# ANALYTICAL, BIOACTIVITY AND STABILITY STUDIES ON *STROBILANTHES CRISPUS* L. BREMEK AND *SONCHUS ARVENSIS* L. EXTRACTS

AFRIZAL

UNIVERSITI SAINS MALAYSIA

2008

# ANALYTICAL, BIOACTIVITY AND STABILITY STUDIES ON *STROBILANTHES CRISPUS* L. BREMEK AND *SONCHUS ARVENSIS* L. EXTRACTS

**by**

**AFRIZAL**

**Thesis submitted in fulfillment of the requirements**

**for the Degree of Doctor of Philosophy**

**June 2008**

**DEDICATED TO**

**Beloved**

**My parents, Itam St. Pamenan and Tinun**

**My wife, Hartati**

**My children, Indah Permata, Ridho Sahary Adha and Nadya Chantika**

## ACKNOWLEDGMENTS

Alhamdulillah, praise to Almighty Allah SWT, who is creator and sustainer of the universe. His blessings be upon Prophet Muhammad SAW.

I respectfully express my sincere and deepest gratitude to my supervisor Professor Dr Zhari Ismail and co supervisor Dr Amin Malik Shah Abdul Majid for their helpful advice, generous help and guidance throughout the course of this my study.

I am grateful to university authorities and Dean of School of Pharmaceutical Sciences, University Sains Malaysia, for the facilities provided during the course of this work.

I am thankful to all of my fellow colleagues who assisted me to complete my work, especially, Nor Amin, Mohammad Abdul Razak, Suhaimi, Azizan, Rosidah, Saravanan and Khalid Hussain.

In this occasion, I would like to express my special thanks to Mr. Hider who has assisted me doing on antiangiogenic work.

Finally, I am grateful to my university for providing me study leave to complete my education.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## LIST OF APPENDICES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 3D | Three of dimension |
| BAW | Buthanol-1: Acetic acid: Water |
| BHA | Butylated Hydroxyl Anisole |
| BHT | Butylated Hydroxyl Toluene |
| DMSO | Dimethylsulfoxide |
| DPPH | 1,1-Diphenyl-2-picrylhydrazyl |
| E7ac | 70% acetone extract |
| Eac | Acetone extract |
| Em | Methanol extract |
| Em-sox | Methanol fraction from soxhlet |
| Ew | Water extract |
| FTIR | Fourier Transform Infra Red |
| GC | Gas Chromatography |
| GC–MS | Gas Chromatography–Mass Spectrometry |
| GC/TOF-MS | Gas Chromatography/Time-of- Flight Mass Spectrometry |
| HPLC | High Performance Liquid Chromatography |
| HPTLC | High Performance Thin Layer Chromatography |
| MeOH | Methanol |
| NMR | Nuclear Magnetic Resonance |
| $Ox^{-2}$ | anion of oxalate |
| PCA | Principal Component Analysis |
| Rf | Retention factor |
| RH | Relative humidity |
| Sa | *Sonchus arvensis* |
| Sc | *Strobilanthes crispus* |
| SOM | Self-Organizing Map |
| TLC | Thin Layer Chromatography |
| TOF-MS | Time-of-flight mass spectrometry |
| UV | Ultraviolet |
| VIS | Visible |
| XO | Xanthine oxidase |

**KAJIAN ANALITIKAL, BIOAKTIVITI DAN STABILITI TERHADAP EKSTRAK *STROBILANTHES CRISPUS* L. BREMEK DAN *SONCHUS ARVENSIS* L.**


## ABSTRAK

Tujuan kajian ini adalah untuk memiawaikan ekstrak-ekstrak (Ew, E7ac, Em, Eac dan Em-sox) daun *Strobilanthes crispus* L. Bremek dan *Sonchus arvensis* L. untuk tujuan kajian praklinikal. Kaedah pemiawaian dibahagikan kepada tiga bahagian iaitu profil kimia (kajian analitikal dan fitokimia), profil biokimia (kajian perencatan pertumbuhan kristal, antioksidan dan kestabilan) dan profil biologi (kajian antiangiogenik).

Sebatian metabolit sekunder yang dikesan dalam daun *S. crispus* ialah α-sitosterol, campesterol, phytol dan stigmasterol, sementara lupeol, phytol dan α-sitosterol dikesan dalam daun *S. arvensis*. Sebatian tritriakontana dan stigmasterol telah diasingkan daripada daun *S. crispus*, sementara lupeol dan kuersetin telah diasingkan daripada daun *S. arvensis*.

Indeks perencatan ekstrak Ew, E7ac, Em, Eac, dan Em-sox *S. crispus* terhadap perencatan pertumbuhan kristal kalsium oksalat masing-masing adalah 0.2233 ± 0.0875, 0.1861 ± 0.0124, 0.1587 ± 0.0264, 0.1830 ± 0.0335, dan 0.2081 ± 0.0166. Manakala indeks perencatan ekstrak yang sama daripada *S. arvensis* masing-masing adalah 0.3375 ± 0.0157, 0.1994 ± 0.0257, 0.1938 ± 0.0662, 0.1347 ± 0.0439, dan 0.3157 ± 0.0457.

Aktiviti antioksidan tertinggi ke atas aktiviti radikal bebas, aktiviti xantina oksidase dan pelunturan β-karotena oleh asid linoleik daripada ekstrak *S. crispus* masing-masing adalah E7ac, Em dan Em-sox. Manakala bagi yang sama daripada ekstrak *S. arvensis* masing-masing adalah E7ac, Eac dan Em.

Peratusan fenolik daripada ekstrak tumbuh-tumbuhan ini juga ditentukan. Koefisien penentuan ($R^2$) antara kandungan fenolik dan aktiviti radikalnya adalah 0.93 (*S. arvensis*) dan 0.40 (*S crispus*). Dalam perencatan pelunturan β-karotena oleh asid linoleik, didapati kesan protein lebih besar berbanding kesan polisakarida.

Dalam kajian kestabilan dipercepat, kesemua ekstrak (Ew, Em, and Eac) yang disimpan pada suhu bilik (25 $^0$C, 60% RH) adalah tertinggi berbanding ekstrak yang disimpan pada suhu 40 $^0$C (75%RH), 50 $^0$C (85% RH) dan 60 $^0$C (85% RH). Jangka hayat ($t_{90}$) ekstrak Eac, Em dan Ew daripada *S. crispus* yang disimpan pada suhu bilik, masing-masing adalah 2.14, 2.17 dan 1.94 bulan. Manakala $t_{90}$ ekstrak yang sama daripada *S. arvensis* masing-masing adalah 2.10, 7.89 dan 3.50 bulan. $t_{90}$ stigmasterol dalam Em dan Eac daripada *S. crispus* yang disimpan pada suhu bilik, masing-masing adalah 3.60 dan 2.63 bulan. Manakala $t_{90}$ lupeol dalam ekstrak Em dan Eac daripada *S. arvensis*, masing-masing adalah 2.51 dan 2.22 bulan.

Dalam kajian awal antiangiogenik, keputusan menunjukkan bahawa peratus perencatan Ew dan Em daripada *S. crispus*, masing-masing adalah 16.67 dan 6.25%, manakala peratus perencatan Ew dan Em daripada *S. arvensis*, masing-masing adalah 11.06 dan 8.65%, memperlihatkan bahawa kedua tumbuhan ini mempunyai kemampuan mencegah atau menyembuh penyakit-penyakit yang berkaitan dengan angiogenik.

**ANALYTICAL, BIOACTIVITY AND STABILITY STUDIES ON *STROBILANTHES CRISPUS* L. BREMEK AND *SONCHUS ARVENSIS* L. EXTRACTS**

## ABSTRACT

The purpose of this study was to standardize the leaf extracts (Ew, E7ac, Em, Eac and Em-sox) of *Strobilanthes crispus* L. Bremek and *Sonchus arvensis* L. for preclinical studies. The standardization work was divided into three steps: chemical profiling (analytical and phytochemical studies), biochemical profiling (crystal growth inhibition, antioxidant and stability studies) and biological profiling (antiangiogenic studies).

Secondary metabolites detected in *S. crispus* leaves were α-sitosterol, campesterol, phytol and stigmasterol, whereas lupeol, phytol and α-sitosterol were detected in *S. arvensis* leaves. Tritriacontane and stigmasterol were isolated from *S. crispus* leaves whilst lupeol and quercetin were isolated from *S. arvensis* leaves.

The inhibition indices of Ew, E7ac, Em, Eac, and Em-sox from *S. crispus* to inhibit the growth of calcium oxalate crystals were 0.2233 ± 0.0875, 0.1861 ± 0.0124, 0.1587 ± 0.0264, 0.1830 ± 0.0335, and 0.2081 ± 0.0166, respectively. The values for similar extracts for *S. arvensis* were 0.3375 ± 0.0157, 0.1994 ± 0.0257, 0.1938 ± 0.0662, 0.1347 ± 0.0439, and 0.3157 ± 0.0457, respectively. The highest antioxidant activity on FRSA to DPPH, xanthine oxidase activity and prevention the bleaching of β-carotene by linoleic acid of *S. crispus* extracts are E7ac, Em and Em-sox, respectively whilst those of *S. arvensis* extracts are E7ac, Eac and Em, respectively. The percentages of phenolic content from these plants extract were also determined. Coefficient value ($R^2$) between their phenolic content and FRSA were 0.93 (*S. arvensis*) and 0.40 (*S crispus*). In the

prevention of bleaching of β-carotene by linoleic acid, effect of protein was more than that of polysaccharide.

In accelerated stability studies, the extracts (Ew, Em, and Eac) stored at room temperature (25 $^0$C, 60% RH) was highest when compared to stored at 40 $^0$C (75%RH), 50 $^0$C (85% RH) and 60 $^0$C (85% RH). Shelf life ($t_{90}$) of Eac, Em and Ew from *S. crispus* stored at room temperature was 2.14, 2.17 and 1.94 months, respectively. Meanwhile the $t_{90}$ of similar extracts from *S. arvensis* was 2.10, 7.89 and 3.50 months, respectively. The $t_{90}$ of stigmasterol in Em and Eac from *S. crispus* stored at room temperature was 3.60 and 2.63 months respectively, whilst lupeol in Em and Eac from *S. arvensis* was 2.51 and 2.22 months respectively.

In preliminary antiangiogenic studies, the results showed that inhibition percentages of Ew and Em from *S. crispus* are 16.67 and 6.25% respectively, whilst those of Ew and Em from *S. arvensis* are 11.06 and 8.65% respectively, exhibiting that these plants possess the potential to prevent or cure angiogenesis related diseases.

# CHAPTER 1

# INTRODUCTION

## 1. 1 The Usage of Medicinal Plants

Plants have been used as source of medicines for thousands of years in maintaining health as an alternative to or in conjunction with modern medicines. The majority of the world's population in developing countries used herbal medicines to meet their health needs, following traditional beliefs and practices adopted from their elders and ancestors. The World Health Organization (WHO) estimated about 70% of the world population uses medicinal plants for medicines, and they are highly used mainly in Asia, South America and Africa (Chapman, K. and Chomchalow, N., 2005). Mamedov *et al*. (2005) reported the flora of Russia and Central Asia contains approximately 300 species of plants that have been used in prescription and non-prescription pharmaceutical preparations, while nearly 2500 plants are known to have been used in traditional medicine. A study from Kenya showed that patients had a clear sense of which diseases when they visit a traditional healer although previously they would go to a western clinic. In South Africa, traditional healers are flourishing in urban areas where western health care is also available (Van der Geest, 1997; Mander *et al*., 1997 cited in Jäger, A. K., 2005). Another study reported that the rate of having used an alternative treatment method is 42.1% in the U.S, 48% in Australia, 70% in Canada, 38% in Belgium, 90% in Germany, 75% in France and 75.9% in Turkey (Recai *et al*., 2006). Meanwhile, Lai *et al*. (2007) reported that over two-thirds of the older Chinese immigrants in Canada use traditional Chinese medicine in combination with Western health services. About half (50.3%) of the older Chinese immigrants used Chinese herbs, 48.7%

used Chinese herbal formulas, and 23.8% consulted a Chinese herbalist. In Indonesia, Sulaksana *et al*. (2004) reported that at least 1,845 of medicinal plants have been identified and inventoried, and at least 400 ethnic communities have experiences in use of medicinal plants.

The use of herbal medicine is extensive, increasing and complex. In England, from a survey of the use of complementary and alternative medicine (CAM) reported that purchasing of herbal medicine product (HMPs) have increased almost 20% per year (Heinrich *et al*., 2004). In 2002, the global trade in herbal product to have a value of US$12 billion, with trades in crude medicinal plants exceeding US $800 M., herbal extracts and semi-finished raw materials exceeding US $8 billion and herbal cosmetics about US$1.5 billion (Parke and Tikasingh, 2002). The demand for medicinal plants is increasing everyday and the World Health Organization (WHO) has projected that the global herbal market will grow to $5 trillion by 2050 from the current level of $62 billion with growth rate of 7 to 30 per cent annually (Reddy, 2003). According to Malaysian Deputy Minister of Natural Resources and Environment, currently the value of the local herbal market in Malaysia is estimated to be around 3.8 billion Ringgit (1.03 billion U.S. dollars) and this amount is expected to reach 8 billion Ringgit (2.16 billion U.S. dollars) by 2010, a handsome annual growth rate of 15 to 20 percent (http://english.people.com.cn/ 200609/13/eng20060913_302302.html).

In addition, combination of traditional and modern medicine has an important role in promoting health care system. In many countries, herbal medicine is

making a strong comeback and the world of medicine today embraces both single pure chemical entities and herbal medicine side by side (WHO, 2001).

There are many medicinal herbs used in health care, such as *Eurycoma longifolia*, *Orthosiphon stamineus, Phyllanthus niruri, Andrographis paniculata,* and *Catharanthus roseus* (Wiart, 2002; Zakaria and Ali, 1994; Dalimartha, 1999). To enable medicinal plants to be use in modern medicine, researches and development are important for the advancement of traditional medicines.

My research work will focus on two widely used medicine plant species, *Strobilanthes crispus* and *Sonchus arvensis.* Both of the latter has their origins from Padang Sumatera.

### 1.2 *Strobilanthes crispus* Plant

#### 1.2.1 Botanical Description

*Strobilanthes crispus* L. Bremek is an annual plant, which grows easily in the forest, riverbanks and abandoned fields. It is commonly used as fence hedges. The plant is native to countries from Madagascar to Indonesia, which can be grown 50 to 1200 meters above sea level. This bush-like plant can attain a height between 1 to 2 m. The circular bark can be divided into segments and similar to its branches, they are hairy and green. The leaf is oblong-lanceolate, rather obtuse, and shallowly crenate-crispate. The top surface of the leaf is darker green in color and less rough compared to the under surface. The leaves are very scabrous on both surfaces and covered with short hairs, whereas the flower is short, dense, and consists of penciled spikes. The leaf is 9-18 cm in length and 3-8 cm in width. The plant can be propagated using cut steams.

The classification for *S. crispus* is as follows, Division is Spermatophyta, Sub division is Angiospermae, Class is Dicotyledonae, Sub class is Solanales, Family is Acanthaceae, Genus is *Strobilanthes* and Species is *Strobilanthes crispus*.

The local name is daun picah beling (Jakarta), enyoh kelo, kecibeling, kejibeling, ngokilo (Java), pecah kaca or jin batu (Malay). The Latin synonym is *Sericocalyx crispus* L. Bremek (Departemen Kesehatan Republik Indonesia, 1977[1]; Syamsuhidayat and Hutapea, 1991[1]; Wijayakusuma *et al.*, 2000; Heyne, 1987; Fadzelly *et al.*, 2006). Picture of *S. crispus* is presented in Plate 1.1.

Plate 1.1 Picture of *S. crispus* plant

### 1.2.2 Biological Activity

Studies in Indonesia have found that infusion of the dried leaves has been used as antidiabetic, diuretic, antilithic, and laxative (Perry and Metzger, 1980; Syamsuhidayat and Hutapea, 1991[1]; Wijayakusuma *et al.*, 2000). They suggested boiling 25 – 50g of fresh leaves in 200 ml boiling water, and then drinking the infusion after filtration. For external use, poultice of the fresh leaves can be directly applied on to wounds caused by the bite of poisonous snakes or other animals (Wijayakusuma *et al.*, 2000). Ismail *et al.*, (2000) reported that the extract showed antioxidant activity using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. Jaksa *et al.* (2004) reported that the extract showed anti hepatocarcinogenesis effect on rats. The hot water-extract of fermented and unfermented leaves was found to reduce blood glucose in hyperglycemic rats, while unfermented leaves also reduced glucose level in normal rats. Both fermented and unfermented leaves also exhibited improved lipid profiles (Fadzelly *et al.*, 2006). Rahmat *et al.* (2006) reported that the methanolic extract displayed strong cytotoxic effect on colon cancer (Caco-2), human breast cancer hormone non-dependent (MDA-MB-231) and liver cancer (HepG-2). The chloroform extract of this plant was also shown to have cytotoxic effect against Caco-2 and HepG-2.

### 1.2.3 Phytochemistry

Soediro *et al.* (1983, 1988) isolated and identified verbacoside, glycosidic ester of caffeic acid and seven phenolic acids; namely p-hydroxy benzoic, p-coumaric, caffeic, vanilic, gentinic, ferulic, and syryngic acids in the leaves. Besides, the leaves also contained tannin, saponin, salt of potassium, sodium and silicate (Departemen Kesehatan Republik Indonesia, 1977[1], 1980;

Syamsuhidayat and Hutapea, 1991[1]; Wijayakusuma *et al.*, 2000). Rahmat *et al.* (2006) reported the presence of β-sitosterol, and stigmasterol in the leaves. The chemical structures of the constituents are presented in Fig. 1.1.

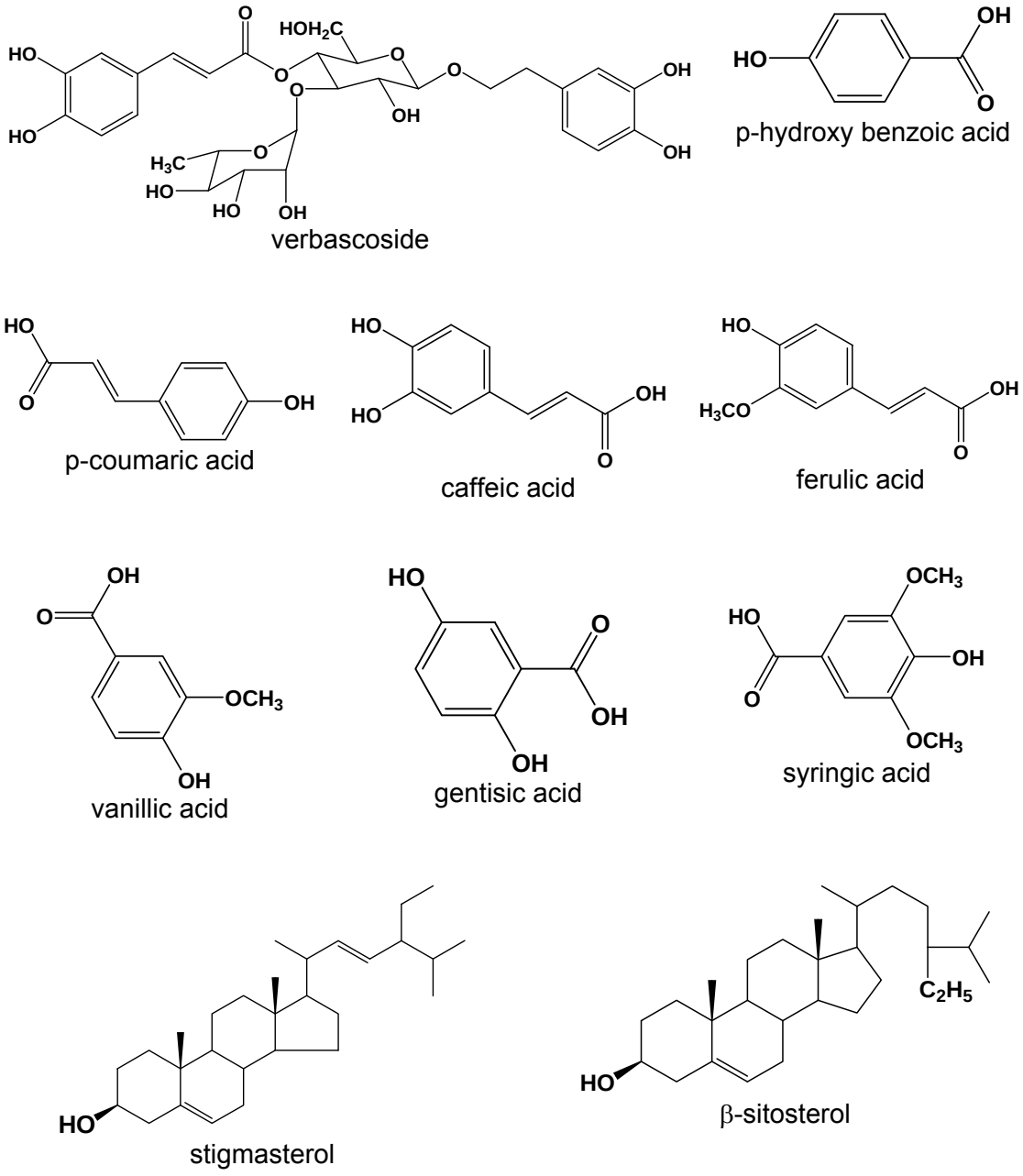


Fig. 1.1 Chemical structures of *S. crispus* constituents

### 1.3 *Sonchus arvensis* Plant

#### 1.3.1 Botanical Description

*S. arvensis* L. is an annual plant that is easy to grow in rainy and sunshine areas, such as on riverbanks, ridges of rice field and abandoned fields 50 – 1650 meters above sea level. The plant is a native of Eurasia with a tapered root and produces bitter latex. The stem is hollow inside. The leaves are single, 6 – 48 cm in length and 3 – 12 cm in width, elliptical, and lanceolate in shape, highly variable, entire to deeply pinnate-lobed, clasping the stem at the base with rounded basal lobes (auricles), sharp-pointed at end side, while at the base is like heart, and green in color.

Flowers are humped shape and having a long stalk, light yellow in color and turns brownish red on maturity. The fruits are thin box shape with five sides, 4 mm in length, hairy and yellowish brown in color. The plants can be propagated using the seeds.

The classification for *Sonchus arvensis* is as follows: Division is Spermatophyta, Sub division is Angiospermae, Class is Dicotyledonae, Sub class is Asterales, Family is Asteraceae, Genus is *Sonchus*, and Species: *Sonchus arvensis.*

The local name is lempung, rayana, jombang and galibug, lalakina (Sunda), tempuyung (Jawa). Other names are Niu she tou (China), Laitron des champs (France), Sow thistle (British) (Dalimartha, 2001; Departemen Kesehatan Republik Indonesia, 1977[2]; Sulaksana *et al.*, 2004; Syamsuhidayat and Hutapea, 1991[2]; Wijayakusuma *et al.*, 2001). Picture of *S. arvensis* is presented in Plate 1.2.

### 1.3.2 Biological Activity

*S. arvensis* L. is one of the medicinal herbs used in traditional medicines, in which the leaf extract was used as a diuretic, lithotriptic and antiurolithiasis agent; also indicated for fever, poisoning and swelling or abscess (Dalimartha, 2001; Syamsuhidayat and Hutapea, 1991[2]). Dalimartha (2001) recommended using 15 – 60 g fresh leaves, boiled in water, and the filtered infusion taken as medicine. For external use, the ground fresh leaves were applied directly on the wounds or the pressed liquid can be used as a compress for abscess, injured skin and wasir (Dalimartha, 2001).

Plate 1.2 Picture of *S. arvensis* plant.

### 1.3.3 Phytochemistry

From the leaves of *S. arvensis* several compounds have been isolated and identified, including luteolin, luteolin-7-O-glucoside (Bondarenko *et al.*, 1973), isocinaroside (Bondarenko *et al.*, 1974), luteolin-7-O-glucoside, linarin (Bondarenko *et al.*, 1975), quercetin, isorhamnetin, chrysoeriol, isorhamnetin-7-β-D-glucoside, quercetin-7-β-D-glucopyranoside (Bondarenko *et al.*, 1976), sonchoside (Bondarenko *et al.*, 1978), and apigenin, luteolin-7-O-glucoside (Qu Guirong *et al.*,1993), acacetin, kaempferol, chrysoeriol, luteolin, isorhamnetin (Qu Guirong *et al.*, 1995), quercetin-3-O-α-L-rhamnoside, kaempferol-3,7-α-L-dirhamnoside (Qu, Guirong *et al.*, 1996), α-amyrin, β-amyrin, lupeol, taraxasterol (lactuserol), pseudo-taraxasterol (Hooper *et al.*, 1982). The leaves also contain manitol, inositol, silica, potassium and saponin (Fig. 1.2).

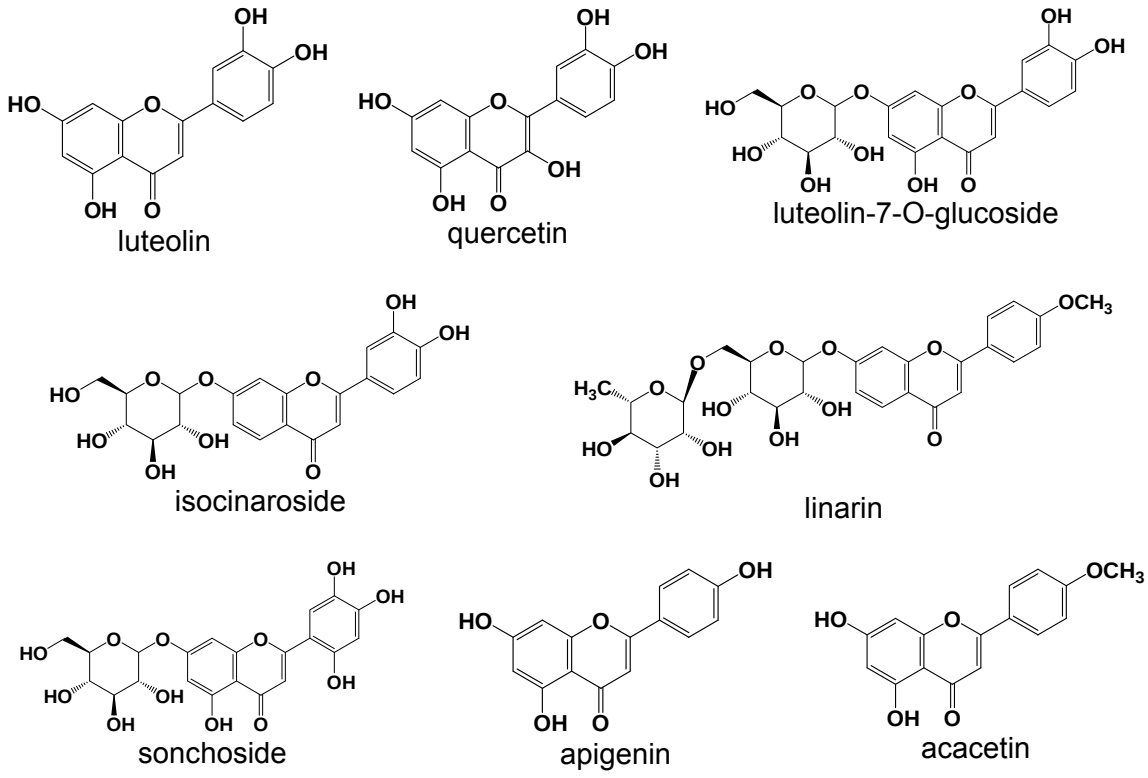


Fig. 1.2 Chemical structures of *S. arvensis* constituents

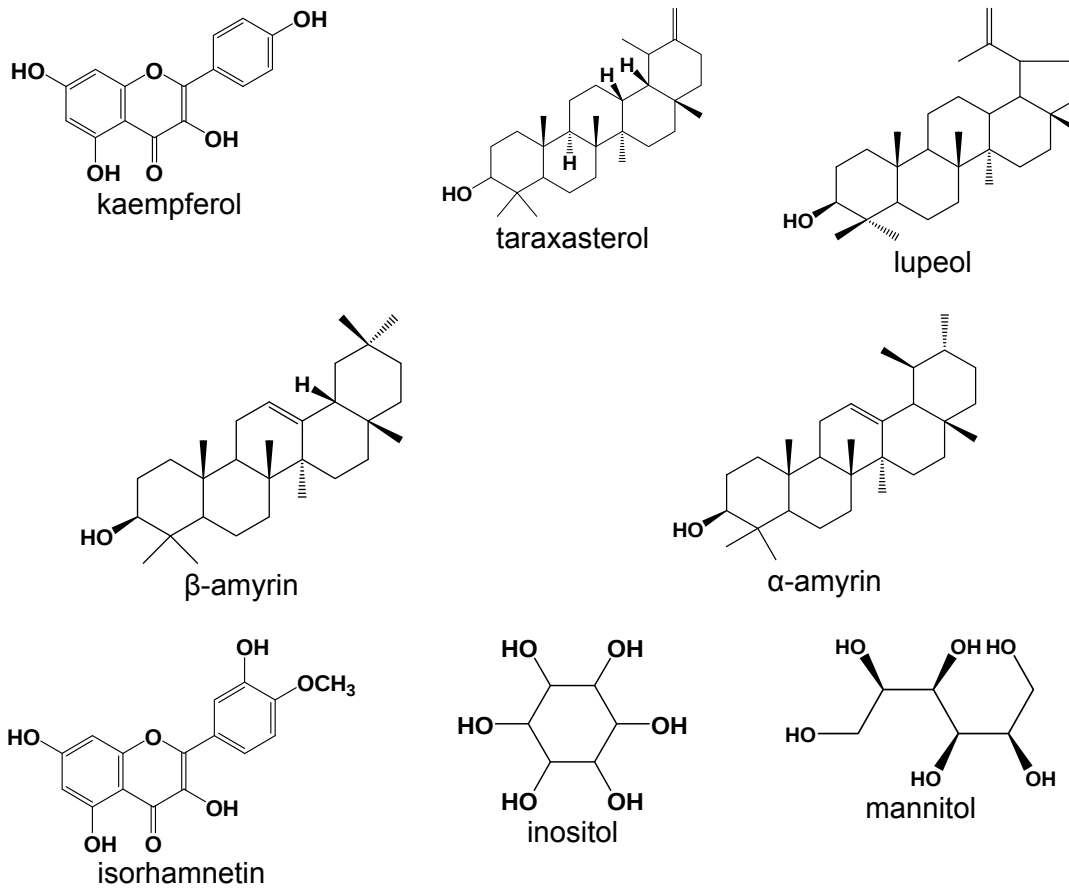


Fig. 1.2 (continued)

### 1.4. Analysis and Isolation of the Chemical Constituents

The purpose of this analysis is to determine the presence of substances in a sample, qualitative or quantitatively. In this study, the aim of the isolation is to obtain compound/s useful as chemical marker.

"Markers are constituents of medicinal plant material that are chemically defined and are of interest for control purpose. Markers are generally used when constituents with known therapeutic activity are not found or are uncertain and may serve to calculate the quantity of plant material or preparation in the finished product. However, the marker has to be quantitatively determined in the

plant material or preparation when the starting materials are tested" (WHO, 1993).

"Chemical constituents in plant vary depending on the genetic heterogeneity of plant species, part of plant, differences in conditions of growth, the age of the plant, the time and manner of collection or harvest, method of processing and storage, shelf life and interaction with the other plant constituents. Furthermore, identification and characterization of the structure of unknown substances are an important part of natural product drug analysis" (Cannell, 1998).

### 1.5 Kidney Stone Disease

#### 1.5.1 Aspects of Kidney Stone Disease

Kidney stones are not a product of modern life, but the Scientists have found evidence of kidney stones in a 7,000-year-old Egyptian mummy. In year 2000, 2.7 million of patients visited health care centers and of these more than 600,000 patients were found to suffer from kidney stone. Men tend to be affected more frequently than women. Prevalence of kidney stone rises for men in their 40s and this continues until they are in their 70s, whilst women tend to suffer disease during their 50s (Coe, 2004).

A kidney stone is a hard mass developed from crystals that separate from the urine and build up on the inner surfaces of the kidney. Urine normally contains chemicals that can inhibit the crystal formation. These chemicals may not function effectively in certain cases leading to stone formation. Fine stone may pass out of the body through the urinary tract. The chemical composition of kidney stones depends on the chemical imbalance in the urine. There are four

types of kidney stones i.e. calcium, uric acid, struvite and cystine stones. Calcium type is predominantly stone, approximately 80%, and the most common type of stone in combination with either oxalate or phosphate. Struvite called infection stone is a less common followed by uric acid stone which is the least common of all. Cystine stones are also very rare (Coe, 2004; Hesse *et al.*, 1976)

### 1.5.2 Natural Product Inhibitor of Urinary Calculi

Traditionally, some plants were found to be acceptable in treating kidney stone and related kidney disorders, for example, *Orthosiphon stamineus* Benth, *Strobilanthes crispus* L. Bremek, *Soncus arvensis* L., *Malpighia coccigera* and genus *Phyllanthus* were used (Perry and Metzger, 1980). The plants used traditionally in kidney stone diseases are presented in Table 1.1.

Table 1.1 Plants used for treatment of kidney stone and related diseases

| No | Plant Name | Constituent | Reference |
|---|---|---|---|
| 1 | *Plantago major* | Glycoside aucubin, plantagin, plantenolic, succinic acid, adenine, cholin, and aucubin. Polysaccharides, lipids, cafeic acid derivatives, flavonoids, iridoid glycosides, terpenoids, alkaloid, organic acid. | Perry and Metzger, 1980; Samuelsen, 2000. |
| 2 | *Zea mays* | Galactan, xylan, dextrose, sugar, zeaxanthin, protein, inosite, hexaphosphoric acid, maizenic acid, resins, potassium and calcium salt. Anthocyanins cyanidin-3-glucoside, pelargonidin-3-glucoside etc. Phenolic acid p-coumaric acid, vanillic acid etc. | Perry and Metzger, 1980; Pedreschi and Cisneros, 2007; Pozo-Insfran *et al.*, 2006 |
| 3 | *Raphanus sativus* | Acylated anthocyanin (as pelargonidin), alkaloids pyrolidine, isoquinoline, phenethylamine, sulphuric compounds glucoparin, sinigrin, allylisothiocyanate. Flavonoids apigenin, apigenin-7-O-triglycoside etc. | Otsuki *et al.*, 2002 ; Vargas *et al.*, 1999; Basile *et al.*, 2003. |
| 4 | *Phylanthus niruri* | Potassium, phyllanthin, hypophyllanthin, triacontanal, triacontanol, lignans, glycosides, flavonoids, alkaloids, tannins, phenylpropanoids, saponins | Perry and Metzger, 1980; Syamasundar *et al.*, 1985 |
| 5 | *Orthosiphon stamineus* | High potassium salt, glucoside, diterpenes, orthosiphols, rosmarinic acid, salvigenin, orthosiphols, flavonoids (eupatorin, sinensitin, 3′-hydroxy-5,6,7,4′-tetramethoxyflavone,TMF) | Perry and Metzger, 1980; Takeda *et al.*, 1993; Akowuah *et al.*, 2005 |

## 1.6 Antioxidant

### 1.6.1 Aspect and Process of Antioxidation

An antioxidant is a chemical or any substance or any enzyme that prevents or reduces the oxidation or oxidative damage due to oxygen or other chemicals. All living organisms contain complex systems of antioxidant enzymes and chemicals. Antioxidants in biological systems have multiple roles and these include deterring oxidative damage and participating in the major signaling pathways of the cells. One major action of antioxidants in cells is to prevent

damage due to the action of reactive oxygen species (ROS) involved hydrogen peroxide ($H_2O_2$), the superoxide anion ($O_2^{\bullet -}$), and free radicals such as the hydroxyl radical (•OH). These molecules are unstable and highly reactive, and can damage cells by chemical chain reactions such as lipid peroxidation, or formation of DNA adducts that could cause cancer-promoting mutations or cell death (Ames *et al.*, 1993; Finkel and Holbrook, 2000). Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases, such as cancer (Muramatsu *et al.*, 1995), atherosclerosis (Steinberg *et al.*, 1989), gastric ulcer (Das *et al.*, 1997).

The classification of antioxidants is one of two ways, i.e. chain-breaking and preventive. In the chain-breaking event, a free radical releases or steals an electron leading to the formation of a second radical. This molecule in turn follows the same path that leads to the formation of a third molecule. This process repeats itself leading to the generation of more unstable products. The process continues until the radical is stabilized by a chain-breaking antioxidant or it simply decays into a harmless product. In preventive, antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase prevent oxidation by reducing the rate of chain initiation (Parnes, 1998). In the works, antioxidants reduce the free radical energy, stop the free radical from forming in the first place, or interrupt an oxidizing chain reaction to minimize the damage caused by free radicals (Ames *et al.*, 1993).

### 1.6.2 Systems of Antioxidation

These systems can be divided into enzymatic and non enzymatic. The enzymatic involved superoxide dismutase (SOD), which catalyses such as the conversion of $O_2^{\bullet -}$ to $H_2O_2$ and $H_2O$, and then convert $H_2O_2$ to $H_2O$ and $O_2$.

Meanwhile non enzymatic involved the lipid-soluble vitamin for example vitamins E and A or provitamin A (β-carotene) (Fouad, 2007).

The example of enzymatic antioxidant is xanthine oxidase (XO), which is a very important enzyme in the purine metabolism involved in the formation of uric acid in the body, i.e. catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. XO is responsible for the medical condition known as gout. Gout is caused by deposition of uric acid in the joints leading to painful inflammation, with inhibition of XO leading to a remission in gout.

The active site of XO is composed of a molybdopterin unit with the molybdenum atom also coordinated by terminal oxygen (oxo) and sulfur atoms and a terminal hydroxide. In the reaction with xanthine to form uric acid, an oxygen atom is tranferred from molybdenum to xanthine. The reformation of the active molybdenum center occurs by the addition of water. Like other known molybdenum-containing oxidoreductases, the oxygen atom introduced to the substrate by XO originates from water rather than from dioxygen ($O_2$) (Chiang *et al.*, 1994; Hille, 2005; Harrison, 2002; Rastelli *et al.*, 1997; Parnes, 2006). Xanthinuria, hypouricemia, hypercalcinuria, and decreased bone density are the diseases caused by insufficient function of xanthine oxidase. Similar symptoms are increased xanthine excretion, decreased uric acid excretion, and mental retardation. One proposed hypothesis says that drinking tea decreases the risk of cancer because of the presence polyphenols which are know as inhibitors of xanthine oxidase (Xu *et al.*, 1994). Some dietary phenolic compounds might function as natural biological response modifier (BRM) by protecting cells or

tissues against injuries especially those caused by lipid peroxidation and/or enzyme mediated oxidation (Nakagami *et al.*, 1995).

The followings are example of non enzymatic antioxidant:

a. Free Radicals

Free radicals are believed to play a role in different health conditions, including the aging process, cancer, and atherosclerosis. Reducing exposure to free radicals and increasing intake of antioxidant nutrient has the potential to reduce the risk of free radical-related health problem.

In the free radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, the purple colored DPPH constitute the stable free radical, which is reduced to 1,1- diphenyl-2-picrylhydrazine (yellow colored) by reacting with an antioxidant The antioxidant donates hydrogen from the hydroxyl group to free radical (DPPH) to inhibit the chain oxidation by the free radical. The product is a stable molecule, which will not initiate or propagate further oxidation of lipids (Sherwin, 1978; Blois, 1958).

b. β-Carotene

β-Carotene is a member of a class of substance called carotenoids is a vitamin that acts as an antioxidant, protecting cells against oxidation damage. Some studies have showed differences in the *in vitro* activities of the β-carotene isomers. One study showed that 9-*cis* β-carotene that isolated from *Dunaliella bardawil* has higher potency to protect methyl linoleate from oxidation than that of the all-*trans* β-carotene isomer (Levin and Mokady, 1994). Another study demonstrated that 9-*cis* β-carotene and all-*trans* β-carotene had equal

antioxidant activities when assessed by enhanced human neutrophil chemiluminescence (Liu *et al*., 2000).

### 1.6.3 Nutritional Antioxidants

The following substances are example of nutritional antioxidant:

a. Vitamins: Vitamin A, C (ascorbic acid), E.

The example of food containing high levels of these antioxidants is fruits, vegetables and vegetable oils. Vitamins are believed to play a role in preventing the development of such chronic diseases as cancer, heart disease, stroke, memory loss, rheumatoid arthritis, and cataracts (Parnes, 1998). Low dietary intake of antioxidant vitamins and minerals increase the incidence rate of cardiovascular disease and cancer (Hercberg *et al.*, 2004)

b. Carotenoid terpenoids (α-carotene, β-carotene).

Carrot is the example of food containing carotenoids.

c. Flavonoid and polyphenolics.

Food containing of these antioxidant are tea, coffee, chocolate, fruits and soy. Flavonoids have a variety of biological effects in numerous mammalian cell systems, *in vitro* as well as *in vivo*. Recently much attention has been paid to their antioxidant properties and to their inhibitory role in various stage of tumor development in animal studies (Hollman *et al.*, 1996; Miller, 1996).

In addition, Yu *et al.* (2006) reported that in a β-carotene-linoleate system, crude protein showed antioxidant activity and Li *et al.* (2007) and Kishk *et al.* (2007) reported that polysaccharides showed also inhibitory activity in β-carotene-linoleate model system. Chuanguang *et al.* (2002) reported that *Misgurnus anguillicaudatus* polysaccharides have ability to remove $O_2^{\bullet -}$, $HO^{\bullet}$, $H_2O_2$ and other oxygen active compounds. Polysaccharides, which are widely

distributed in animals, plants, and microorganisms, have been demonstrated to play an important role as dietary free-radical scavenger for the prevention of oxidative damage (Blander *et al.*, 2003; Harman, 1993; Liu *et al.*, 1997).

As we known, both, *S. crispus* and *S. arvensis* contain such as phenolic and flavonoids, thereby, the purpose of this work is to evaluate the antioxidant activity of the extracts on the oxidative potential.

## 1.7 Angiogenesis

### 1.7.1 Definition and Process of Angiogenesis

Angiogenesis can be defined as the process by which new blood vessel form from pre-existing vessel, which is controlled by certain chemicals produced in the body. The other chemicals stopped the process called angiogenesis inhibitors. The angiogenesis process consists of the following steps, beginning with activation of endothelial cells by growth factors, followed by enzymatic degradation of basement membrane, detachment of endothelial cells from adhesion proteins, endothelial cell migration into the perivascular spaces and proliferation, and final new vessels formation. The process is regulated by various growth factors and cytokines. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), tumor necrosis factor alpha (TNF-a) and interleukin-8 (IL-8) are the potent angiogenic growth factors (Brooks *et al.*, 1999; Huang and Zheng, 2006; Mochizuki *et al.*, 2007).

Angiogenesis plays an important role in the growth and metastasis of tumor and several chronic inflammatory diseases including rheumatoid arthritis and proliferative diabetic retinopathy. Meanwhile many ischemic diseases for examples ischemic coronary artery disease, critical limb ischemia and brain

infarction may benefit from the induction of angiogenesis. Inhibition of angiogenesis has been recognized as a promising therapeutic approach for the control of tumor or cancer growth and metastasis and chronic inflammatory diseases. Tumor or chronic inflammatory diseases cannot grow or spread without the formation of the new blood vessels, the oxygen and nutrients be brought into cells via blood vessels, allowing the cells to grow, invade nearby tissue, spread to other part of the body, and form new cells colonies (Huang and Zheng, 2006, Sheeja *et al.*, 2007, Sylvia *et al.*, 2003, Tsuneki *et al.*, 2005).

### 1.7.2 The Relationship between Antioxidants and Antiangiogenic Agents

A number of antiangiogenesis compounds have been recognized and many have antioxidative properties. Matsubara *et al.* (2005) reported that nasunin; an antioxidant anthocyanin isolated from eggplant peels was demonstrated as an angiogenesis inhibitor. They also implied that nasunin may also be useful to prevent angiogenesis related diseases. Huang and Zheng (2006) reported that rosmarinic acid inhibited angiogenesis of human umbilical vein endothelial cells. Rosmarinic acid, a water soluble polyphenolic compound which is isolated from medicinal plants has been reported to have biological activities such as anti-oxidative, anti-inflammatory and anti-depressive activities.

Several flavonoids that are more widely distributed in the plant kingdom, including 3-hydroxyflavone, 3′,4′-dihydroxyflavone, 2′,3′-dihydroxyflavone, fisetin, apigenin and luteolin have ability to inhibit the *in vitro* angiogenesis process (Fotsis *et al.*, 1997 cited in Mukherjee *et al.*, 1999; Engelmann *et al.*, 2002). Mukherjee *et al.* (1999) reported other flavonoids including genistein and daidzein, an isoflavone have ability in inhibiting of angiogenesis process.

Meanwhile Kim *et al.* (2006) reported flavonol of myricetin, quercetin, kaempferol and galangin can also inhibit angiogenesis process. Previously, Tan *et al.* (2003) reported that quercetin which is found in many fruits and vegetables, as well as olive oil, red wine, and tea, possesses antiangiogenic potential. Various pharmacological activities of quercetin have been demonstrated including antioxidation by scavenging free radicals, prevention of atherosclerosis, and chronic inflammation. Some of the earlier antiangiogenic compounds identified were steroids, including progestin, medroxyprogesterone acetate (MPA), the glucocorticoids, dexamethasone and cortisone (Williams *et al.*, 1999). The other antiangiogenesis compound is squalamine, a natural amino sterol purified and characterized from tissues of the dogfish shark (Williams *et al.*, 1999).

Antiangiogenic activity of the herb extracts was recently reported by Song *et al.* (2003). In this study it was reported that *Phellinus linteus* extract showed strong antiangiogenic and antioxidant activity. The researchers suggested that antioxidant and anti-angiogenic activities of *Phellinus linteus* would be partly responsible for its anti-tumor effect. *In vitro* assay using human endothelial cells of edible berry extracts showed that the extracts impaired angiogenesis (Bagchi *et al.*, 2004). Berries are rich in anthocyanins, compounds that provide pigmentation to fruits and serve as natural antioxidants. Anthocyanins also serve as anti-inflammatory, anti-mutagenic agents and natural antioxidant. Extracts of *Gastrodia elata* rhizome demonstrated potent anti-angiogenic activity in the CAM assay (Ahn *et al.*, 2007). Rhizome of *Gastrodia elata* Blume is a traditional herbal medicine in Oriental countries. Several phenolic

compounds, such as 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde and gastrodin [4-(β-D-glucopyranosyl)benzyl alcohol] have been identified from this plant.

Chemical structures of a variety of phytochemicals exhibiting antiangiogenic activity are presented in Fig. 1.3.

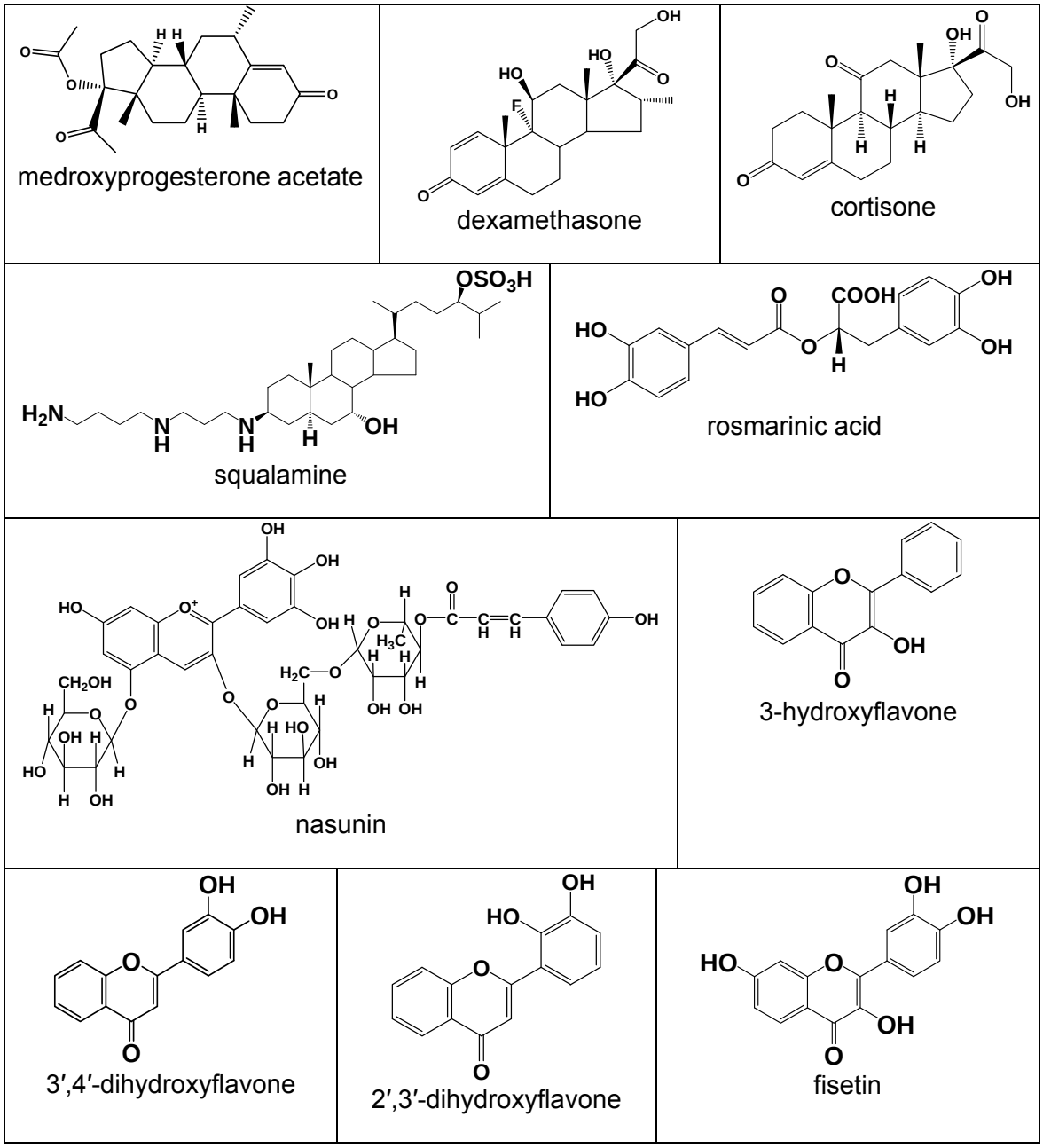


Fig. 1.3 Chemical structures of a variety of phytochemicals exhibiting antiangiogenic activity

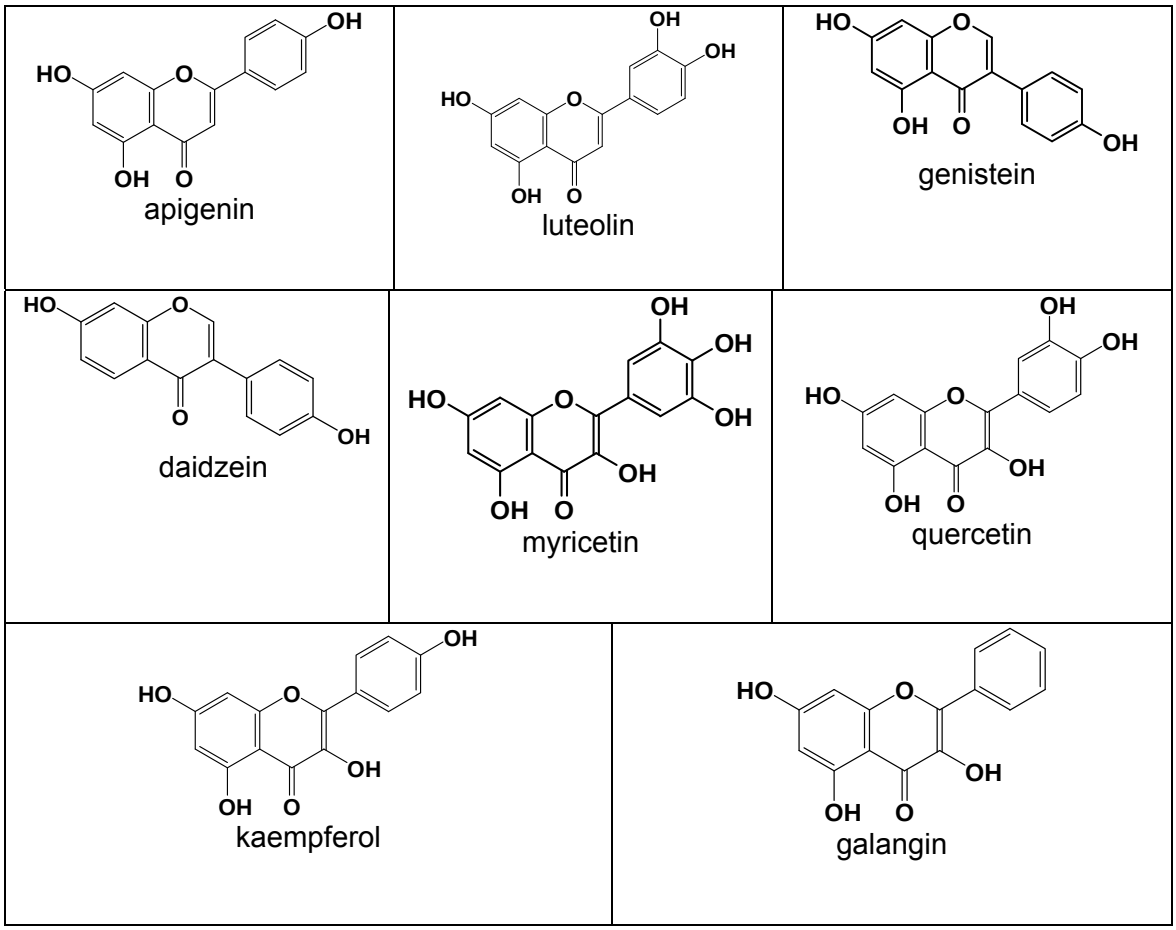

Fig. 1.3 (*continued*).

## 1.8 Drug Stability

### 1.8.1 General Concept in Drug Stability

"The purpose of stability tests is to provide evidences on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, as well as to establish a re-test period for the drug substance or a shelf life for the drug product and recommended storage conditions" (ICH, 2003).

"Stability is one of the most important factors, which determine whether a compound or mixture of compounds can be developed into a therapeutically useful pharmaceutical product. The recognition of this concept, along with ability

to optimize drug stability and product shelf life has been among the most significant achievements in drug research and development. The stability of a pharmaceutical preparation may be defined as its degree of resistance to chemical and physical changes. The efficacy of the preparation must remain constant (or change only within the limits specified by legal provision) until the date of expiration" (Racz, 1989).

Since the herbal drug or herbal drug preparation in its entirety is regarded as the active substance, a mere determination of the stability of the constituents with known therapeutic activity will not suffice. It must also be shown, as far as possible e.g. by means of appropriate fingerprint chromatogram, that other substances present in the herbal drug or in the herbal drug preparation are likewise stable and that their proportional content remains constant. If herbal medicinal product contains several herbal drugs or preparation of several herbal drugs and if it is not possible to determine the stability of each active substance, the stability of the medicinal product should be determined by appropriate fingerprint chromatograms, appropriate overall methods of assay and physical and sensory tests or other appropriate tests.

The variation in content during the proposed shelf-life of herbal medicinal product containing a herbal drug or herbal drug preparation whose constituents of known therapeutic activity should not more than 5% of the initial assay value whilst those whose constituents of unknown therapeutic activity should not exceed 10 % of the initial assay value (CPMP, 2001).

### 1.8.2 Guideline for Stability Testing of Drug Substance and Drug Product

Stress tests of the drug substance can help to identify the likely degradation products, which in turn can help to establish the degradation pathway and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedure used. The nature of the stress tests will depend on the individual drug substance and the type of drug product involved. Stress tests are likely to be carried out on a single batch of the drug substance. It should include the effect of temperature in 10 $^{0}$C increment above that for accelerated testing (e.g. 50°C, 60°C etc.) and humidity at 75 % or greater. The design of the formal stability studies for the product should be based on knowledge of the behavior and properties of the drug substance and from stability studies on the drug substance and on experience gained from clinical formulation studies. The likely changes on storage and the rationale for the selection of attributes to be tested in the formal stability studies should be stated. For long term studies, frequency of testing should be sufficient to establish the stability profile of the drug substance or drug product. Either for drug substances with a proposed re-test period or for drug product with a proposed shelf life of at least 12 months, the frequency of testing at the long term storage condition should normally be every 3 months over the first year, every 6 months over the second year, and annually thereafter through the proposed re-test period for the drug substance or the proposed shelf life for the drug product. At the accelerated storage condition, a minimum of three time points, including the initial and final time points (e.g., 0, 3, and 6 months), from a 6-month study is recommended. In general, a drug substance should be evaluated under storage conditions (with appropriate tolerances) that test its thermal stability and, if applicable, its