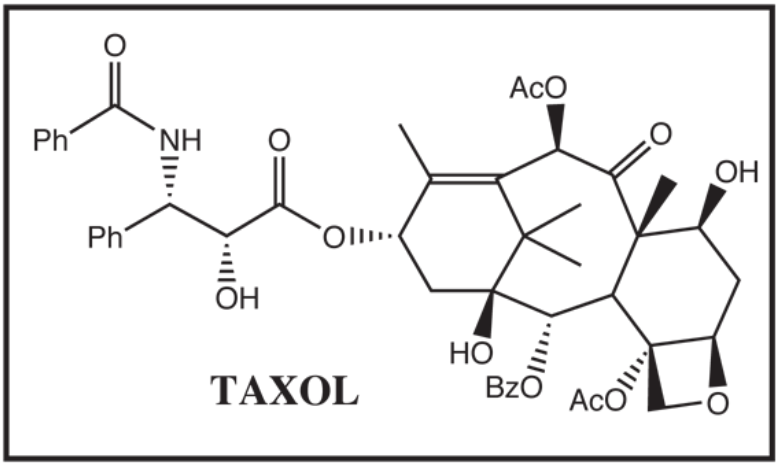


Figure 1.1. Taxol, a diterpene from *Taxus spp.* has very strong anticancer activity

The pathways for modifying and synthesizing carbohydrates, proteins, fats and nucleic acids are the same in all organisms, apart from minor variations. Also, the biosynthesis of most secondary metabolites begins from a relatively small group of compounds, which are modified into an unlimited number of compounds through various synthesis pathways. The most important of these metabolic pathways are those with acetyl coenzyme A (acetyl-CoA), shikimic acid, or mevalonic acid intermediates, and known as the acetate, shikimate, and mevalonate pathways, respectively. These key intermediates are formed from products of the glycolytic pathway or their intermediates (Figure 1.2). These major metabolic pathways are briefly described below (Dewick, 1997; Samuelsson, 1992; Mann *et al.*, 1994). In addition to these key intermediates, a wide variety of antibiotics, peptides, proteins, and alkaloids are formed from amino acids derived from acetyl-CoA via citric acid (Krebs) cycle (Dewick, 1997; Murray *et al.*, 1990), or via the shikimate pathway (Tolonen, 2003).

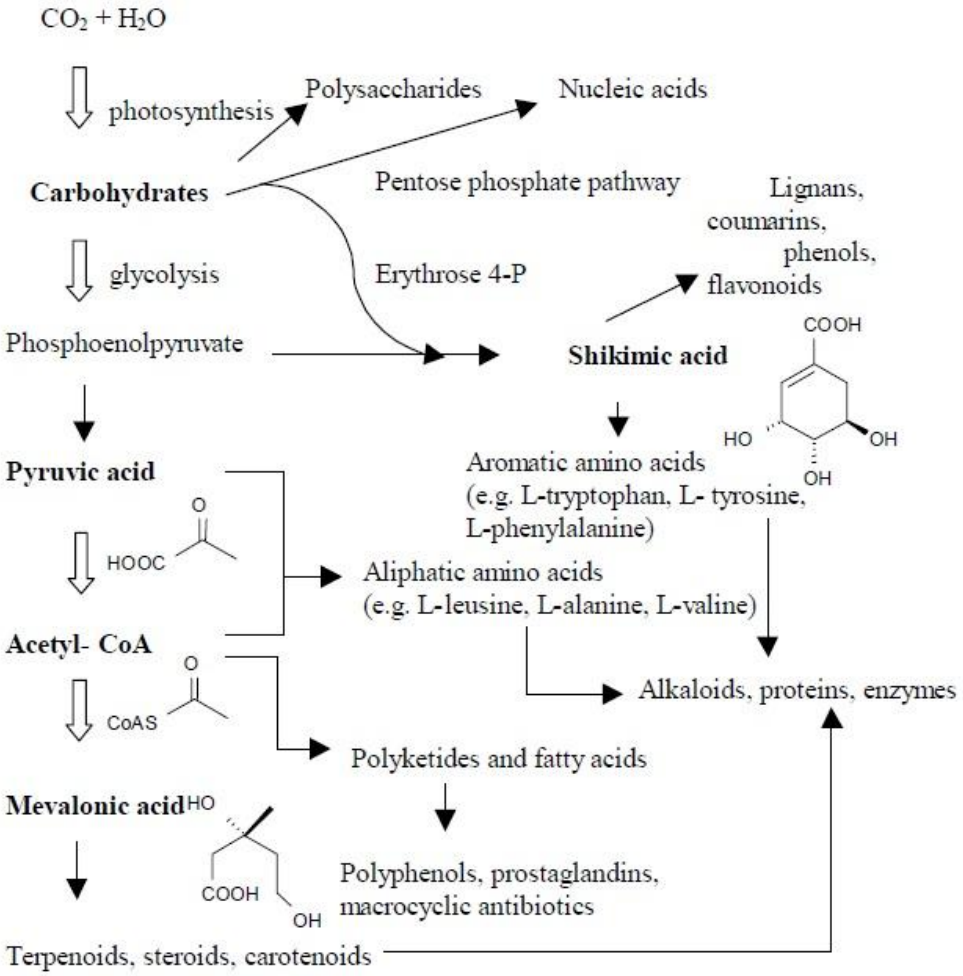


Figure 1.2. Common biosynthetic pathways to secondary metabolites via acetyl-CoA, skikimic acid, mevalonic acid and amino acids (Tolonen, 2003.)

1.2 The Classes of Secondary Metabolites

The structures of secondary metabolites may seem to be bewilderingly diverse. However, the majority of these compounds belong to one of a number of families, each of which have particular structural characteristics arising from the way in which they are built up in nature, i.e. from their biosynthesis. The classes of secondary metabolites are:

* Polyketides and fatty acids
* Terpenoids and steroids
* Phenylpropanoids
* Alkaloids
* Specialized carbohydrates (Hanson, 2003).

1.3 Terpenes

The terpenes, or isoprenoids, are one of the most diverse classes of metabolites. The Dictionary of Natural Products (Buckingham 2004) lists over 30.000, mainly of plant origin, encompassing flavours and fragrances, antibiotics, plant and animal hormones, membrane lipids, insect attractants and antifeedants, and mediators of the essential electron-transport processes which are the energy-generating stages of respiration and photosynthesis. Some of these key molecules are illustrated in Figure 1.3. In addition, terpenoid motifs are recurring structural and functional features of a host of bioactive natural products, fromthe phytol side chain of the chlorophylls and the diterpenoid skeleton of the anti-cancer drug Taxol to the core structural unit of tetrahydrocannabinol, the major bioactive component of marijuana (Crozier *et al.*, 2006).

Conifer wood, balm trees, citrus fruits, coriander, eucalyptus, lavender, lemon, grass, lilies, carnation, caraway, peppermint species, roses, rosemary, sage, thyme, violet and many other plants or parts of those (roots, rhizomes, stems, leaves, blossoms, fruits, seed) are well known to smell pleasantly, to taste spicy, or to exhibit specific pharmacological activities. Terpenes predominantly shape these properties (Breitmaier, 2006).

In order to enrich terpenes, the plants are carved, e.g. for the production of incense or myrrh from balm trees; usually, however, terpenes are extracted or steam distilled, e.g. for the recovery of the precious oil of the blossoms of specific fragrant roses. These extracts and steam distillates, known as ethereal or essential oils are used to create fine perfumes, to refine the flavor and the aroma of food and drinks and to produce medicines of plant origin (phytopharmaca). The biological and ecochemical functions of terpenes have not yet been fully investigated (Breitmaier, 2006).

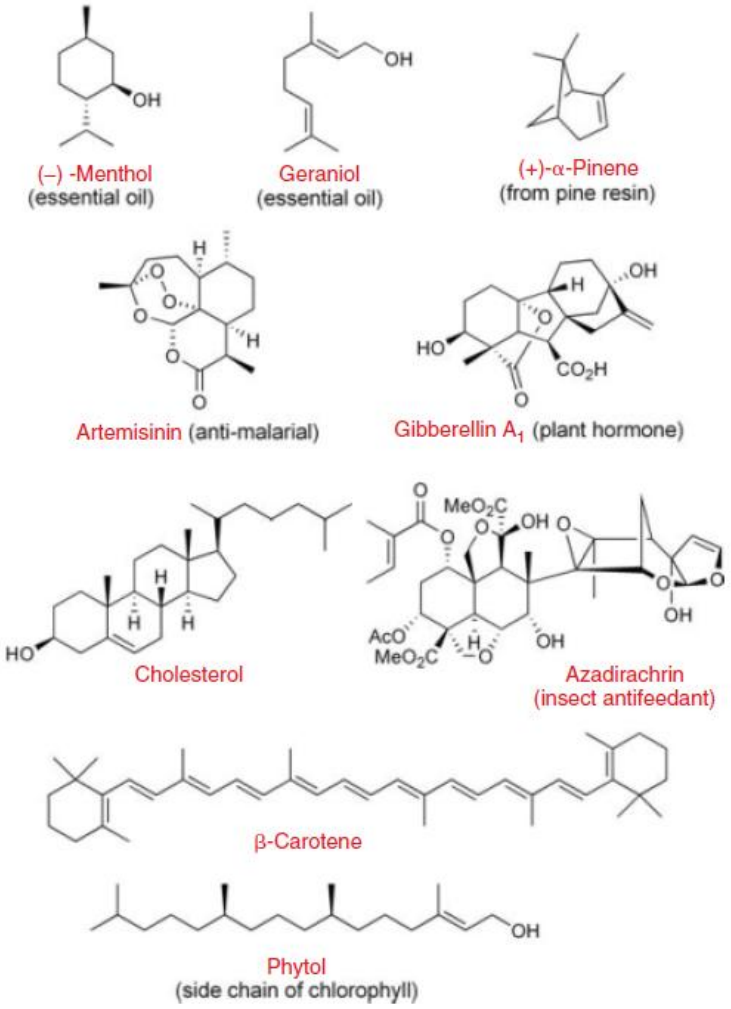


Figure 1.3. Examples of terpenes

Many plants produce volatile terpenes in order to attract specific insects for pollination or otherwise to expel certain animals using these plants as food. Less volatile but strongly bitter-tasting or toxic terpenes also protect some plants from being eaten by animals (antifeedants). Last, but not least, terpenes play an important role as signal compounds and growth regulators (phytohormones) of plants, as shown by preliminary investigations (Breitmaier, 2006).

About 30.000 terpenes are known at present in the literature.Their basic structure follows a general principle: 2-*Methylbutane* residues, less precisely but usually also referred to as *isoprene* units, $(C_5)_n$, build up the carbon skeleton of terpenes; this is the isoprene rule found by RUZICKA and WALLACH (Table 1.1). Therefore, terpenes are also denoted as *isoprenoids.* In nature, terpenes occur

predominantly as hydrocarbons, alcohols and their glycosides, ethers, aldehydes, ketones, carboxylic acids and esters. (Breitmaier, 2006).

Depending on the number of 2-methylbutane (isoprene) subunits one differenatiates between *hemi-* (C_5), *mono-* (C_{10}), *sesqui-* (C_{15}), *di-* (C_{20}), *sester-* (C_{25}), *tri-* (C_{30}), *tetraterpenes* (C_{40}) and *polyterpenes* $(C_5)_n$ with n>8 according to Table 1.1. (Breitmaier, 2006).

The isopropyl part of 2-methylbutane is defined as the *head*, and the ethyl residue as the *tail* (Table 1.1). In mono-, sesqui-, di- and sesterterpenes the isoprene units are linked to each other from *head-to-tail;* tri- and tetraterpenes contain one *tail-to-tail* connection in center (Breitmaier, 2006).

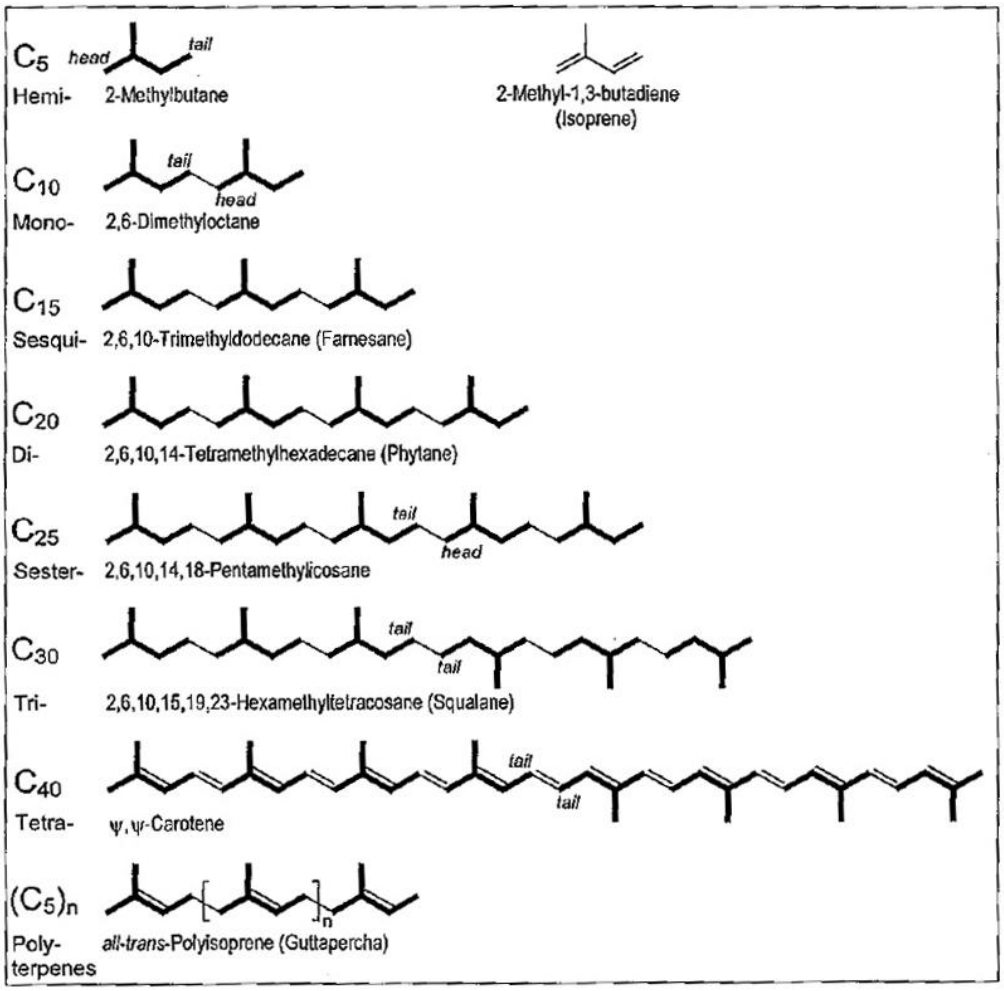


Table 1.1. Parent hydrocarbons of terpenes (Isoprenoids)

Steroids (C27) belong to the group of isoprenoids as they originate from triterpenes. Labeling experiments showed that the formation of tetracyclic triterpenoids from 2,3-epoxysqualene is an introductory step in the formation of steroids (Breitmaier and Teubner, 1999). The loss of carbon atoms and rearrangement reactions during their biosynthesis from triterpenes makes the isoprene units of steroids unrecognizable. Therefore, they are classified separately from terpenes (Thiele and Verlag, 1979).

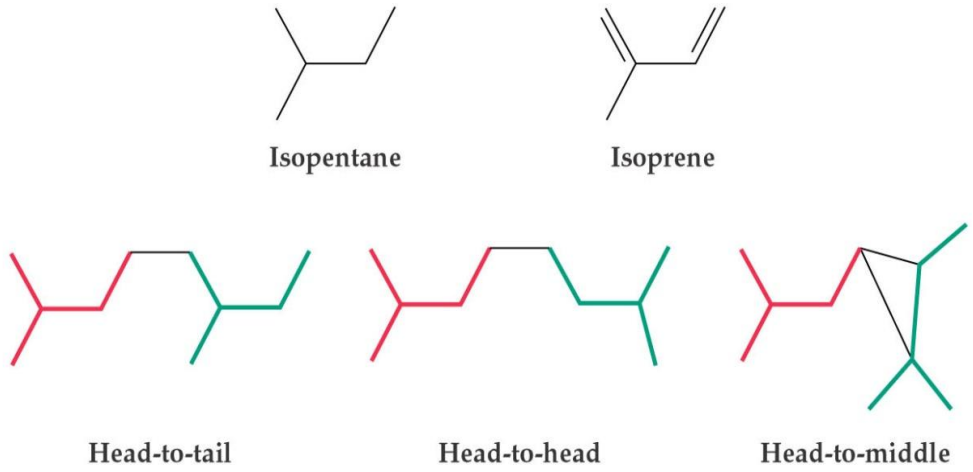


Figure 1.4. The terms head-to-tail and tail-to-tail often used to describe how the isoprene units are joined

1.4 Saponins

The name ‘saponin’ is derived from the Latin word sapo, which means ‘soap’, because molecules form soap-like foams (Oleszek, 2002; Vincken *et al.*, 2007) in aqueous solution (Osbourn, 2003).

Saponins, generally known as non-volatile (Oleszek, 2002; Lanzotti, 2006; Vincken *et al.*, 2007), are a major family of secondary metabolites that occur in a wide range of plant species (Hostettmann and Marston, 1995).

Saponins are high-molecular-weight glycosides, consisting of a sugar moiety linked to a triterpene or steroid aglycone. The classical definition of saponins is based on their surface activity; many saponins have detergent properties, give stable foams in water, show haemolytic activity, have a bitter taste and are toxic to fish (piscicidal). Such attributes, while not common to all

saponins, have frequently been used to characterize this class of natural products (Hostettmann and Marston, 1995).

In the Orient, these compounds were used as soap and many trivial names of saponin-rich species are derived from this feature, e.g. soapwort (*Saponaria of.cinalis*), soaproot (*Chlorogalum pomeridianum*), soapbark (*Quillaja saponaria*), soapberry (*Sapindus saponaria*) or soap- nut (*Sapindus mukurossi*) . Some of them find a commercial application as drugs and medicines, adjuvants, taste modifiers, emulsifiers, precursors of hormone synthesis and sweeteners (Oleszek *et al*., 2006).

Mostly seeds, leaves, stem bark, roots and rhizomes of higher plants containing saponins and some marine animals e.g. seaslug and starfish also contain saponins. Many saponins showed characteristic antimicrobial and antifungal activities (Tschesche *et al*., 1965; Wagner and Horhamer, 1971).

Most saponins isolated to date are triterpenoid (C30 aglycone-based) saponins (750 saponins with 360 different aglycones). These structures have been well characterized and are published in the literature making structure elucidation of atypical aglycone relatively straightforward (Berger *et al*., 2001).

Saponins have been shown in both in vitro and in vivo experimental test systems during the last decade to possess a broad spectrum of biological and pharmacological activities such as cancer-related activity, immunostimulating, immunoadjuvant, antihepatoxic, antiplogistic, antiallergic, molluscicidal, hemolytic, antifungal, antiviral, and hypoglycemic activities (Lacaille-Dubois *et al*., 2000).

1.4.1. Biosynthesis

In general, very little is known about the enzymes and biochemical pathways involved in saponin biosynthesis. Triterpenoid saponins are synthesised via the isoprenoid pathway by cyclisation of 2,3-oxidosqualene to give primarily oleanane (ß amyrin) or dammarane triterpenoid skeletons (Haralampidis *et al.*, 2002).

A brief biosynthetic summary of triterpenes and steroids is shown in Figure 1.5. They are built up of six isoprene units and have a common biosynthetic origin

in that they are all derived from squalene, presumably via ring opening of squalene-2(3-epoxide (oxidosqualene), followed by a concerted cyclization. It is only recently that the corresponding cyciases have been characterized (Abe *et al*., 1993). While the true triterpenes have 30 carbon atoms, the steroids have only 27 carbons by virtue of the oxidative cleavage of three methyl groups from a Cjo intermediate. Other details of the biosynthesis of steroids have been reviewed (Heftmann, 1968; Takeda, 1972). Much work remains to be done to clarify the intermediates but at least the distinction between the cholesterol (C27) and the triterpene (C30) pathways is evident (K. Hostettmann and A. Marston, 1995).

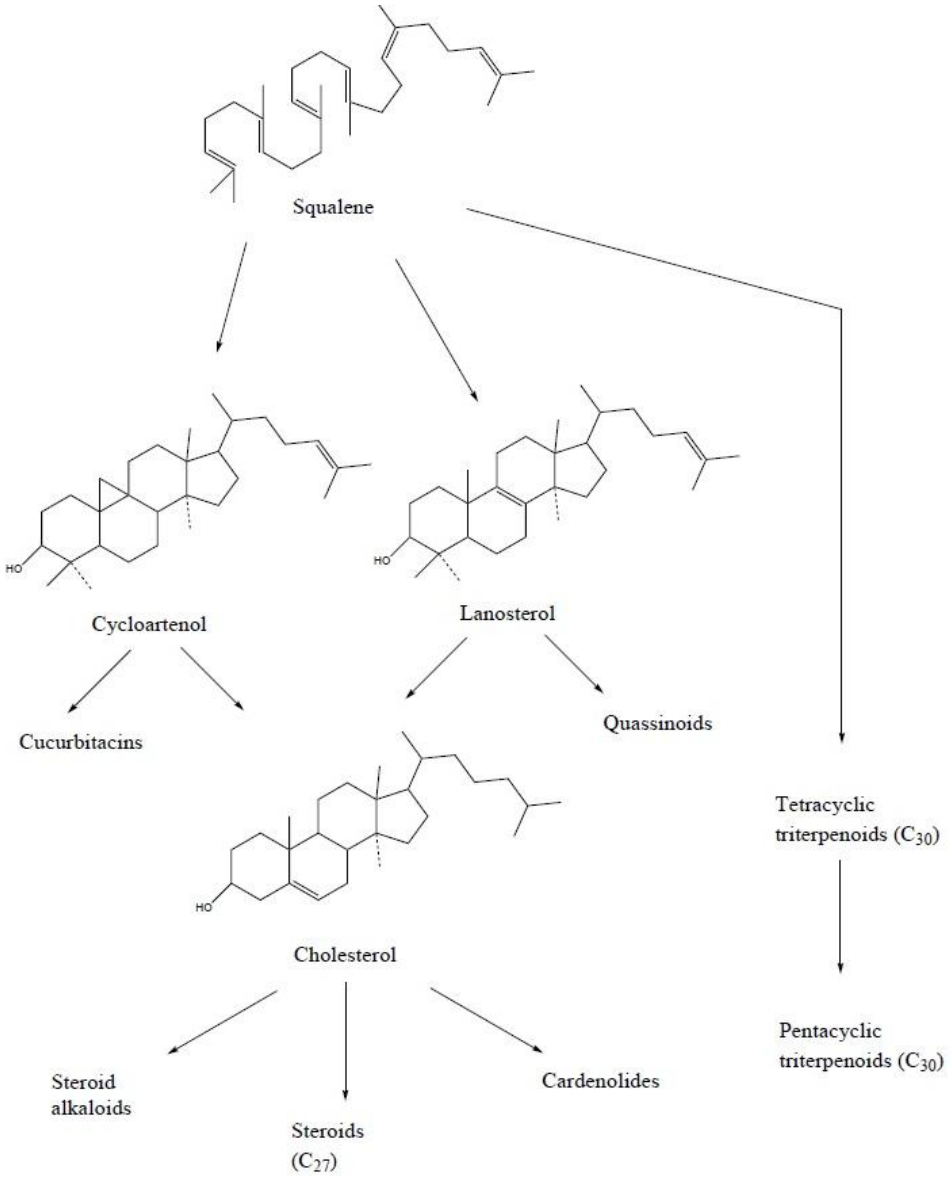


Figure 1.5. Biosynthesis of pentacyclic triterpenes and steroids from oxidosqualene

Figure 1.6 shows the cyclization of the chair-chair-chair-boat conformation of squakne-2,3-epoxide. Following the rearrangement of the tetracych'c carbonium ion, either a pentacyclic (eg. 0-amyrin) or tetracychc (e.g. dammarenol) triterpene is formed (Luckner, 1990).

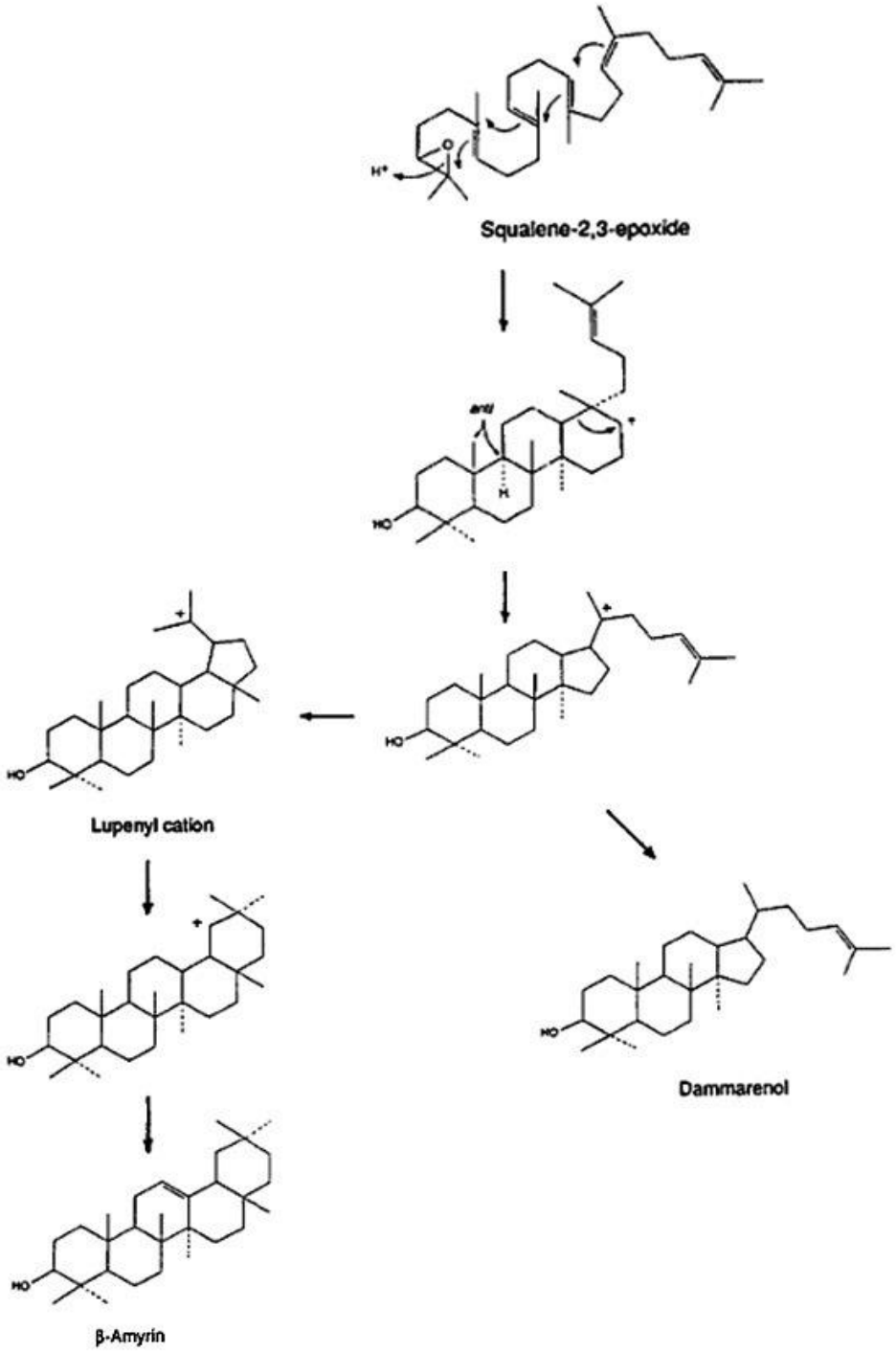


Figure 1.6. Cyclization of squalene-2,3-epoxide to C30 triterpenes

In contrast, cyclization of the chair-boat-chair-boat conformation of squalene-2,3-epoxide, followed by several 1,2-rearrangements, leads to the formation of either cycloartenol or lanosterol (Figure 1.7).

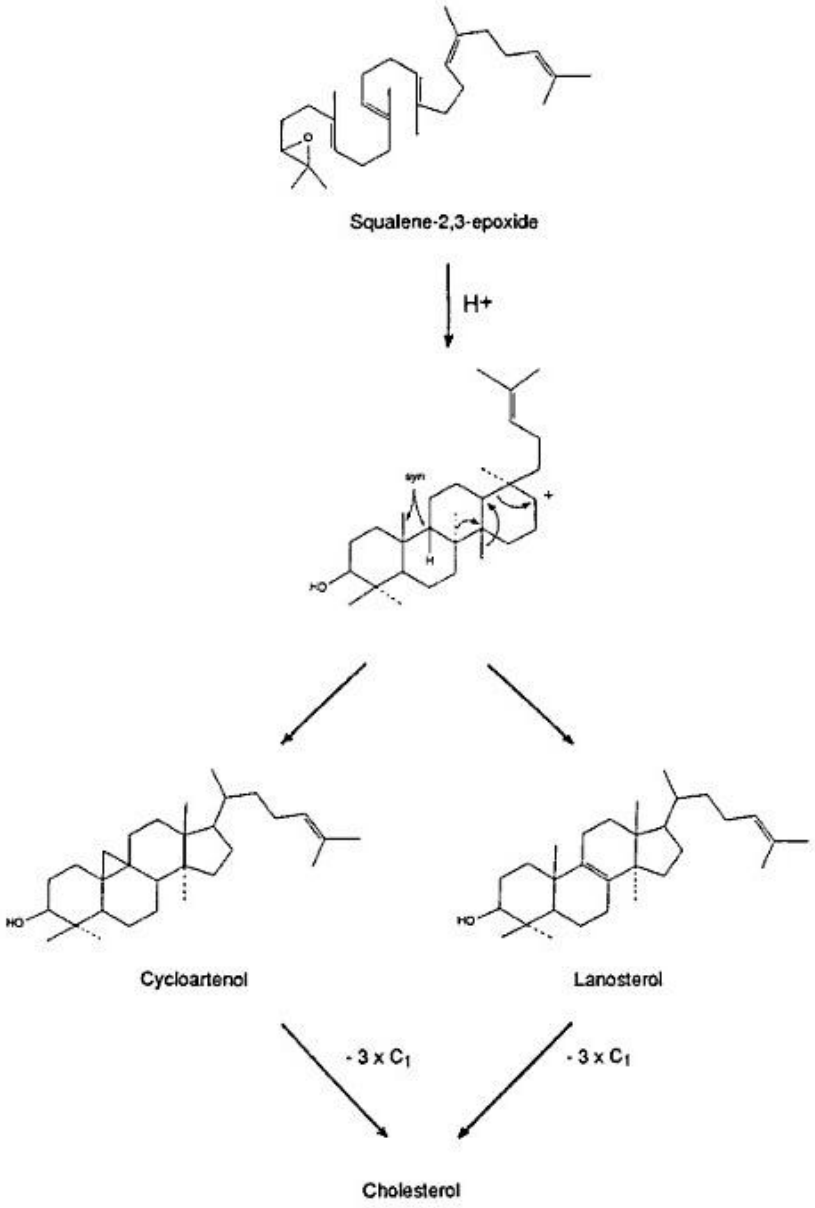


Figure 1.7. Cyclization of squalene -2,3-epoxide and formation of cholesterol

Other important classes of secondary metabolites such as phytosterols, cardenolides, cucurbitacins, quassinoids and fimonoids are also derived from squalene. There are similarities in the biosynthesis of cardenolides 3-keto intermediate and both processes occur actively in Digitalis spp. (Heftmann, 1974). Like the saponins, many cardenolides give foams in aqueous solution but are

classified separately because of their different biological activities (Hostettmann and Marston, 1995).

Oxidosqualene cyclases (OSCs) convert oxidosqualene to one or more cyclic triterpene alcohols with up to six carbocyclic rings (Figure 1.7). Plants biosynthesize diverse triterpenoids and encode multiple OSC enzymes to form these skeletons. Cycloartenol synthase (CAS) converts oxidosqualene to cycloartenol through the protosteryl cation intermediate (Figure 1.8). and was the basal plant OSC from which others derived. It can suggests that the known cycloartenol synthases are orthologs, and that cycloartenol synthase predates the emergence of plants. Lanosterol is the initial carbocyclic sterol precursor in animals, fungi, and trypanosomatids. Although substantial labeling experiments support cycloartenol rather than lanosterol as the major plant sterol precursor, lanosterol biosynthesis has been demonstrated in a fewplants (e.g. in the latex of several Euphorbia species). β-Amyrin synthases forms the lupyl cation, but allow further ring expansion and some rearrangement before deprotonation to β-amyrin. Several OSCs from eudicots and monocots produce β-amyrin accurately. These β-amyrin synthases are considerably more distant from one another (48–50% identical) than are the CAS enzymes (70–79% identical), and independent origins of β-amyrin synthases in eudicots and monocots have been proposed. Lupeol, β-amyrin, and their diverse metabolites are implicated in various plant processes. β-Amyrin is a precursor of saponins, which are triterpene glycosides such as the antifungal saponin avenacin found in Avena roots (Phillips *et al.*, 2006).

A fair of information is available about the location of saponin biosynthesis in plants. While *Calendula officinalis* saponins are biosynthesized in the green parts of the plant and than transported to the roots, glycyrrhizin is biosynthesized in the roots of *Glycyrrhiza glabra* (Leguminosae). It is shown thet glycosylated triterpenes are stored in the roots and rhizomes of *Saponaria officinalis* (Caryophyllaceae), *Gypsophila paniculata* (Caryophyllaceae) and *G. altissima*. Labeling studies have shown that saponins are biosynthesized only in the roots of *G. Paniculata* and are not translocated in the shoots. They accumulate mainly in the secondary phloem, while in the roots of *Buplerium falcatum* (Umbelliferae) they are localized in the outer layer of the phloem and in Panax ginseng (Araliaceae) the ginsenosides are found located outside the root cambium, particularly in the periderm and outer cortex (Hostettmann and Marston, 1995).

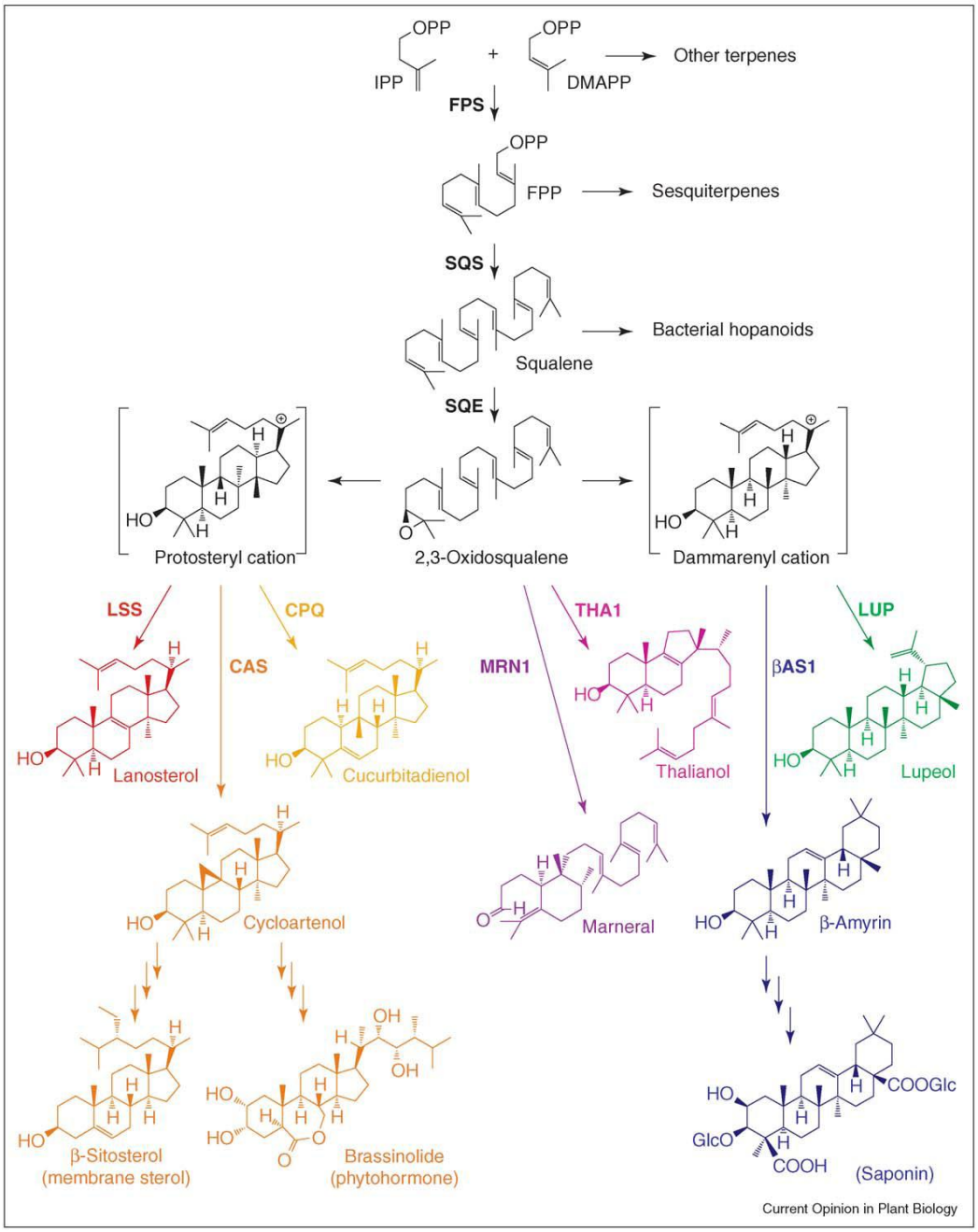


Figure 1.8. Simplified scheme of plant triterpenoid biosynthesis

Farnesyl diphosphate synthase (FPS) isomerizes isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to farnesyl diphosphate (FPP), which squalene synthase (SQS) converts to squalene. Squalene epoxidase(SQE) oxidizes squalene to 2,3-oxidosqualene. OSC enzymes cyclize 2,3-oxidosqualene through cationic intermediates (e.g. the protosteryl cation,the dammarenyl cation and others not shown) to triterpene alcohols or aldehydes. OSC products can be further modified by multiple enzymes to form membrane sterols, brassinosteroids,

saponins,and other compounds. bAS1, b-amyrin synthase; LUP, lupeol synthase; MRN1, marneral synthase; PP, diphosphate; THA1, thalianol synthase (Phillips *et al*., 2006).

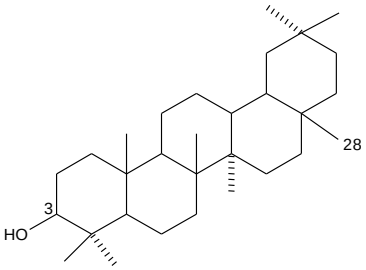
1.4.2. Classification, Occurance and Role in Plants

The aglycone or non-saccharide portion of the saponin molecule is called the genin or sapogenin. Depending on the type of genin present, the saponins can be divided in to three major classes:

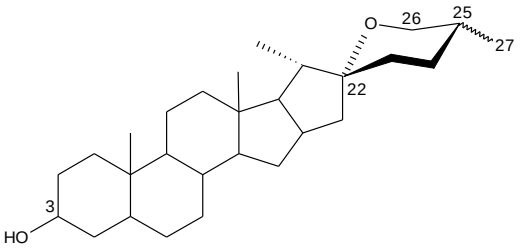
1) Triterpene glycosides
2) Steroid glycosides
3) Steroid alkaloid glycosides

The genin of these 3 classes can be depicted as shown in Figure 1.9.

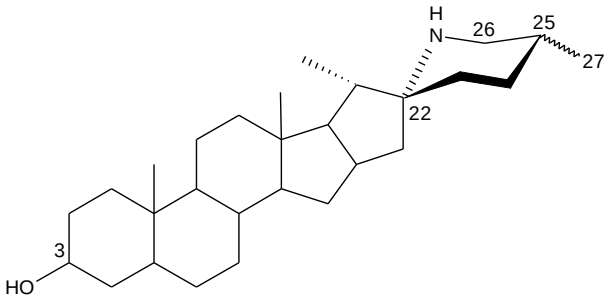
The aglycones are normally hydroxylated at C-3 and certain methyl groups are frequently oxidized to hydroxymethyl, aldehyde or carboxyl functionalities. When an acid moiety is esterified to the aglycone, the term ester saponin is often used for the respective glycosides (Hostettmann and Marston, 1995).



Triterpene class



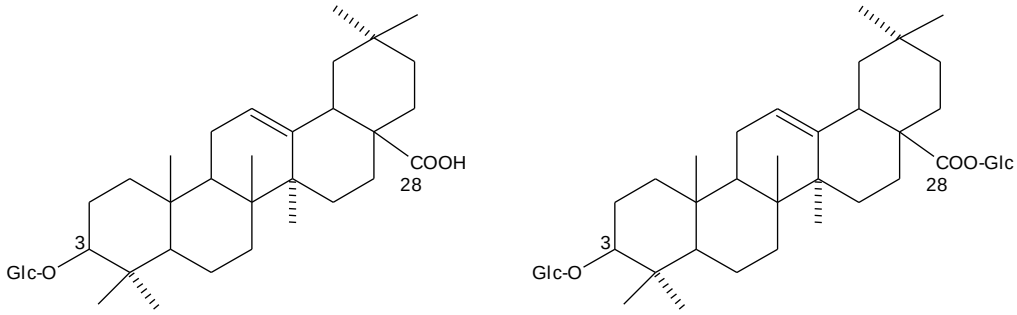
Steroid class



Steriod alkoloid class

Figure 1.9. Skeletal types of genin found in the three principal classes of saponin

All saponins have in common the attachment of one or more sugar chains to the aglycone. Monodesmosidic saponins have a single sugar chain, normally attached at C-3, bidesmosidic saponins have too sugar chains, often with one attached through an ether linkage at C-3 and one attached through an ester linkage (acyl glycoside) at C-28 (triterpene saponins) (Figure 1.10) or and ether linkage at C-26 (furostanol saponins). Tridesmosidic saponins have three sugar chains and are seldom found. Bidesmosidic saponins are easily transformed into monodesmosidic saponins by, for example, hydrolysis of the esterified sugar at C-28 in triterpene saponins; they lack many of the characteristic properties and activities of monodesmosidic saponins (Hostettmann and Marston, 1995).



Monodesmosidic Bidesmosidic

Figure 1.10. Monodesmosidic and bidesmosidic saponins

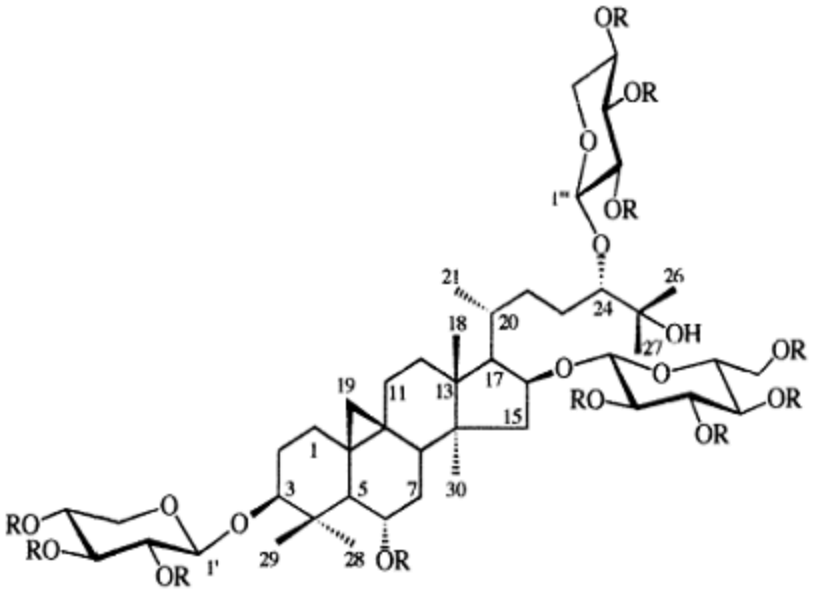


Figure 1.11. Cephalotoside A, a tridesmosidic cycloartane type glycoside

The saccharide moiety may be linear or branched, with 11 being the highest number of monosaccharide units yet found in a saponin (Clematoside C from *Clematis manshurica* (Raminculaceae); Khorlin *et al.,* 1965). As a rule, however, most of the saponins so far isolated tend to have relatively short (and often unbranched) sugar chains, containing 2-5 monosaccharide residues. Kochetkov and Khorlin (1966) have introduced the term *oligoside* for those glycosides containing more than 3-4 monosaccharides.

The most common monosaccharide moieties found. These are D-glucose (Glc), D-galactose (Gal), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), L-rhamnose (Rha), L-arabinose (Ara), D-xylose (Xyl) and D-fucose (Fuc). Saponins from marine organisms often contain D-quinovose (Qui) (K. Hostettmann and A. Marston, 1995).

1.4.2.1. Steroidal Saponins

Steroidal saponins (C_{27} based aglycones) are structurally similar to triterpene saponins. In general, they possess similar physical and biological properties to the triterpene saponins. Fewer steroidal saponins have been found compared to the triterpene saponins (Berger *et al.,* 2001).

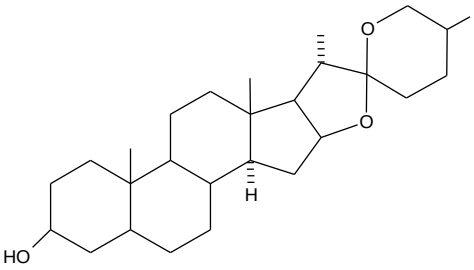
Steroid saponins are widely distributed in nature than the triterpenoid type. Monocotyledons are the main source of steroid saponins, with the Liliacea, Dioscoreaceae and Agavaceae providing many representatives. The most important saponin-bearing genera are; in the Liliaceae family *Trillium, Chlorogalum, Smilax, Nolina, Agapanthus*; in the Agavaceae the *Agave*, *Yucca* and *Manfreda;* and in the Dioscoreaceae the *Dioscorea* (Hostettmann and Marston, 1995).

Over 100 steroid sapogenins are known and most are derived from the furostan or spirostan skeleton (Hostettmann and Marston, 1995).

The steroid saponins are divided into four groups depends on their aglycones (Figure 1.12) (Hostettmann and Marston, 1995).

A. SPIROSTANOL

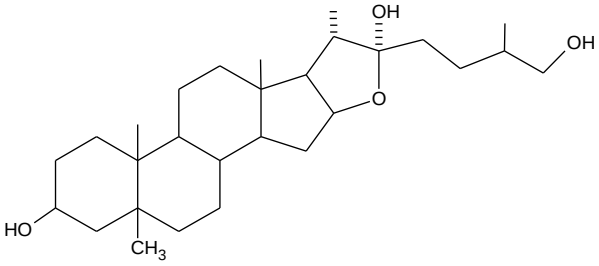
Sarsasapogenin



B. FUROSTANOL

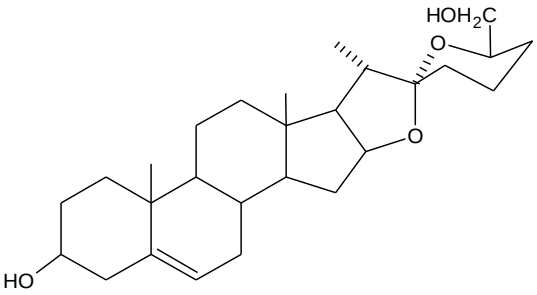
(25 S) 5β-furostan-3 β,

22α, 26-triol



C. NUATIGENIN

Nuatigenin



D. POLIPODO

Polipodogenin

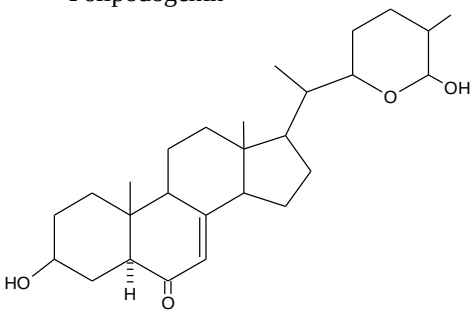


Figure 1.12. Classifications of steroidal saponins

Steroid saponins consequently lack most of the characteristic saponin properties. Spirostanol glycosides are found mainly in the seeds, roots or bulbs of plants, whereas furostanol glycosides are in the asshnilatory parts (Hostettmann and Marston, 1995).

1.4.2.2. Triterpene Saponins

This subdivision forms by far the most extensive collection of saponins, comprising, to date, over 750 triterpene glycosides with 360 sapogenins.

Triterpene glycosides have either oleanane (β-amyrin) or dammarane skeletons, with ursanes, hopanes, lanostanes or lupanes assuming secondary importance with respect to distribution (Figure 1.13).

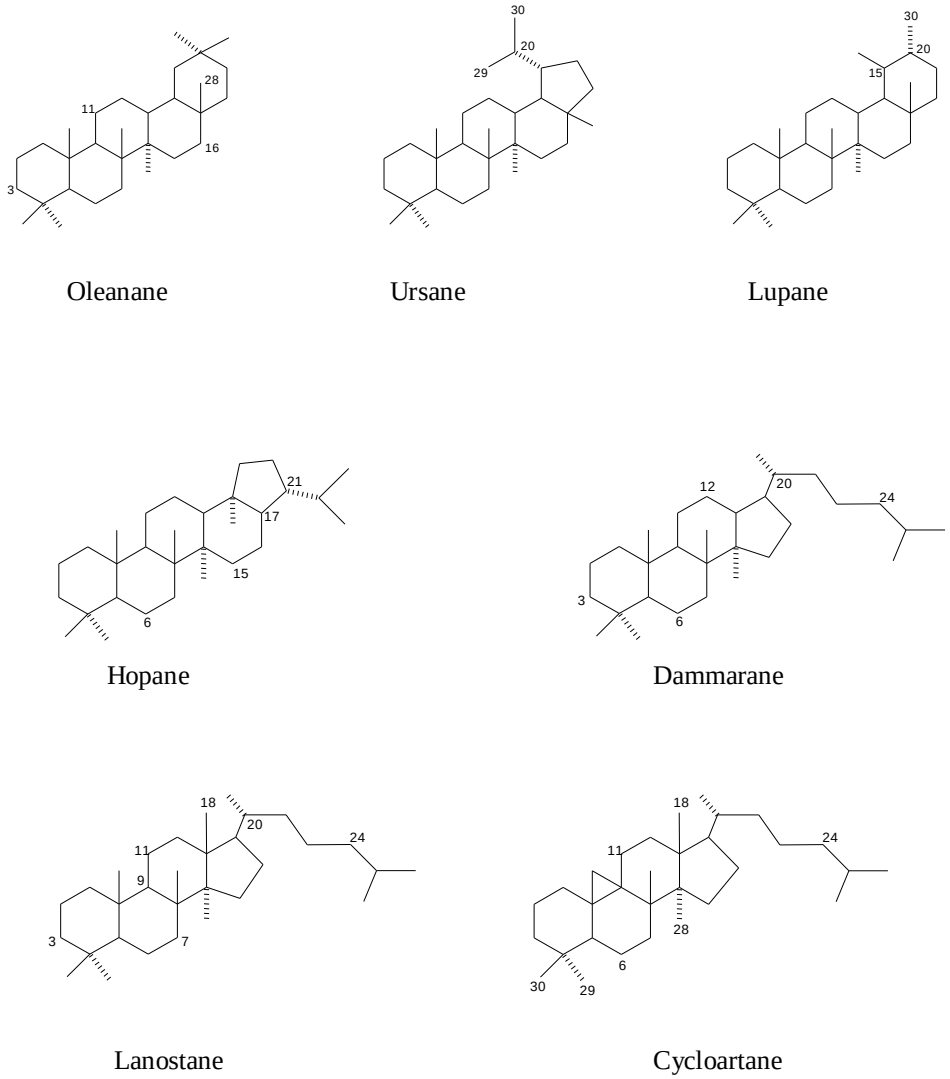


Figure 1.13. Triterpene aglycones (other than olean-12-en type)

There are numerous structural variants of the oleanane (or, more precisely, the olean-12-en) skeleton (Figure 1.14).

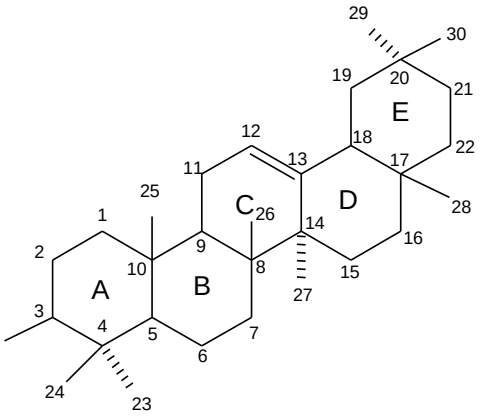


Figure 1.14. Olean-12-en skeleton

The triterpene saponins can be mono, bi, or even tridesmosidic. One sugar chain is often attached at C-3 and a second is frequently found to be esterified to the carboxyl group at C-17 of the aglycone (except certain dammarane glycosides and lanostane glycosides which have second or even a third glycosidically bound sugar chain)(Hostettmann and Marston, 1995). Steroidal saponins are typically monodesmosidic. The most common sugars possessed by saponins are D-glucose, L-rhamnose, L-arabinose, and D-xylose, D-apiose, D-quinovose, and D-allose have also been reported but are less common. Typically, a glycosyl moiety is attached to the aglycone at the 3-O position. However if the aglycone possesses additional oxygenated functionalities then additional glycosidal moieties may be present (Figure 1.15). The glycosidal moieties can be can be linear, branched, nitrogenated, oxidized, reduced, acetylated, or any combination thereof (Berger *et al.*, 2001).

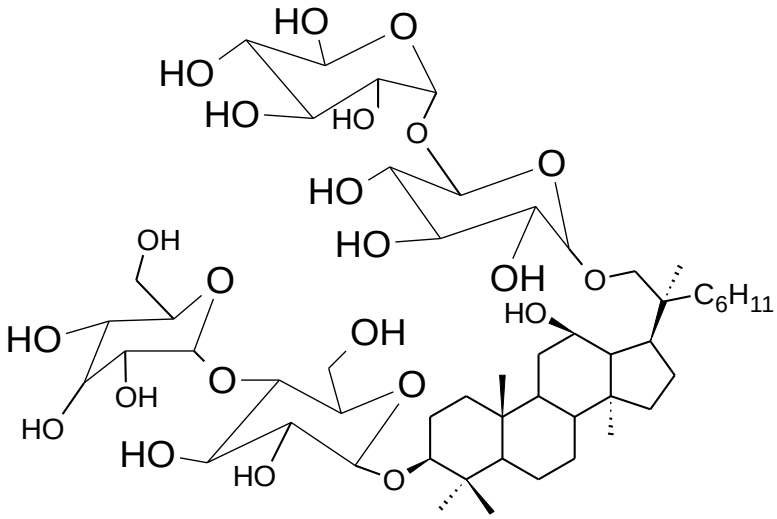


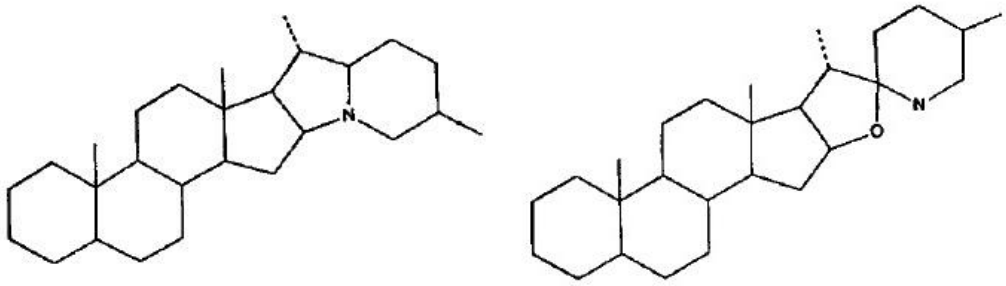
Figure 1.15. Gingengoside RB_2

1.4.2.3. Steroid alkaloids

Glycoalkaloids are usually found in all organs of the plant, with the highest concentrations in regions of high metabolic activity, such as flowers, unripe berries, young leaves and sprouts.

Solanaceae family (which includes many agricultural crop plants important to humans, such as potato, tomato, eggplant, capsicum and naranjilla) constituting the largest source (Schreiber, 1968).

There are two basic skeletal types; the spirosolans and the solanidans. Examples of both types are given in Figure 1.16 (Hostettmann and Marston, 1995).



Solanidan Spirosolan

Figure 1.16. The two classes of steroid alkaloid sapogenin (conventional representation)

The physiological role of saponins in plants is not yet fully understood. While there is a number of publications describing their identification in plants, and their multiple effects in animal cells and on fungi and bacteria, only a few have addressed their function in plant cells. Many saponins are known to be antimicrobial, to inhibit mould, and to protect plants from insect attack. Saponins may be considered a part of plants' defence systems, and as such have been included in a large group of protective molecules found in plants named 'phytoanticipins' or 'phytoprotectants' (Morrissey & Osbourn, 1999). It has also been suggested that saponins could be a source of monosaccharides (Barr *et al.*, 1998).

Saponins occur constitutively in a great many plant species, in both wild plants and cultivated crops. In cultivated crops the triterpenoid saponins are

common in plants used as herbs or for their health-promoting properties (Fenwick *et al.* 1991). Triterpenoids saponins have been detected in many legumes such as soyabeans, beans, peas, Lucerne, etc. and also in alliums (Lanzotti, 2006), tea, spinach, sugar beet, quinoa, liquorice, sunflower, horse chestnut, and ginseng. Steroid saponins are found in oats, capsicum peppers, aubergine, tomato seed, alliums, asparagus, yam, fenu-greek, yucca and ginseng. One example of an extensively studied group of triterpenoid saponins is produced from *Quillaja saponaria*, a tree native to the Andes region. The bark was peeled off and extracted with water by the indigenous peoples as a shampooing agent, and by the shamans as an overall curing agent. Yucca schidigera is the most common commercial source of steroids saponins (Francis *et al.*, 2002).

Saponins are found in a large number of plants and some animals (such as the sea cucumber). In plants, they occur in different parts such as root, tuber, bark, leaves, seed, and fruit. Young leaves contain more saponins than mature leaves, but foliage saponins were found to be less haemolytic than root saponins (Wina *et al.*, 2005). Alfalfa Triterpenoid saponins are found principally in dicotyledons while steroidal saponins occur in monocots. Alfalfa saponins are induced by insect attack and act as a deterrent to subsequent attacks. When alfalfa saponins were administered in the diet of larval *Spodoptera littoralis*, they caused prolongation of the larval and pupal stages, retarded growth, increased mortality, and decreased fecundity and fertility (Adel *et al.*, 2000). However, some plant species contain both triterpenoid and steroidal saponins. Avenacoside (steroidal), for example, occurs in oat leaves while avenacin (triterpenoid) is found in oat roots. Avenacin, is a saponin found in large quantities in root tips, but only at low levels in the rest of the root system, and acts as a means of controlling the zoosporic fungus, *Gaeumannomyces graminis.*(Osbourne, 1996). Besides, the simplest triterpene, squalene, was first isolated from fish liver oils. Subsequently, it has been found in plant oils and mammalian fats. The common tetracyclic triterpene lanosterol is a major constituent of wool fat and its esters are found in wood resin and the bark of many trees. A triterpene lactone, abietospiran, crystallizes on the surface of the bark of the silver fir, Abies alba, giving it a grey-white appearance. Glycyrrhetinic acid is a triterpene found in liquorice, and has healing properties in the treatment of peptic ulcers (Hanson, 2000). Saponins also control rhizosphere bacteria in the soil (Fons *et al.*, 2003).

1.4.3. Biological and Pharmacological Properties of Saponins

There are many biological activities associated with saponins. Most of those arise from the chemical nature of saponins which have two constitutional moieties: The hydrophilic sugar and the usually lipophilic sapogenin. These properties are responsible for the interaction between saponins and cell membranes. Attributes such as their fungicidal and piscicidal (a chemical substance that is poisonous to fish) effects have been known for years, while new activities are continually being discovered (Hostettmann and Marston, 1995).

Haemolytic activity

Saponins have the ability to rupture erythrocytes. The haemolytic properties are generally attributed to the interaction between the saponins and the sterols of the erythrocyte membrane. As a result, the membrane bursts, causing an increase in permeability and a loss of haemoglobin. Baumann *et al*. (2000) investigated the effect of saponins on the membrane structure through haemolysis of human erythrocytes. The findings showed that saponin-lysed erythrocytes do not reseal, and therefore indicates that saponin damage to the lipid bilayer is irreversible.

Oda *et al*. (2000) reported on the haemolytic activity of 47 different plant derived saponins, purified from food and medicinal plants. It has been suggested that there is a relationship between the adjuvant and haemolytic activity of saponins, however, the results indicated that the adjuvant activity does not relate with haemolytic activity. A substance or compound is said to have adjuvant activity if when used with another active compound, it enhances the activity of the active compound. The level of haemolytic activity was attributed to the type of aglycone and the presence of sugar side chains. Saponins with an acyl residue or oxide-ring moiety tended to show haemolytic activity, except for lablaboside D, which did not show haemolytic activity despite possessing a acyl residue. Escin saponins found in *Aesculus hippocastanum* L. (Hippocastanaceae) and jujuboside saponins from *Zizyphus jujuba* Mill. (Rhamnaceae) had strong haemolytic activity Sindambiwe *et al.* (1998).

Molluscicidal activity

Although toxic to cold-blooded species, if taken orally by warm-blooded species, saponins have only a weak toxicity (Bruneton, 1995), which is probably

attributed to low absorption rates. The toxicity towards cold-blooded species has lead to the use of saponin containing drugs to catch fish. Saponins are also highly toxic to molluscs and have been investigated as molluscicides in the control of schistosomiasis (Sindambiwe *et al.,* 1998; Abdel-Gawad *et al*., 1999).

Anti-inflammatory activity

There are a number of reports of saponins with anti-inflammatory properties. Many saponins isolated from plant sources produce an inhibition of inflammation in the mouse carrageenan-induced oedema assay. In a study by Just *et al.* (1998), Fruticesaponin B, a bidesmosidic saponin with an unbranched saccharide moiety isolated from *Bupleurum fruticescens* L. (Apiaceae), was shown to have the highest anti-inflammatory activity of the all the saponins tested in the mouse oedema assays.

Antifungal activity

Saponins have high toxicity against fungi (Delmas *et al.,* 2000; Wang *et al.,* 2000a). Fungicidal activity against *Trichoderma viride* was previously used as an identification method for saponins. Kalopanaxsaponins A and I isolated from Kalopanax pinctus exhibited strong and specific antifungal activity against Candida albicans and Cryptococcus neoformans (Kim *et al.,* 1998*b*). Asparagus saponin-1, from the lower leaves of Asparagus officinalis, has antifungal properties in concentrations of 0.5-8.0 μg/ml depending on the type of fungus (Shimoyamada *et al.,* 1990). The monodesmosidic spirostanol saponins from Y. schidigera destroy certain food-deteriorating yeasts, film-forming yeasts, and dermatophytic yeasts and fungi (Miyakoshi *et al.,* 2000). The major mechanism suggested for the antifungal activity of saponins is their interaction with membrane sterols. Synthetic steroid saponins prepared by Takechi *et al.* (1999) were both antifungal and haemolytic but in many cases haemolytic triterpenoid saponins show little antifungal activity. It was observed that those saponins having a branched-chain trisaccharide moiety without any oxygen-containing groups at C_2 and C_{12} exhibited the anti-yeast activity, while saponins with 2,3-hydroxyl or 12 keto groups showed very weak or no activity (Miyakoshi *et al.,* 2000).

Antibacterial/Antimicrobial activity

Saponins have also been reported to have antimicrobial activity (Killeen *et al*., 1998). Three butanol-extractable 5ß-spirostan-3ß-ol saponins were shown to have antimicrobial activity on both prokaryotic and eukaryotic organisms, but only at low cell densities. The saponins did not inhibit microbial growth of dense populations.

Three new triterpenoid saponins, Nudicaucins A, B, and C and a known saponin guaiacin D were isolated from *Hedyotis nudicaulis* Wight & Arn. (Rubiaceae) were tested against *Bacillus subtilis* (Konishi *et al.*, 1998). All four of the isolated saponins showed weak antibacterial activity. Results indicated that the tetraglycoside saponins have stronger activity than the triglycoside saponins.

Antiparasitic activity

Two new triterpenoid saponins, glinoside A and B, isolated from the aerial parts of *Glinus oppositifolius* L. (Molluginaceae) were shown to have antiprotozoal activity against *Plasmodium falciparum* (Traore *et al.*, 2000). Results showed that the saponin fractions had slightly better antiplasmodial activity (IC_{50} of 31.8 µg/ml) than pure glinoside A (IC_{50} of 42.3 µg/ml).

Cytotoxicity and antitumor activity

Numerous reports highlight the highly cytotoxic properties of many saponins. However, saponins with high cytotoxicity do not always have antitumor properties as cytotoxic compounds can potentially be used as antitumor agents.

Many isolated steroidal saponins have been shown to be either cytostatic or cytotoxic to HL-60 human leukemia cell lines (Mimaki *et al.*, 1998b, 1998c, 1999a, 1999c, 2001b; Yokosuka *et al.*, 2002b) Mimaki *et al*. (1998b).

Triterpenoid saponins have also shown cytotoxicity against human cell lines. A novel triterpene saponin, isolated from the root bark of Aralia dasyphylla Miq. (Araliaceae) showed significant cytotoxic activity against KB and HeLa-S_3 cells (Xiao *et al.*, 1999).

Mixtures of saponins have also been shown to have cytotoxic activities. Marquina *et al.* (2001) reported on a mixture of monodesmoside saponins, which were not active as pure compounds, to be highly cytotoxic against P388 and colon cell lines (ED_{50} values of 2.3 and 3.6 μg/ml, respectively).

Antiviral activity

Saponins have also been reported to have antiviral activities. Simões *et al.* (1999) tested two triterpenoid saponins isolated from Brazilian and Chinese plants for their antiviral activity. Both triterpenoid saponins exhibited antiviral activity. The oleanane-type inhibited herpes simplex virus type 1 DNA synthesis, while the ursane-type saponin inhibited viral capsid protein synthesis of herpes simplex virus type 1.

1.5 *Nigella* genus

The genus *Nigella* L. (Ranunculaceae) includes about 20 species distributed from the Mediterranean regions to west Asia (Hegnauer, 1973). In folk medicine as well as in horticulture, some *Nigella* species (e.g. *Nigella sativa* L., *Nigella damascena* L. and *Nigella arvensis* L.) are of interest. The three species have been in use in many Middle Eastern and Far Eastern countries as a natural remedy for over 2000 years (Fatima *et al.*, 2002). *N. sativa* is widely cultivated throughout southern Europe, Syria, Egypt, Saudi Arabia, Turkey, Iran, Pakistan and India for culinary and medicinal purposes. The seeds, commonly known as black cumin, or their oil have been traditionally used as a carminative, diuretic, lactagogue and vermifuge (Hegnauer, 1973; Burits and Bucar, 2000). They have also been used for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and hypertension. This plant has been extensively studied pharmacologically and seeds or their extracts have anti-inflammatory, CNS depressant and analgesic, antitumor, immunostimulant, antihistaminic, antidiabetic, antihypertensive and antimicrobial activities. Some of these activities have been predominantly attributed to the volatile and fixed oils (Houghton *et al.*, 1995; Burits and Bucar, 2000; El-Dakhakny *et al.*, 2000; Zaoui *et al.*, 2002; Nickavar *et al.*, 2003; Ali and Blunden, 2003).

Previous work on nigella seed extracts has shown that it inhibits the growth of the bacteria *Escherichia coli, Bacillus subtilis* and *Streptococcus faecalis* (Saxena and Vyas, 1986). The antimicrobial activity of N. sativa against several

other species of pathogenic bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa*) and pathogenic yeast (*Candida albicans*) has also been established (Hanafy and Hatem, 1991; Fatima *et al.,* 2002).

For a long time, plant remedies, including nigella, have been used to treat diabetes. It has been proposed that the antidiabetic action of the nigella extracts may, at least partly, be mediated through decreased hepatic gluconeogenesis (al-Awadi *et al.,* 1991). Traditionally, these seeds are well known for their action on stone dissolution in the kidney and bladder (Fatima *et al.,* 2002).

The genus Nigella comprises about 13 species in Turkey (Davis, 1978, 1988). N. sativa seeds are also used as seasoning for foodstuffs like bread and pickles among Turkish people (Kokdil and Yilmaz, 2005).

1.5.1. *Nigella arvensis* L.

Nigella arvensis L. (vernacular names ‘black bread weed’ or ‘wild fennel’) is an erect annual herb, up to 0.3 m tall. Leaves are soft, pinnatisect into linear segments. Flowers are bluish, 20–30 mm in diameter. It is a wide-spread weed, distributed from Central and South East Europe to the Russian Federation, Near East Region and Northern Africa.13 Black granulate seeds are locally used as a flavouring for cakes and bread, raw or cooked (Facciola *et al.*, 1990).

Only few reports on the pharmacological activity or chemistry of *N. arvensis* have been published. The seed extract was found to have a stimulatory effect on Na^+ transport in renal epithelia (Atia *et al.,* 2002).

Figure 1.17. *Nigella arvensis* (Fungoceva, 2015)

1.6 Analysis and Isolation

An important aspect of the modern use of plant extracts as pharmaceutical preparations is the characterization and determination of the individual active constituents. Such is also the case for saponin preparations, which often require sophisticated techniques for the isolation, structure elucidation and analysis of their component triterpene and steroid glycosides. When biological testing of the pure compounds is to be performed, it is necessary to isolate them in sufficient quantity and purity. As many foodstuffs contain saponins, their isolation and characterization is vital in order to investigate their biological activities and possible toxic effects separations (Hostettmann and Marston, 1995).

A large body of work on the isolation of saponins comes from Japan where there is great interest in the constituents and pharmacological properties of Oriental drugs, many of which contain either triterpene glycosides or steroid glycosides (Hostettmann and Marston, 1995).

Saponins are complex molecules consisting of non-sugar aglycone coupled to sugar chain units. The aglycones can be divided into the groups of saponins containing triterpene or steroidal aglycones—sapogenins. Both triterpene and steroidal aglycones have a number of different substituents (-H, -COOH, -CH_3).

The sugars can be attached to the aglycone either as one, two or three side chains. Thus, number of constituents and different possibilities of sugar chain composition and attachment causes great natural diversity of saponin structures. Even within one plants species different parts may harbour saponins with different structures (Oleszek and Bialy, 2006)

Due to the fact that saponins usually occur in plants as a mixture of structurally related forms with very similar polarities, their separation still remains a challenge. It is a usual practice in isolation of these compounds that a number of different separation techniques (TLC, column chromatography, flash chromatography, liquid– partition chromatography, Sephadex chromatography and HPLC) should be used to obtain pure compounds for the structure and biological activity determination. In most cases, certain of the above steps have to be repeated with a change of support or eluent to achieve high purity (Fenwick, *et al.,* 1991).

Early work on saponins included hot extraction of plant material with alcohol–water solutions followed by evaporation of alcohol and extraction of saponins into butanol (liquid–liquid extraction). However, hot extraction may disintegrate some labile functions (acylated forms) and produce artifacts rather than genuine saponins. Besides, extraction with methanol in some cases, especially for steroidal saponins may result in formation of methyl derivatives, not found originally in plants. Thus, for obtaining real composition of saponins, cold extraction with ethanol–water solutions should be rather recommended. In liquid–liquid extraction, some highly polar saponins (bidesmosides, tridesmosides) can be lost or extraction may not be quantitative (Oleszek and Bialy, 2006).

Once the saponin has been purified, it may be subjected to analytical methods including MS (Mass Spectrometry), proton and carbon NMR (Nuclear Magnetic Resonance) spectroscopy, and FTIR (Fourier Transform Infrared Spectroscopy). Other classical methods are used to ascertain the presence of saponins in a crude plant extract, and to elucidate their composition throughout purification steps. TLC (Thin Layer Chromatography) and staining with dehydrating reagents containing aromatic aldehydes (such as anisyl aldehyde in sulfuric acid) are commonly used. The pure saponin may also be hydrolysed to verify the nature of its glycosidic moieties (Fenwick *et al.,* 1991).

1.6.1. Chromatography

All chromatographic techniques involve the distribution of extract components between two phases-a moving mobile phase that is passed over an immobile stationary phase. Separation depends on the difference in affinity of the components toward stationary phase and mobile phase (Sarker *et al*., 2006).

The isolation of pure saponins requires one or (as is almost always the case) more chromatographic separation steps in order to remove other polar constituents of alcoholic or aqueous plant extracts (Hostettmann and Marston, 1995).

A variety of modern separation techniques (Hostettmann *et al*., 1986, 1991-Marston and Hostettmann, 1991b) such as flash chromatography; DCCC, low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC) and HPLC are available, but a large number of the separations (especially the preliminary fractionation work) reported in the literature are still carried out by conventional open column chromatography.

1.6.1.1. Open-column chromatography

Open-column chromatography is often used as a first fractionation step for a crude saponin mixture but in certain cases may yield pure products. In general, though, the resolution is not high and complex mixtures are only partially separated. Other problems are the loss of material because of irreversible adsorption and the length of time required to perform the separations (Hostettmann and Marston, 1995).

Silica gel chromatography with chloroform-methanol-water eluent is the most popular method and is still used in the majority of separations (Hostettmann, and Marston, 1995).

As an addition to normal silica gel, chemically derived silica packing have obtained increasing popularity due to their chemical stability and good separation efficiency. RP-8 and RP-18 sorbents are the most frequent packing used. The commercially available particle sizes from 3 microns to 60 microns allow the use of vacuum chromatography (VLC) or medium to high pressure (MPLC or HPLC) chromatography depending on the desired load/resolution result. Thus, analytical and preparative separations can be performed, the analytical method can be easily

transferred onto a preparative separation, if the chemistry of the sorbents is similar. The solvents of choice are mixtures of methanol-water or acetonitrile-water using gradient conditions (Verotta *et al.*, 2001).

RP chromatography is generally introduced after an initial silica gel separation step and enables a change in selectivity for the substances being separated.(rabia)

The use of dextran supports, as found in Sephadex column packing, has been current practice for several years. Sephadex LH-20 finds the most frequent application but the 'G' series of polymers is not without interest. Typically, the polymeric supports are washed with water after loading the sample in order to elute monosaccharides, small charged molecules, such as amino acids, and other highly water soluble substances. Elution with a methanol-water gradient (or with methanol alone) is then commenced to obtain the saponin fractions. Other chromatographic techniques are employed for the isolation of pure saponins (Hostettmann and Marston, 1995).

1.6.1.2.Thin-layer Chromatography (TLC)

Thin-layer chromatography is becoming rather a supporting technique in analysis of saponin fractions obtained from column chromatography. This has been also used for confirmation of purity and identity of isolated compounds (Oleszek and Bialy, 2006).

The qualitative analysis of saponins by TLC is of great imporance for all aspects of saponin investigations. TLC plates (usually silicagel) are inexpensive, rapid to use and require no specialized equipment A number of visualization reagents are available for spraying onto the plates. Spraying with vanillin-sulphuric acid in the presence of ethanol and perchloric acid (Godin, 1954), for example, gives a blue or violet coloration with triterpene saponins. With anisaldehydesulphuric acid, a blue or violet-blue coloration is produced on heating the TLC plate (Hostettmann and Marston, 1995).

Reversed-phase TLC plates provide an excellent analytical method for saponins, complementary to TLC on silica gel plates. Almost exclusive use of methanol-water and acetonitrile-water mixtures is made for developing reversed phase (RP-8 or RP-18) plates (Hostettmann and Marston, 1995).

1.6.1.3. Low-pressure liquid chromatography (LPLC)

LPLC (Low-pressure liquid chromatography) is fast becoming one of the most popular methods for the isolation of pure saponins because of the speed of separation and ease of manipulation (Hostettmann and Marston, 1995).

In LPLC, a mobile phase is allowed to flow through a densely packed sorbent. The separation mechanism is adsorption or size exclusion depending on the choice of packing material for the stationary phase (adsorption: silica gel, bonded-phase silica gel, alumina, polystyrene; size-exclusion: polyacrylamide, carbohydrates). Silica gel is the most commonly used stationary phase in LPLC for the separation of natural products. Silica gel may be regarded as a typical polar sorbent. For LPLC, the particle size of the silica gel is normally in the range of 40–60 mm, which allows one to achieve high flow-rates with low pressures (Sticher, 2007).

LPLC is generally used in combination with other separation methods and may form the intermediate or final steps of purification (Sticher, 2007).

1.6.1.4. Medium-pressure liquid chromatography (MPLC)

When relatively large amounts of pure saponins are required, mplc (medium-pressure liquid chromatography) is very useful. Unlike commercially available lplc equipment, gram quantities of sample can be loaded onto the column, while separations are run at pressures of up to 40 bars. The granulometry of the support normally lies in the 25-40 μm range (Hostettmann and Marston, 1995).

MPLC involves longer columns with large internal diameters and requires higher pressures than LPLC to enable sufficiently high flow-rates. MPLC fulfils the requirement for a simple complementary or supplementary method to open-column chromatography (CC) and flash chromatography (FC) with both higher resolution and shorter separation times (Nyiredy *et al*., 1990). MPLC isolation procedure is simpler, more convenient, more cost-effective, and less time-consuming (Sticher, 2007).

1.6.1.5. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography is the most powerful and the most frequently used technique for saponin determination due to the fact that it can deal effectively with non-volatile, highly polar compounds. It has been used extensively for de termination of both aglycones and intact saponins. The separations are performed usually on normal (silica gel) and reversed-phase (C, C) columns, of which C has been definitely preferred.

The main problem in HPLC analysis of saponins is detection. Since only a few saponins, for instance glycyrrhetinic acid and its glycosides and cucur bitacins, have absorption maxima in UV range, they are the only compounds that can be easily detected at 254 nm. The majority of saponins do not possess chromophores necessary for UV detection, and the separation of intact glycosides or their aglycones has to be traced at lower UV wavelengths ranging from 200 to 210 nm. The sensitivity with this detection mode has been satisfactory and depending on the nature of the saponin ranges from 50 ng for avenacoside B (Kesselmeiner *et al.,* 1981) to 300 ng for ginseng saponins (Soldati and Sticher, 1980). The detection at lower wavelengths, however, limits the selection of solvents and the gradients that can be used. Since acetonitrile gives much lower absorption at lower wavelengths than methanol, the selection of acetonitrile–water gradients is the mode of choice. Similarly, the gradient cannot cover a wide range of concentrations due to the baseline drift, which creates an additional problem with saponin analysis. The bidesmosidic saponins elute in water–acetonitrile gradient at relatively low con centrations of MeCN while monodesmosides elute later and are consequently much more difficult to quantify. Some plant extracts may contain large numbers of glycosides differing in polarities due to the number of sugars attached. Analysis of such a complicated mixture would need wide range of solvent concentration. Application of a gradient completely excludes detection with a refractive index detector (RI) and this is why this type of detection has been rarely used (Oleszek, 2002).

The alternative to low wavelength UV or RI detection has been pre-column derivatisation of saponins in order to attach a chromophore that facilitates UV detection at higher wavelength (254 nm) (Oleszek, 2002).

No matter which method of detection is used for HPLC determination of saponins, a solid-phase clean-up step is highly desirable prior to analysis. The C18

for saponins and NH_2 Sep-Pak cartridges for glycoalkaloids have been used successfully, with recovery of glycosides higher then 95%.(Oleszek, 1988). The solid-phase clean-up procedures by simplifying extraction and purification ensure mild conditions for these processes and protect saponins from multiple artefact formation (Massiot *et al.,* 1996; Oleszek, 2002).

1.6.1.6. Vacuum liquid chromatography (VLC)

VLC may be considered as a preparative TLC run as a column, with a vacuum applied to speed up eluent flow-rates. As opposed to flash chromatography, the column is allowed to run dry after each fraction is collected. VLC has been increasingly used in the field of natural products because of its simplicity of operation. Separations of up to 30g of extract are possible (Sticher, 2007).

This technique involves the use of reduced pressure to increase the flow rate of a mobile phase through a short bed of solvent The chromatography column is packed with silica gel (generally 10-40 urn TLC grade) and the sample is eluted with appropriate solvent mixtures, starting with solvent of low polarity and gradually increasing the elution strength (Hostettmann and Marston, 1995).

Different chromatographic supports have been employed in VLC: silica gel (both normal and reversed-phase), Al_2O_3, CN, diol and polyamide. VLC is mainly used for the fractionation of natural products prior to other separation steps such as RPC, MPLC, and HPLC (Sticher, 2007).

1.6.2. Structure Determination

Saponins have a very complex structure, which is hard to elucidate. The benefit of using nuclear magnetic resonance (NMR) in combination with mass spectrometry (MS) is that this allows an examination of the intact saponin, instead of using cleavage reactions to cleave off sugar moieties from the sapogenin and analyze them separately (Hostettmann and Marston, 1995).

1.6.2.1. NMR Spectrometry

Of all these modern methods for the structure elucidation of oligosaccharides and glycosides, NMR spectroscopy provides the most complete

information, with or without prior structural knowledge (Agrawal, 1992). It is the only approach which can, in principle, give a complete structure without resort to any other method (Hostettmann and Marston, 1995).

Proton and carbon-13 NMR spectrometry is widely used to determine the structure of saponins. By analysing the spectra, the following aspects can be ascertained: Where the glycosidic linkages to the aglycone are positioned; the number, sequence and nature of monosaccharide units; configuration of the interglycosidic linkages; presence of acyl glycosides in the chains; what kind of aglycone the saponin has, and the structures of eventually attached esters (Madland, 2013).

In the ^{1}H NMR spectra, diffculties arise. The proton resonances are prone to overlap, due to the majority of signals from the carbohydrate moieties that appear in the range from 3.0 to 4.2 ppm. The chemical shifts are similar, even though they derive from the same bulk of non-anomeric sugar methyne and methylene protons. Luckily, it is possible to assign proton shifts by combining different 1D and 2D NMR-techniques. By using 2D NMR the spectral crowding will be limited (Hostettmann and Marston, 1995).

1.6.2.2. Mass spectrometry

The ionization method in MS depends on the polarity, lability and molecular weight of the compound to be analysed. Previously, ionization techniques such as fast atom bombardment (FAB) and chemical ionization (CI) have been applied to find important structural information, like molecular weight and sugar sequence, for naturally occuring glycosides. These techniques enabled analysis without derivatization of the glycosides. (Hostettmann and Marston, 1995). However, the matrix background generates a chemical noise, which reduces the sensitivity of the FAB method (Lui *et al*., 2004).

For the most common MS method, electron impact (EI), samples need to be volatile and thermally stable. Saponins require conversion to permethyl or peracetyl derivatives in order to be analyzed by EI. This derivatization also has its limitations, since it is not applicable to saponins containing more than three sugar moieties (Schulten *et al.*, 2013).

Electrospray ionization (ESI) has been reported as a powerful tool in determining the molecular weight of saponins due to its high sensitivity, rapid analysis time and low levels of sample consumption. This ionization technique combined with collision induced dissociation (CID) can aid in identification of the backbone and glycosidic linkage sites of the saponins (Li *et al.,* 2005). CID is a process where energy is transferred to an ion through collision with a neutral collision gas (He, N_2, Ar). This energy transfer is sufficient to result in bond cleavages and rearrangements of the selected ion. Fragmentation will be possible for gaseous ions that are otherwise perfectly stable (Gross, 2006).

2 MATERIALS AND METHODS

2.1 General

The NMR spectra were recorded on a Agilent DD2 Premium compact NMR spectrometer at 600 MHz (^{1}H) and 150 MHz (^{13}C) in *d6*-DMSO, using TMS as internal standard. 2-D NMR spectra (COSY, HMQC, HMBC) were run using a standard Agilent pulse programs.

Column chromatography was carried out a silica gel 60 (Merck 7734) and Li Chroprep RP (C-18, Merck 9303). Analytical TLC was conducted on pre-coated silica gel 60 F_{254} aluminium sheets (Merck 5554) and RP-18 F_{254} (Merck) plates.

Compounds were detected at 254 and 366 nm UV lamp by using Desaga Uvis device. The spots were detected by 20 % H_2SO_4 water spraying reagent onto the TLC plates followed by heating the plates to 110 $^{\circ}C$ until the spots become visible.

During the chromatographic studies (CC, MPLC and TLC controls) the following solvent systems were used:

I	CH_2Cl_2:MeOH:H_2O	95:5:0,5
II	CH_2Cl_2:MeOH:H_2O	90:10:1
III	CH_2Cl_2:MeOH:H_2O	85:15:1,5
IV	CH_2Cl_2:MeOH:H_2O	80:20:2
V	CH_2Cl_2:MeOH:H_2O	75:25:2,5
VI	CH_2Cl_2:MeOH:H_2O	70:30:3
VII	CH_2Cl_2:MeOH:H_2O	61:32:7
VIII	MeOH:H_2O	8:2

IX	MeOH:H_2O	7:3
X	MeOH:H_2O	6:4
XI	MeOH:H_2O	5:5
XII	MeOH:H_2O	4:6
XIII	MeOH:H_2O	2:8

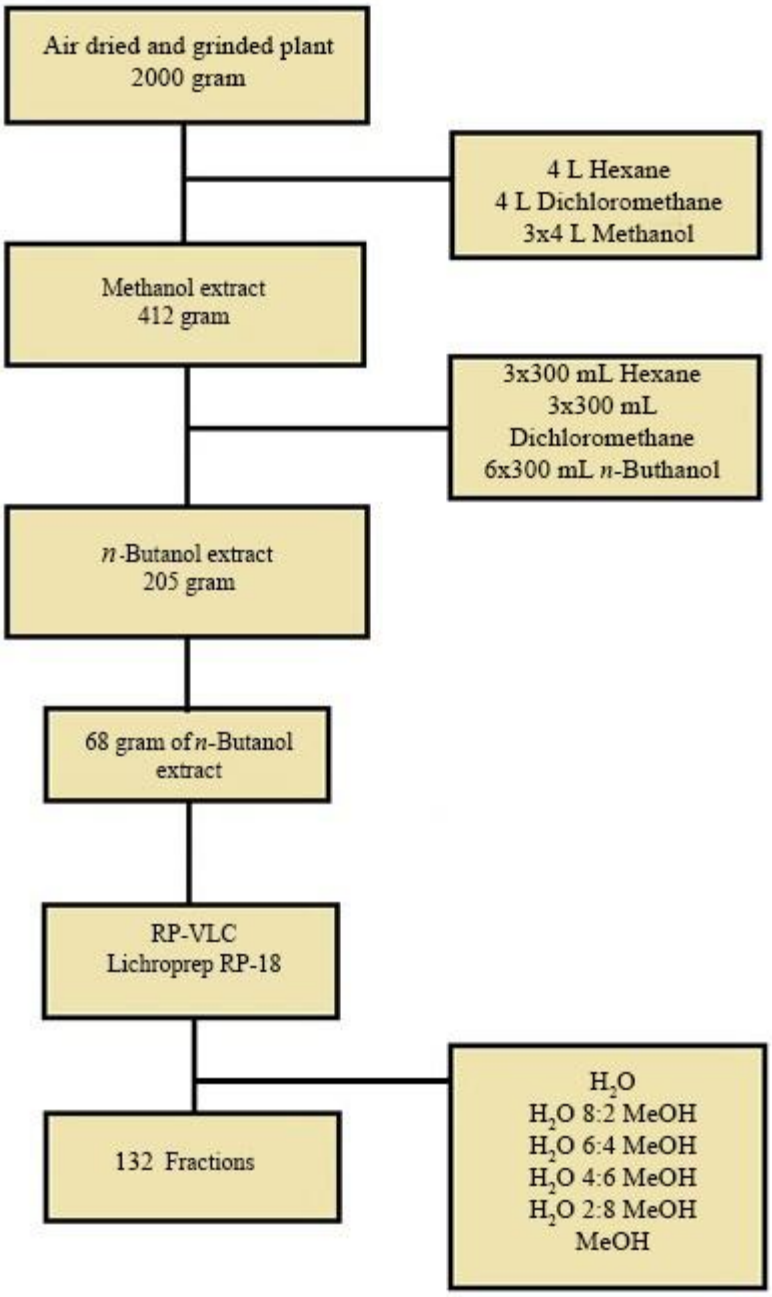
2.2 Plant material

Nigella arvensis L. was collected from Güme-Tire-İzmir in June 2013. Plant material was identified by Volkan EROĞLU (Department of Biology, Faculty of Sciences, Ege university, Turkey).

2.3 Isolation and Purification

The dried and grinded plant of *Nigella arvensis* (2000 g) were extracted with *n*-hexane (4 L),dichloromethane (4 L) and methanol (MeOH) (3x4 L) at room temperature. After filtration, the solvent was removed by rotary evaporation yielding 412 gram of extract. The MeOH extract was dissolved in H_2O (350 mL), and successively partitioned with *n*-hexane (3x300 mL), CH_2Cl_2 (3x300 mL), and *n*-BuOH saturated with H_2O (6x300 mL).

68 gram of *n*-BuOH extract was subjected to vacuum liquid chromatograpy (VLC) using reversed-phase material (Lichroprep RP-18, 25-40 μm) employing H_20 (1400 mL), H_2O-MeOH (8:2, 1400 mL; 6:4 , 7800 mL; 4:6, 10200 mL; 2:8, 2600 mL; 2:8, 2700 mL) and MeOH (2200 mL) to give 132 main fractions (Scheme 2.1).

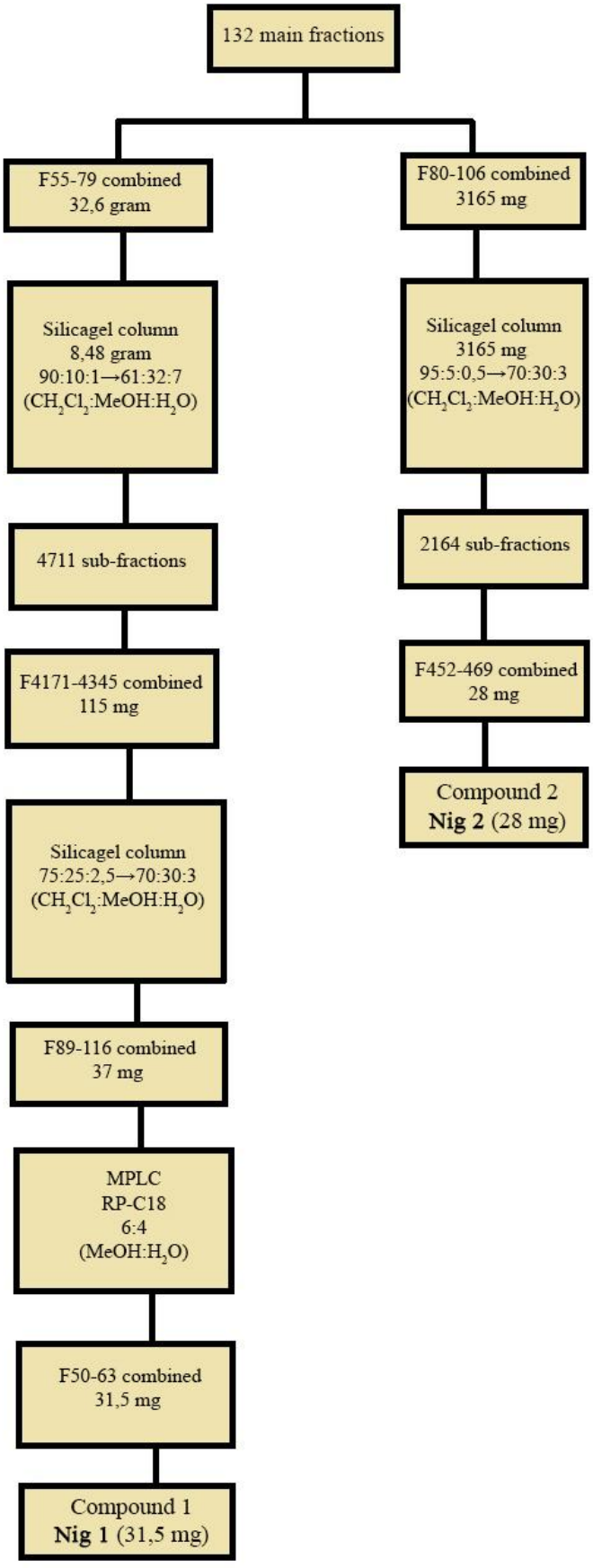


Scheme 2.1. Extraction procedure of *Nigella arvensis*

Fractions (55-79) (32,6 g) that rich in saponins were combined. 8,48 gram of fractions 55-79 applied to an open column chromatography using silica gel (743 g) as stationary phase. Elution performed with CH_2Cl_2-MeOH-H_2O (90:10:1→61:32:7), mixtures to yield 4711 sub-fractions. This from column, fractions 4171-4345 (115 mg) were combined and fractionated over silica gel (32g). Elution performed with CH_2Cl_2-MeOH-H_2O (75:25:2,5→70:30:3), mixtures to yield 188 sub-fractions. Fractions (89-116)(37 mg) were combined

and subjected to MPLC by using RP-C18 as an adsorbent eluted with MeOH:H_2O (6:4). The sub-fractions 50-63 gave **Nig 1** (31,5 mg).

Secondly, fractions 80-106 (3165 mg) from VLC-RP were combined and subjected to silica gel (298 g) column employing with CH_2Cl_2-MeOH-H_2O (95:5:0,5→70:30:3) mixtures to give 2164 sub-fractions. Fractions 452-469, afforded **Nig 2** (28 mg) (Scheme 2.2).



Scheme 2.2. Isolation procedure of compounds

3 RESULTS AND DISCUSSION

3.1 Structural Identification of Compound 1

The ^{1}H NMR showed signals for the anomeric protons at δ 5.27 (d, J = 7.8 Hz), 4.38 (d, J = 7.5 Hz), 4.46 (d, J = 3.5 Hz), 4.61 (d, J = 7.5 Hz), 4.69 (d, J = 1.2 Hz) and 5.21 (d, J = 7.5 Hz), which showed correlations in the HSQC spectrum with the anomeric carbon signals at δ 102.2, 104.2, 102.6, 101.0, 100.2 and 93.6, respectively. These data, in combination with 1D-TOCSY, HSQC, HMBC, DQF-COSY correlations, showed that Nig1 differed from Nig2 in the presence of an additional two β-glucopyranosyl and one rhamnopyranosyl units.. HMBC spectrum allowed us to deduce the sugar sequence. A cross-peak between the signals of H-1rib (δ 4.46) and C-3 (δ 88.0), H-1glcI (δ 4.61) and C-2rib (δ 77.3), H-1glcII (δ 4.38) and C-2glcI (δ 83.2), allowing us to determine a β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-ribopyranosyl chain linked at C-3. Similarly the sequence of the trisaccharide chain at C-28 was established by the cross-peaks between H-1glcIII (δ 5.21) and C-28 (δ 174.8), H-1glcIV (δ 4.27) and C-6glcIII (δ 67.4), H-1rha (δ 4.69) and C-4glcIV (δ 76.1). Therefore, compound Nig1 was identified as the new 3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-ribopyranosyl-28-O-[α-L rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] oleanolic acid.

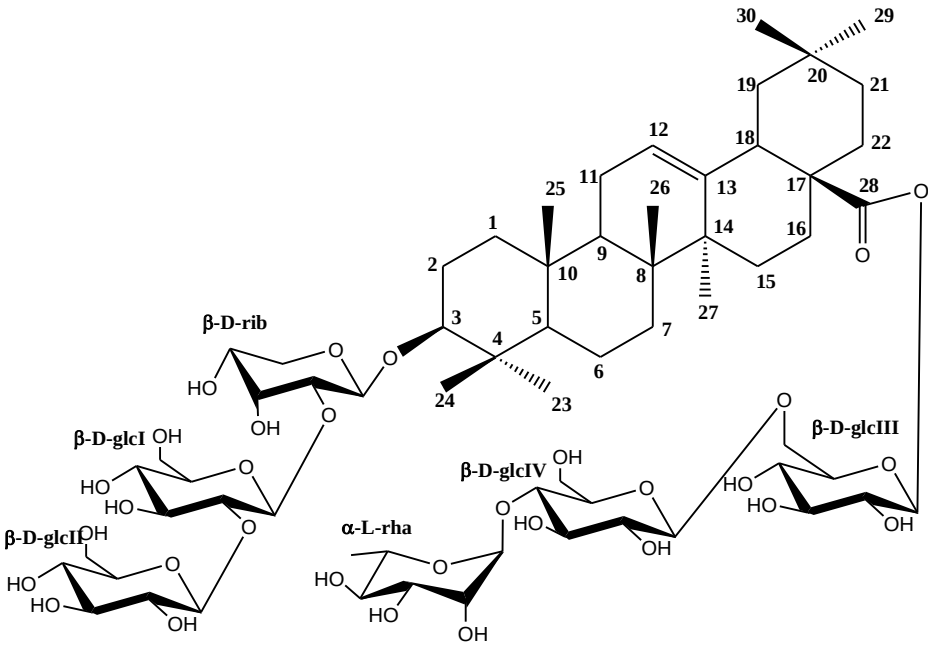
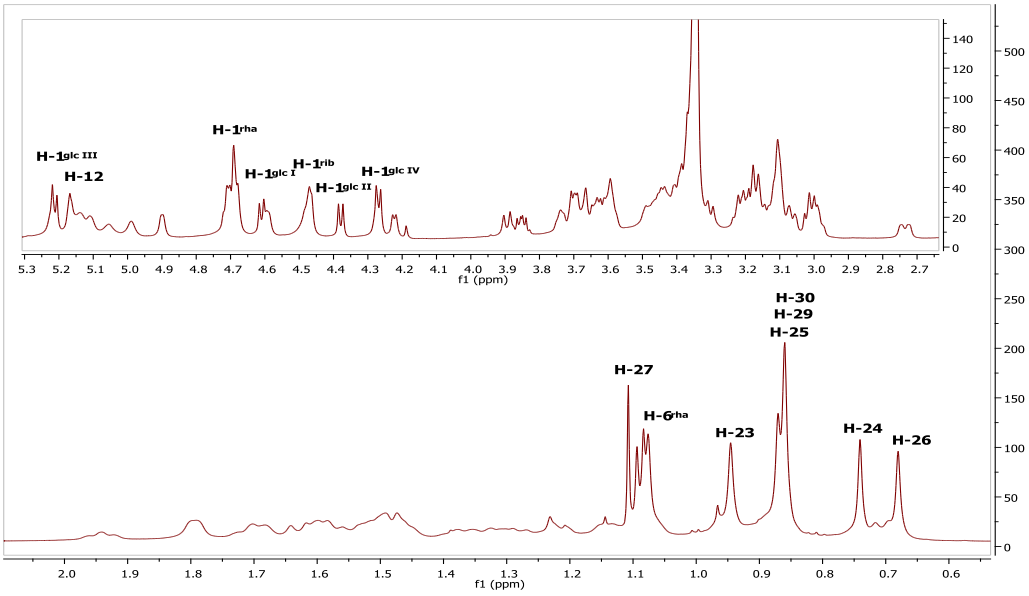


Figure 3.1. Structure of compound 1 (nig1)

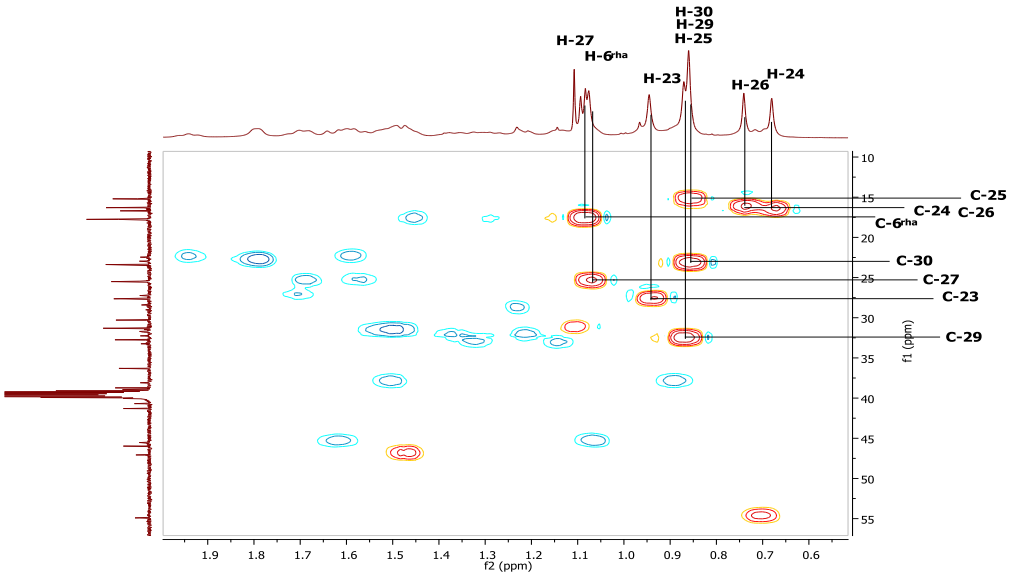
Table 3.1. ^{1}H and ^{13}C assignments of **nig1** (in *d*-DMSO)

Position	δ_C **(ppm)**	**Position**	δ_H **(ppm)**
1	39.7	**H_2-1**	1.64, 1.04, m
2	25.7	**H_2-2**	2.18, 1.80, m
3	88.4	**H-3**	3.35, dd (11.5, 4.5)
4	38.5	**-**	-
5	55.5	**H-5**	0.97, m
6	17.6	**H_2-6**	1.67, 1.41
7	32.2	**H_2-7**	1.61,1.38, m
8	41.0	**-**	-
9	47.7	**H-9**	1.62, m
10	37.5	**-**	-
11	22.8	**H_2-11**	1.92, (2H) m
12	121.3	**H-12**	5.27, t (3.5)
13	144.0	**-**	-
14	41.1	**-**	-
15	27.3	**H_2-15**	1.90, 1.02, m
16	22.7	**H_2-16**	1.91, 1.00, m
17	39.7	**-**	-
18	40.8	**H-18**	2.29, m
19	45.6	**H_2-19**	2.12, 0.99, m
20	41.5	**-**	-
21	33.0	**H-21**	3.83, brs
22	32.0	**H-22**	3.36, d (3.5)
23	27.2	**H_3-23**	1.24, s
24	16.1	**H_2-24**	4.12, d (11.5), 3.23, d (11.5)
25	15.0	**H_3-25**	0.90, s
26	16.6	**H_3-26**	1.00, s
27	25.8	**H_3-27**	1.23, s
28	180.0	**H_3-28**	0.98, s
29	32.6	**H_2-29**	3.45, d (10.5), 3.12, d (10.5)
30	23.2	**H_3-30**	1.03
β-D-Rib (at C-3)-1	102.6	**H-1**	4.46, d (3.5)
2	77.3	**H-2**	3.70, m
3	70.4	**H-3**	3.73, m
4	65.8	**H-4**	3.68, m
5	61.9	**H-5**	3.65, m 3.31, m
β-D-GlcI (at C-2_{rib})-1	101.0	**H-1**	4.61, d (7.5)
2	83.2	**H-2**	3.17, dd (7.5, 9.2)
3	75.0	**H-3**	3.21, dd (9.0, 9.0)
4	69.4	**H-4**	3.10, dd (9.0, 9.0)
5	76.7	**H-5**	3.10, m
6	60.9	**H-6**	3.46, dd (3.5, 12.0) 3.70, dd (4.5, 12.0)
β-D-GlcII (at C-2_{glcI})-1	104.2	**H-1**	4.38, d (7.5)
2	74.6	**H-2**	3.01, dd (7.5, 9.2)
3	75.6	**H-3**	3.16, dd (9.0, 9.0)
4	69.0	**H-4**	3.16, dd (9.0, 9.0)
5	75.4	**H-5**	3.06, m
6	60.9	**H-6**	3.46, dd (3.5, 12.0) 3.70, dd (4.5, 12.0)
β-D-GlcIII (at C-28)-1	93.6	**H-1**	5.21, d (7.5)
2	71.8	**H-2**	3.13, dd (7.5, 9.2)
3	75.0	**H-3**	3.21, dd (9.0, 9.0)

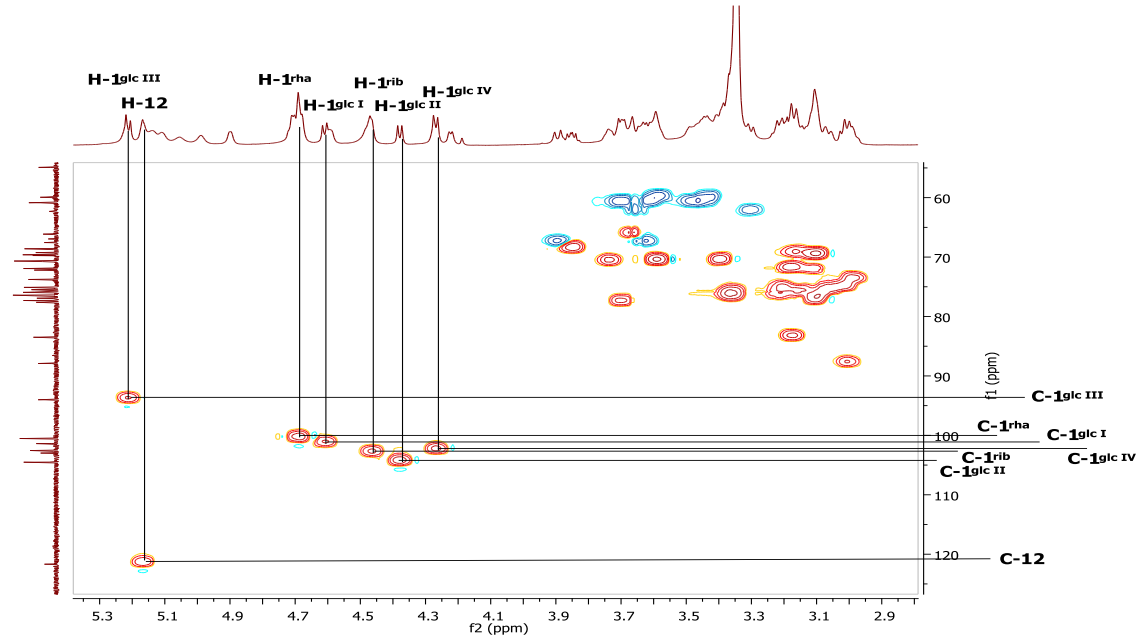
4	69.0	**H-4**	3.16, dd (9.0, 9.0)
5	76.1	**H-5**	3.36, m
6	67.4	**H-6**	3.61, dd (3.5, 12.0) 3.89, dd (4.5, 12.0)
β-D-GlcIV (at C-6glcIII)-1	102.2	**H-1**	4.27, d (7.5)
2	73.5	**H-2**	2.99, dd (7.5, 9.2)
3	76.0	**H-3**	3.20, dd (9.0, 9.0)
4	76.1	**H-4**	3.36, dd (9.0, 9.0)
5	76.0	**H-5**	3.08, m
6	60.1	**H-6**	3.59, dd (3.5, 12.0) 3.46, dd (4.5, 12.0)
α-L-Rha (at C-2xyl)-1	100.2	**H-1‴**	4.69, d (1.2)
2	70.3	**H-2‴**	3.59, dd (1.2, 3.2)
3	71.7	**H-3‴**	3.18, dd (3.2, 9.7)
4	70.3	**H-4‴**	3.40, t (9.7)
5	68.3	**H-5‴**	3.85, m
6	17.6	**H-6‴**	1.06, d (6.5)



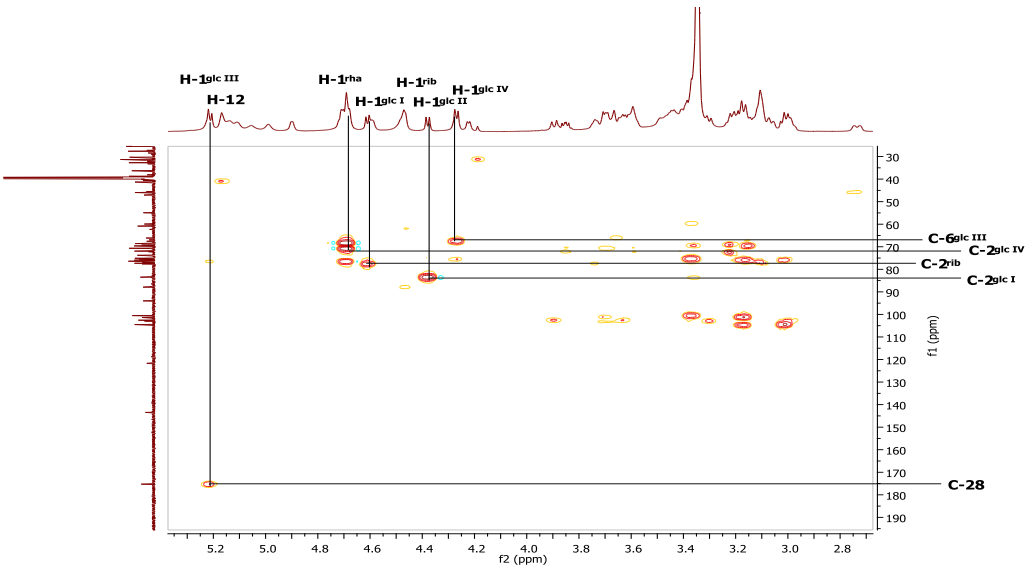
Spectrum 3.1. ^{1}H NMR spectrum of **nig1** (600 MHz, d-DMSO)



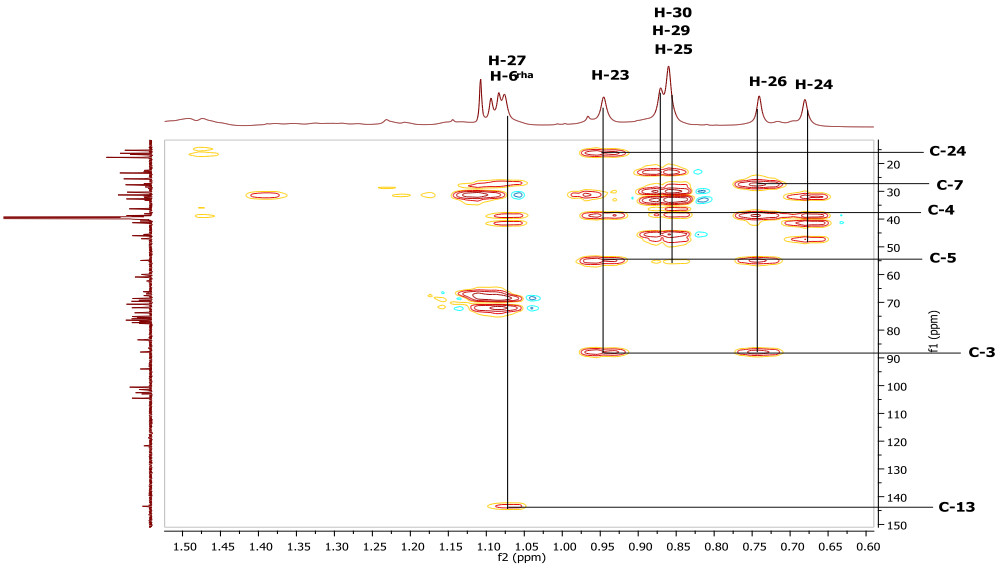
Spectrum 3.2.a. HSQC spectrum of nig1



Spectrum 3.2.b. HSQC spectrum of nig1



Spectrum 3.3.a. HMBC spectrum of nig1



Spectrum 3.3.b. HMBC spectrum of nig1

3.2 Structural Identification of Compound 2

A detailed comparison of the NMR data (1H, 13C, HSQC, HMBC, COSY) of compounds Nig2–Nig1 showed that the aglycone moiety was identical in all the two compounds. The 1H NMR spectrum displayed signals for seven tertiary methyl groups at δ 0.72, 0.73, 0.86 (9H), 0.93, and 1.08 and for a vinyl proton at δ 5.12 (1H, t, J = 3.5 Hz). These signals along with the carbon resonances in the

13C NMR spectrum for the methyl groups at δ 15.0, 16.1, 16.6, 23.2, 25.4, 27.2, 32.6 and the two olefinic carbons at δ 121.3, 144.0, suggested that compound Nig2 possessed an olean-12-ene skeleton as aglycon. Detailed NMR studies allowed us to identify the aglycon as oleanolic acid (Seebacher et al., 2003). The downfield shifts of C-3 (δ 88.4) of the aglycon suggested that compound Nig2 was a monodesmosidic glycoside. The 13C NMR spectrum showed 47 carbon signals, of which 30 were assigned to the aglycon moiety (see Table 3.2) and 17 to a sugar portion. The 1H NMR spectrum displayed in the sugar region signals corresponding to three anomeric protons at δ H 4.31 (d, J = 7.5 Hz), 4.32 (d, J = 3.5 Hz), and 4.62 (d, J = 7.5 Hz), which were unambiguously correlated by HSQC experiment to the corresponding carbon resonances at δ 105.0, 103.7, and 101.1, respectively.

Complete assignments of the resonances of each sugar unit were achieved by extensive 1D-(^{1}H, ^{13}C, TOCSY) and 2D-(HSQC, HMBC) NMR analyses. These data showed the presence of two β-glucopyranosyl units (δ 4.31 and 4.62) and one β-arabinopyranosyl unit (δ 4.32).

In the HMBC spectrum of Nig2 the long-range correlations between H-1rib (δ 4.32) and C-3 (δ 88.4), confirmed the monodesmosidic nature of Nig2. The sequence of the oligosaccharidic chain of Nig2 was determined by the HMBC spectrum, which showed key cross-peaks between H-1glcI (δ 4.62) and C-2rib (δ 77.1), H-1glcII (δ 4.31) and C-2glcI (δ 84.6), allowing us to determine a β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-ribopyranosyl chain linked at C-3. Therefore, compound Nig2 was identified as the new oleanolic acid 3-O-β-D glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-ribopyranoside.

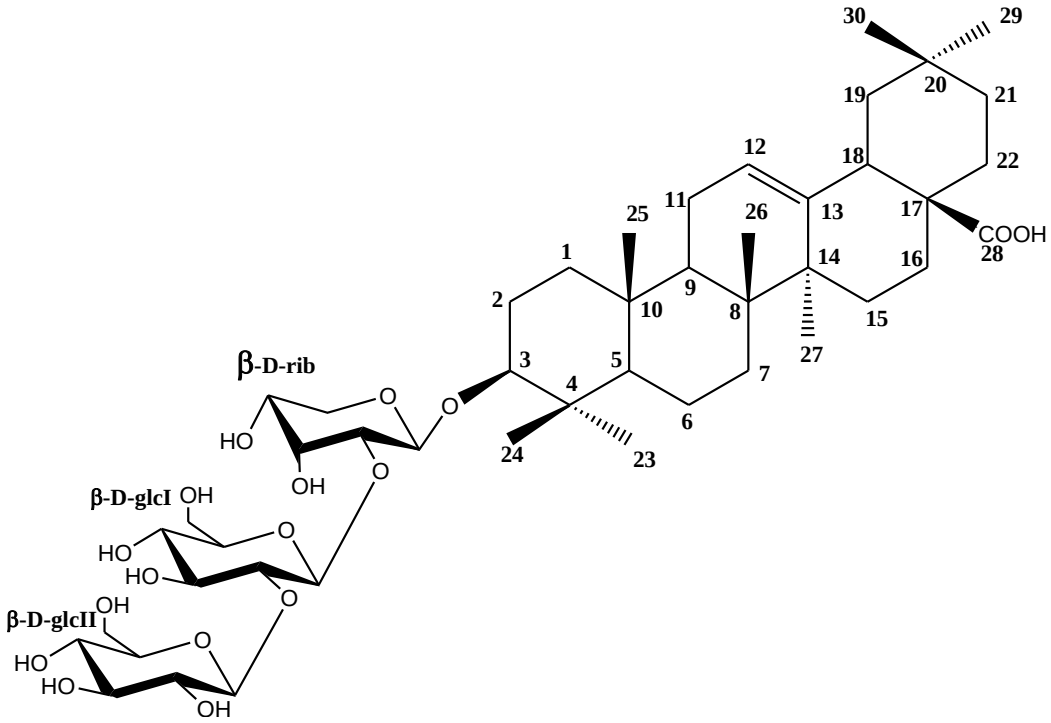
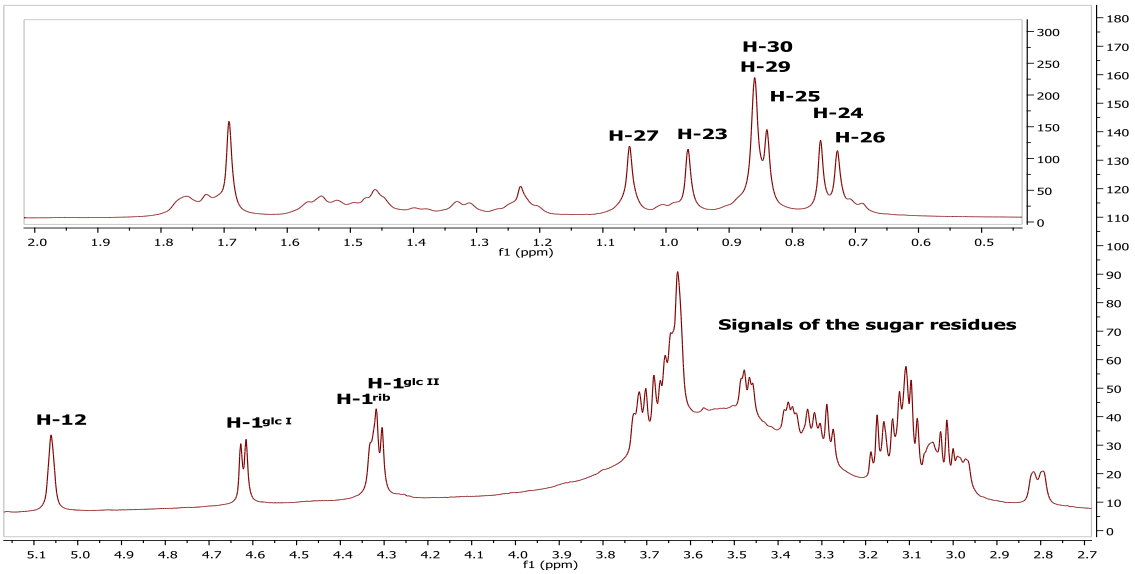


Figure 3.2. Structure of compound 2 (nig2)

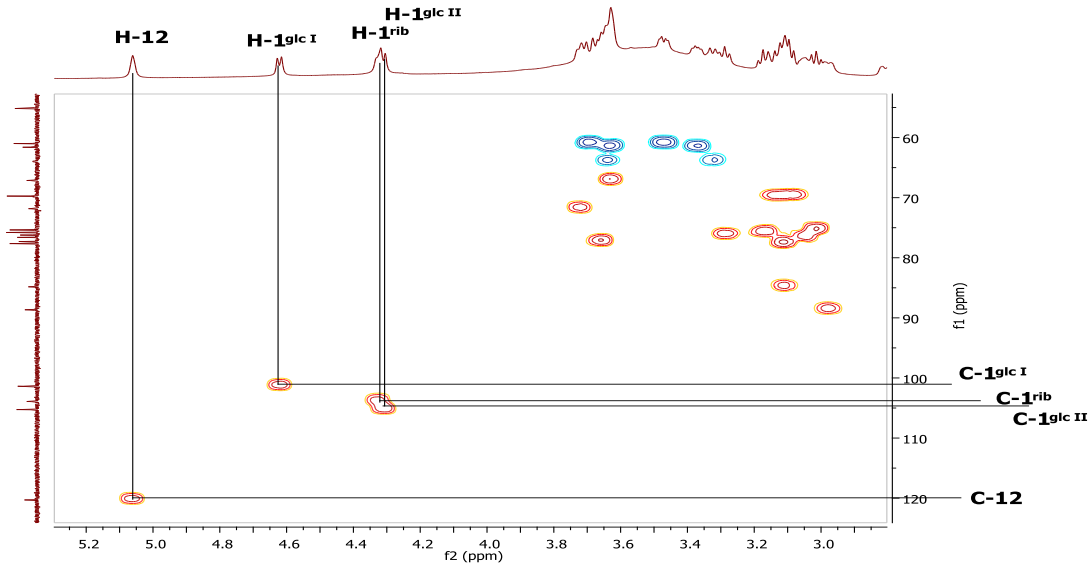
Table 3.2. ^{1}H and ^{13}C assignments of **nig2** (in *d*-DMSO)

Position	**δ_C (ppm)**	**Position**	**δ_H (ppm)**
1	39.7	**H_2-1**	1.64, 1.04, m
2	25.7	**H_2-2**	2.18, 1.80, m
3	88.4	**H-3**	3.35, dd (11.5, 4.5)
4	38.5	**-**	-
5	55.5	**H-5**	0.97, m
6	17.6	**H_2-6**	1.67, 1.41
7	32.2	**H_2-7**	1.61,1.38, m
8	41.0	**-**	-
9	47.7	**H-9**	1.62, m
10	37.5	**-**	-
11	22.8	**H_2-11**	1.92, (2H) m
12	121.3	**H-12**	5.27, t (3.5)
13	144.0	**-**	-
14	41.1	**-**	-
15	27.3	**H_2-15**	1.90, 1.02, m
16	22.7	**H_2-16**	1.91, 1.00, m
17	39.7	**-**	-
18	40.8	**H-18**	2.29, m
19	45.6	**H_2-19**	2.12, 0.99, m
20	41.5	**-**	-
21	33.0	**H-21**	3.83, brs
22	32.0	**H-22**	3.36, d (3.5)
23	27.2	**H_3-23**	1.24, s
24	16.1	**H_2-24**	4.12, d (11.5), 3.23, d (11.5)
25	15.0	**H_3-25**	0.90, s
26	16.6	**H_3-26**	1.00, s

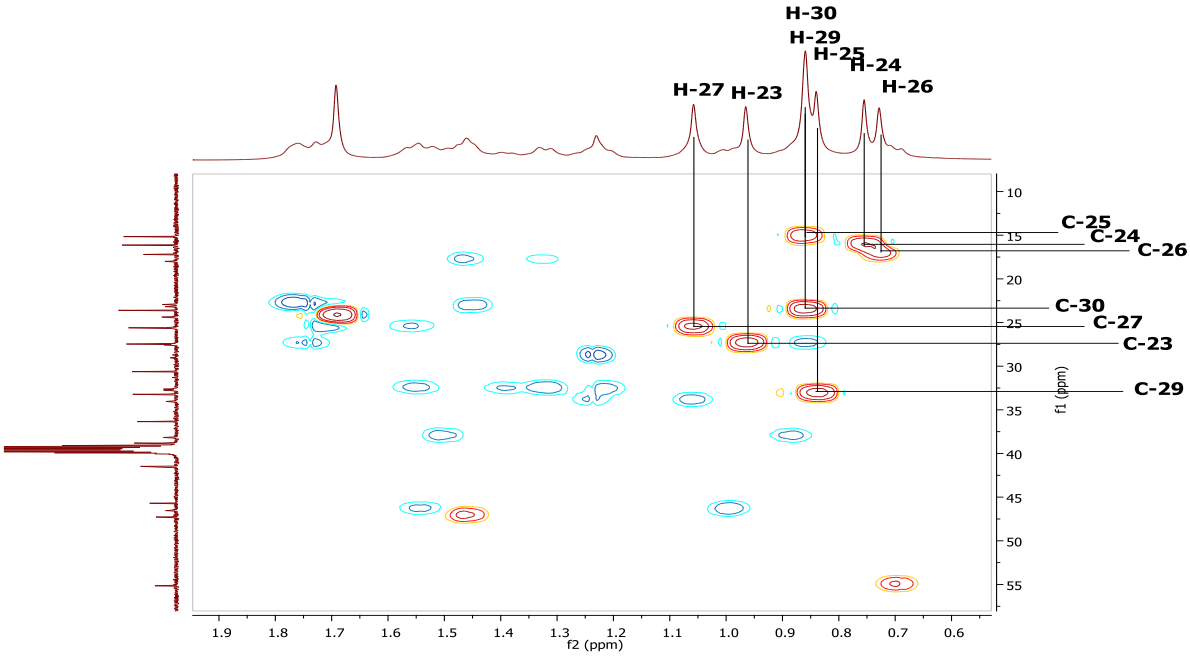
27	25.8	**H_3-27**	1.23, s
28	180.0	**H_3-28**	0.98, s
29	32.6	**H_2-29**	3.45, d (10.5), 3.12, d (10.5)
30	23.2	**H_3-30**	1.03
β-D-Rib (at C-3)-1'	103.7	**H-1'**	4.32, d (3.5)
2'	77.1	**H-2'**	3.66, m
3'	71.6	**H-3'**	3.72, m
4'	66.8	**H-4'**	3.63, m
5'	63.7	**H-5'**	3.64, m 3.32, m
β-D-GlcI (at C-2_{rib})-1''	101.1	**H-1''**	4.62, d (7.5)
2''	84.6	**H-2''**	3.11, dd (7.5, 9.2)
3''	75.9	**H-3''**	3.28, dd (9.0, 9.0)
4''	69.6	**H-4''**	3.08, dd (9.0, 9.0)
5''	77.3	**H_2-5''**	3.11, m
6''	60.8		3.47, dd (3.5, 12.0) 3.69, dd (4.5, 12.0)
β-D-GlcII (at C-2_{glcI})-1'''	105.0	**H-1'''**	4.31, d (7.5)
2'''	75.2	**H-2'''**	3.01, dd (7.5, 9.2)
3'''	75.5	**H-3'''**	3.17, dd (9.0, 9.0)
4'''	69.5	**H-4'''**	3.13, dd (9.0, 9.0)
5'''	76.3	**H-5'''**	3.04, m
6'''	61.4	**H_3-6'''**	3.36, dd (3.5, 12.0) 3.63, dd (4.5, 12.0)



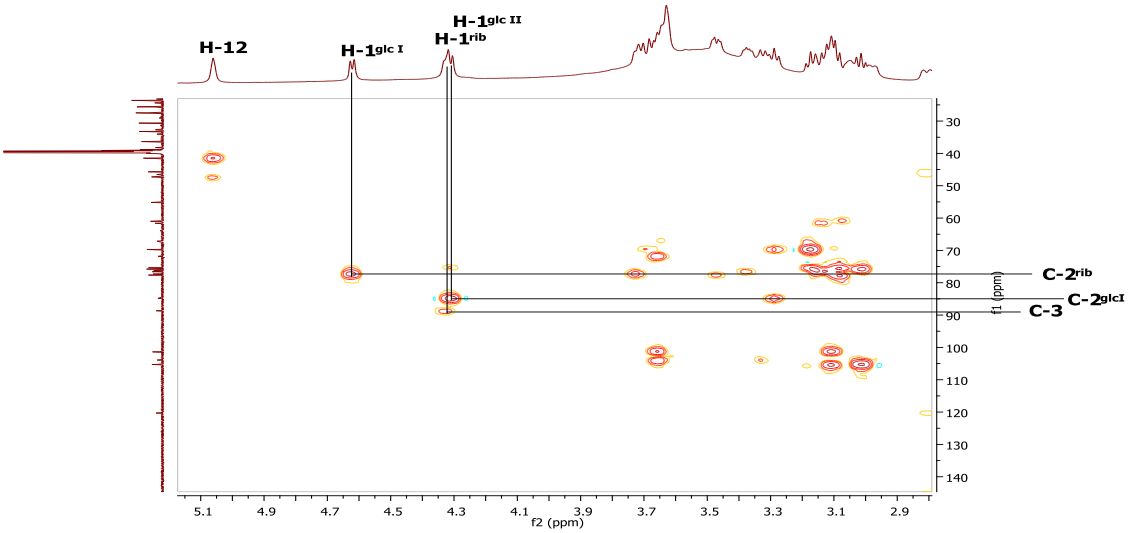
Spectrum 3.4. ^{1}H NMR spectrum of **nig2** (600 MHz, d-DMSO)



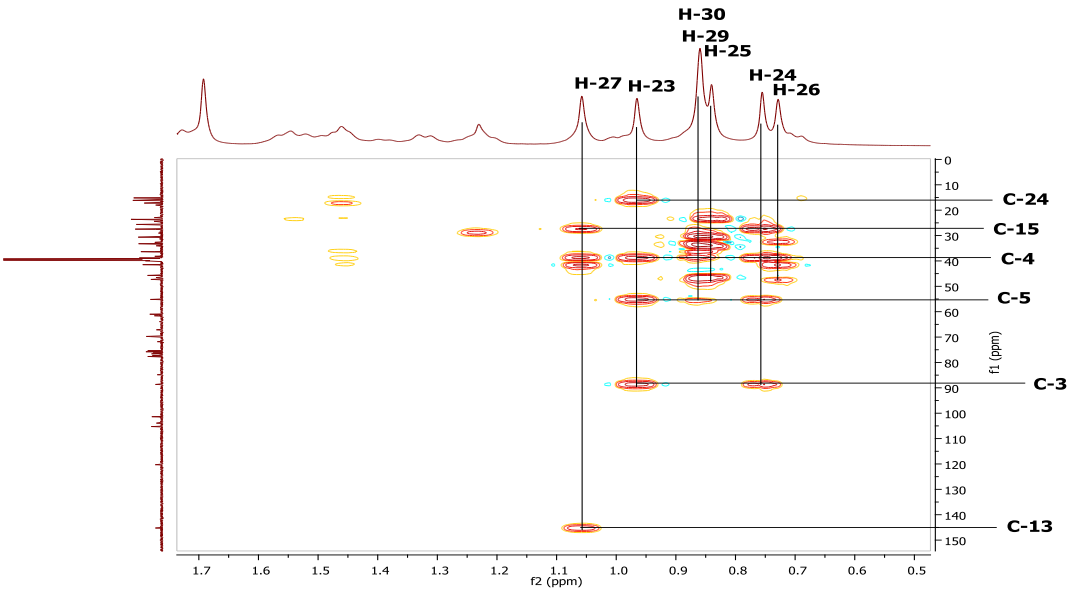
Spectrum 3.5.a. HSQC spectrum of nig2



Spectrum 3.5.b. HSQC spectrum of nig2



Spectrum 3.6.a. HMBC spectrum of nig2



Spectrum 3.6.b. HMBC spectrum of **nig2**

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