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PHYTOCHEMICAL ANALYSIS of *Nerium oleander*

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Aygün YAVUZ tarafından Yüksek Lisans tezi olarak sunulan "**Phytochemical Analysis of *Nerium oleander* (*Nerium oleander*'in Fitokimyasal Analizi)**" 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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EGE ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ ETİK KURALLARA UYGUNLUK BEYANI

E.Ü. Lisansüstü Eğitim ve Öğretim Yönetmeliğinin ilgili hükümleri uyarınca Yüksek Lisans Tezi olarak sunduğum "**Phytochemical Analysis of *Nerium oleander* (*Nerium oleander*'in Fitokimyasal Analizi)**" 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

Aygün YAVUZ

ÖZET

Nerium oleander'in FİTOKİMYASAL ANALİZİ

Aygün YAVUZ

Yüksek Lisans Tezi, Kimya Anabilim Dalı

Tez Yöneticisi: Doç. Dr. Tamer KARAYILDIRIM

Şubat 2015, 34 sayfa

Bu çalışmada *Nerium oleander* türünden altı tane glikozid izole edilmiş, izole edilen bileşiklerden iki tanesinin yapısı spektroskopik teknikler kullanılarak oleandrin ve deasetil oleandrin belirlenmiştir.

Anahtar Kelimeler: *Nerium oleander*, kardiyak glikozit, oleandrin

ABSTRACT

PHYTOCHEMICAL ANALYSIS of *Nerium oleander*

YAVUZ Aygün

Master of Science Thesis, Chemistry Department

Supervisor: Assoc. Prof. Dr. Tamer KARAYILDIRIM

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In this study, six natural products were isolated from *Nerium oleander* specie and structures of the two of the isolated compounds determined as oleandrin and deacetyl oleandrin by using spectroscopic techniques.

Keywords: *Nerium oleander*, cardiac glycoside, oleandrin

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1. INTRODUCTION

Natural products are an excellent source of complex chemicals possessing a wide variety of biological activities and, therefore, having great potential therapeutic value. For sheer chemical diversity, biologically derived compounds are unrivalled. Such compounds are also invaluable springboards to new chemistries. Furthermore, natural products can define new drug targets. For example, different toxins have revealed numerous classes of ion channels. Many natural products have been developed into drugs which have revolutionized medicine. A classic example of a plant-based medicine is the discovery of taxol from the bark of the Pacific yew tree, which has been referred to as the most important anticancer drug of the past two decades. Today's plant-based prescription medicines come from plants belonging to only 95 of the estimated 250.000 species worldwide. More than 1000 of these plants have already been described as having significant anticancer properties but there is still a huge amount of screening and characterization to be done. The ability to exploit fully the active components from natural resources for their therapeutic value has previously been hampered by technical limitations. New enabling technologies that are being utilized by the pharmaceutical industry, coupled to a growing trend of consumer preference for naturally derived products, is spurring renewed efforts toward developing therapeutic agents from natural sources. In the past, the ability to exploit the active components from natural resources has been hampered in several ways. One of the obstacles that previously impeded herbal drug development was the lack of bioassays for screening efficacies of pharmaceutical interest.

Natural products have and will continue to be a major resource for therapeutic products. They present researchers with complete libraries of compounds of such diverse chemical structure that are virtually impossible to replicate in a synthetic chemistry laboratory. In addition, the structural elucidation of natural products, with identified activity or otherwise, will continually provide new scaffolds for synthetic chemists and structural biochemists.

Until recent years, chance discoveries have been the prime driving force in the identification of new natural products. This is set to change, not only because of the increasing awareness of the immensity of the resources available in the natural world but also because of the application of high-throughput methods to the generation of natural product libraries, the screening process and the structural elucidation of active compounds (New et al., 2003).

1.1. General Information about Saponins

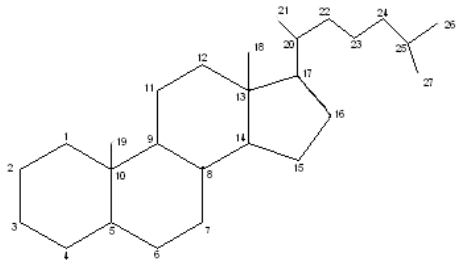
Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources, and found in particular abundance in various plant species. Saponins are high-molecular-weight triterpene glycosides, consisting of a sugar moiety linked to a triterpene or steroid aglycone. They are now more conveniently defined on the basis of their molecular structure, namely as triterpene or steroid glycosides.

Saponins are widely distributed in the plant kingdom and composed of two parts: glycone and aglycone or genin (triterpene) (Figure 1.1) (Kaufman, 1999). The aglycones or non-saccharide portion of the saponin molecule is called the genin or sapogenin. The classical definition of saponins is based on their surface activity; many saponins have detergent properties, stable foams in water, show haeomolytic activity, have a bitter taste and are toxic to fish (piscicidal) (Hostettmann and Marston, 1995).

Saponins are steroid or triterpenoid glycosides, common in a large of plants and plant products that are important in human and animal nutrition (Francis et al, 2002). Yucca and quillaja saponins, for example, have both current and potential importance in animal and human nutrition (Singh et al. 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).

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).



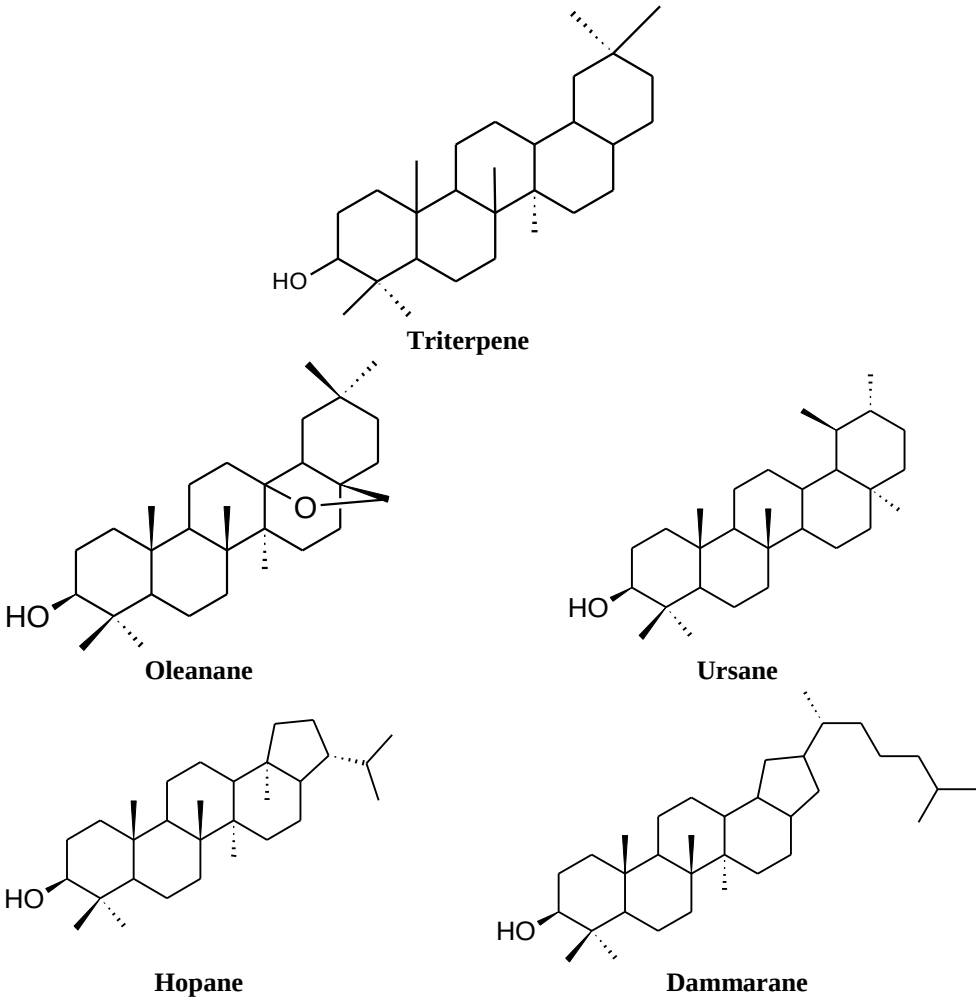


Figure 1.1. A Triterpene Skeleton and Four Common Triterpene Skeletons

Some saponins-containing plants have been employed for hundreds of years as soaps and this fact reflected in their common names; soapwort (*Saponaria officinalis*), soaproot (*Chlorogalum pomeridianum*), sopapbark (*Quillaja saponaria*), soapberry (*Sapindus saponaria*), soapnut (*Sapindus makurossi*) (Hostettmann and Marston, 1995). Indeed, 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). That is, saponins are a special category of isoprenoid glycosides that form colloidal solutions with water and foam when shaken. Removing the glycosides fraction yields aglycones known as sapogenins, which have either a terpenoid or steroid structure (Figure 1.2) (Barken, 2001).

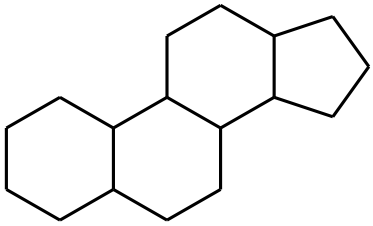


Figure 1.2. Basic Skeleton of steroid

1.2. General Information about Flavonoids

The flavonoids are a large group of natural products which are widespread in higher plants but also found in some lower plants including algae. The anthocyanidins are responsible for flower colour in the majority of angiosperms, but colourless flavonoids are also widespread and abundant. A variety of biological functions is fulfilled by various members of the series, but many metabolic and extracellular roles doubtless remain to be discovered. Flavonoids fall into two major categories according to whether the central heterocyclic ring is unsaturated or not. When unsaturation is present, as in anthocyanins, flavones and flavonols, the molecule is planar (occasionally distorted, e.g. by the substitution of the 2′-hydroxyl group in a 3-O-methyl flavonol). Saturated flavonoids (flavanones, flavans) have one or more chiral centres. Optical activity may also be present in flavonoids due to the presence of glycosidic substituents. Flavonoids can be classified according to their biosynthetic origin. Some flavonoid types are both intermediates in biosynthesis as well as end-products, which can accumulate in plant tissues. These include chalcones (the first formed C15 structure derived from malonyl coenzyme A and p-coumaryl coenzyme A), flavanones, flavanon-3-ols and flavan-3,4-diols. Other classes are only known as end-products of biosynthesis, e.g. anthocyanins, flavones and flavonols. Two further classes of flavonoid are those in which the 2-phenyl sidechain of flavonoid isomerises to the 3-position (giving rise to isoflavones and related isoflavonoids) and then to the 4-position (giving rise to the neoflavonoids). (Dictionary of Natural Products, Volume 7)

1.3. General Information about Tannins

Plant polyphenols (vegetable tannins) are secondary metabolites widely distributed in the plant kingdom. They are based upon two broad structural themes: (a) Condensed proanthocyanidins in which the fundamental structural unit is the phenolic flavan-3-ol (catechin) nucleus. (b) Galloyl and hexahydroxydiphenoyl esters and their derivatives. These metabolites are almost invariably found as multiple esters of 3,4,5-

trihydroxybenzoic (gallic) acid with D-glucose and a great many can be envisaged as derived from the key biosynthetic intermediate β-1,2,3,4,6-pentagalloyl-Dglucose. Derivatives of hexahydroxydiphenic acid are assumed to be formed by oxidative coupling of vicinal galloyl ester groups in a galloyl-D-glucose ester. (Dictionary of Natural Products, Volume 7)

1.4. General Information about Lignans

The lignans are a group of plant phenols whose structures are determined by the union of cinnamic monomers or their biogenetic equivalents. The lignan dimers linked at β-positions in the side chain were first defined in 1936 by R.D. Haworth and these are discussed first. This group of about 900 compounds has since been extended to include some 500 neolignans, which are dimerised in other ways, and higher oligomers of cinnamyl alcohols. Higher lignoid polymers occur in wood as lignin and are degraded during papermaking processes. (Dictionary of Natural Products, Volume 7)

1.5. General Information about Terpenoids

The immense variety of structural types found in the terpenoids was rationalised by the isoprene rule of Ruzicka. However, the number of exceptions to the regular arrangement of isoprene units led to the biogenetic isoprene rule which encompassed the possibility of rearrangements during biosynthesis. Terpenoids are thus seen as being formed from linear arrangements of isoprene units followed by various cyclisations and rearrangements of the carbon skeleton. They can also be biosynthetically modified by the loss or addition of carbon atoms. It is useful to classify terpenoids according to the number of isoprene units from which they are biogenetically derived, even though some carbons may have been added or lost. (This sometimes causes some uncertainty if it is believed that more than five carbons have been lost; only a biosynthetic study can resolve this issue. For example the irones (C15) are derived biosynthetically from the iridial group (C31)). The biogenetic isoprene rule implies the involvement of a branched five carbon unit in the biosynthesis of terpenoids. Isoprene, although a natural product, is not a precursor of the terpenoids. The biosynthetic origin of this five carbon unit is well established. The pathway involves mevalonic acid which is converted into isopentenyl diphosphate, the five-carbon precursor of the terpenoids. However alternative biosynthetic pathways to isopentenyl diphosphate are now becoming common. (Dictionary of Natural Products, Volume 7)

1.6. General Information about Steroids

For general information on the biogenesis of steroids, see the preceding terpenoid section.

The steroid structure is based on four carbocyclic rings arranged as in cyclopenta[a]phenanthrene, which is normally fully or partially reduced so that only limited unsaturation, if any, is present. The four steroid rings are labelled, and their carbon atoms are numbered according to the universal convention illustrated.

The great majority of steroids also have one or two methyl groups present at the bridgehead positions C-10 and C-13; the methyl carbon atoms are numbered C-19 and C-18, respectively.

Methyl groups, hydrogen atoms, or substituents at the bridgehead positions C-8,9,10,13, and 14 are assumed to have the 8β, 9α, 10β, 13β, 14α configurations unless otherwise specified. C-5 is a special case, as there are many steroids of each of the 5α and 5β configurations, and it is therefore necessary to specify the C-5 configuration for any steroid which is saturated at C-5. (e.g. 5α-Androstane or 5β-Androstane).

It is worth noting here some changes in Chemical Abstracts indexing policy. Prior to the 8th Collective Index (1967), the indexing of steroid stereoisomers gave priority to the C-5 configuration which effectively led to a separation of 5α- and 5β-steroids. Users should be alert to this when searching the literature before 1967.

The hydrogen atoms at C-8,9, and 14 are generally omitted from formulae if they have the natural configurations shown here.

Any side-chain at C-17 is assumed to have the 17β-configuration unless otherwise indicated. This is shown either by using a wedge bond or, where there is any possibility of uncertainty owing to substitution at C-20, by drawing in the C-17 α-hydrogen atom.

Configurations of substituents in the side chain were formerly also indicated by α or β, according to a convention devised by Fieser, whereby the side-chain is drawn in Fischer projection, with the highest numbered locant at the top.

However, use of this arbitrary system demands either a good memory or reference to printed texts. The unambiguous Cahn-Ingold-Prelog sequence rule descriptors (R or S)

are now recommended for side-chain configurations. Designations according to Fieser's system are also given in DNP entries where these are or have been in widespread use.

The presence of substituents at C-17 or C-21 may change the priority of groups so that 20S is no longer equivalent to 20α. This happens for example in the pregnane-20,21-diols.

Difficulty in labelling configurations unambiguously can also occur in secosteroids, in which a ring bond has been broken. Parts of the molecule which are normally constrained when the rings are intact become free to rotate in relation to each other so that α and β lose their defined meanings. The sequence rule is again recommended to overcome this problem. The compounds of the Vitamin D series (9,10-secosteroids) are the most important in this class.

The sequence rule descriptors (E-) and (Z-) are required for defining sidechain double bond configurations. (Dictionary of Natural Products, Volume 7)

1.7. General Information about Alkaloids

Alkaloids are a large group of nitrogen-containing secondary metabolites of plant, microbial or animal origin. The term originally implied pharmacologically active bases of plant origin, but the definition has subsequently been broadened so that it is now generally considered to include the majority of nitrogencontaining natural products with the exception of the simple aminoacids, proteins and nitrogen-containing substances of polyketide origin such as the aminoglycoside antibiotics. Basic properties may be weak or absent as in the various types of amide alkaloids. The class of microbial alkaloid overlaps considerably with that of the nitrogenous antibiotics, and substances such as the cytochalasans which show antibiotic properties are in DNP classified as alkaloidal, the definition being a matter of semantics.

Biogenetically and structurally the alkaloids are diverse and it is usual to discuss them in terms of biogenetic origin rather than purely on the basis of structural features. The organisation of alkaloid groups within the Type of Compound Index follows the order given below. (Dictionary of Natural Products, Volume 7)

1.8. Chromatography

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.

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).

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).

Flash Chromatography

Flash chromatography is a preparative pressure liquid chromatography method which enables a considerable time saving when compared with conventional open-column chromatography. Ordinary glass columns are used but eluent is driven through a

sorbent by compressed air or nitrogen, reaching a maximum pressure of about 2 bars at the top of the column. The granulometry of the sorbent is somewhat reduced because solvent is being delivered under pressure; resolution is consequently higher. Flash chromatography can be employed as a fast alternative to open-column chromatographic methods of preliminary fractionation; separations of 10 mg to 10 g of sample can be achieved in as little as 10 min.

Although most applications have involved silica gel sorbents, there is an increasing trend towards RP materials. RP flash chromatography enables the easy separation of saponins from other, more polar, components such as oligosaccharides (Hostettmann, and Marston 1995).

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. LPLC employs columns containing sorbents with a particle size of 40-60 μm. High flow rates at pressures of up to 10 bar are possible and columns are mostly made of glass. Commercially available pre-packed columns in different sizes are ideal for the preparative chromatography of saponins in the 50-500 mg sample range. A high and uniform packing density guarantees good separation efficiency.

It is relatively easy to transpose analytical HPLC conditions onto an LPLC separation, given that the chemistry of the sorbents is similar.

Most applications have been performed on RP sorbents, eluted with methanol-water mixtures. It is generally only pre-purified samples which are injected in this case.

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 and separations are, rapid, requiring considerably less time than open-column chromatography. A direct transposition of separation conditions from analytical HPLC to MPLC can be achieved on reversed-phase supports, thus facilitating the choice of solvent (Hostettmann, and Marston, 1995).

High-Performance Liquid Chromatography (HPLC)

Chromatography by HPLC (High-performance liquid chromatography) is a powerful technique for obtaining multi-milligram quantities of saponins from mixtures of closely related compounds and, in this respect, is very frequently employed as a final purification step (Hostettmann, and Marston, 1995). But, the absence of a chromophore in saponins hampers their detection in ultraviolet light and allows non-specific detection in at 200-210 nm. Thus, most of published data are based on recording HPLC profiles at 200-210 nm. However, at this wavelength other than saponin components of the analyte may overlap with saponins making determination difficult. Only for 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP) conjugated soyasaponins, which have an UV absorption maximum at 295 nm, glycyrrhetinic acid glycosides and cucurbitacins detection with UV–vis detectors could be successful. To overcome these problems and to be able to develop validated analytical methods for quality control of some products, several trials were performed to apply evaporative light scattering detection (ELSD) for detection of saponins. This detector was successfully applied for measuring soya sapogenols A and B, separated on C18 column with MeCN:PrOH:H_2O:AcOH (80:6:13.9:0.1) in soybean. Validated HPLC method with ELSD was also developed for determination of major ginsenosides in samples of Chinese traditional medicine. Saponins were successfully separated on Spherisorb ODS2, C18 column in MeCN:H_2O gradient and quantified using calibration curves, with detection limits of 50 mg. (Oleszek et al., 2006).

Vacuum Liquid Chromatography (VLC)

The technique can be considered as preparative TLC run as a column, with a vacuum provided to speed up eluent flow rates. It differs from flash chromatography in that the column is allowed to run dry after each fraction is collected. This is similar to preparative TLC because plates can be dried after a run and then re-eluted. The chromatography column is packed with silica gel (generally 10-40 micrometer TLC grade) and the sample is eluted with appropriate solvent mixtures, starting with solvent of low polarity and gradually increasing the elution strength. Application of a vacuum to the bottom of the column pulls the solvent through the sorbent.

In the last few years VLC has been increasingly used in the field of natural products because of its simplicity of operation. Separations of up to 30 g extract are possible (Hostettmann and Marston, 1995).

Over-Pressured Layer Chromatography (OPLC)

This is a modified planar chromatographic technique in which the vapor phase is eliminated by covering the sorbent layer with an elastic membrane under variable external pressure. The mobile phase is forced through the sorbent layer with a pump.

In over-pressured chromatography a horizontal thin-layer chromatography plate is covered by an elastic cushion. Pressure is applied to the cushion so that separations can performed on the TLC plate in the absence of vapor phase. Under forced-flow conditions of eluent, sample development is rapid. High efficiencies can be achieved due to the use of fine-particle sorbents and longer plates than those found in capillary-controlled systems. As separation times are short, diffusion effects are reduced. It is also possible to employ mobile phases with poor solvent wetting characteristics.

Applications to the separation of natural products include following: Anthraquinones, furocoumarins, secoiridoid glycosides, sesquiterpenes, diterpenes, cucurbitacins, steroid saponins and essential oils (Hostettmann and Marston, 1995).

1.9. Structure Determination

The complexity of many bioactive natural products containing sugar residues dictates the use of a combination of modern techniques dominated by those involving mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy for structure elucidation. In more and more cases the use of a single technique precludes a satisfactory unambiguous determination.

1.9.1. Mass Spectrometry

The choice of ionization method in MS depends on the polarity, lability and molecular weight of the compound to be analysed. It is principally the so-called 'soft' ionization techniques such as FAB and desorption/chemical ionization (D/CT) which are employed to obtain molecular weight and sugar sequence information for naturally occurring glycosides (Wolfender et al. 1992). These permit the analysis of glycosides without derivatization. In certain cases, fragmentations of aglycones are observed, but electron impact mass spectra (EI-MS) are more useful for this purpose.

Electron Impact MS (EI-MS)

The most common MS method, samples need to be volatilized and, of course, saponins cannot be analysed by this technique unless they are converted to permethyl or peracetyl derivatives; such derivatization is not applicable to saponins possessing more than four sugar residues (Komori et al. 1975). However, mention should be made here of the application of EI-MS to the structure elucidation of the aglycones obtained from saponins. It is possible to observe the molecular peak of the aglycone and also to arrive at conclusions about the structure of the terpenoid skeleton from the fragmentation pattern. One of the diagnostically important features of the EI-MS of aglycones possessing 12(13)-double bonds is the retro-Diels- Alder rearrangement which cleaves the molecule at ring C and enables information to be furnished about substitution in the ring systems (Djerassi et al. 1962; Budzikiewicz et al. 1963; Karliner and Djerassi, 1966). An especially good indication of the distribution of hydroxyl groups between rings A and B, and rings D and E can be obtained.

The EI-MS of peracetylated saponins gives useful information as to the construction of the sugar chain. For example, fragment ions corresponding to terminal glucose, (Glc)Ac4 (m/z 331), to terminal rhamnose, (Rha)Ac3 (m/z 273) and to (Rha-Glc)Ac6 (m/z 561) aided in the structure elucidation of a cyclamiretin A tetrasaccharide from *Ardisia crenata* (*Myrsinaceae*) (Wang et al. 1992).

1.9.2. Nuclear Magnetic Resonance (NMR)

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.

^{13}C-Nuclear Magnetic Resonance

Carbon-13 NMR spectroscopy, now widely used for the structure determination of saponins, is a fast and non-destructive method but requires quite large quantities of sample (mg amounts). Analysis of the spectra allows conclusions to be drawn about the following aspects:

- positions of attachment of the glycosidic chains to the aglycone,
- sequence, nature and number of monosaccharides,

- configuration and conformation of the inter glycosidic linkages,
- presence of acylglycosides in the chains (Ishii et al 1978,1984),
- nature of the aglycone (Doddrell et al. 1974; Tori et al. 1974; Blunt and Munro, 1980),
- structures of attached ester acids (Okada et al. 1980).

For assigning chemical shifts, it is very helpful to compare observed data with data reported for model and related compounds.

^{1}H-Nuclear Magnetic Resonance

The ^{1}H-NMR spectra have characteristically proved complex and tedious to analyse. The vast majority of proton resonances of the carbohydrate moiety appear in a very small spectral width of 3.0-4.2 ppm, with subsequent problems of overlapping. These derive from the bulk of non-anomeric sugar methine and methylene protons which have very similar chemical shifts in different monosaccharide residues.

However, the methyl peaks of triterpenes are readily discernible and most proton resonance positions in oleanene, ursene and related skeletons have been assigned since the 1960s (Kojima and Ogura, 1989) by a variety of techniques.

Different 2-D NMR techniques

The use of HMQC and HMBC ^{13}C multiple- quantum coherence spectra is valuable not only for aglycone assignments but also for sugar sequence details. These experiments are analogous to ^{13}C–^{1}H heteronuclear correlated spectroscopy (HETCOR) but instead of observing ^{13}C, the more abundant ^{l}H is detected. It was possible to assign all the chemical shifts in the ^{1}H-NMR spectrum by considering ^{13}C-NMR data in conjunction with 2-D ^{l}H-detected HMQC and HMBC spectra. Cross peaks corresponding to two and three bond couplings were observed for nearly all possible correlations in the molecule. Similarly, long-range ^{1}H-^{13}C correlations in the HMQC and HMBC spectra enabled the determination of the sequence and positions of attachment of the sugar moieties.

1.10. Previous Chemical Studies on *Nerium* species

Chemical Name	Part of plant	Reference
3β-O-(β-D-Sarmentosyl)-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2007
3β,14-Dihydroxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2007
3β-O-(β-D-diginosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2006
3β-O-(β-D-Diginosyl)-16β-acetoxy-14-hydroxy-5α,14β-card-20-(22)-enolide	Trunk and brunches	Zhao et al., 2007
3β,14-Dihydroxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2007
3β-O-(β-D-diginosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2006
3β-O-(β-D-Diginosyl)-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2007; Takada et al., 2009; Rashan et al., 2011; Siddiqui et al., 2012
3β-O-(β-D-Diginosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2007
3β-O-(β-D-Diginosyl)-8,14-dihydroxy-5β,14β-card-20(22)-dienolide	Trunk and brunches	Zhao et al., 2007
3β-O-(β-D-Sarmentosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20-(22)-enolide	Trunk and brunches	Zhao et al., 2007
16β-Acetoxy-3β,14-dihydroxy-5β,14β-card-20-(22)-enolide	Trunk and brunches	Zhao et al., 2007
3β-O-(β-D-Diginosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2007
3β-O-(β-D-Diginosyl)-8,14-epoxy-5β,14β-card-16,20(22)-dienolide	Trunk and brunches	Zhao et al., 2007
3β-O-(β-D-Diginosyl)-14-hydroxy-5R,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al., 2007
3β-O-(D-Diginosyl)-5β,14β-dihydroxy-card-20(22)-enolide	Leaf	Begum et al., 1999

3β-O-(D-2-O-Methyldigitalosyl)-14β-hydroxy-5β-carda-16,20(22)-dienolide	Leaf	Siddiqui et al., 1997
3β-Hydroxy-8,14-epoxy-5β-carda-16,20(22)-dienolide	Leaf	Siddiqui et al., 1997
3β-O-(D-Digitalosyl)-14β-hydroxy-16β-acetoxy-5β-card-20(22)-enolide	Leaf and breastin	Siddiqui et al., 1997; Rashan et al., 2011; Bai L., et al., 2011
3β-O-(D-Digitalosyl)-14β-hydroxy-5β-card-20(22)-enolide	Leaf, branch and breastin	Siddiqui et al., 1997; Rashan et al., 2011; Bai L., et al., 2011
Oleandrigenin-3-O-α-oleandroside	Leaf, branch and breastin	Rashan et al., 2011; Newman et al., 2007; Dunn et al., 2011
Oleandrigenin-3-O-β-D-sarmentoside	Breastin	Rashan et al., 2011
3β-Hydroxy-5α-carda-14(15),20(22)-dienolide	Root	Huq et al., 1999a
7β,14β-Dihydroxy-5α-card-20(22)-enolide	Root	Huq et al., 1999a
3β-O-(D-Digitalosyl)-21-hydroxy-5β-carda-8,14,16,20(22)-tetraenolide	Root	Huq et al., 1999a
12β-Hydroxy-5β-carda-8,14,16,20 22-tetraenolide	Root	Huq et al., 1999b
3β-O-(β-D-Digitalosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk and branches	Bai L., et al., 2011
3β-O-(β-D-Diginosyl)-7β,8-epoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk and branches	Bai L., et al., 2011
(8R)-3β-Hydroxy-14-oxo-15(14→8)abeo-5β-card-20(22)-enolide	Trunk and branches	Bai et al., 2010, Bai L., et al., 2011
3β-O-(β-D-Glucosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk and branches	Bai L., et al., 2011
3β-O-(β-D-Diginosyl)-14,16β-dihydroxy-5β,14β-card-20(22)-enolide	Trunk and branches	Bai L., et al., 2011
3β-O-(β-D-Digitalosyl)-14-hydroxy-5α,14β-card-20(22)-enolide	Trunk and branches	Bai L., et al., 2011
3β-O-(β-D-Digitalosyl)-8,14-epoxy-5β,14β-card-16,20(22)-dienolide	Trunk and branche	Bai L., et al., 2011

3β-O-(β-D-Diginosyl)-14-hydroxy-5β,14β-card-16,20(22)-dienolide	Trunk and branche	Bai L., et al., 2011
(8R)-3β-O-(β-D-Diginosyl)-14-oxo-15(14→8)abeo-5β-card-20(22)-enolide	Trunk and branches	Bai L., et al., 2011
3β-O-(β-D-Diginosyl)-8,14-seco-14α-hydroxy-8-oxo-5β-card-20(22)-enolide	Trunk and branches	Bai L., et al., 2011
3β-O-(β-D-Digitalosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk and branche	Bai L., et al., 2010
5-β-Card-20(22)-enolide, 8,14-epoxy-3-β-hydroxy	Leaf	Siddiqui et al., 2012
Adynerigenin 3-O-β-D-diginoside	Leaf	Siddiqui et al., 2012
3β-[(3-O-Methyl-2,6-dideoxy-β-D-lyxo-hexopyranosyl)oxy]-14-hydroxy-5α,14β-carda-20(22)-enolide	Leaf	Siddiqui et al., 2012
3β-O-[β-D-Diginosyl-(1→4)-α-L-oleandrosyl]-8,14-β-epoxy-5β-card-16,20(22)-dienolide	Leaf	Siddiqui et al., 2012
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-diginopyranosyl]-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Zhao et al, 2011
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-sarmentopyranosyl]-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Zhao et al, 2011
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-diginopyranosyl]-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Zhao et al, 2011
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-digitalopyranosyl]-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Zhao et al, 2011
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-digitalopyranosyl]-8,14-epoxy-5β,14β-card-16,20(22)-dienolide	Trunk, branches and leaves	Zhao et al, 2011
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-digitalopyranosyl]-14-hydroxy-5α,14β-card-20(22)-enolide	Trunk, branches and leaves	Zhao et al, 2011
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-diginopyranosyl]-16β-acetoxy-14-hydroxy-5α,14β-	Trunk, branches and leaves	Zhao et al, 2011

card-20(22)-enolide		
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-digitalopyranosyl]-16β-acetoxy-14-hydroxy-5α,14β-card-20(22)-enolide	Trunk, branches and leaves	Zhao et al, 2011
3β-O-[β-D-Glucopyranosyl-(1→4)-β-D-digitalopyranosyl]-16β-acetoxy-14-hydroxy-5α,14β-card-20(22)-enolide	Trunk, branches and leaves	Zhao et al, 2011
3β-O-(4-O-Gentiobiosyl-D-diginosyl)-7β,8-epoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Zhao et al, 2011
3β-O-(4-O-Gentiobiosyl-D-diginosyl)-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-digitalosyl)-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-diginosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-digitalosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-L-oleandrosyl)-16β-acetoxy-14-hydroxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-diginosyl)-14-hydroxy-5α,14β-card-20(22)-enolide	Trunk and brunches	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-digitalosyl)-14-hydroxy-5α,14β-card-20(22)-enolide	Trunk and brunches	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-diginosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk, branches and leaves	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-digitalosyl)-8,14-epoxy-5β,14β-card-20(22)-enolide	Trunk and brunches	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-diginosyl)-8,14-epoxy-5β,14β-card-16,20(22)-enolide	Trunk and brunches	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-digitalosyl)-8,14-epoxy-5β,14β-card-16,20(22)-enolide	Trunk, branches and leaves	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-L-oleandrosyl)-14,16β-dihydroxy-5β,14β-card-20(22)-enolide	Leaves	Bai Y., et al., 2011

3β-O-(4-O-Gentiobiosyl-D-diginosyl)-8β,14-dihydroxy-5β,14β-card-20(22)-enolide	Leaves	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-diginosyl)-14α-hydroxy-8-oxo-8,14-seco-5β-card-20(22)-enolide	Trunk, branches and leaves	Bai Y., et al., 2011
3β-O-(4-O-Gentiobiosyl-D-diginosyl)-14-oxo-15(15→8)abeo-card-20(22)-enolide	Trunk, branches and leaves	Bai Y., et al., 2011
1β,3β-Dihydroxyurs-12-ene-28-oic acid	Leaves	Siddiqui et al., 1986
3β,28-Dihydroxy- 12-ursene	Leaves	Siddiqui et al., 1986
3β,20α-Dihydroxyurs-21-en-28-oic acid	Leaves	Fu et al., 2005
3β,12α-Dihydroxyoleanan-28,13β-olide	Leaves	Fu et al., 2005
3β,27-Dihydroxy-12-ursen-28-oic acid	Leaves	Fu et al., 2005
3β,13β-Dihydroxyurs-11-en-28-oic acid	Leaves	Fu et al., 2005
3β-Hydroxyurs-12-en-28-aldehyde	Leaves	Fu et al., 2005
28-Norurs-12-en-3β-ol	Leaves	Fu et al., 2005
Urs-12-en-3β-ol	Leaves	Fu et al., 2005
Urs-12-ene-3β,28-diol	Leaves	Fu et al., 2005
3β,27-Dihydroxy-12-oleanen-28-oic acid	Leaves	Fu et al., 2005
(20S,24R)-Epoxydammarane-3β,25-diol	Leaves	Fu et al., 2005
(20S,24S)-Epoxydammarane-3β,25-diol	Leaves	Fu et al., 2005
20β,28-Epoxy-28α-methoxytaraxasteran-3β-ol	Leaves	Zhaoet al., 2006
20β,28-Epoxytaraxaster-21-en-3β-ol	Leaves	Zhaoet al., 2006
28-Nor-urs-12-ene-3β,17β-diol	Leaves	Zhaoet al., 2006
Oleanderol	Fresh leaves	Siddiqui et al., 1988
Lup-20(29)-ene-3β,28-diol	Fresh leaves	Siddiqui et al., 1988; Fu et al., 2005

(3β)-3-Hydroxy-lup-20(29)-en-28-oic acid	Fresh leaves	Siddiqui et al., 1988; Fu et al., 2005
3-β-Hydroxyurs-12-en-28-oic acid	Fresh leaves	Siddiqui et al., 1988; Fu et al., 2005
3β-Hydroxy-12-oleanen-28-oic acid	Fresh leaves	Siddiqui et al., 1988; Fu et al., 2005
24-Nor-3β, 5-dihydroxyursa-4(23), 18-dien-28-oic acid	Fresh and unground leaves	Siddiqui et al., 1989
3β-Hydroxyursa-28-oic acid	Fresh and unground leaves	Siddiqui et al., 1989
3β-Hydroxy-27-(4-acetyl)-(Z)-cinnamoyl-lup-20(29)-en-28-oic acid	Leaves	Siddiqui et al., 2012
3β,27-Dihydroxy-urs-18-en-13,28-olide	Fresh, unground leaves	Begum et al., 1997
3β,22α,28-Trihydroxy-25-nor-lup-1(10),20(29)-dien-2-one	Fresh, unground leaves	Begum et al., 1997
3α-Acetophenoxy-urs-12-en-28-oic acid	Leaves	Siddiqui et al., 2009
3β-Acetophenoxyurs-12-en-28-oic acid	Leaves	Siddiqui et al., 2009
12-β-Hydroxypregna-4,6,16-triene-3,20-dienone	Root	Huq et al., 1999a ; Kumar et al., 2005
Quercetin-5-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside	Leaves	Siddiqui et al., 2012
Kaempferol-5-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside]	Leaves	Siddiqui et al., 2012
3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	Commercial standard	Ibrahim et al., 2008; Tu et al., 2007; Parveen et al., 2007; Hosseinzadeh et al., 2007
4H-1- Benzopyran-4- one, 3-[[6-O-(6- deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl] oxy]-2-(3,4-dihydroxypheny l)-5,7-dihydroxy	Leaves	Rajyalaksh mi et al., 2011

2. MATERIAL AND METHODS

2.1 General

The NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 500 MHz (1H) in DMSO, using TMS as internal standard. Column chromatography was carried out on 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 Merck 5569). Compounds were detected by UV and 1% vanillin/H2SO4 followed by heating 105 C for 1-2 min. During the isolation process and TLC controls the following solvent system were used:

CH_2Cl_2: MeOH: H_2O	99:1:0.1
CH_2Cl_2: MeOH: H_2O	95:5:0.5
CH_2Cl_2: MeOH: H_2O	90:10:1
CH_2Cl_2: MeOH: H_2O	80:20:2
CH_2Cl_2: MeOH: H_2O	70:30:3
CH_2Cl_2: MeOH: H_2O	61:32:7
CH_2Cl_2: MeOH: H_2O	64:50:10
MeOH: H_2O	9:1
MeOH: H_2O	8:2
MeOH: H_2O	7:3
MeOH: H_2O	6:4
MeOH: H_2O	5:5

2.2 Plant Material

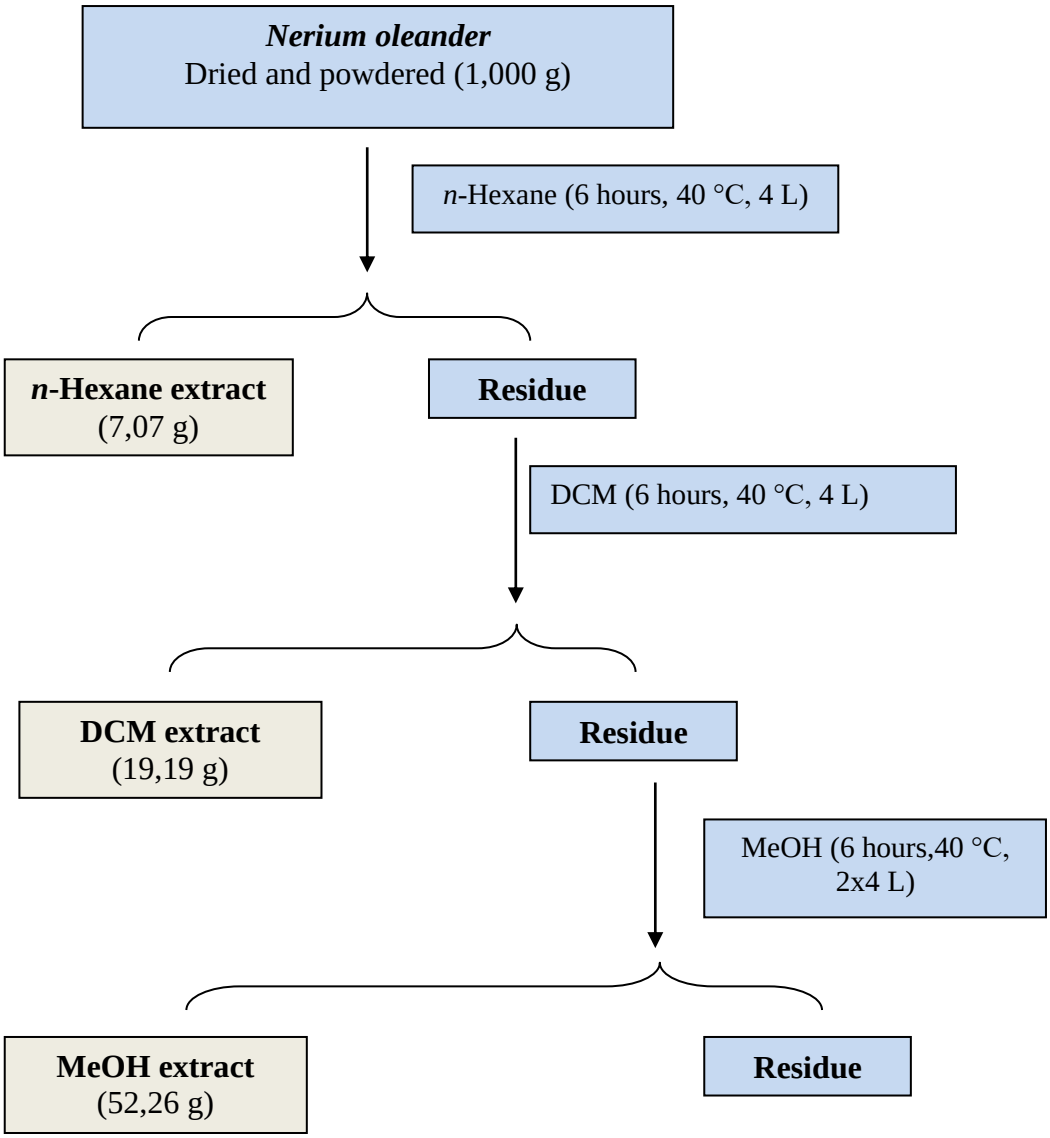
Nerium oleander was collected from Manisa in June 2012. Plant material was identified by Assoc. Prof. Dr. Serdar G. ŞENOL (Biology Department, Faculty of Sciences, Ege University).

2.3. Isolation and Purification

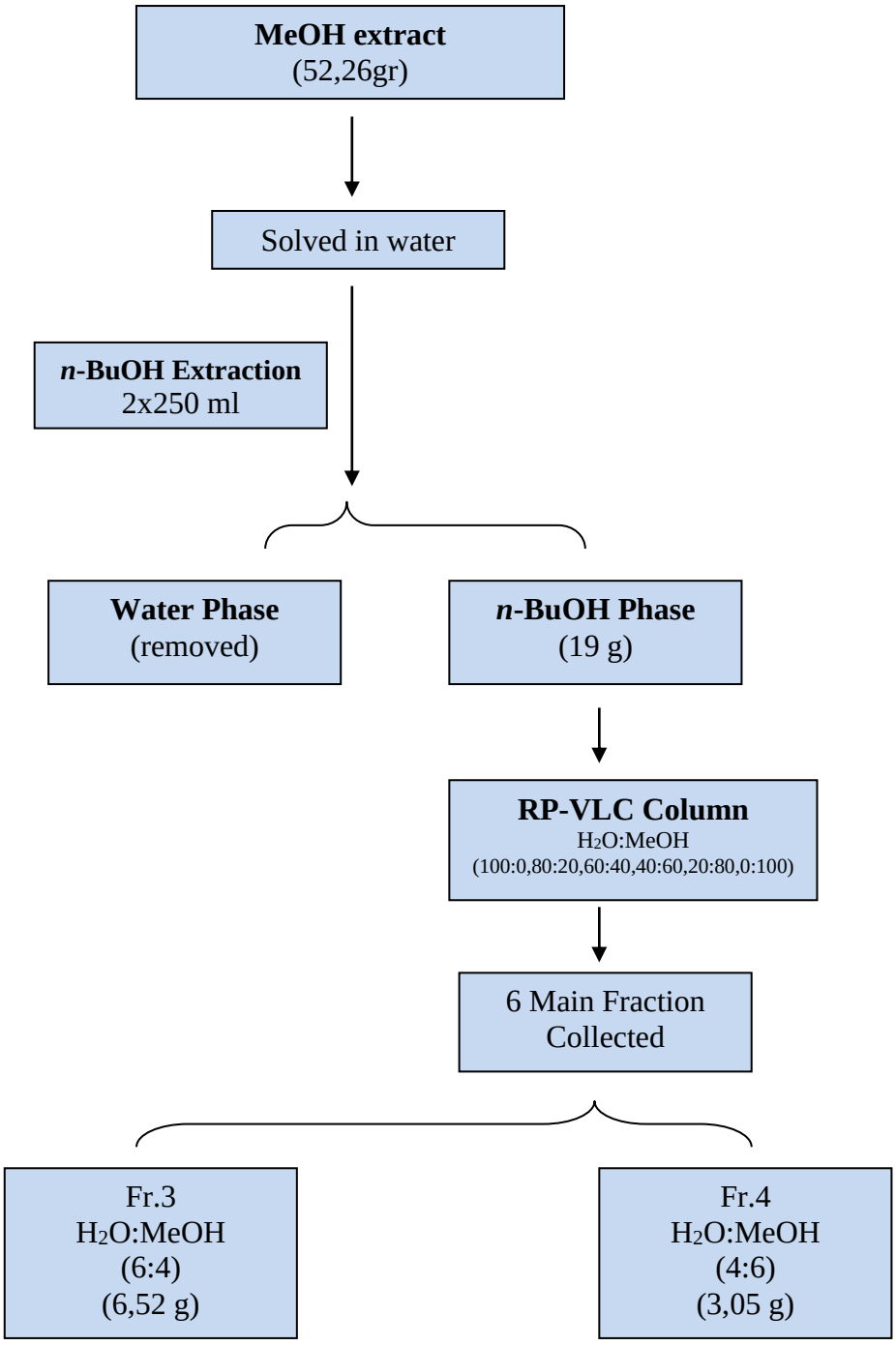
A 1000 g of dried and grinded root of *Nerium oleander* was extracted with 4 litter *n*-hexane, 4 litter dichloromethane (DCM) and 2 times with 4 litter methanol at 40 °C simultaneously. In each extraction step, the solvent was removed by rotary evaporator and at the end of extraction 52.26 gram MeOH was obtained. The MeOH extract was dissolved in 250 mL H_2O, and successively partitioned with *n*-BuOH saturated with H_2O (2 x 250 mL) (Scheme 2.1).

19 gram of *n*-BuOH extract was subjected to vacuum liquid chromatography (VLC) using reversed-phase material (Lichroprep RP-18, 25-40 μm) employing H_2O, H_2O:MeOH and MeOH. Saponin rich fractions Fraction 3 and Fraction 4 are obtained (Scheme 2.2).

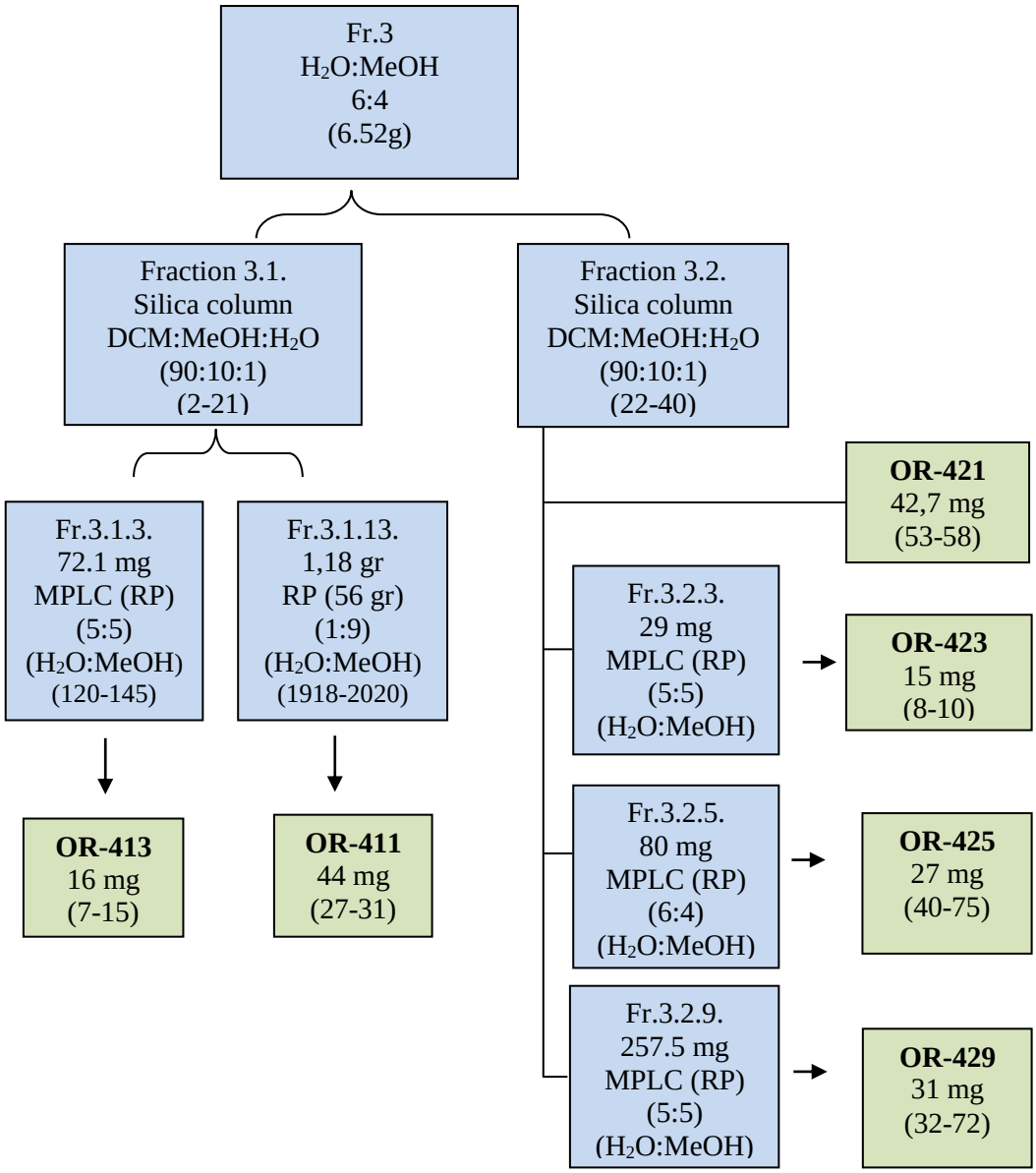
Firstly, 4.53 g of Fraction 3 was subject to silica-gel column with H_2O:MeOH (6:4) solvent system and Fraction 3.3 (72.1 mg) and 3.13 (1.18 g) were obtained. Fraction 3.3 was purified via RP in MPLC with H_2O:MeOH (6:4) solvent system to give Compound **2** OR 413 (16 mg). Compund **1** (OR 411) was obtained as a result of purification of Fraction 3.13 over RP in open column chromatography with H_2O:MeOH (1:9) solvent system (Scheme 2.3).



Scheme 2.1. Extraction process of *Nerium oleander*



Scheme 2.2. Obtaining of Fraction 3 and Fraction 4



Scheme 2.3. Purification of Fraction 3

3. RESULTS and DISCUSSION

3.1. Structural Identification of Compound 1 (OR 411)

In ^{1}H-NMR spectrum of Compound **1**, at δ 0.92, 0.95 and 1.17 ppm signals are corresponded three methyl groups (H-3, H-18 and H-19) in aglycone. As a proof of one singlet at δ 5.93 ppm which is correspond one hydrogen, and *dd* at δ 5.10 (H-21a) and 5.15 (H-21b), which are correspond one hydrogen of each, it could be concluded that aglycon has a α-β unsaturated lactone. H-17 and H-15 are splitted H-16 signal and signal for H-16 at δ 4.64 ppm is observed as *ddd*. As a comparing these result with literature data, the structure of aglycone is determined as 16-β-acetoxy-14-hydroxy-5α,14β-card-20(22)-enolide (Bai L., et al. 2011). The anomeric proton of sugar is observed at δ 4.98 ppm as *d* with J= 2.8 Hz and important signals sugar hydrogens are observed at δ 3.49 (-OC<u>H</u>$_3$) and 1.18 (H-6′). Taking NMR signals and comparing with literature data, the structure of sugar is identified as α-L-oleandrosile.

As a result of the above mentioned data comparing with literature, the structure of compound **1** was identified as deacetyl oleandrin.

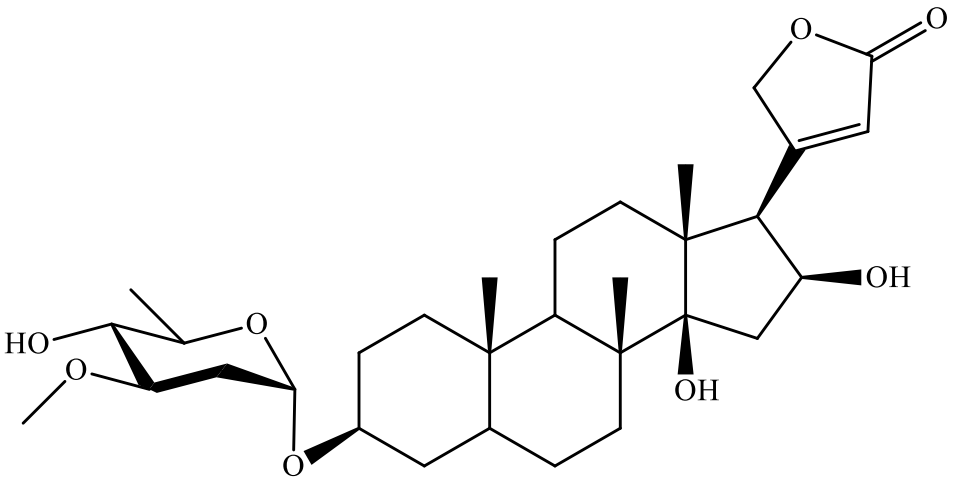
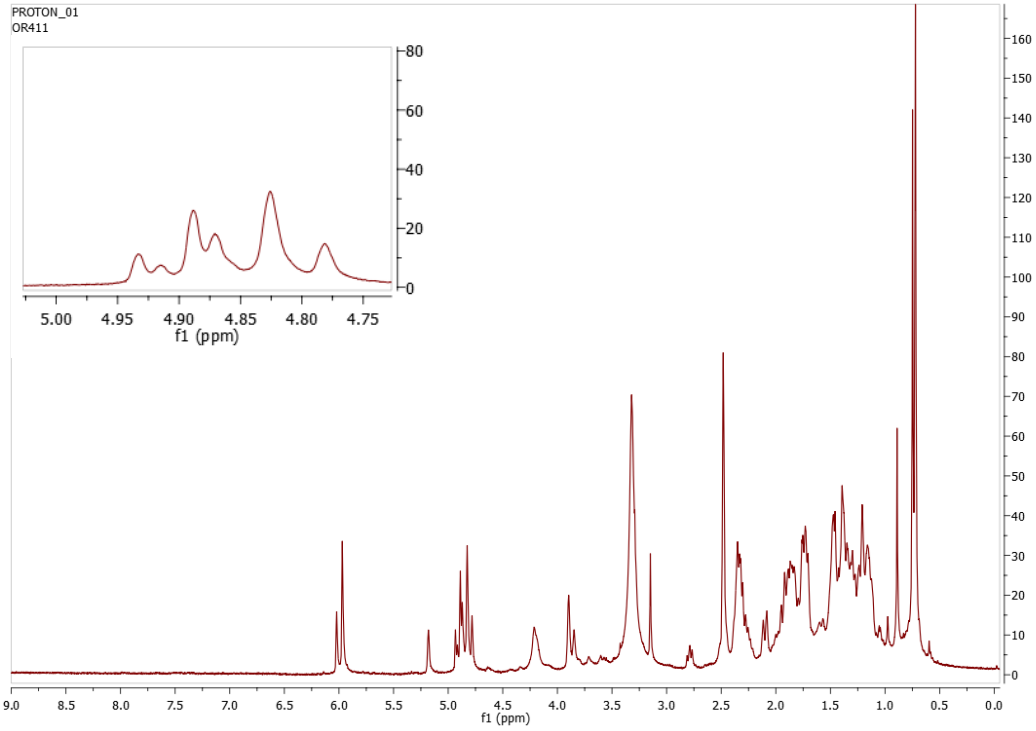


Figure 3.1. Structure of Compound **1** (**OR411**)



Spectrum 3.1. [1]H-NMR Spectrum of Compound **1** (**OR411**)

3.2. Structural Identification of Compound 2 (OR 413)

In [1]H-NMR spectrum of Compound **2**, the signals at δ 5.97, 5.0 and 4.85 are corresponded lactone, at δ 0.90, 1.25 and 2.74 are corresponded aglycone methyl, at δ 4.64 is corresponded anomeric proton and at δ 3.40 is corresponded methoxy proton of sugar. As a result of these, structure of aglycone is same as Compound **1**. However, shifting of H-16 signal from δ 4.64 to 5.47 and observing singlet at δ 1.97 for three protons indicated that acetylation is took place at C-16.

As a result of the above mentioned data, the structure of compound **2** was identified as oleandrin (Abe et al., 1996; Wahyuningsih et al., 2008).

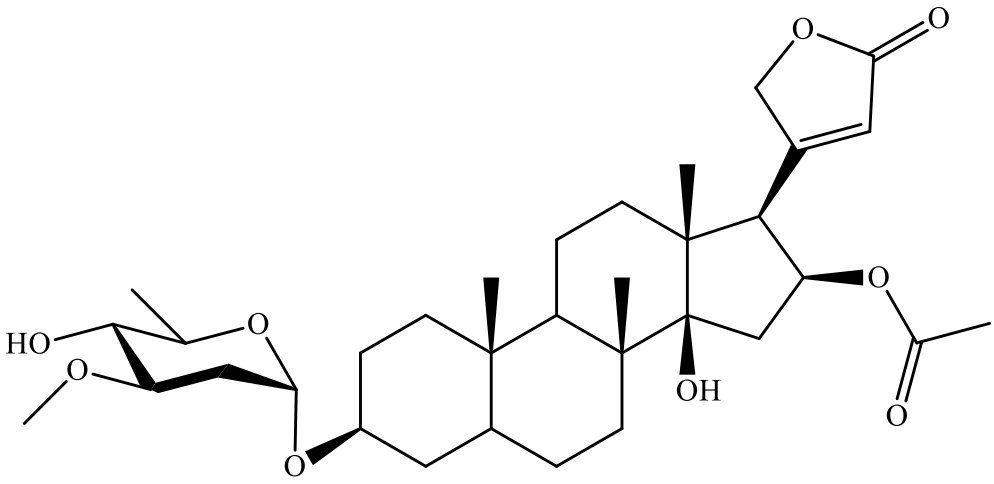
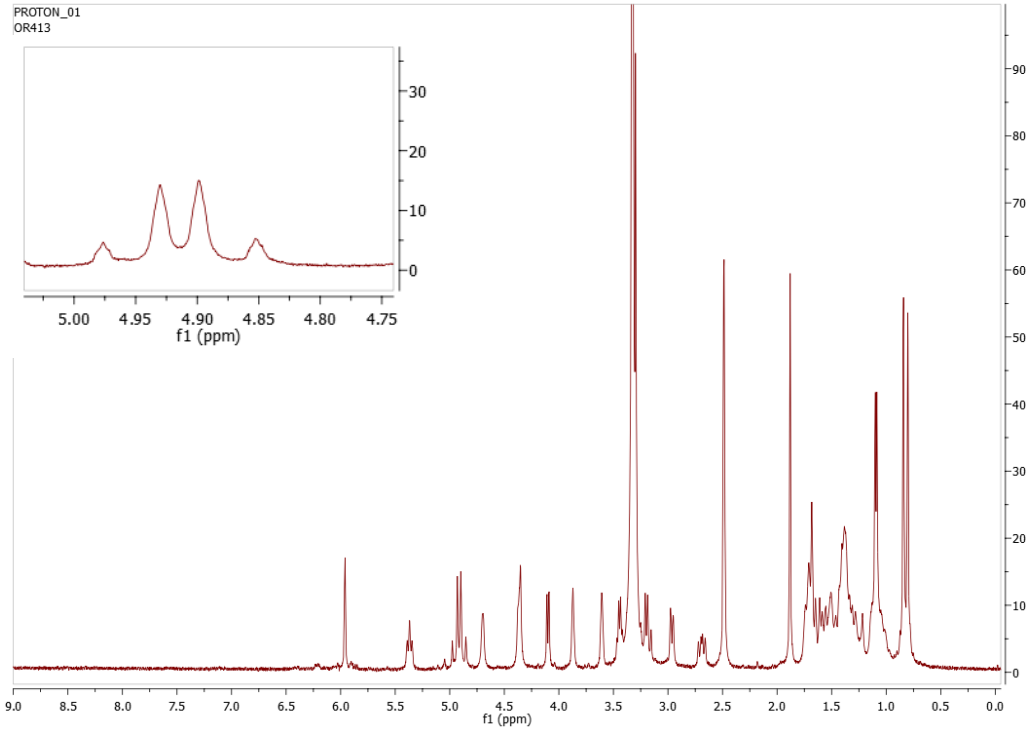


Figure 3.2. Structure of Compound **2** (**OR413**)



Spectrum 3.2. ^{1}H-NMR Spectrum of Compound **2** (**OR413**)

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