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ISOLATION and CHARACTERIZATION of SAPONINS from ASTRAGALUS STRICTIPINUS

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Recep BAŞTÜRK tarafından Yüksek Lisans tezi olarak sunulan ‘Isolation and Chracterization of Saponins from *Astragalus Strıctıpınus* (*Astragalus Strıctıpınus*’dan Saponinlerin İzolasyonu ve Karakterizasyonu) başlıklı bu çalışma E.Ü.Lisansüstü Eğitim Yönetmeliği ile E.Ü. Fen Bilimleri Enstitüsü ve Öğretim Yönergesi’nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve 11.02.2011 tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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ÖZET

ASTRAGALUS STRICTIPINUS'DAN SAPONİNLERİN İZOLASYONU VE KARAKTERİZASYONU

Recep BAŞTÜRK

Yüksek Lisans Tezi, Kimya Bölümü

Tez Danışmanı: Doç. Dr. Özgen ÇALIŞKAN

Şubat 2011, 41 Sayfa

Bu çalışmada *Astragalus Strictipinus* türünden dokuz tane saponin elde edilerek, izole edilen bileşiklerden üç tanesinin yapısı spektral teknikler kullanılarak Cyclotrisectoside (I), Cyclocephaloside (II), ve Astragaloside IV(III) olarak belirlenmiştir

Anahtar Kelimeler: *Astragalus Strictipinus, Saponin,* Cyclotrisectoside (I), Cyclocephaloside (II), and Astragaloside IV(III).

ABSTRACT

ISOLATION and CHARACTERIZATION of SAPONINS *ASTRAGALUS STRICTIPINUS*

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In this study, nine saponins were ısolated from *Astragalus Strictipinus* and structures of the three of the isolated compounds determinated as, Cyclotrisectoside (I), Cyclocephaloside (II), and Astragaloside IV(III)by using spectroscopic techniques.

Keywords: *Astragalus Strictipinus,* Saponin, Cyclotrisectoside (I), Cyclocephaloside (II), and Astragaloside IV (III).

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ABBREVIATIONS

Kısaltmalar

HPLC	High Performance Liquid Chromatography
UV	Ultraviolet
LC-MS	Liquid Chromatography-Mass Spectrometry
DMSO	Dimethylsulfoxide
NMR	Nuclear Magnetic Resonance
COSY	^{1}H-^{1}H Correlated Spectoscopy
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
MPLC	Medium Pressure liquid Chromatography
HMBC	Heteronucleer Multiple Bond Correlation
CC	Column Chromatography
RP-VLC	Reverse Phase Vacuum Liquid Chrom
FT-IR	Fourrier Transformation Ifrared
FAB-MS	Fast Atom Bombardment Mass Spectrometry
TLC	Thin Layer Chromatography

1.INTRODUCTION

1.1. Natural products

Natural products are products from various natural sources, plants, microbes and animals. Natural products can be an entire organism (e.g. a plant, an animal or a micro-organism), a part of an organism (e.g. leaves or flowers of a plant, an isolated animal organ), an extract of an organism or part of an organism and an exudate, or pure compound (e.g. alkaloids, coumarins, flavonoids, lignans, steroids and terpenoids) isolated from plants, animals or micro-organisms. However, in practice, the term natural product refers to secondary metabolites, small molecules (molecular weight < 1500 amu), produced by an organism, but not strictly necessary for the survival of the organism.

1.1.1. Natural products in medicine

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 and the Akkadian civilizations in about the third millenium BC. Hippocrates (ca. 460–377 BC), one of the ancient authors who described medicinal natural products of plant and animal origins, listed approximately 400 different plant species for medicinal purposes. Natural products have been an integral part of the ancient traditional medicine systems, e.g. Chinese, Ayurvedic and Egyptian. Even now, continuous traditions of natural product therapy exist throughout the third world, especially in the orient, where numerous minerals, animal substances and plants are still in common use. According to the World Health Organisation (WHO), some 3,4 billion people in the developing world depend on plantbased traditional medicines. This represents about 88 per cent of the world’s inhabitants, who rely mainly on traditional medicine for their primary

health care. In China alone, 7295 plant species are utilized as medicinal agents. Nature has been a potential source of therapeutic agents for thousands of years. An impressive number of modern drugs have been derived from natural sources. Over the last century, a number of top selling drugs have been developed from natural products. Anticancer drug vincristine from Vinca rosea, narcotic analgesic morphine from Papaver somniferum, antimalarial drug artemisinin from Artemisia annua, anticancer drug Taxol1 from Taxus brevifolia and antibiotic penicillins from Penicillium ssp. are just a few examples.

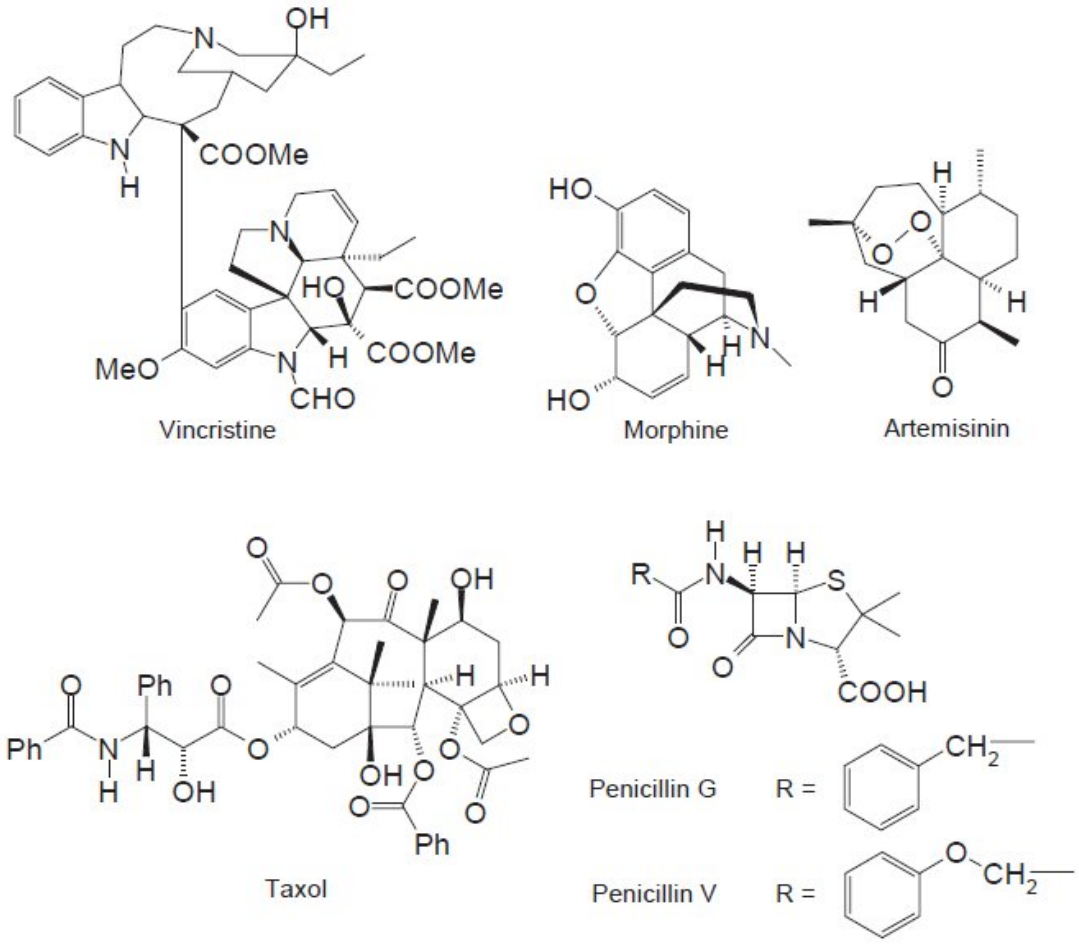


Figure 1 A number of top selling drugs have been developed from natural products

Apart from natural-product-derived modern medicine, natural products are also used directly in the 'natural' pharmaceutical industry that is growing rapidly in Europe and North America, as well as in the traditional medicine programmes being incorporated into the primary health care systems of Mexico, The People's Republic of China, Nigeria and other developing countries.

1.1.2 Saponins

Saponins are compounds whose active portions form colloidal solutions in water, which produce lather on shaking and precipitate cholesterol. They occur as glycosides whose aglycones are triterpenoid or steroidal structures. The combination of lipophilic (fat-soluble) aglycones at one end of the molecule and hydrophilic (water-soluble) sugars at the other end gives them the ability to lower surface tension, producing the characteristic.

Saponin glycosides possess 'soaplike' behaviour in water, i.e. they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. There are two types of sapogenin: steroidal and triterpenoidal. Usually, the sugar is attached at C-3 in saponins, because in most sapogenins there is a hydroxyl group at C-3.

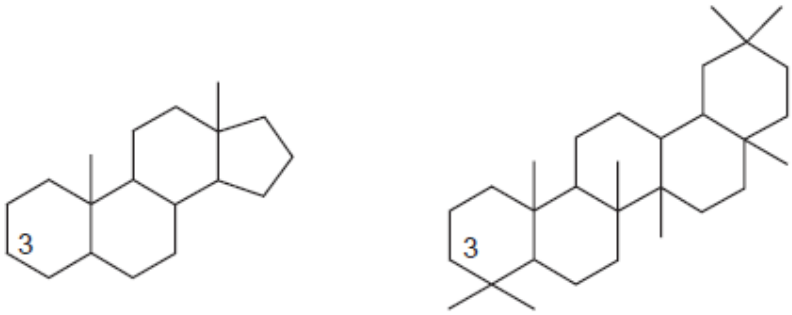
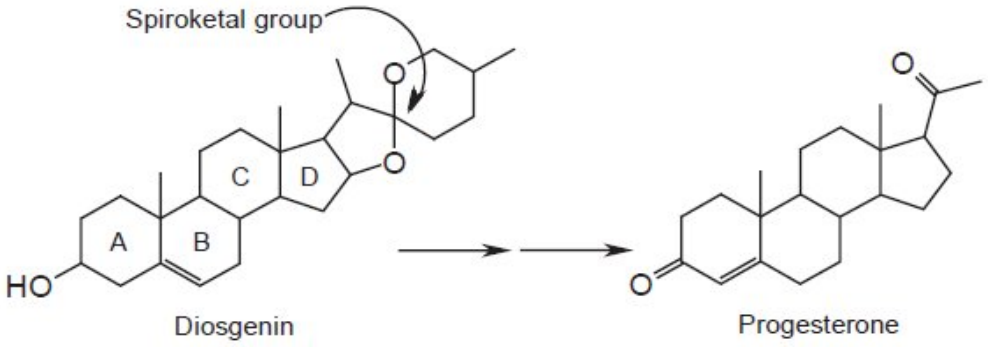


Figure 2 Two types of sapogenin: steroidal and triterpenoidal

The two major types of steroidal sapogenin are diosgenin and hecogenin. Steroidal saponins are used in the commercial production of sex hormones for clinical use. For example, progesterone is derived from diosgenin.



The most abundant starting material for the synthesis of progesterone is diosgenin isolated from Dioscorea species, formerly supplied from Mexico, and now from China. The spiroketal group attached to the D ring of diosgenin can easily be removed. Other steroidal hormones, e.g. cortisone and hydrocortisone, can be prepared from the starting material hecogenin, which can be isolated from Sisal leaves found extensively in East Africa. In triterpenoidal saponins, the aglycone is a triterpene. Most aglycones of triterpenoidal saponins are pentacyclic compounds derived from one of the three basic structural classes represented by a-amyrin, b-amyrin and lupeol. However, tetracyclic triterpenoidal aglycones are also found, e.g. ginsenosides. These glycosides occur abundantly in many plants, e.g. liquorice and ginseng roots contain glycyrrhizinic acid derivatives and ginsenosides, respectively. Most crude drugs containing triterpenoid saponins are usually used as expectorants. Three major sources of triterpenoidal glycosides along with their uses are summarized below.

Plants Botanical names (Family)	Main constituents	Uses
Liquorice root *Glycyrrhiza glabra* (Fabaceae)	Glycyrrhizinic acid derivatives	In addition to expectorant action, it is also used as a flavouring agent.
Quillaia bark *Quillaja saponaria* (Rosaceae)	*Several complex* triterpenoidal saponins, e.g. senegin II	*Tincture of this plant is* used as an emulsifying agent.
Ginseng *Panax ginseng* (Araliaceae)	Ginsenosides	As a tonic, and to promote the feeling of well being.

Table 1 Three major sources of triterpenoidal glycosides along with their uses

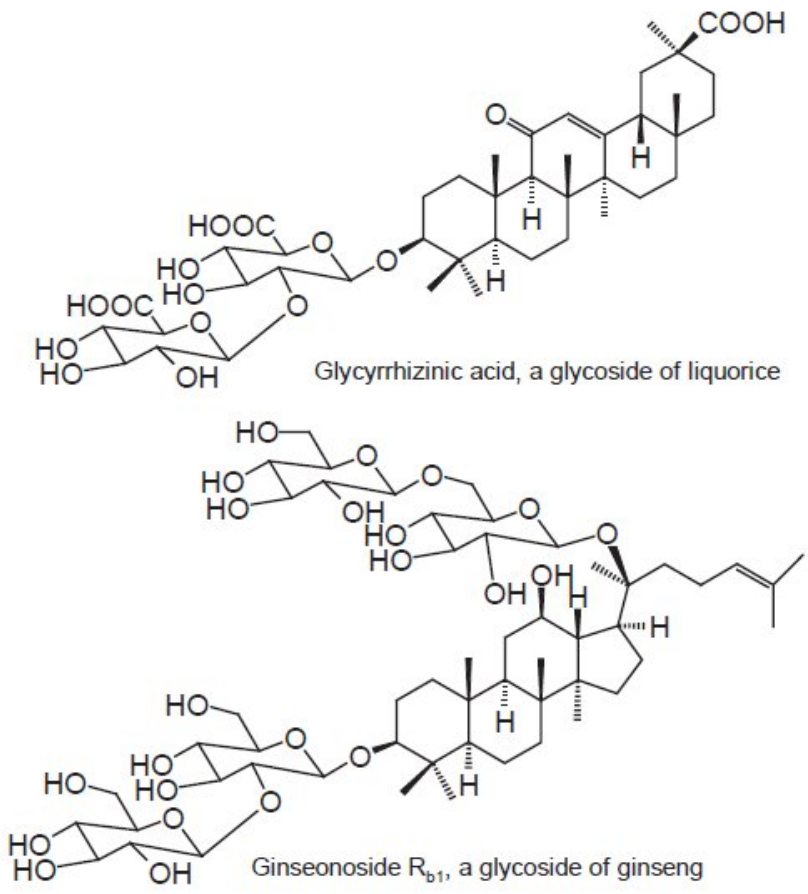


Figure 3 Glycyrrhizinic acid derivatives and ginsenosides

Saponins are the glycosides of triterpenes or steroids and include the group of cardiac glycosides and steroidal alkaloids. Some of them are stored as bidesmosidic compounds in the vacuole, which are cleaved to the active monodesmosidic compounds by glucosidase upon wounding-induced decompartmentation (Wink and VanWyk, 2008). Monodesmosidic saponins are amphiphilic compounds, which can complex cholesterol in biomembranes with their lipophilic terpenoid moiety and bind to surface glycoproteins and glycolipids with their sugar side chain. This leads to a severe tension of the biomembrane and leakage. This activity can easily be demonstrated with erythrocytes, which lose their haemoglobin when in contact with monodesmosidic saponins. This membrane activity is rather unspecific and effects a wide set of organisms from microbes to animals. Some saponins have additional functional groups, such as cardiac glycosides (carrying a five- or sixmembered cardenolide or bufadienolide ring), which enable them to inhibit one of the most important molecular targets of animal cells, the Na+-, K+- ATPase (Ashour et al., 2010; Kreis and M¨ uller-Uri, 2010; Wink and Van Wyk, 2008). Among steroidal glycosides, the cucurbitacins (occurring in members of the Cucurbitaceae) express substantial cytotoxic activities (Wink and Van Wyk, 2008).

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).

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).

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).

The percentage of saponins has been reported in more than 100 families of plants, out of which at least 150 kinds of natural saponins have been found to possess significant anticancer properties. The steroidal saponins are mainly found in Agavaceae, Dioscoreaceae, Liliaceae, Solanaceae, Scrophulariaceae, Amaryllidaceae, Leguminosae and Rhamnaceae; while triterpene saponins are predominantly present in Acanthopanax, Leguminosae, Araliaceae, Scrophulariaceae, Campanulaceae and Caryophyllaceae. There are more than 11 mainly distinguished classes of saponins including dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes, and steroidals. Among these saponins, cycloartanes, dammaranes, oleananes, lupanes and steroids showed strong antitumor effect on kinds of cancers

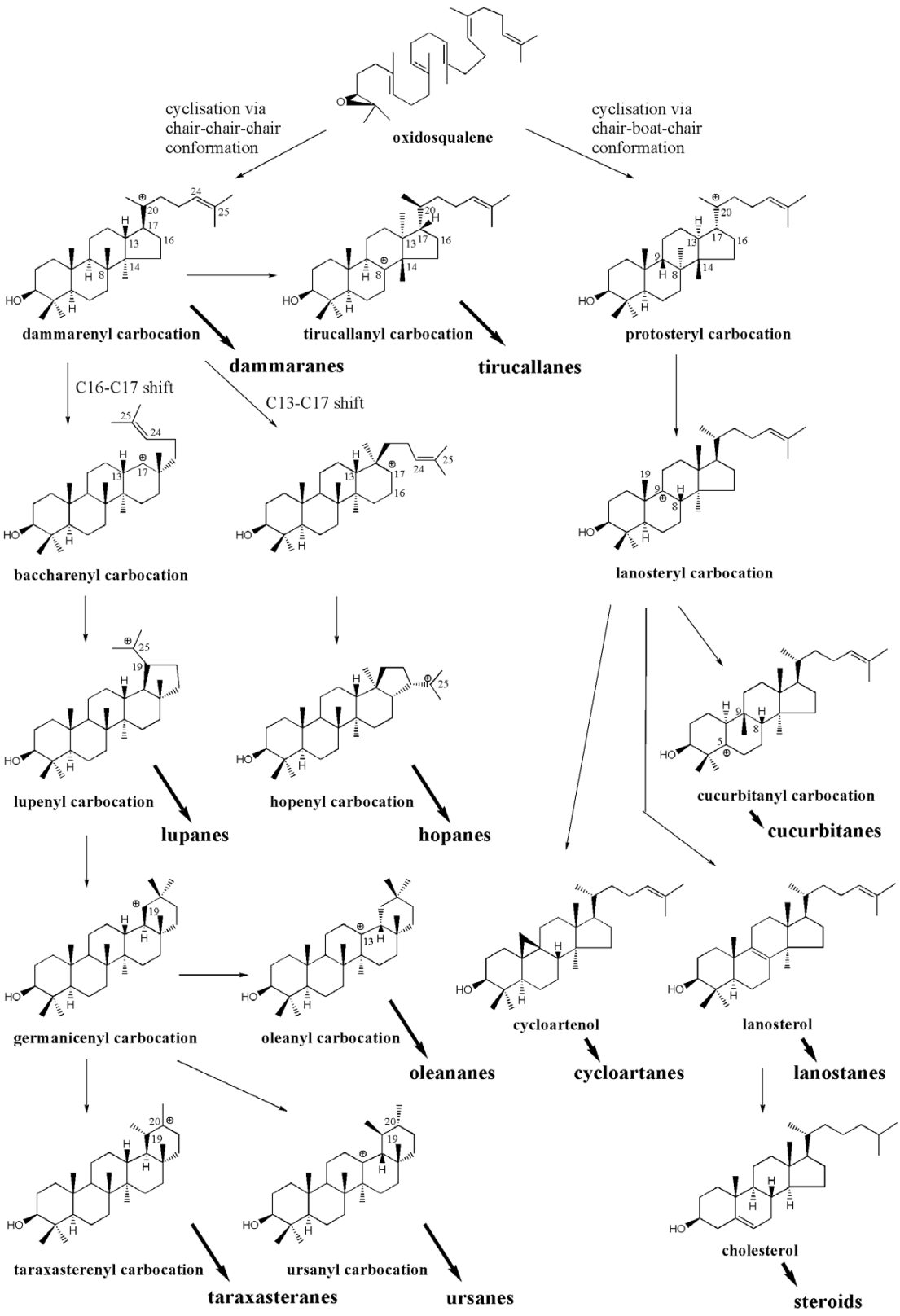
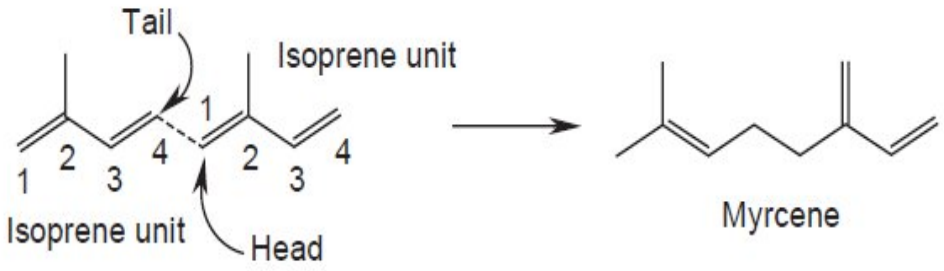


Figure 4 The cyclization of oxidosqualene to the various saponin skeletons

1.1.3.Terpenoids

Terpenoids are compounds derived from a combination of two or more isoprene units. Isoprene is a five carbon unit, chemically known as 2-methyl-1,3-butadiene. According to the isoprene rule proposed by Leopold Ruzicka, terpenoids arise from head-to-tail joining of isoprene units. Carbon 1 is called the 'head' and carbon 4 is the 'tail'. For example, myrcene is a simple 10-carbon-containing terpenoid formed from the headto- tail union of two isoprene units as follows.



Terpenoids are found in all parts of higher plants and occur in mosses, liverworts, algae and lichens. Terpenoids of insect and microbial origins have also been found.

1.1.3.1 Classification

Terpenoids are classified according to the number of isoprene units involved in the formation of these compounds.

Type of terpenoids	Number of carbon atoms	Number of isoprene units	Example
Monoterpene	10	2	Limonene
Sesquiterpene	15	3	Artemisinin
Diterpene	20	4	Forskolin
Triterpene	30	6	α-amyrin
Tetraterpene	40	8	β-carotene
Polymeric terpenoid	Several	Several	Rubber

Table 2 Classification of terpenoids

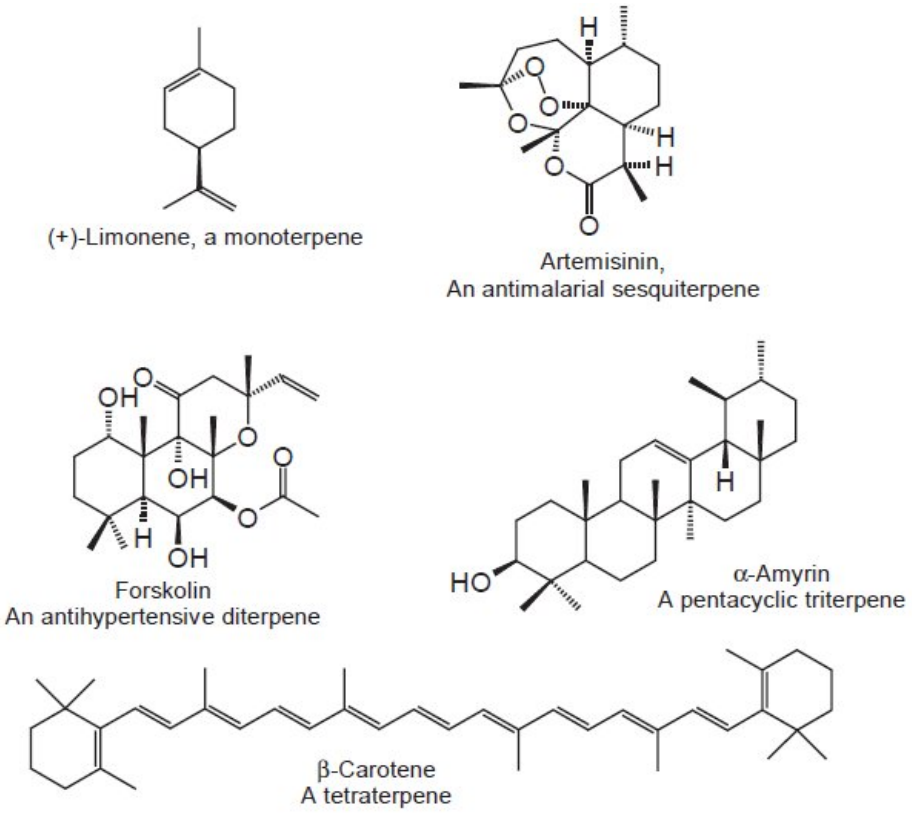


Figure 5 Types of terpenoids

1.1.4. Biosynthesis of Saponins

3R-(ş)-mevalonic acid is the precursor of all terpenoids. The enzymesmevalonate kinase and phosphomevalonate kinase catalyse phosphorylation of mevalonic acid to yield 3R-(ş)-mevalonic acid 5-diphosphate,which is finally transformed to isopentenyl diphosphate, also known as isopentenyl pyrophosphate (IPP), by the elimination of a carboxyl and a hydroxyl group mediated by mevalonate 5-diphosphate decarboxylase. Isopentenyl pyrophosphate is isomerized by isopentenyl isomerase to dimethylallylpyrophosphate (DMAPP). A unit of IPP and a unit of DMAPP are combined together head to tail by dimethylallyl transferase to form geranyl pyrophosphate, which is finally hydrolysed to geraniol, a simple monoterpene. Geranyl pyrophosphate is the precursor of all monoterpenes. In similar fashions, the core pathway up to C25 compounds (five isoprene units) is formed by sequential addition of C5 moieties derived from IPP to a starter unit derived from DMAPP. Thus, sesquiterpenes are formed form the precursor 2E, 6E-farnesyl pyrophosphate (FPP), and diterpenes from 2E, 6E, 10E-geranylgeranyl pyrophosphate (GGPP). The parents of triterpenes and tetraterpenes are formed by reductive coupling of two FPPs or GGPPs,respectively. Rubbers and other polyisoprenoids are produced from repeated additions of C5 units to the starter unit GGPP.

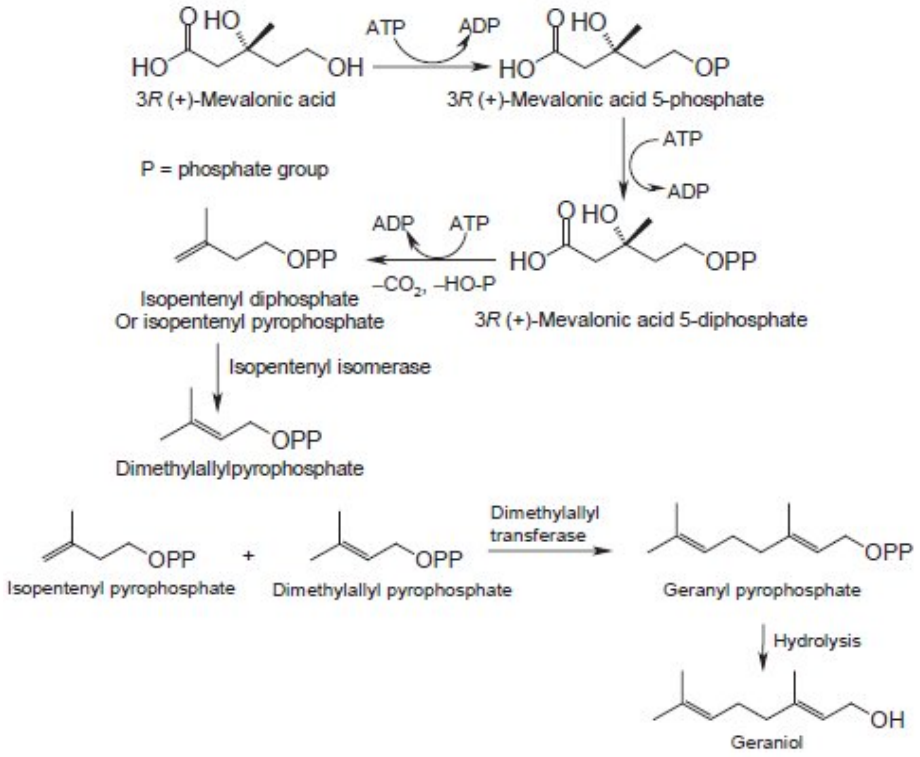


Figure 6 Terpenoid biosynthesis

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).

The biosynthesis of saponins in the genus Medicago has not been investigated extensively (Morris and Tankersley, 1963; Nowacki et al ., 1976; Suzuki et al ., 2002). It is known that the first committed step in triterpenoid biosynthesis in plants involves the cyclization of 2,3-oxidosqualene to give different triterpene polycyclic skeletons, including α – and β-amyrin and lupeol. These conversions are catalysed by specific oxidosqualene cyclases (OSCs) (Hostettman and Marston, 1995). In recent years, a number of OSCs that catalyse the biosynthesis of triterpene have been cloned and characterized from several plant species (Herrera et al . 1998; Kushiro et al . 1998; Shibuya et al ., 1999; Morita et al . 2000; Hayashi et al . 2001; Haralampidisis et al ., 2002; Suzuki et al . 2002; Iturbe-Ormaetxe et al . 2003; Cammareri et al. 2008), but only a few OSCs have been used in plant genetic engineering to modify triterpene production. Using RNA interference (RNAi) to silence the dammarenediol synthase, a specific OSC, Han et al . (2006) produced antisense transgenic Panax ginseng with 84.5% reduction of triterpene saponins in the roots. Furthermore, transgenic P. Ginseng overexpressing a squalene synthase (PgSS1) coding sequence exhibited enhanced phytosterols and saponin content (Lee et al ., 2004). The PgSS1 coding sequence has also been transferred into Siberian ginseng (Eleutherococcus senticosus Rupr. and Maxim.), and transgenic plants showed an increased production of phytosterols and triterpenoids (Seo et al . 2005).

As the effects of OSC expression on triterpene saponin biosynthesis in a legume species are currently unknown, in this study we used Agrobacterium-mediated transformation to introduce a novel Aster sedifolius β-amyrin synthaseencoding gene (AsOXA1) in barrel medic (M. Truncatula). We demonstrated that the ectopic expression of AsOXA1 in transgenic plants led to a greater accumulation of triterpenic compounds and enhanced root nodulation.

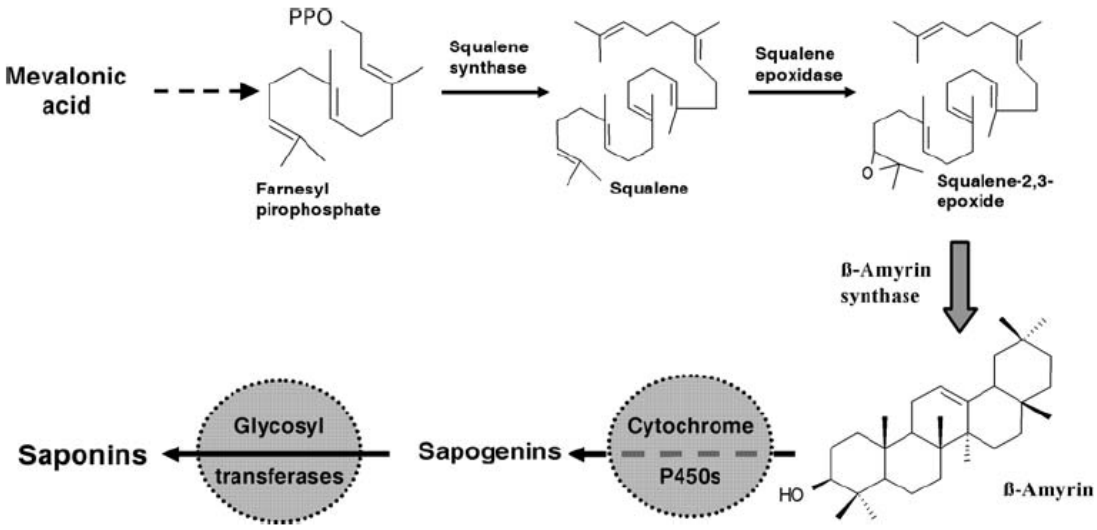


Figure 7 Biosynthetic pathway of triterpene saponins

1.1.5. Occurence and Distribution

Saponins are found in a wide variety of foods including asparagus, beans, blackberries, peas, potatoes, sugar beet and tea (Dini et al., 2001a). They occur in many different plant families, as evidenced by the isolation of saponins from phytochemical studies of many plant species over the years. Table 1 provides a list of species from which saponins have been isolated in the last 5 years (1998–2003). Although this list is not exhaustive, it does give a good indication of the plant species and families which have formed the focus of research in saponin chemistry in recent years. Many of these species have been chosen for phytochemical research based on ethnobotanical use. Of the roughly 200 species listed, 40% of the species were investigated based their traditional usage. Two triterpenoid saponins, eliciting the typical response in cancer cells, were isolated from the root bark of Becium grandiflorum (Lam.) Pichi-Serm. var. obovatum (E.Mey. ex Benth) Sebald (Lamiaceae) due to the traditional use of the plant (along with four others) for the treatment of breast and buccal cancer (Burger et

al., 1998). A further 10% of the species listed were selected based on previous ethnobotanical studies on related species either of the same genus or family. Allium nutans L. (Alliaceae), a wild species of onion, was investigated because of the many reports on the steroidal saponins from Allium species (Akhov et al., 1999). This approach to drug discovery has received renewed interest in recent years, and potentially increases the chances for the discovery of novel therapeutic agents (Fabricant and Farnsworth, 2001)

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. Triterpenoid saponins are found principally in dicotyledons while steroidal saponins occur in monocots. 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 .Young leaves contain more saponins than mature leaves, but foliage saponins were found to be less haemolytic than root saponins .Soyasapogenols are mainly concentrated in the axis of the seeds rather than in the cotyledons and seed coat. In the seedlings, the root (radicle) contains the highest concentration of soyasapogenol A, while the plumule has the greatest amounts of soyasapogenol B (Wina et al., 2005).

As mentioned earlier, steroidal saponins are almost exclusively found in the monocotyledonous angiosperms. This trend is confirmed by the presence of steroidal saponins in the monocotyledonous families of the Agavaceae, Alliaceae, Asparagaceae, Dioscoreaceae, Dracaenaceae, Liliaceae and Taccaceae. Interestingly, although the family Solanaceae is dicotyledonous, all the species studied contained steroidal saponins. Other exceptions include the presence of steroidal saponins in Aspilia montevidensis (Asteraceae), Balanites aegyptiaca (Balanitaceae), Trigonella foenum-graecum (Leguminosae) and Tribulus terrestris (Zygophyllaceae).

The Leguminosae have also been extensively investigated for saponins, in particular, species of Acacia, Albizia and *Astragalus*. Table 3 lists 23 species of the Leguminosae from which saponins have been isolated.

A list of plant species from which saponins have been isolated in recent years (1998–2003)

Family	Species	Saponin type	Reference
Leguminosae	*Acacia concinna* Wall.	Triterpenoid	Tezuka et al., 2000
	Acacia tenuifolia (L.) Willd.	Triterpenoid	Seo et al., 2002b
	Acacia victoriae Benth.	Triterpenoid	Hanausek et al., 2001
			Mujoo et al., 2001
			Jayatilake et al., 2003
	Albizia gummifera C.A.Sm.	Triterpenoid	Debella et al., 2000
	Albizia julibrissin Durazz.	Triterpenoid	Zou et al., 2000
	Albizia lebbeck Willd.	Saponin mixture	Une et al., 2001
	Albizia myriophylla Benth.	Triterpenoid	Yoshikawa et al., 2002
	Albizia procera Benth.	Triterpenoid	Yoshikawa et al., 1998
	Albizia subdimidiata (Splitg.) Barneby & J.W.Grimes	Triterpenoid	Abdel-Kader et al., 2001
	Astragalus kahiricus DC.	Triterpenoid	Verotta et al., 2002
	Astragalus trigonus DC.	Triterpenoid	Shaker et al., 2001
	Baptisia australis (L.) R.Br.	Triterpenoid	Udayama et al., 1998
	Gleditsia sinensis Lam.	Triterpenoid	Zhang et al., 1999b, 1999c, 1999d
	Gliricidia sepium (Jacq.) Walp.	Triterpenoid	Kojima et al., 1998
	Gliricidia sepium (Jacq.) Steud.		Rastrelli et al., 1999
	Lathyrus japonicus Willd. [= *Lathyrus maritimus* Bigel.]	Triterpenoid	Kang et al., 1998
	Lupinus oreophilus Phil.	Triterpenoid	Woldemichael et al., 2003
	Medicago sativa L.	Triterpenoid	Bialy et al., 1999
	Spartium junceum L.	Triterpenoid	Yeşilada and Takaishi, 1999
	Swartzia schomburgkii Benth. var. *schomburgkii*	Triterpenoid	Abdel-Kader et al., 2000
	Trifolium spp.	Triterpenoid	Oleszek and Stochmal, 2002
	Trifolium resupinatum L.	Triterpenoid	Simonet et al., 1999
	Trigonella foenum-graecum L.	Steroidal	Murakami et al., 2000a
	Vigna angularis (Willd.) Ohwi & H.Ohashi	Triterpenoid	Iida et al., 1999

Table 3 A list of Leguminosae species from which saponins have been isolated in recent years

1.1.6. Role in Plants

Generally, saponins are found in tissues that are most vulnerable to fungal or bacterial attack or insect predation. Therefore, one of their roles is to act as a chemical barrier or shield in the plant defense system. 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 and pupal stages, retarded growth, increased mortality, and decreased fecundity and fertility.Saponins also control rhizosphere bacteria in the soil (Wina et al., 2005).

Saponins are a diverse family of secondary metabolites that are produced by many plant species, particularly dicots. These molecules commonly have potent antifungal activity and their natural role in plants is likely to be in protection against attack by pathogenic microbes. They also have a variety of commercial applications including use as drugs and medicines. The enzymes, genes and biochemical pathways involved in the synthesis of these complex molecules are largely uncharacterized for any plant species. Cereals and grasses appear to be generally deficient in saponins with the exception of oats, which produce both steroidal and triterpenoid saponins. The isolation of genes for saponin biosynthesis from oats is now providing tools for the analysis of the evolution and regulation of saponin biosynthesis in monocots. These genes may also have potential for the development of improved disease resistance in cultivated cereals.(Anne E. Osbourn,2002)

1.1.7. Isolation and characterization

The developments during 2002–2005 in the methods used for saponin analyses in plant material are presented. There were number of papers published on isolation and identification of new saponins by chromatographic techniques. Some new developments can be found in separation techniques or solid and

mobiles phases used. Separation of individual saponins is still complicated and time consuming. This is due to the fact that in most of the plant species saponins occur as a multi-component mixture of compounds of very similar polarities. Thus, to isolate single compound for structure elucidation or biological activity testing, a combination of different chromatographic techniques has to be used, e.g. first separation of the mixture to simpler sub-fractions on reversed phase C18 has to be followed by further purification on normal phase Silica gel column. Especially difficult is determination of saponins in plant material as these compounds do not posses chromophores and their profiles cannot be registered in UV. Most HPLC methods apply not only specific registration at 200–210 nm, but these methods are not applicable for determination of many saponins in plant material at levels lower than 200–300 mg/kg. Some new or improved techniques for quantification of saponins in plant material were published in reviewed period. These include further progress in the application of evaporative light scattering detection (ELSD) for saponin profiling and quantification, which is also not only specific but also more sensitive in comparison to 200–210 nm detection. Some progress in development of new applications for liquid chromatography-electrospray mass spectrometry (LC/ESI/MS) for saponin determination has also been done. This method gives highest sensitivity and on line identification of separated saponins and should be recommended for specialized analyses of extracts and pharmaceutical formulas like the validation of a new assay. From non-chromatographic techniques for saponin determination, a sensitive and compound specific ELISA tests for some saponins were developed.(W. Oleszek , Z. Bialy, Journal of Chromatography A, 1112 (2006) 78–91)

1.1.7.1. Choromatograghy

Non-chromatographic techniques of saponin analysis

Immunoassays which use monoclonal antibodies (MAbs) against drugs and low molecular weight natural bioactive compounds are becoming an important

tool in saponin analyses. They show high specificity and sensitivity for receptor bindinganalyses, enzyme assays and qualitative and quantitative analytical techniques. They find an application in new Eastern blotting and in immuno-affinity column chromatography, for diagnosis, therapy and drug monitoring. Enzyme-linked immunosorbent essay (ELISA) based on MAbs are in many cases more sensitive than conventional HPLC methods. Number of MAbs were also developed for saponins.

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.

The qualitative analysis of saponins by TLC is of great importance for all aspects of saponin investigation. TLC plates (usually silica gel) can handle both pure saponins and crude extracts, are inexpensive, rapid to use and require no specialized equipment. The detection reagents most generally applicable to all types of saponins and sapogenins, and therefore the most widely used, are the vanillin-sulphuric acid (Godin reagent), antimony(III) chloride (Carr-Price reagent), anisaldehyde-sulphuric acid and Liebermann-Burchard reagents. Spraying with vanillin-sulphuric acid in the presence of ethanol and perchloric acid gives a blue or violet coloration with triterpene saponins. With anisaldehyde-sulphuric acid, a blue or violet coloration is produced on heating the TLC plate. Spraying TLC plates with a solution of cerium sulphate in sulphuric acid gives violet-red, blue or green fluorescent zones under 365 nm UV light. Steroid saponins can be identified by their characteristic colour reaction with antimony (III) chloride. Occasionally, simply spraying the plates with water is sufficient to reveal the saponins present.

Vacuum liquid chromatography

The tecnique 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).

Low-pressure column chromatography (LPCC) for isolation of saponins

A preparative separation of individual saponins for structure determination and biological activity evaluationwas performed by combinations of low-pressure liquid chromatography For this purpose proper careful selection of stationary and mobile phases was essential for successful work. There were still a limited number of possibilities as regard to stationary phases. Most frequently, the first step of crude extract separation employed Sephadex LH-20 molecular filtration, which allowed preliminary separation of complex matrix of the extract into saponins and into accompanying impurities.(G.M. Woldemichael, G. Montenegro, B.N. Timmermann, Phytochemistry 63 (2003) 853.) In some cases during this preliminary separation, simultaneous partition of mixtures of saponins into simpler subfractions could also be achieved. Similar goal could also be accomplished by passing water solution of plant crude extract trough a porous polymer gel column Diaion HP-20 (synthetic polyaromatic adsorbent with pore volume 1.3 ml/g, surface area 500m2/g, pore radius >200).(F.R. Melek, T.

Miyase, N.S. Ghaly, Phytochemistry 62 (2003) 557). Washing this column with water removed some impurities and adsorbed saponins which were eluted with MeOH. Plant extract could also be separated to different classes of phytochemicals by selective solid phase extraction/fractionation on RP-18 short column(I. Kapusta, A. Stochmal, A. Perrone, S. Piacente, C. Pizza, W. Oleszek, W.J. Agric. Food Chem. 53 (2005) 2164.)

Next step of saponin isolation includes column chromatography (CC) on silica gel with mobile phase composed of different combinations of $CHCl_3$–MeOH–H_2O and EtOAc–MeOH–H_2O, or on reversed phase silica gel RP-18, with mobile phase composed of MeOH–H_2O or MeCN–H_2O and their acidified (HOAc, TFA) modifications. In most cases, one column separation was not efficient enough and combination of normal and reversed phase separation were needed. Final purification could also be accomplished using HPLC systems with normal or reversed phase columns, but this procedure was rather tedious and did not allow to obtain bigger amounts of pure saponins.

High-performance liquid chromatography (HPLC)

Chromatography by hplc (high-performance liquid chromatography) is a powerful technique for obtaining multi-miligram 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 soyasapogenols a and b, separated on C18 column with MeCN–PrOH–H_2O–HOAc (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)

1.1.8 *Astragalus* Genus

The world *Astragalus* is derived from two Greek words “Astrone” means a star and “Gala” means milk, for the belief that the presence of *Astragalus* plants in grass-land improved milk yield of livestock (Verotta et al., 2001).

Astragalus L. (Leguminosae) is a genus widely distributed throughout the temperate regions of the world, located principally in Europe, Asia and North America. About 2000 species have been described, 372 of them in North America and 133 in Europe. Forty two of the European species are located in the Iberian peninsula. The European species were classified in nine subgenera: Trimeniaeus Bunge, Epiglottis (Bunge)Willk., Hypoglottis Bunge, Phaca (L.) Bunge, Caprinus Bunge, Tragacantha Bunge, Calycophysa Bunge, Cercidothrix Bunge, and Calycocystis Bunge. Recent views of thetaxonomy of *Astragalus* include Tietz and Zarre (1990);Wenninger (1992) on section Chlorostachys (including thesections Sesbanella, Diplotheca, Phlebophace, Coluteocarpus , and Robusti), the section Phyllobium (including thesections Bibracteolati and Dolochochaete), and the section Skytropos; Podlech‘s (1990) on the section Platyglottis, and Liston’s (1990) on the section Leptocarpi. (Rios, and Waterman., 1997).

1.1.8.1 Medicinal and Biological Properties

Several *Astragalus* species are used as medicinal plants, especially A. membranaceus, which is the one most widely used and studied. It is officially listed in the Chinese Pharmacopoeia, as the species A. complanatus and A.mongholicus (A. membranaceus var. mongholicus). Radix Astragali is the dried roots of *Astragalus* membranaceus (Fisch.) Bge. or A. meinbranaceus var. mongholicus (Bge.) Hsiao, belonging to the non-toxic medicine. It can strenghthen general health and it is used as a common tonic for vital energy (Qi) and Yin. The "sweet" and "warm" drug enters the lung and spleen meridians. Its functions are to replenish vital energy and cause Yang to ascend, to benefit vital energy and stabilize the exterior, to remove toxins, to promote healing and water metabolism and to reduce edema. Recent pharmacological studies have shown that radix Astragali can inhibit the biosynthesis of glycogen and dilate coronary as well as renal vessels. It also serves as a cardiac tonic, diuretic and antibacterial agent. More interestingly, it possesses an antiinfluenza action and may prevent respiratory infections. Both effects are believed to be related to the enhancement of immunological functions.

Saponins occur in numerous herbal remedies--both oriental and occidental—such as liquorice, sarsaparilla and ginseng (Shibata, 1977). In general they seem to have expectorant and antiinflammatory properties, but probably the most significant property of saponins, considered as dietary components for humans, is their effect on plasma lipid concentrations.

1.1.8.2 Toxicity of saponins

Before proceeding to feeding trials with humans or recommending saponins as a prophylactic to reduce the risk of heart disease it is clearly essential to know

something about both the short term and the chronic toxicity of saponins. This is a complex problem because saponins are a class of compounds, not a single compound.

The Merck Index (1976) states that saponins are practically non-toxic to man upon oral ingestion. Plant extracts, such as liquorice and sarsaparilla, are permitted as food flavourings in Australia (National Health and Medical Research Council, 1976), Britain (George, 1965) and the USA (George, 1965). Quillaia, a commercial preparation of saponin from quillay bark (Quillaia saponaria), is permitted for food use in Britain and 'generally recognised as safe' (GRAS) in the USA (George, 1965). (The levels in use in the USA are given in Table 4 and in Britain quillaia extract is permitted in soft drinks at not more than 200ppm under the Emulsifiers and Stabilisers in Food Regulations 1975 Act (Statutory Instrument 1975, no. 1486)). There is clearly some uncertainty about the safety ofsaponins, though, since quillaia

is prohibited as a food additive in West Germany (George, 1965) and all saponins are prohibited in foodstuffs in Spain and Morocco (George, 1965). Neither Britain nor the USA has legislation controlling the use of saponins as a class, presumably because of the considerable variation in the toxicity of different saponins. The oral toxicity of saponins from a number of plant sources is given in Table 10. The lethal dose varies from 25 to 3000 mg/kg body weight. Saponins normally remain within the digestive tract (Birk, 1969) but in severe poisoning gastrointestinal lesions may occur, allowing saponins to enter the blood stream. It is when saponinsenter the blood stream that damage occurs (the intravenous lethal dose can be one thousandth of the lethal oral dose (George, 1965)). The result is liver damage, haemolysis of red blood cells, respiratory failure, convulsions and coma (Martindale, 1972). Irritation of the gastrointestinal tract by some other cause may increase an animal's susceptibility to saponin poisoning (Ewart, 1931) and it has been suggested that continued ingestion of sublethal doses of saponins can lead to 'corrosion of the intestine' so that saponins eventually enter the blood stream (Solman, 1957). Ewart (193 l) advised that people using drastic purgatives or having inflamed intestines or cirrhotic livers should avoid all foods containing saponins, whether of natural or artificial origin.

ORAL TOXICITY OF SOME SAPONINS[a]

Source of saponin	*Animal*	*Dose*	*Dosage (mg/kg)*	*Reference*
Sapindus sapindus	mouse	LD	3000	(Spector, 1956)
	mouse	LD	1000	(Spector, 1956)
Agrostemma githago (common corn cockle)	rat	LD_{50}	>50	(Vogel & Marek, 1962)
	rabbit	LD	56–62	(Spector, 1956)
	dog	LD	20–25	(Spector, 1956)
Saponaria vaccaria	mouse	LD_{50}	960	(Abubakirov *et al.*, 1960)
Aesculus hippocastanum (horse chestnut)	rat	LD_{50}	>50	(Abubakirov *et al.*, 1960)
Hedera helix (ivy)	rat	LD_{50}	>100	(Abubakirov *et al.*, 1960)
Gypsophila paniculata	rat	LD_{50}	50	(Vogel & Marek, 1962)
Cyclamen europaeum	rat	LD_{50}	>160	(Vogel & Marek, 1962)
Digitalis purpurea (fox glove)	rat	LD_{50}	>50	(Vogel & Marek, 1962)
	mouse	LD	90	(Spector, 1956)

[a] A condensed version of data compiled by George (1965).

Table 4 Toxicity of some saponins

Unfortunately these are all relatively short term studies. One long term toxicity study has recently been reported (Phillips et al., 1979). Quillaia saponin at the level of 0.5~ of the diet had no adverse effects on mice over an 84 week period. Martindale (1972) states that quillaia saponin is too irritant to the gastrointestinal tract to be used internally and most pharmacopoeias advise against the internal use of saponins (George, 1965). The chronic toxicity of saponin would have to be examined further before saponins could be recommended for human consumption as a means of lowering the serum cholesterol level. An exception would be saponins consumed in the form of a traditional foodstuff such as soybeans, consumed in 'normal' quantities.

There seems little doubt, though, that the saponins from soybeans, lucerne, or quillaia are safe for short term human feeding trials at levels of below 50 mg/kg body weight--about 3 g/day.

2. MATERIAL AND METHODS

2.1. General

In Spectroscopic identifications, optic rotations were measured using a Perkin Elmer 341 Model Polarimeter. The NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 500 MHz (^{1}H) and 125 MHz (^{13}C) in C_5D_5N using TMS as internal standart. Column chromatography was carried out an Silica gel 60 (Merck 7734) and Li Chroprep RP (C-18, Merck 9303). 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 lamb by using Desaga Uvis. The spots were detected by 20 % H_2SO_4 /water spraying reagent onto the TLC plates followed by heating the plates to 110 ^{o}C until the spots become visible.

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

I	CH_3Cl:MeOH	90:10
II	CH_3Cl:MeOH:H_2O	80:20:2
III	CH_3Cl:MeOH:H_2O	75:25:2.5
IV	CH_3Cl:MeOH:H_2O	70:30:3
V	CH_3Cl:MeOH:H_2O	61:32:7

VI	CH_3Cl:MeOH:H_2O	64:50:10
VII	MeOH:H_2O	8:2
VIII	MeOH:H_2O	7:3
IX	MeOH:H_2O	6:4
X	MeOH:H_2O	5:5
XI	MeOH:H_2O	4:6
XII	MeOH:H_2O	2:8

2.2. Plant Material

Astragalus strictipinus was collected from …….in……Plant material was identified by Serdar G. Senol (Department of Biology, Faculty of Sciences, Ege University, Izmir, Turkey). A voucher specimen was deposited in the Herbarium of Ege University, Izmir, Turkey (EGE ……)

2.3. Isolation and Purification

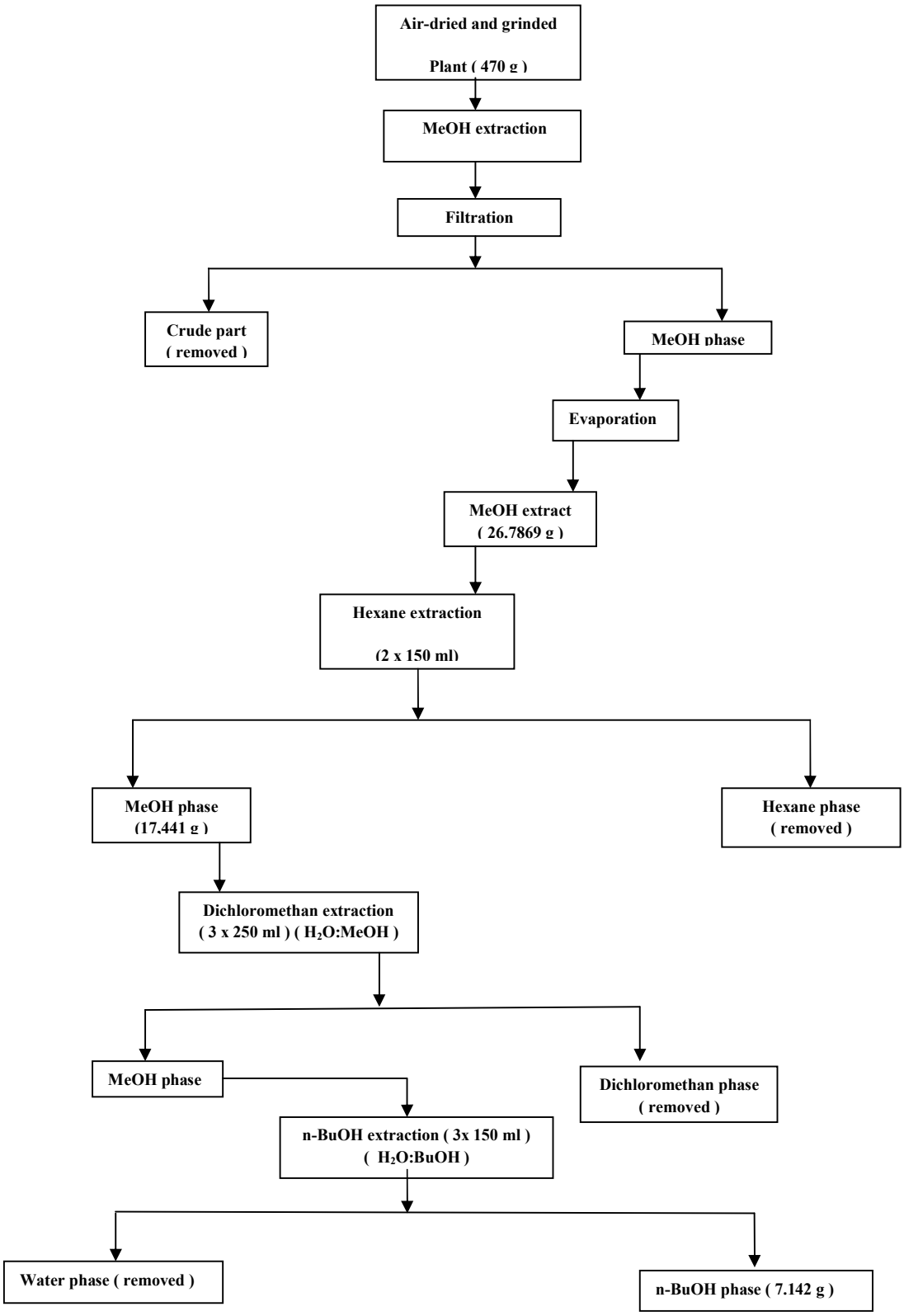
Air-dried and grinded plant (470 g) were extracted with MeOH (2x3 L) at 60 ^oC. After filtration, the solvent was removed by rotary evaporation yielding 26.7869 g of extract. The MeOH extract was dissolved in H_2O (200 ml), and successively partitioned with n-hexane (2x200 ml), CH_2Cl_2 (3x200 ml), and n-BuOH saturated with H_20 (3x150 ml) **(scheme 2.1)**.

The n-BuOH extract (7.142 g) was subjected to vacuum liquid chromatograpy (VLC) using reversed-phase material (Lichroprep RP-18, 25-40 μm) employing H_20 (600 ml), H_2O-MeOH (8:2, 1150 ml; 6:4 , 3250 ml; 4:6, 2800 ml; 3:7, 3150 ml; 2:8, 2400 ml) and MeOH (1600 ml) to give 118 main fractions(1-118). **(Scheme 2.1)**First of all, Fractions 66-72 (162 g) that rich in saponins were combined and applied to an open column chromatograpy using Silica gel (25 g) as stationary phase. Elution performed with CH_3Cl-MeOH-H_2O (80:20:2, 3200 ml) and CH_3Cl-MeOH-H_2O (70:30:3, 2600 ml) mixtures to yield 860 main fractions. Fractions 38-43 gave compound **1** (9 mg), Fractions 90-100 gave compound **2** (2 mg), Fractions 150-220 gave compound **3** (32 mg) and Fractions 410-495 gave compound **4** (24 mg). Secondly, Fractions 72-80 eluted with H_2O:MeOH (4:6) from VLC-RP also were rich in saponins. These were combined (285 mg) and applied to Silica gel (75 g). Elution was started with CH_3Cl:MeOH:H_2O (80:20:2) to yield 320 main fractions.Fractions 150-220 gave compound **5 (**146 mg) .

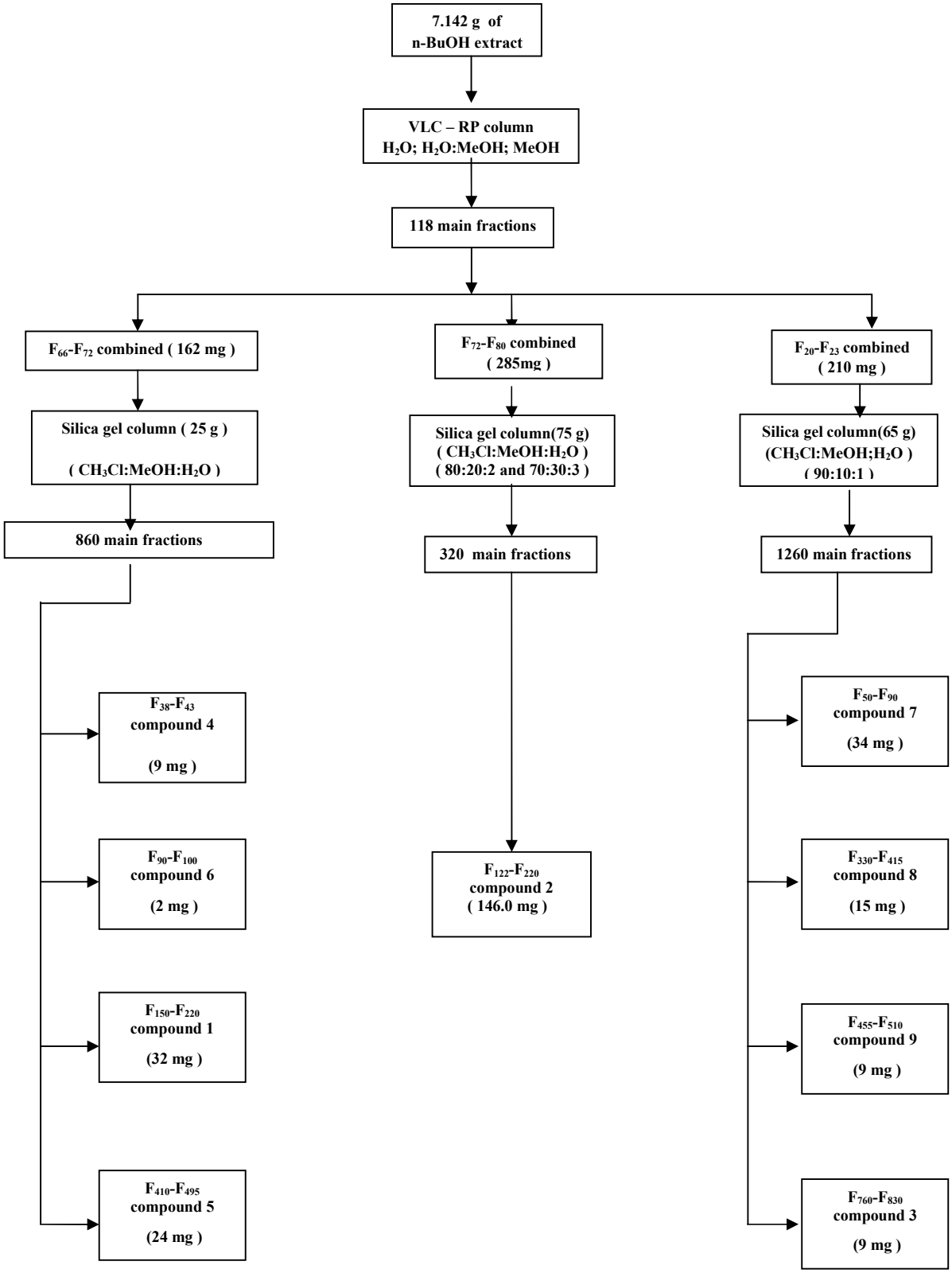
Lastly, Fractions 20-23 eluted with H_2O:MeOH (8:2) from VLC-RP also were rich in saponins. These were combined (210 mg) and applied to Silica gel (65 g). Elution was started with CH_3Cl-MeOH-H_2O (90:10:1 ; 5250 ml) to yield 1260 main fractions.

Fractions 50-90 gave compound **6** (34 mg), fractions 330-415 gave compound **7**

(15 mg), fractions 455-510 gave compound **8** (9 mg) and fractions 760-830 gave compound **9** (9 mg).**(Scheme 2.2)**



Scheme 2.1 Extraction of *Astragalus* Strictipinus



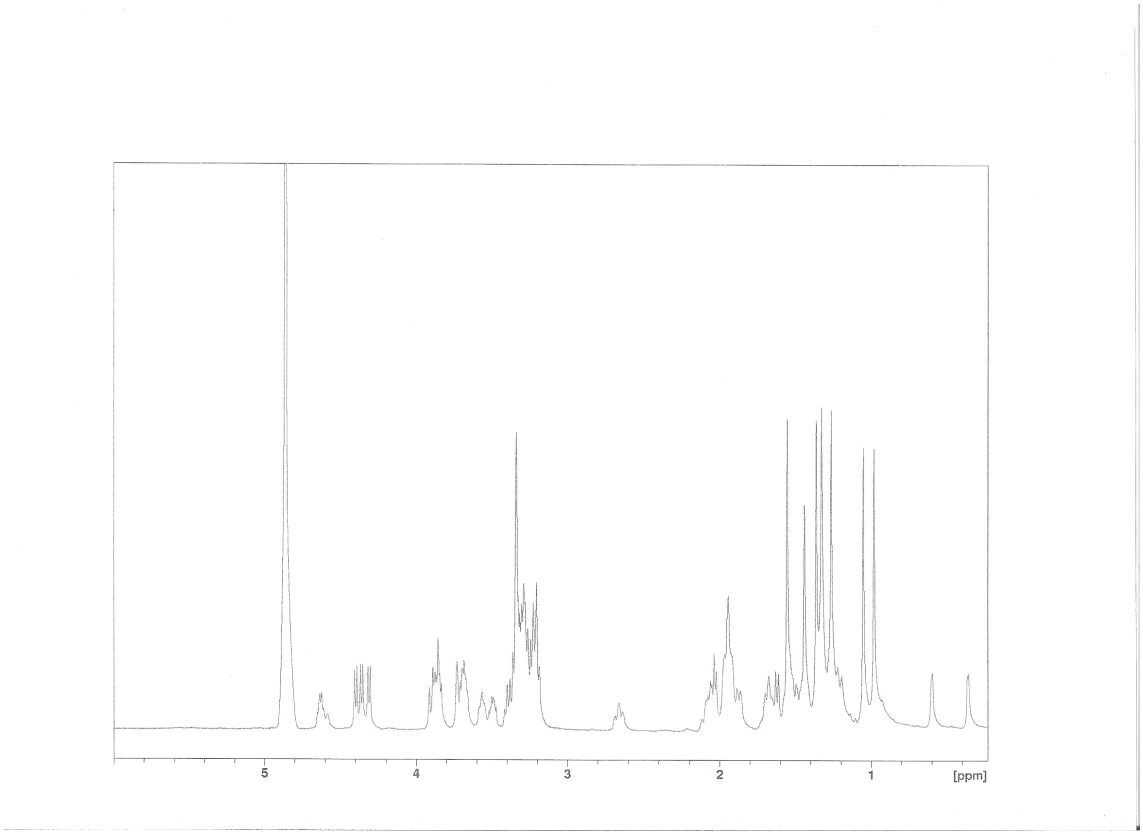
Scheme 2.2 Isolation of compounds from 1 to 9

3. RESULT AND DISCUSSIONS

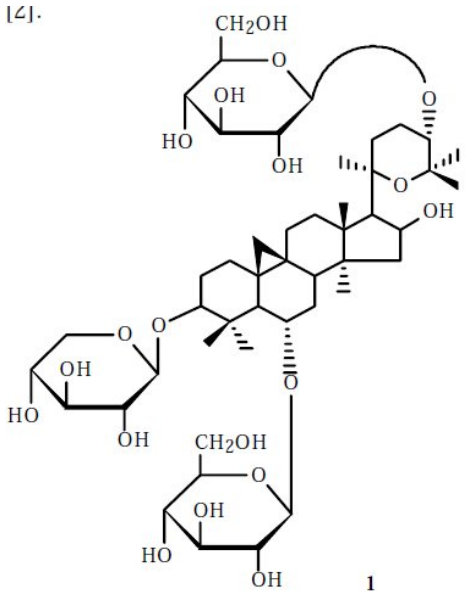
3.1 Structural Identification of Compound 1(*Cyclotrisectoside*)

Signals of tertiary C atoms bonded to an O atom in the J-modulated ^{13}C NMR spectrum of **1** were observed at δ78.62 and 74.62 and belonged to C-20 and C-25. This indicated that a 20*R*,25-epoxy was present. A multiplet at δ 4.75 in the PMR spectrum and a doublet at δ74.14 in the ^{13}C NMR spectrum of this same glycoside indicated that it contained a 16*β*-hydroxyl. A doublet for the resonance of H-17 at δ 2.09 confirmed this conclusion. One of the H-22 protons in the PMR spectrum of **1** resonated at δ 2.98, like in spectra of cyclocephalogenin glycosides. This indicated that C-24 in **1** was singly bonded to an O atom and that C-24 had the *S*-configuration. This C atom gave a signal at δ 79.50, proving that it was glycosylated. Atom C-24 of cyclodissectoside and cyclocanthoside F had a free hydroxyl and resonated in the ^{13}C NMR spectra at δ 68.77 and 68.84, respectively. In fact, the chemical shift of the anomeric C atom of a single D-glucopyranose is δ 100.67 and confirms that the hexose was located on C-24. Taking into account the chemical shifts of anomeric C atoms of the remaining monosaccharides, *β* -D-xylopyranose (δ 107.67) and b-D-glucopyranose (δ 105.01) , and biogenetic considerations that six cycloartane glycosides with a C-3 D-xylose, the C-1 atom of which resonates in the range δ 107.38-107.65 , have been isolated from *A. dissectus*, it can be concluded that the pentose was located on C-3 and the hexose on C-6.

Thus, the new glycoside **1**, which we called cyclotrisectoside, was a trisdesmoside trioside of cyclocephalogenin with the structure 20*R*,25-epoxy-24*S*-cycloartan-3 *β*,6α,16 *β*,24-tetraol 3-*O*- *β* -D-xylopyranoside-6,24-di-*O*- *β* -D-glucopyranoside.



^{1}H NMR spectra of *Cyclotrisectoside*



1

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

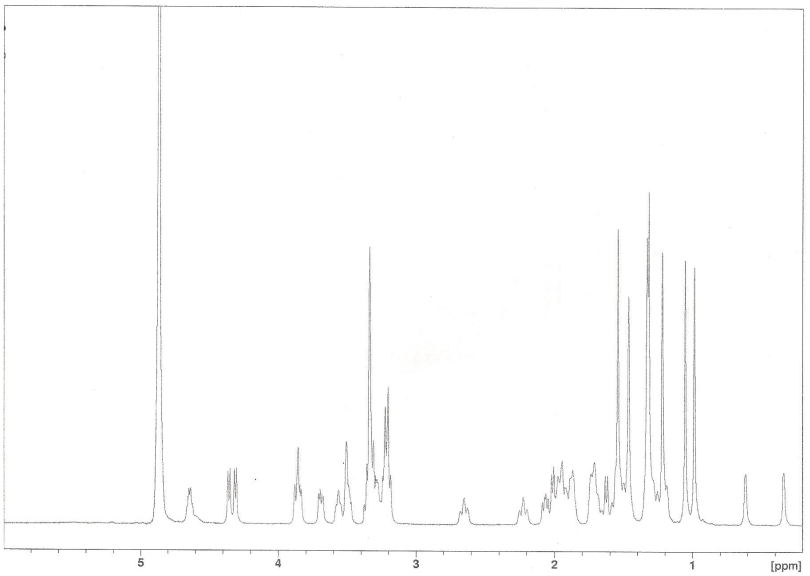
3.2 Structural Identification of Compound 2(*Cyclocephaloside* I)

The IR spectrum of **2** showed hydroxyl absorption bands. The FABMS of **2** exhibited quasimolecular ion peaks at *m/z* 807 $[M + Na]^+$ and at *m/z* 1569 $[2 M + H],^+$ which are compatible with the molecular formula $C_{41}H_{68}O_{14}$. The ^1H NMR spectrum of **2** showed characteristic signals due to cyclopropane-methylene protonsas an AX system (δ 0.16 and 0.54, AX system, J_{AX} = 4.0 Hz; H2-19) and seven tertiary methyl groups. Additionally, the resonances for two anomeric protons were observed at δ 4.80 (d, *J* = 7.4 Hz) and 4.87 (d, *J* = 7.6 Hz). Thus, compound **2** was considered to be a cycloartane- type triterpene diglycoside. This observation was supported by the ^{13}C NMR spectral data of **2**. The NMR signals were analyzed by the use of COSY and TOCSY coupled with HMQC. The ^1H and ^{13}C NMR data supported the assignment of the sugar moieties in **2** as *β* -D-xylopyranose and *β* -D-glucopyranose.

The remaining carbon and proton resonances were consistent with $C_{30}H_{50}O_5$ for the aglycon moiety. This implied six saturated ring systems because there were no olefinic protons. Additional functionalities on the aglycon included four geminal methine protons on oxygen-bearing carbon atoms (H-3, H-6, H-16, and H-24). The resonances for the oxygenated carbons also indicated the presence of four oxymethine carbons (δ 88.6, 79.5, 74.0, 68.7; C-3, C-6, C-16, and C-24, respectively) and two oxygenated quaternary carbons (δ 78.9 and 75.2, C-20 and C-25, respectively). To clarify the intermolecular connectivities of the partial structures in **2** HMBC was used. By the help of this experiment, not only connectivities but also interglycosidic linkages were revealed. These data suggested the presence of the partial structure, a monohydroxypyran derivative. The carbon resonances assigned to the side chain consisting of a doublet (δ 68.7, C-24), two triplets (δ 26.3 and 26.7, C-22 and C-23, respectively), two singlets (δ 78.9 and 75.2, C-20 and C-25, respectively), and three quartets (δ 28.8, 28.6, and 28.0; C-21, C-26, and C-27, respectively). The HMBC correlations from C-17 (to Me- 18 and Me-21), C-20 (to H-17 and Me-21), C-22 (to H-17 and Me-21), C-24 (to Me-26 and Me-27), and C-25 (to Me-26) confirm this proposal The relative

stereochemistry of **1** was established based on ROE data from a 500-ms ROESY experiment. The stereochemistry of the ring fusions and at the substituents could be unambiguously determined from ROE involving diaxial (1,3) correlations. Figure 1 displays the correlations that were used to elucidate the relative stereochemistry. Consequently,the structure of **1** was established as 20,25-epoxy-3*â*- (*β* -D-xylopyranosyl)oxy-6R-(*β* -D-glucopyranosyl)oxycycloartane- 16 *β*,24α-diol, for which the trivial name

cyclocephaloside I is proposed.



^{1}H NMR spectra of *Cyclocephaloside* I

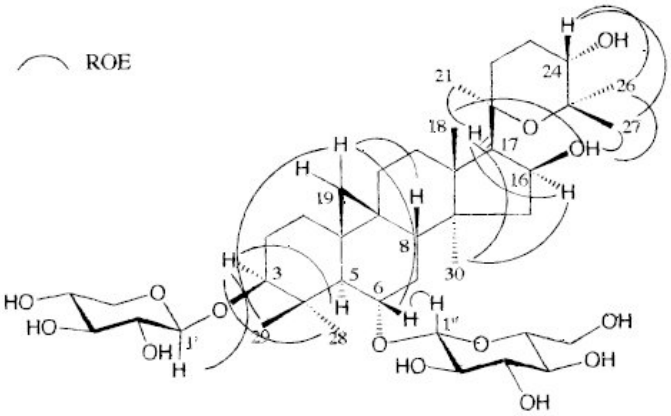


Figure 1. ROE correlations observed for compound **1**.

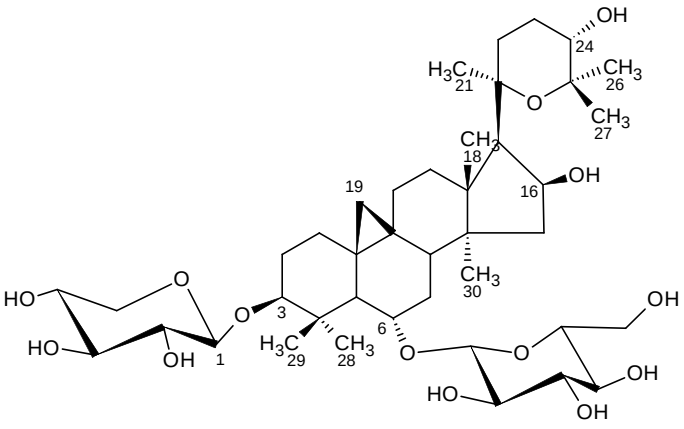
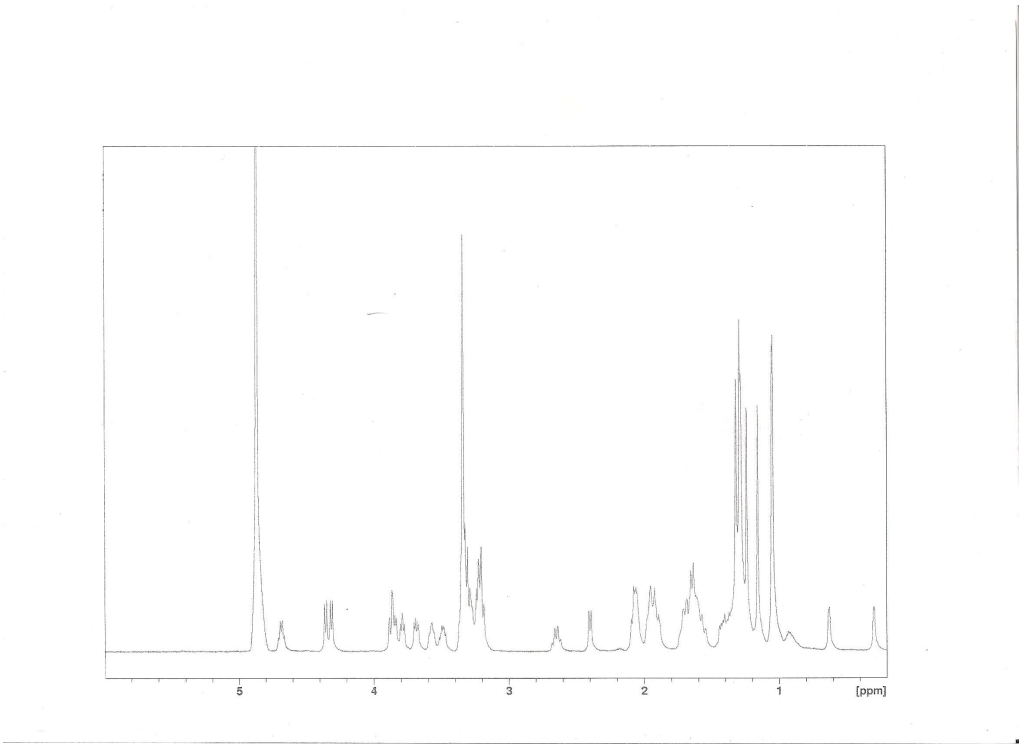


Figure 3.2. Structure of Compound 1 **(*Cyclocephaloside I*)**

3.3 Structural Identification of Compound 3(*Astragaloside* IV)

Compound was obtained as colourless needles from methanol. The NMR spectra of compound were characteristic of cycloartane glycosides.The ESI/MS of the compound exhibited the quasi-molecular ion peak at m/z 807 [M+Na]+, which is well-matched with the molecular formula C41H68O14 .The 1H NMR spectrum showed signals typical of cyclopropane–methylene protons, such as an AX system at δ 0.28 and 0.60 (each d, J_{AX}=4 Hz, H2-19) and seven tertiary methyl groups (δ 0.95, 1. 27, 1.30, 1.31, 1.39, 1.43, 1.60; H-30, H-27, H-21, H-26, H-18, H-28 and H-29, respectively) . Additionally, the resonances for two anomeric protons were observed at δ 4.87 (d, J=4.70 Hz) and 4.82 (d, J=4.60 Hz). Thus, compound 3 was considered to be a cycloartane triterpene diglycoside. The NMR signals were analysed by the use of 1D and 2D NMR. Inspection of the ^{13}C NMRdata showed 41 signals, 30 of which were attributed to a triterpenic aglycone and the remaining 11 resonances indicated the presence of pentosyl and hexosyl moieties. The^{1} H and ^{13}C NMR data supported the assignments of the sugar moieties as *β*-xylopyranose and *β* -glucopyranose. The xylose group was assigned to C-3 and the glucose group was assigned to C-6 by low-field signals at δ 88.6 and δ 79.4, respectively. On the basis of COSY, HSQC and HMBC spectra, all signals were assigned . Accordingly, the aglycon of compound 3 was identified as a cycloastragenol. Consequently, by spectral data and comparisons with previous reports, the structure of compound 3 was established as astragaloside IV (Bedir et al., 1998; Özipek et al., 2005; Tabanka, Bedir, Alankus-Caliskan, & Khan 2005; Yao et al., 2000). Astragaloside IV has been previously identified in *Astragalus membranaceus* and some other *Astragalus* species, and is well-known for its immunoregulatory, antiinflammatory and antifibrotic actions (Du et al., 2008). It has also been commonly used for the treatment of cardiovascular diseases (Zhang et al., 2006). Thus, the identification of this bioactive constituent in the roots of A. *caspicus* opens new perspectives on its probable pharmacological application.



^{1}H NMR spectra of *Astragaloside* IV

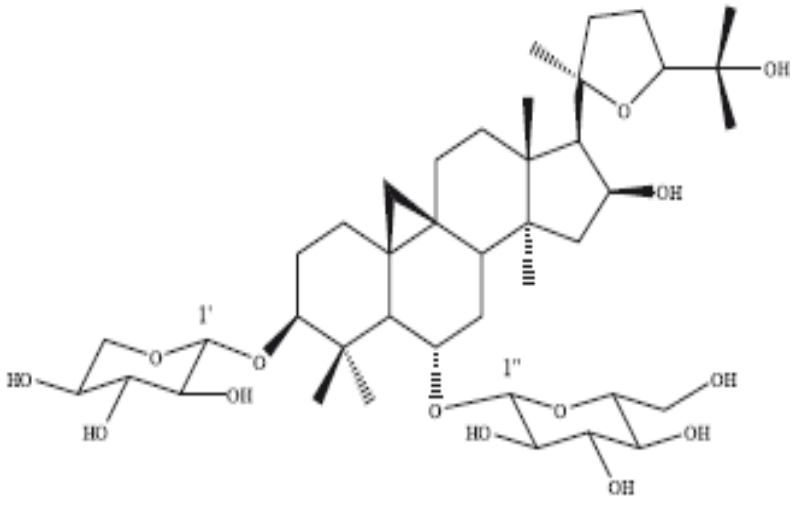


Figure 3.3. Structure of Compound 3 **(***Astragaloside IV***)**

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