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**QUALITY CONTROL OF *CISTUS INCANUS*
CONTAINING PHARMACEUTICAL
PREPARATIONS**

**By
Hiba Hani Mohammed Ali Al-Sheikh Hamed**

**A thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of Master of Science**

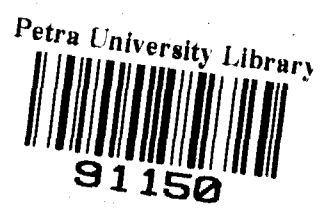
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Amman- Jordan

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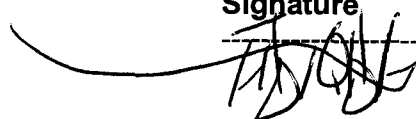
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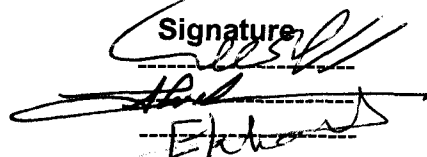
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Abstract
QUALITY CONTROL OF CISTUS CONTAINING
PHARMACEUTICAL PREPARATIONS

by

Hiba Hani Mohammed Ali Al-Sheikh Hamed

Petra University, 2009

Under the supervision of Dr. Fadi Q´adan

Cistus incanus L. (Cistaceae), a shrub widely distributed in the Mediterranean area, is traditionally used in various skin diseases, Rheumatism, fever, diarrhea, anti-inflammatory agents, antimicrobial, antiviral and antitumor, while in Jordan traditionally used for the treatment of gout. Recent research in Turkey shows that, of seven plants used as folk remedies for ulcers, the one with the greatest efficacy was *Cistus incanus*.

Several polyphenols were isolated from the air-dried herb material of *Cistus incanus* and were characterized. Among the flavan-3-ols as; catechin, galocatechin, epicatechin, epigallocatechin, epicatechin-3-*O*-gallate and epigallocatechin-3-*O*-(4-hydroxybenzoate) were isolated. The presence of the

dimeric prodelphinidins such as epigallocatechin-(4 β →8)-epigallocatechin, epigallocatechin-3-*O*-gallate-(4 β →8)-epigallocatechin, and epigallocatechin-(4 β →6)-epigallocatechin-3-*O*-gallate were also reported. This knowledge is of great importance for being interested in *Cistus* herb due to their role in the traditional use as antiviral, antibacterial, anti-inflammatory agents and antioxidant activity of the extract.

Reviewing the available data about the phenolic composition of *Cistus* extract indicate high medicinal importance of *Cistus* containing products, and since then up to now few studies have been carried out using *Cistus* extract, the present work developed two formulations, liquid dosage form and capsule from *Cistus incanus* extract and accelerated stability studies were done on them according to ICH for preclinical studies to ensure quality of final *Cistus* extract products has to go through battery of quality control parameters. A proper integration of physical, chemical, microbial, spectral and chromatographic analysis will ensure proper analysis of *Cistus* herb by comprising of organoleptic, microscopical, physical, chemical, chromatographic testing.

To
My Family

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I respectfully express my sincere and deepest gratitude to my supervisor Dr. Fadi Qa'dan for supervising this work. His wide knowledge and his logical way of thinking have been of great value for me. His understanding, encouraging and personal guidance have provided a good basis for the present thesis.

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Hiba, Jordan, Dec. 2009

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CHAPTER ONE

INTRODUCTION

Chapter One

1. Introduction

1.1. Cistaceae

The Cistaceae, a medium-sized family, consists of eight genera and 180 species (herbaceous plants and shrubs). The common name is rockrose. In open areas on poor soils Cistaceae occur. Distributed in temperate and subtropical regions of the northern hemisphere, Cistaceae show the highest genus and species in the Mediterranean region. Five of the eight genera (*Cistus*, *Fumana*, *Halimium*, *Helianthemum*, *Tuberaria*) are native to this region while the remaining three (*Crocanthemum*, *Hudsonia*, *Lechea*) inhabit temperate regions in America (Guzmán and Vargas, 2006).

The taxonomy of Cistaceae has been based on vegetative and reproductive characters. Phylogenetic relationships among Cistaceae genera indicate that *Cistus* is related to *Halimium* and *Helianthemum* (Guzmán and Vargas, 2005; Guzmán and Vargas, 2009).

1.2. *Cistus* genus

The genus *Cistus* is one of the most characteristic Genera containing about twenty species (Table1) commonly found in the Mediterranean region from Morocco and Portugal through to the Middle East, and also on the Canary Islands which are perennial shrubs found on dry or rocky soils (Fig. 1.1.). The history of this plant starts already in the 4th century before Christ (Guzmán and Vargas, 2005; Attaguile et al., 2006; Bouamama et al., 2006).

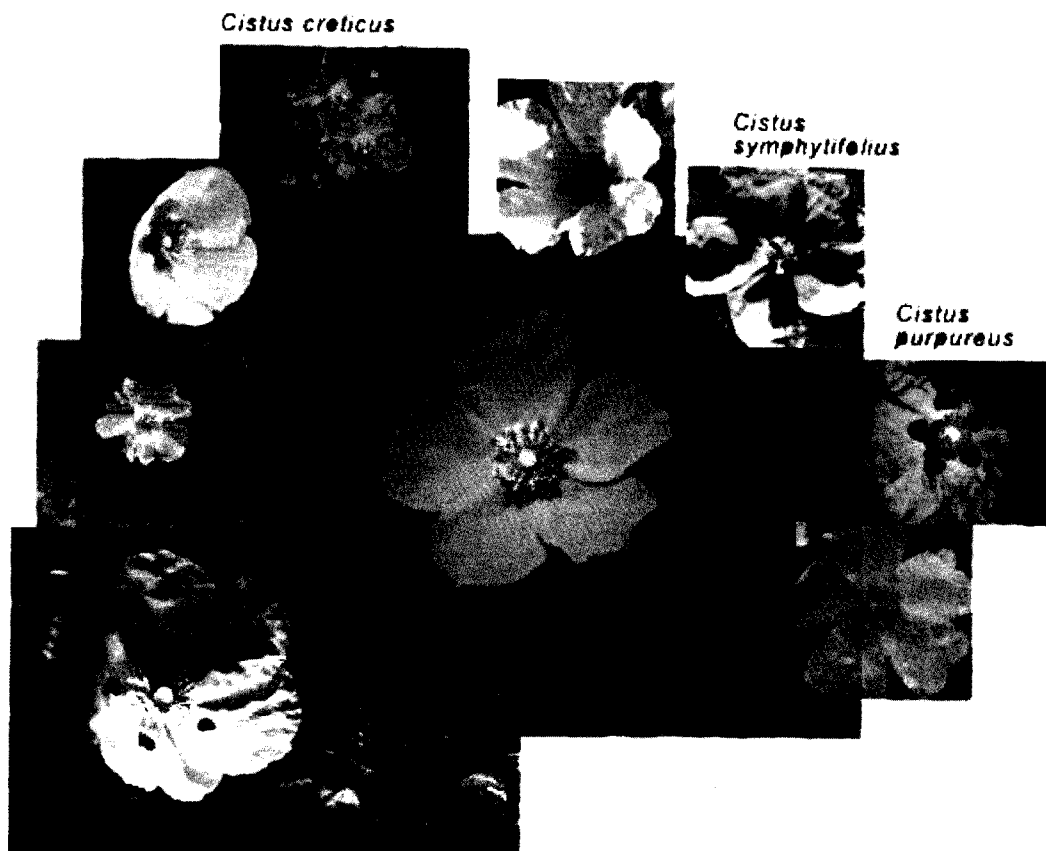


Figure1.1. Cistus species

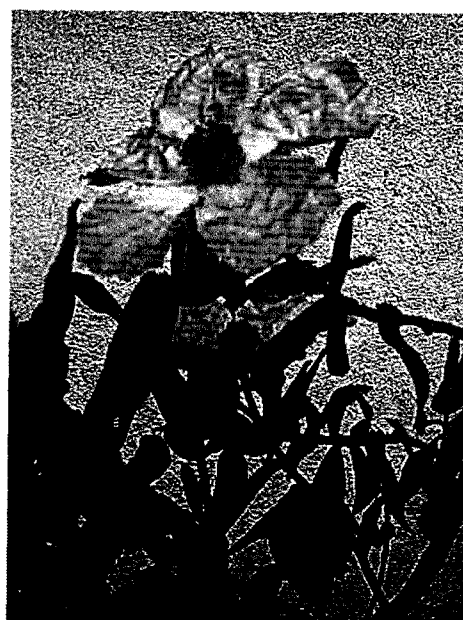


Figure1.2. Cistus incanus

Table 1.1. some *Cistus* species

<i>Cistus</i> species	Origin
<i>Cistus incanus</i>	Mediterranean region, Greece, Turkey, Jordan
<i>Cistus creticus</i>	Italy, Lebanon, Syria, Turkey, Albania, Bulgaria
<i>Cistus salviifolius</i>	France, Spain, Libya, Morocco, Tunisia, Albania, Bulgaria, Greece, Italy, Croatia
<i>Cistus parviflorus</i>	Turkey, Greece, England
<i>Cistus monspeliensis</i>	Spain, north Africa, south Europe, Balearic Islands, Malta, Albania, Croatia
<i>Cistus clusii</i>	Spain, Italy, Algeria, Tunisia
<i>Cistus populifolius</i>	Spain, France, Morocco, Portugal
<i>Cistus chinamadensis</i>	Canary Islands
<i>Cistus ladanifer</i>	California, The Canary Islands, Spain
<i>Cistus heterophyllus</i>	Spain, Iberian, North-West Africa
<i>Cistus laurifolius</i>	Southwestern Europe, Turkey
<i>Cistus psilosepalus</i>	Portugal, West Spain
<i>Cistus symphitifolius</i>	Canary Islands, Spain
<i>Cistus albidus</i>	Spain, France, Italy, Corsica, Portugal, Algeria, Balearic Islands
<i>Cistus libanotis</i>	Canary Islands
<i>Cistus albanicus</i>	Greece, Albania
<i>Cistus osbeckiaefolius</i>	Canary Islands, Spain
<i>Cistus crispus</i>	Europe, Russia, West Mediterranean region, Portugal, Spain.
<i>Cistus varius</i>	Southwestern Europe, France, Algeria, and Morocco.
<i>Cistus munbyi</i>	Algeria, Morocco

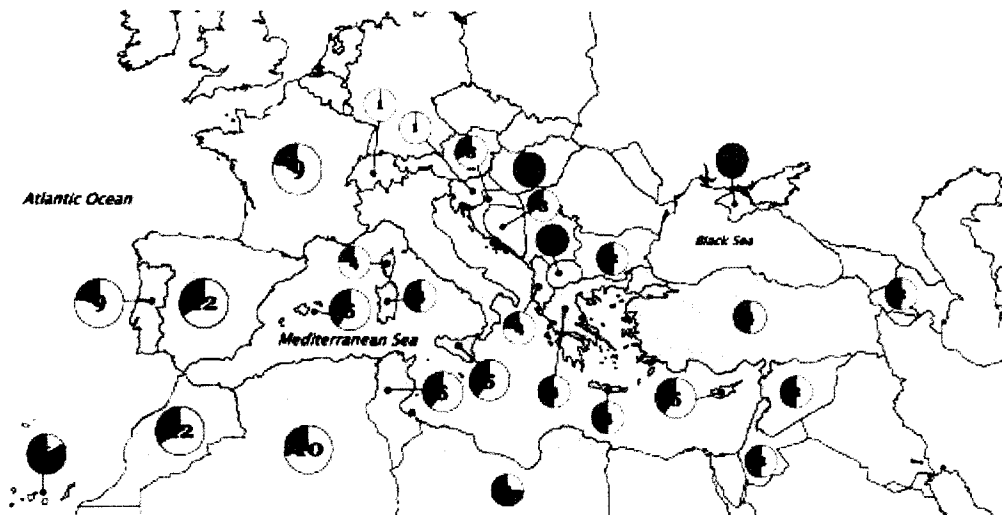


Figure 1.3. Phylogeographic distribution of *Cistus* species include proportion of white-flowered (white) and purple-flowered (grey) species in every country. The highest species diversity in the western Mediterranean (Guzmán and Vargas, 2005).

1.2.1. Description of the herb

Cistus species often have yellow, pink or white to purple flowers. In few species with a conspicuous dark red spot at the base of each petal, which are generally short-lived. The flowers are bisexual, regular, solitary; they usually have five flowers, and sometimes three flowers. The petals are free, usually crumpled in the bud, and sometimes in the flowers open flower as in *Cistus incanus*. It has five sepals, the inner three of which are distinctly wider, and the outer two are narrow and sometimes regarded as bracteoles. The sepal

arrangement is a characteristic property of the family, (Guzmán and Vargas, 2005; Thanos et al., 1992).

The leaves of all *Cistus* species are evergreen, opposite, usually slightly rough-surfaced, covered with glands secreting essential oils and resins called ladanum or labdanum, a gold-colored essential oil with a brownish resin on the surface of the leaves and stems consists mainly of terpenoids, ladanum have a long history of use in folk medicine. The common Greek name for the resin is "Ladano". Seven labdane types had been reported to isolate from extracts of *Cistus incanus* ssp. *creticus* L. The essential oils and resins are secreted from glandular hairs on the leaves and young stems, especially under hot sunshine (Demetzos et al., 1990; Attaguile et al., 1995).

1.2.2. Traditional uses of *Cistus* species

In the Mediterranean folk medicine, all the *Cistus* species are frequently used in medicine for the treatment of various skin diseases, rheumatism, fever and diarrhea, it had shown anti-inflammatory, antimicrobial, antiviral and antitumor activities (Lendeckel et al., 2002; Attaguile et al., 2004; Attaguile et al., 2006; Bouamama et al., 2006).

While in Turkish folk medicine, several *Cistus* species were reported to be effective against a broad range of disorders either internally or externally. Internally it was prepared as an infusion or a decoction of leaves which was used to treat diarrhea and hypoglycaemia. Flower decoction was used to treat gastric pains, it also showed a potent anti-ulcerogenic activity as was reported *in vivo* against various peptic ulcer models. Preparations from the leaves of the plant for external uses were also used as an effective remedy against several

inflammatory diseases such as rheumatic pain, high fever, edema and urinary inflammations. For relieving of rheumatic pain a warm decoction of leaves was used as a bath or wilted leaves were externally applied on joints. For treating urinary inflammations, a poultice was prepared by boiling the leaves and mixing them with flour and it was applied externally as a plaster on the dorsal part of the body in line with the kidneys (Yeşilada et al., 1997; Sadhu et al., 2006; Küpeli and Yesilada, 2007).

In Greece, *Cistus creticus* had a long history in folk medicine for centuries. The herb was used as an emetic, disorders of the spleen and diarrhea. It was also used for hair loss, scurvy, catarrh, asthma, stomach ulcers and cancer, as a protection against the plaque and as a fumigant (Malamas and Marsellos, 1992).

1.2.3. Pharmacological and antimicrobial uses of the genus *Cistus*

Data from recent pharmacological studies provided the beneficial effects of *Cistus* extracts in inflammatory or infective diseases by demonstrating their antiproliferative, cytotoxic activity and strong gastric antiulcer activity, antibacterial, antiviral, antifungal, hypotensive, skin care and spasmolytic activities (Lendeckel et al., 2002; Pomponio et al., 2003; Attaguile et al., 2004; Attaguile et al., 2006; Šarić et al., 2008).

1.2.4. *Cistus incanus*

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Cistus incanus (Pink Rockrose) is one species of the genus *Cistus*. *Cistus incanus* includes two subspecies, *Cistus incanus* ssp. *tauricus* and *Cistus*

incanus ssp. undulatus. *Cistus incanus ssp. tauricus* is preferably used for extraction (Petereit et al., 1991; Pandalis, 2007; Droebner et al., 2007).

1.2.4.1. Pharmacology uses

Cistus incanus has been reported to have a beneficial effect in many diseases as anti-inflammatory, antiulcer, spasmolytic and antioxidant agents and these activities will be discussed in the following paragraphs (Attaguile et al., 1995; Attaguile et al., 2004; Bouamama et al., 2006; Attaguile et al., 2006; Ehrhardt et al., 2007).

1.2.4.1.1 Anti-inflammatory activity

Aqueous extracts of *Cistus incanus* was reported to inhibit human leukocyte function, alanyl aminopeptidase (APN, CD13) and dipeptidylpeptidase IV (DP IV, CD₂₆) enzymatic activities so it is thought to help to prevent damage from excessive immune response and, thereby, promote wound healing. This may explain the beneficial folk medical utilization of *Cistus* extracts for the treatment of various inflammatory diseases (Lendeckel et al., 2002).

1.2.4.1.2. Antiulcer activity

Aqueous *Cistus incanus* extract has been reported to have gastroprotective effect in rats. A study demonstrated that the aqueous extract of *Cistus incanus* protects the gastric mucosa against lesions produced in rats by various gastric mucosal damaging agents (Attaguile et al., 1995).

The mode of action by which the extract prevent ulcer development is not yet understood, but since *Cistus incanus* contain bioflavonoids, this effect might be related to the established cytoprotective and antiulcer activities which are able to counteract mast cells degranulation and to exert vasoprotective action. Also it may be related to their antioxidant and free-radical scavenging properties (Attaguile et al., 1995).

1.2.4.1.3. Spasmolytic agent

Cistus incanus has been reported to exert a relaxant effect in vitro by inhibiting the contractile response in isolated intestinal and vascular rat smooth muscle. A study observed that this pharmacological activity of aqueous *Cistus* extracts may be due to an unspecific "reactive compound" and/or to the combined effects of the several chemical constituents of the plant, such as quercetin, kaempferol, kaempferol-3-methyl ether, aesculin, myricetin, flavan-3-ols and proanthocyanidins (Attaguile et al., 2004).

These results demonstrated the beneficial effects of *Cistus* extracts as spasmolytic agents in local folk medicine in diarrhoea and digestive disorders. Vasorelaxant properties of *Cistus* extracts also suggested their possible use in vascular conditions such as hypertension (Attaguile et al., 2004).

1.2.4.1.4. Antioxidant activity

Plants containing natural antioxidants are utilized as important sources for the development of novel drugs. Chemical medicines have potent activity but long-term administration for treatment of chronic diseases may show various and severe adverse effects. Therefore, many intensified research

efforts to discover and utilize naturally antioxidant agents with few side-effects to substitute the chemical therapeutics (Pokorny, 2007 Fang et al., 2009; Conforti et al., 2009).

Cistus species mainly *Cistus incanus* and *Cistus monspeliensis*, are of the typical Mediterranean flora, and possess flavonoids that are proved to be antioxidants (Auddy et al., 2003).

Cistus incanus extract has been reported to have a protective effect on DNA cleavage and a dose-dependent free radical scavenging capacity. The pharmacological properties of flavonoids are due to its ability to protect against the damaging action of free radicals. These polyphenols not only interfere with the propagation reaction but also prevent the formation of free radicals, either by chelating the transition metal or by inhibiting the enzymes involved in the initiation reaction (Attaguile et al., 2006).

1.2.4.2. Antimicrobial and antiviral activity

Cistus leaf extracts were reported to have antimicrobial properties against many bacteria and fungi responsible for human infections. Whereas in vitro a study done on *Cistus incanus* extract against five strains of bacteria and five strains of fungi, the extracts showed inhibitory activity against microorganisms so it is considered as antibacterial and antifungal activities (Bouamama et al., 1999).

A new study done using *Cistus incanus* showed the antiviral activity of the herb against a highly pathogenic avian influenza A virus (H₅N₁) in cell culture and in a mouse infection model is due to polymeric polyphenols binding to the virus surface and inhibiting binding of the hemagglutinin to cellular receptors.

So local application of *Cistus incanus* at the viral entry routes may be a promising approach that may help to protect from influenza virus infections and also a recent research attend to use *Cistus incanus* for the preparation of a formula for the prevention and treatment of influenza (Droebner et al., 2007; Pandalis, 2007; Ehrhardt et al., 2007).

Although several antiviral compounds had been developed but still their long term efficacy is limited either due to drug resistant virus or due to toxicity.

1.2.4.3. Constituents

1.2.4.3.1. Essential oil

Essential oils contain several compounds which can be used in the industry as in fine perfumery as a component great perfumes such as Chanel. Numerous studies had been conducted on *Cistus* essential oils where some species showed cytotoxic and antibacterial properties. (Gülz et al., 1984; Robles and Garzino, 1997).

Many studies had been conducted on *Cistus* volatile compounds. Oils are mainly composed of sesquiterpenes. The sesquiterpene hydrocarbons are the major components in addition to sesquiterpene alcohols which are also present. The essential oils contain also some alkanes (docosane and pentacosane) but in very small quantities as trace components. The oil yields vary according to the changes in environmental conditions as the study site and also to the sampling month (Robles and Garzino, 1997; Demetzos et al, 2002).

1.2.4.3.3. Phenolic carboxylic acids

Phenolic compounds are widely distributed in the plant kingdom. Phenolics are considered as potential therapeutic agents against a wide range of diseases. Dietary intake of phenolics varies considerably among geographic regions, it is estimated that daily intake range from about 20mg to 1gm. Phenolic acids can exist as free entities or can be attached to other types of polyphenols as gallic acid and gallate groups attached to a flavonoid such as epicatechin. One of the widely known phenolic acids is Gallic acid which frequently occurs as an added moiety as a gallate unit on many other types of polyphenols including catechins as epigallocatechin gallate, and tannins. *Cistus incanus* contain protocatechuic and (-)-shikimic acid (Danne et al., 1993; Srinivasan et al., 2007).

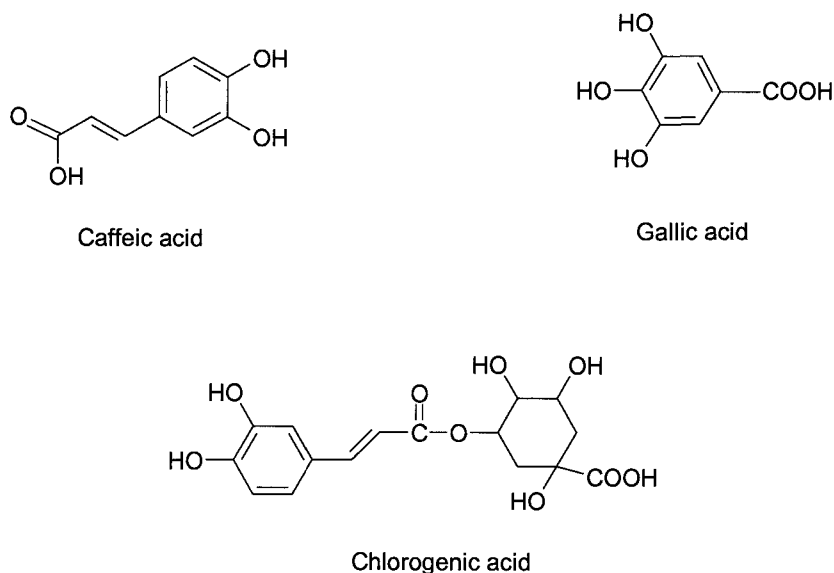


Figure1.5. Phenolic acid examples

1.2.4.3.4. Flavonoids

Flavonoids are biologically active polyphenolic compounds that are widely distributed in plants. More than 6,000 plant flavonoids were found, and they have been classified into at least ten chemical groups according to their structural patterns. Flavonoids are water soluble polyphenolic molecules containing 15 carbon atoms, characterized by a phenylbenzopyran chemical structure. Flavonoids were investigated for their health beneficial activity, which are one of the most numerous secondary plant metabolites of natural constituents in plants. Flavonoids are currently consumed in large amounts in the daily diet, the average consumption of polyphenol flavonoids in the diet is 1 g per day (Chaves et al., 1998; Attaguile et al., 2004; Attaguile et al., 2006; Aron and Kennedy, 2008; Harnafi and Amrani, 2007; Theodoratou et al., 2007; Šarić et al., 2008).

Medicinal and aromatic plants flavonoids possess a high antioxidant potential due to their hydroxyl groups and protect more efficiently against free radical-related diseases. Flavonoids act as antioxidants through several mechanisms including the scavenging of free radicals, chelation of transition metals, as well as the mediation and inhibition of enzymes. Although flavonoids are poorly absorbed in the body after their absorption into the blood but rapidly metabolized in the intestines and liver and become active as antioxidant or antiradical preventing free radical toxicity, oxidative stress and pathophysiology of various diseases (Knekt et al., 2002; Ramiro-Puig and Castell, 2008; Šarić et al., 2008; Aron and Kennedy, 2008).

Cistus incanus extract contain several chemical constituents of flavonoids as quercetin, kaempferol, kaempferol-3-methyl ether, myricetin, apigenin,

luteolin, Flavanone 2R, 3R-dihydromyricetin was also obtained (Petereit et al., 1991; Attaguile, 1995; Lendeckel et al., 2002; Attaguile et al., 2004; Attaguile,1995).

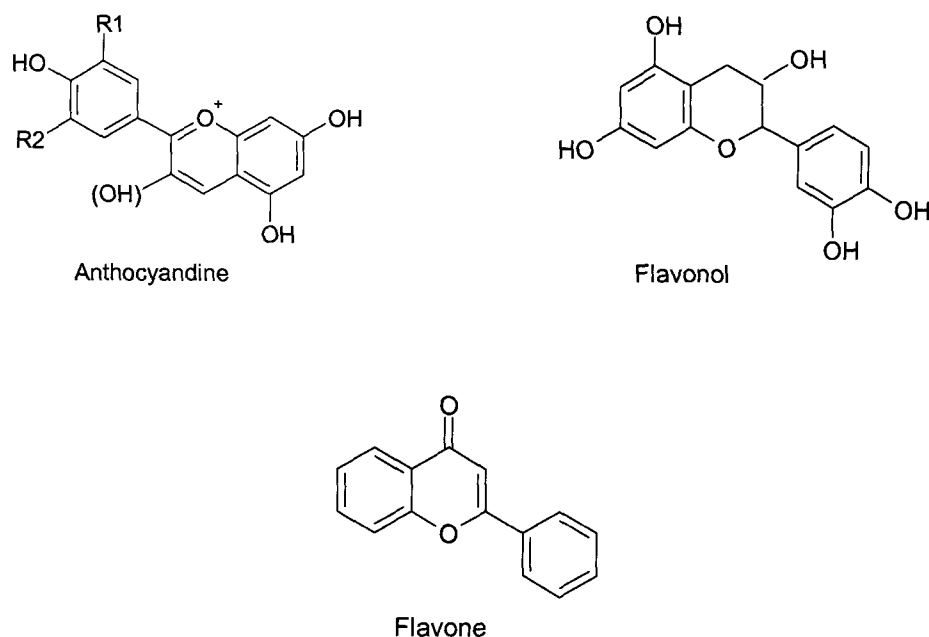


Figure1.6. Flavonoid examples

1.2.4.3.5. Tannins

Tannins are phenolic secondary compounds of plants. The name 'tannin' was derived from the French word 'tanin' (tanning substances). They are usually classified based on their chemical structures into three groups as hydrolysable tannins, condensed tannins and the complexed tannins (Khanbabaee and Ree, 2001; Dentinho et al., 2007).

Condensed tannins are the most common tannin type found in trees and shrubs are all oligomeric and polymeric proanthocyanidins formed by linkage of C-4 of one catechin with C-8 or C-6 of the next monomeric catechin. (Khanbabaee and Ree, 2001; Ansong, 2004).

Tannins can be characterized by their ability to bind proteins. The characteristics of tannin-protein interactions have been well studied both in the hydrolysable tannins and the proanthocyanidins. Several techniques such as equilibrium dialysis, ESI-MS, isothermal titration microcalorimetry, size exclusion chromatography and radiolabeled proteins have been employed to study tannin-protein interactions (Dentinho et al., 2007; Ansong, 2004).

Cistus incanus extract contain condensed tannins as proanthocyanidines and flavan-3-ols (Attaguile et al., 2006; Dentinho et al., 2007).

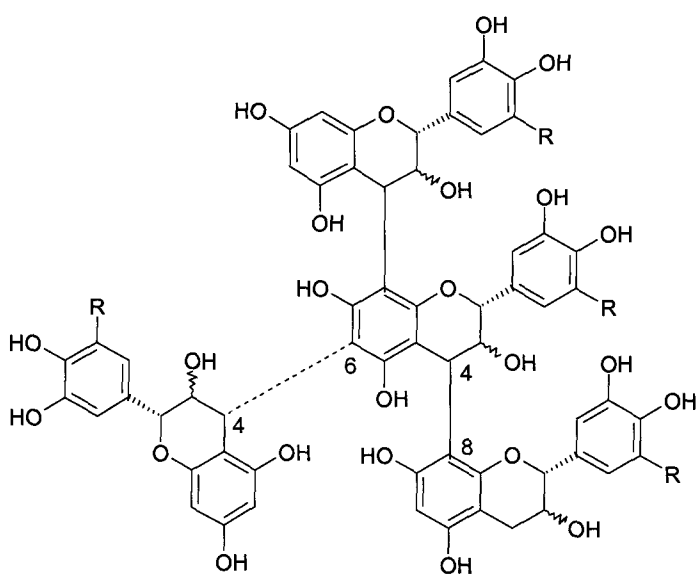


Figure1.7. Condensed tannins

1.2.4.3.5.1. Flavan-3-ols and proanthocyanidins

Major constituents in *Cistus incanus* include flavan-3-ols and proanthocyanidins. The total content of monomeric components is lower than 2% while the polymeric components especially the proanthocyanidines with a molecular weight range of 500–1200 Da, are dominant (Droebner et al., 2007; Ehrhardt et al., 2007).

1.2.4.3.5.1.1 Flavan-3-ols

Flavan-3-ols are considered as functional ingredients in various beverages, foods, herbal remedies and food grains. In food flavan-3-ols affect on many food quality parameters such as astringency, bitterness, sourness, sweetness, salivary viscosity, aroma, and color formation. Flavan-3-ols have been reported to have several health benefits as antioxidant, anticarcinogen as in decreasing the risk of colorectal cancer, cardiopreventive properties, antimicrobial activity in addition to antiviral and antibacterial activity, some plant species utilize flavan-3-ols such as (-)-catechin to prevent proliferation of neighboring plant species (Mink et al., 2007; Tasdemir et al., 2006; Bastianetto et al., 2006; Theodoratou et al., 2007; Aron and Kennedy, 2008; Rauha, 2001)

Many reports showed the deleterious effects of flavan-3-ol on human health including activation of procarcinogens, reactive-oxygen species formation (pro-oxidant activity), hemorrhage formation, initiation of

hepatotoxicity, alteration of pharmacokinetics of therapeutic drugs, increased estrogenic tumor formation, mutagenicity, modification of plasma biochemistry, instigation of gastroenteritis, antinutritive activity and weight loss (Nagao et al., 2007; Aron and Kennedy, 2008).

Major phytochemical compounds in addition to polyphenolic compounds and bioflavonoids in aqueous extracts of *Cistus incanus* are four monomeric and seven oligomeric flavanoids have been identified. Flavan-3-ols are (+)-catechin, (+)-gallocatechin, (+) gallocatechin 3-gallate and the rarely occurring (+)-catechin 3-O- α -L -rhamnoside (Petereit et al., 1991; Lendeckel et al., 2002; Attaguile et al., 2004).

1.2.4.3.5.1.2. Proanthcyandins

Proanthocyanidins (PAs) are polyphenolic naturally-occurring plant metabolites that are oligomeric or polymeric end products of the flavonoid biosynthetic pathway. Proanthocyanidins are increasingly recognized for their beneficial effects on human health. The proanthocyanidine are also known as oligoflavanoids; they comprise condensed polyphenols or condensed tannins. Proanthocyanidins have an important role at the nutritional and physiological level and in pharmacology. It is now established that the proanthocyanidins possess a wide range of potential effects that are beneficial for human health (Santamaria et al, 2002; Cos et al., 2003; Lesschaeve and Noble, 2005; Aron and Kennedy, 2008; Diouf et al., 2009).

proanthocyanidins exist in plants as complex mixtures of polymers with an averages degree of polymerization between (4 to11), although high degrees of polymerization (up to 70) may also be found. The soluble

proanthocyanidin polymers usually have molecular weight averages in the range of 1000-6000 Da, although some polymers have molecular weight as high as 20 000 Da (Cos et al., 2003; Tanner et al.,2003; Haslam, 2007; Aron and Kennedy, 2008).

1.2.4.3.5.1.2.1. A-type proanthocyanidin

The A-type proanthocyanidins have a second ether linkage between the A ring hydroxyl group of the lower unit and C₂ of the upper unit. Compared to the B-type proanthocyanidins, A-type proanthocyanidin dimmer are more rigid in conformation than the B class due to the presence of two interflavan linkage: one C-C and other C-O. This subclass lacks two hydrogens compared to the B-type. A-type proanthocyanidins are not frequently encountered and isolated from plants in nature (Lazarus et al., 1999; Xie and Dixon, 2005; Aron and Kennedy, 2008; Hümmer and Schreier, 2008).

1.2.4.3.5.1.2.2. B-type proanthocyanidin

B-type proanthocyanidin are oligomers or polymers of flavan-3-ols which are characterized by a single interflavan bond, linking the C₄ of the upper extension of naturally occurring unit and the C₈ or the C₆ of the lower starter unit. They are the dominant class of naturally occurring proanthocyanidins B₁, B₂, B₃ and B₄ are the most frequently present in different plants (Xie and Dixon, 2005; Hümmer and Schreier, 2008).

Proanthocyanidins in *Cistus incanus* are procyanidins B₁ and B₃, gallocatechin-(4 α →8)-gallocatechin, (4 α →6)-regioisomer, gallocatechin-(4 α →8)-catechin, catechin-(4 α →8)-gallocatechin and the trimer gallocatechin-

(4 α →8)-gallo catechin-(4 α →8)-catechin. Flavanone 2R, 3R-dihydromyricetin was also obtained (Petereit et al., 1991).

Another study done for chemical investigation an aerial parts of *Cistus incanus* L. ssp. *tauricus* which led to the isolation more proanthocyanidine types as epicatechin-(4 β →6)-catechin, the dimeric prodelphinidins, epigallocatechin-(4 β →8)-catechin and epigallocatechin-(4 β →8)gallo catechin, and galloylated isomers, epigallocatechin-3-O-gallate-(4 β →8)-gallo catechin and epigallocatechin-3-O-gallate- (4 β →6)-gallo catechin, in addition to the trimer gallo catechin-(4 α →8)-gallo catechin-(4 α →8)-gallo catechin (Danne et al., 1993).

Cistus incanus plants which harvested in Greece, were identified to contain (+)-catechin, (+)-gallo catechin, (+)-gallo catechin 3-gallate, (+)-catechin 3-O- α -L -rhamnoside, procyanidin B1 and B3, gallo catechin-(4 α →8)-gallo catechin, its new (4 α →6) regioisomer, gallo catechin-(4 α →8)-catechin, and the trimer gallo catechin-(4 α →8)-gallo catechin-(4 α →8)-catechin (Attaguile et al., 2006).

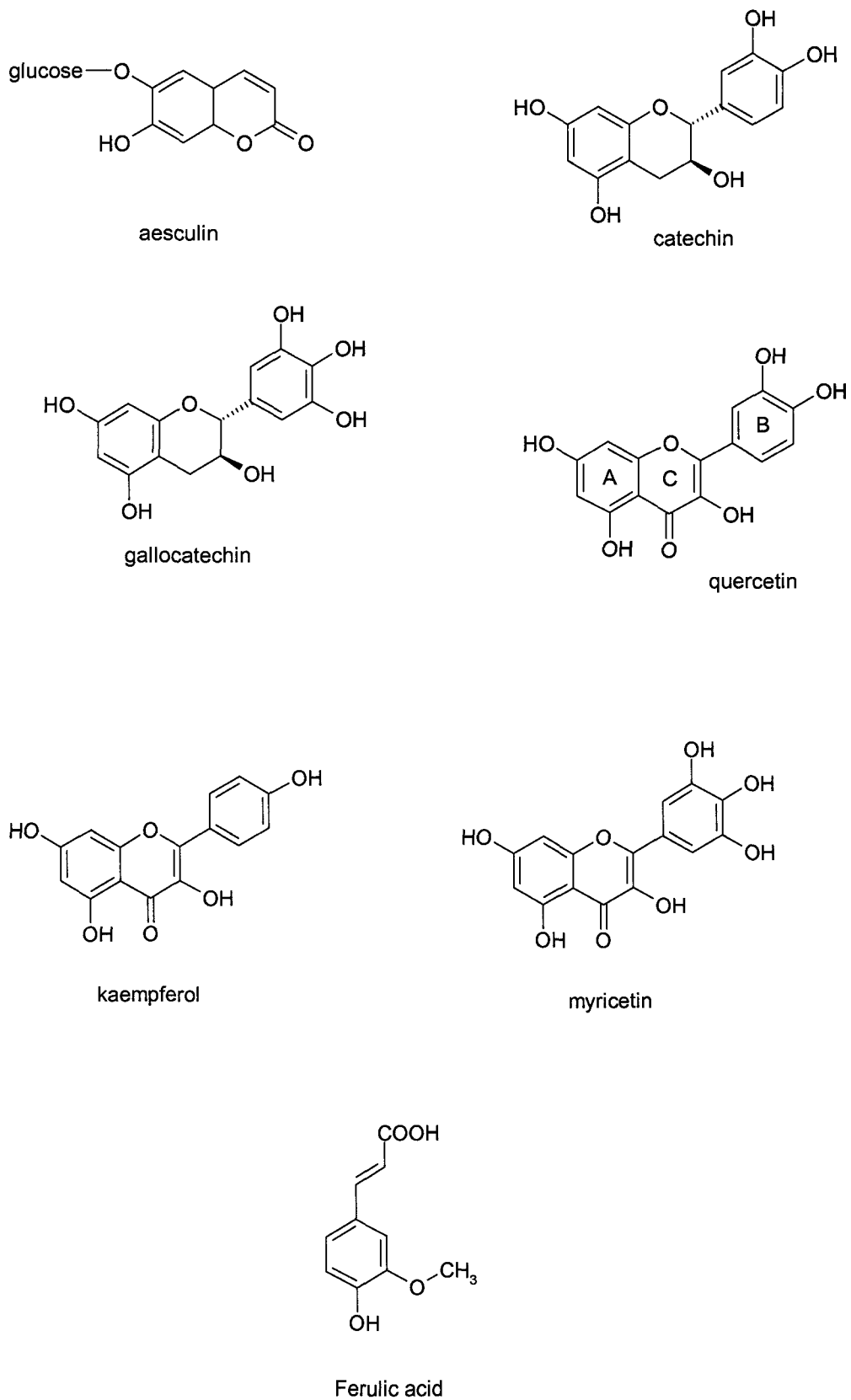


Figure 1.8. Some *Cistus incanus* constituents

1.3. Methods for estimation plant polyphenols

1.3.1. Total phenols

Among the several assays available to quantify total polyphenols, Folin-Denis method (1912) is one of the most commonly used methods and is based on a color reaction. It was later improved by Folin and Ciocalteu in 1927 with an addition of lithium sulfate to the reagent to prevent precipitation in the reaction in order to increase the sensitivity (Qing Zhang et al., 2006).

Folin- Ciocalteu assay, a recommended official method, is a major colorimetric technique that many years it has been used to determine and measure total polyphenols content in medicinal plant extracts and the spectrophotometric assays employed to measure their antioxidant power. Since introduction of the improvements was recommended by Singleton and Rossi (Roura et al., 2006; Harnafi and Amrani, 2008).

The quantitative determination of phenolic compounds using Folin-Ciocalteu reaction which measures the absorbance caused by a specific reaction following the oxidation of polyphenolic compounds with the yellow phosphomolybdic and phosphotungstic acids in a basic medium; the resulting color intensity is blue reaction products, molybdotungstophosphate blue, these blue pigments have a maximum absorption depending on the qualitative and/or quantitative composition of phenolic mixtures and on the pH of solutions usually obtained by adding sodium carbonate and is directly proportional to the concentration of polyphenols (Dka et al., 2002; Harnafi and Amrani, 2008; Fang et al., 2009; Cicco et al., 2009).

Folin-ciocalteu assay is affected by several interfering substances, such as sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, Fe(II), as well as nonphenolic organic substances that can react with the folin-ciocalteu reagent (Roura et al., 2006).

The variations of the method, widely studied by Singleton and Rossi and later by others, concerning the initial and final concentrations of sodium carbonate, the sequence of reagent additions as well as the timing of these additions, and the time and temperature of reaction mixture incubation. Wavelengths in the 700–760 nm range at which absorbance is determined and the final mixture volume in the 2–100 ml range are variable parameters too (Cicco et al., 2009).

Folin-Ciocalteu colorimetry provided satisfactory results for total phenolic content of the *Cistus incanus* (Dka et al., 2002).

In addition to Folin-Ciocalteu method which is characterized as a rapid, accurate, and precise polyphenols analysis with minimal sample preparation. Other conventional methods using reversed-phase high performance liquid chromatography (RP-HPLC), and normal-phase high performance liquid chromatography (NP-HPLC), and dimethylaminocinnamaldehyde (DAC) assays can be used for total phenol estimation (FAO/IAEA, 2000; Beer et al., 2004; Harnafi and Amrani, 2008).

Recently, a method called cyclic voltammetry has been used to measure wine total phenols based on their redox potential. Two other modern methods were able to measure total phenols based on their antioxidant activity, free radical scavenging and lipid peroxidation (Beer et al., 2004).

1.3.2. Flavan-3-ols

Flavan-3-ol can be measured by using reversed-phase high performance liquid chromatography (RP-HPLC) and dimethylaminocinnamaldehyde (DAC) assays. Proanthocyanidins can also be measured using RP-HPLC assay for monomeric phenols (flavan-3-ol) but after cleavage with a specific reagent into their subunits (Beer et al., 2004).

Other detection methods as electrochemical detection (ED), where flavonoids and proanthocyanidins are electroactive compounds can be used in conjugation with HPLC. ED method is more sensitive and has a higher degree of sensitivity than UV or fluorescence detection. Fluorescence detection (excitation at 276 nm and emission at 316 nm) can also be used in conjugation with HPLC for proanthocyanidins detection (Schofield et al., 2001; Kelm et al., 2005).

1.3.3. Tannins

Total tannin content can also be determined by the Folin–Ciocalteu procedure. Determination of total tannins is partly chemical, based on the *reducing property of tannins*, and partly physical because tannins are measured as the reduction in phenolics that occur when a binding agent polyvinylpolypyrrolidone is added to the extract. Tannins can be measured by protein precipitation assay which is based on the ability of tannins to precipitate proteins. The most commonly protein used in this method involves the use of Bovine serum albumin dye with Ramazol Brilliant Blue. Two mechanisms for tannin-protein co-precipitation have been proposed. One is a cross-linking mechanism based on association, in which one tannin molecule

binds to two or more protein molecules. While another mechanism is a two-stage mechanism, initial complexation stage of tannin with protein and subsequent precipitation of the complexes. However, little evidence is still supporting these precipitation mechanisms (Kawamoto et al., 1996; Schofiels; FAO/IAEA, 2000; et al., 2001; Beer et al., 2004; Harnafi and Amrani, 2008).

Many other unspecific methods have been used for tannin quantitative determination, including turbidimetry, ferric chloride, n-butanol-HC₁, and iodate assays (Kawamoto et al., 1995).

1.3.4. Flavonoids

Aluminum chloride colorimetric assay is used to determine the total flavonoid content in plant materials which is based on the quantification of the yellow-green color produced by the interaction of flavonoids with AlCl₃ reagent. Flavonoid content was also determined by the method proposed by *Ough* and *Amerine* using formaldehyde for their precipitation. The flavonoid concentration was calculated as the differences between total phenols and nonflavonoids remaining in the supernatant (Abouzid and Elsherbeiny, 2008; Harnafi and Amrani, 2008).

1.4. International Conference on Harmonization (ICH) Guidelines

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is to