PHYTOCHEMICAL INVESTIGATION ON THE LEAVES OF *BLUMEA BALSAMIFERA* DC AND CORN SILK OF *ZEA MAYS* L AND *IN VITRO* EVALUATION OF THEIR USE IN UROLITHIASIS

by

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LIST OF ABBREVIATIONS

$AlCl_3$	Aluminum Chloride
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
$CDCl_3$	Deuterated Chloroform
^{13}C NMR	Carbon Nuclear Magnetic Resonance
CE	Catechin Equivalent
COSY	Correlational Spectroscopy
1D NMR	One Dimensional (1D) Nuclear Magnetic Resonance
2D NMR	Two Dimensional (2D) Nuclear Magnetic Resonance
DMSO-d_6	Deuterated Dimethylsulfoxide
DEPT	Distortionless Enhancement by Polarization Transfer
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EI	Electron Ionization
ESI	Electrospray Ionisation
GC	Gas Chromatography
^1H NMR	Proton Nuclear Magnetic Resonance
H_2O_2	Hydrogen peroxide
H_3BO_3	Boric Acid
HCl	Hydrochloric Acid
HMQC	Heteronuclear Multiple Quantum Correlation
hr	Hour
IR	Infra Red
LPO	Lipid Peroxidation
MeOH	Methanol
mg	Milligram
MHz	Megahertz
min	Minute
mL, L	Milliliter, Liter
m.p.	Melting Point
MS	Mass Spectrum, Mass Spectrometry
m/z	Mass-Charge ratio
NaOH	Sodium Hydroxide
NaOAc	Sodium Acetate
NaOMe	Sodium Methoxide
NOESY	Nuclear Overhauser Effect Spectroscopy
NP	Natural Products
PPM	Parts Per Million
$O_2^{\bullet-}$	Superoxide radical
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
S.E.M/ S.D	Standard Error Mean/ Standard Deviation
sh	Shoulder (low intensity peak)
TLC	Thin Layer Chromatography
XO	Xanthine Oxidase
XHCORR	Heteronuclear (X, H) shift Correlation

KAJIAN FITOKIMIA DAUN *BLUMEA BALSAMIFERA* DC DAN BENANG SARI JAGUNG *ZEA MAYS* L DAN PENILAIAN *IN VITRO* KEGUNAANNYA DALAM UROLITIASIS

ABSTRAK

Daun *Blumea balsamifera* dan benang sari jagung *Zea mays* digunakan secara tradisional bagi keadaan yang berkaitan dengan urolitiasis. Drug-drug mentah *Blumea balsamifera* dinilai menggunakan parameter kawalan mutu yang termasuk analisis-analisis mikroskopi, makroskopi, gravimetri, kromatografi lapisan nipis dan elusi kecerunan kromatografi cecair prestasi tinggi fasa terbalik (RP-HPLC).

Penyelidikan fitokimia ke atas ekstrak-ekstrak pet-eter, kloroform dan metanol daun *Blumea balsamifera* berjaya memencilkan dua hidrokarbon, dua fitosterol dan sebelas flavonoid. Campuran empat asid lemak, dua fitosterol dan tiga flavonoid telah dipencilkan daripada benang sari jagung *Zea mays*. Identiti struktur sebatian-sebatian ini telah ditentukan dengan menggunakan pelbagai kaedah analitikal seperti UV, IR, 1D NMR, MS, X-Ray dan analisis unsur.

Kapasiti antioksidan pelbagai ekstrak pelarut dan flavonoid *Blumea balsamifera* telah dinilaikan dengan kaedah-kaedah yang telah digunakan secara meluas. Ekstrak metanol menunjukkan aktiviti antioksidan lebih tinggi apabila dibandingkan dengan ekstrak-ekstrak kloroform dan pet-eter. Hubungan struktur-aktiviti yang berbeza juga telah menunjukkan kecekapan antioksidan flavonoid-flavonoid tersebut. Kehadiran pengganti hidroksil pada nukleus flavonoid meninggikan aktiviti antioksidan, manakala penggantian oleh kumpulan metoksi menurunkan aktiviti antioksidan sebatian-sebatian tersebut. Hal ini menunjukkan bahawa struktur kimia ialah penentu aktiviti antioksidan sebatian-sebatian tersebut. Keputusan daripada kajian ini memberikan bukti bahawa

ekstrak daun *Blumea balsamifera* dan flavonoid-flavonoidnya menunjukkan sifat-sifat antioksidan yang menarik yang dinyatakan samada dengan mempunyai kapasiti untuk mengaut radikal bebas atau radikal superoksida, atau mempunyai kapasiti untuk merencat aktiviti xantine oksidase, suatu enzim yang terlibat dalam penghasilan asid urik.

Kesan ekstrak-ekstrak daun *Blumea balsamifera,* benang sari jagung *Zea mays* dan flavonoid-flavonoid terpencil *Blumea balsamifera* terhadap kadar pertumbuhan *in-vitro* kristal kalsium oksalat telah ditentukan dengan menggunakan kaedah Schneider slaid gel terubahsuai yang digabungkan dengan sistem penganalisis imej (Image Analyzer System). Ekstrak metanol dari kedua-dua sumber tumbuhan memberikan aktiviti perencatan lebih tinggi berbanding dengan ekstrak-ekstrak kloroform dan pet-eter dalam pertumbuhan in vitro kristal kalsium oksalat dalam urin manusia dan tanpa urin. Tambahan pula, dalam ujian saringan antimikrobial, ekstrak-ekstrak *Blumea balsamifera,* benang sari jagung dan empat flavonoid terpencil *Blumea ba*lsamifera menunjukkan aktiviti spekturm luas terhadap bakteria patogen.

Suatu kaedah isokratik RP-HPLC dengan pengesan UV yang mudah, peka dan mempunyai kebolehulangan telah dibangunkan bagi penentuan kuantitatif lima flavonoid major dalam daun *Blumea balsamifera.* Kaedah yang telah dibangunkan diaplikasikan untuk penentuan serentak flavonoid-flavonoid tersebut dalam plasma tikus. Prosedur pengekstrakan bagi flavonoid dari plasma tikus adalah mudah dan purata pemulihan flavonoid tersebut dari plasma adalah dari 90 hingga 93%. Variasi dalam sehari dan variasi hari ke hari adalah dalam julat nilai yang boleh diterima (<10%). Parameter farmakokinetik bagi flavonoid ditentukan selepas dos 1 g/kg ekstrak diberikan secara oral kepada tikus. Keputusan menunjukan penyerapan dan pengesanan yang baik.

ABSTRACT

The leaves of *Blumea balsamifera* DC and corn silk of *Zea mays* L are traditionally used in conditions related to urolithiasis. The crude drugs of *Blumea balsamifera* were evaluated using quality control parameters, which includes microscopic, macroscopic, gravimetric, thin layer chromatographic and gradient reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis.

Phytochemical investigation on pet-ether, chloroform and methanol extracts of the leaves of *Blumea balsamifera* resulted in the isolation of two hydrocarbons, two phytosterols and eleven flavonoids. Mixtures of four fatty acids, two phytosterols and three flavonoids were isolated from the corn silk of *Zea mays*. The structural identity of these compounds was determined by means of different analytical methods such as UV, IR, 1D NMR, 2D NMR, MS, X-Ray and elemental analysis.

The antioxidant capacity of different solvent extracts and isolated flavonoids of *Blumea balsamifera* was evaluated using widely used methods. Methanol extracts exerted higher antioxidant activity as compared to chloroform and pet-ether extracts. The four classes of isolated flavonoids of *Blumea balsamifera* exhibited various ranges of antioxidant activities. Distinct structure-activity relationships were also revealed for the antioxidant abilities of the flavonoids. The presence of hydroxyl substituents on the flavonoid nucleus enhanced activity, whereas the substitution by methoxyl groups diminished antioxidant activity. It appeared that the chemical structure of these compounds was a determinant to their antioxidant activity. The results of this study provided evidence that *Blumea balsamifera* leaves extract and their flavonoids exhibited interesting antioxidant properties, expressed either by its capacity to scavenge free

radicals or superoxide radicals or its capacity to inhibit xanthine oxidase activity, an enzyme involved in uric acid production.

The effects of extracts of the leaves of *Blumea balsamifera* and corn silk of *Zea mays*, and isolated flavonoids of *Blumea balsamifera* on the *in vitro* growth rate of calcium oxalate crystals were determined using modified Schneider's gel slide method in association with the Image Analyzer System. Methanol extracts of both plant sources exerted higher inhibitory activity as compared to chloroform and pet-ether extracts on *in vitro* growth of calcium oxalate crystals in absence and presence of human urine. Flavonoids with free hydroxyl group at C-3, C-3′ and C-4′ position showed higher inhibitory activity than their methoxylated compounds. In addition, in antimicrobial screening test, extracts of *Blumea balsamifera* and corn silk, and isolated four flavonoids of *Blumea balsamifera* exerted broad-spectrum activity towards pathogenic bacteria.

A simple, sensitive and reproducible isocratic RP-HPLC method using UV detection was developed for the quantitative determination of five major flavonoids in leaves of *Blumea balsamifera*. The developed method was applied for the simultaneous determination of these flavonoids in rat plasma. The extraction procedure for the flavonoids from the rat plasma was simple and the mean recoveries of these flavonoids from plasma range from 90 to 93%. The within-day and the day-to-day variations were within the acceptable value (<10%). The pharmacokinetic parameters of the flavonoids were determined following oral dosing of 1g/kg extracts in rats. The results showed good absorption and detectability.

Chapter 1: INTRODUCTION

1.1 PREAMBLE

Kidney stone disease is a multi-factorial disorder resulting from metabolic abnormalities influencing the composition of body fluids and urine. It affects about 1-3% of the population and the recurrence rate is quite high, about 50% at 10 years and 75% at 15 if untreated (Balaji & Menon, 1997). Several factors such as heredity, age and sex, geographical factors, climate, race, and diet have been suggested for the etiology of stone disease (Smith, 1989). The majority of upper urinary stones are composed of calcium oxalate and calcium phosphate and usually occur in men, while most stones of magnesium and ammonium phosphate occur in the bladder, mostly in women (Smith, 1978).

The principal causative factor for the formation of calcium salt stones is attributed to the supersaturation of precipitating salts (Robertson, 1976; Menon & Koul, 1992). Even though urine is supersaturated with calcium and oxalate ions in normal subjects, they do not form stones because of the presence of smaller sized crystals and qualitatively improved inhibitors and/or larger quantities of inhibitors (Robertson & Peacock, 1972; Backman *et al.*, 1984) that are normally deficient in stone formers.

Calcium oxalate crystal itself was found to injure the membrane (Hackett *et al.*, 1994) that facilitated the fixation of calcium oxalate crystals and subsequent kidney stone (Khan *et al.*, 1990; Khan & Hackett, 1991). Membrane injury was also mediated by lipid peroxidation reaction through the generation of oxygen free radicals and increased production of superoxide anion and hydroxyl radicals (Bijikurien & Selvam, 1989), and increased both superoxide and H_2O_2 generating enzymes such as xanthine oxidase (Ravichandran & Selvam, 1990; Selvam & Ravichandran, 1991; Selvam &

Bijikurien 1992a; Adhirai & Selvam, 1998). Free radicals positively correlated with cellular oxalate, oxalate binding and calcium level and correlated negatively with antioxidant system (Ravichandran & Selvam, 1990; Selvam & Ravichandran, 1991). It appears that compounds having free radical scavenging properties could be promising remedy in calcium oxalate urolithiasis.

A number of medicinal plants have been used traditionally in patients with kidney stone diseases including leaves of *Blumea balsamifera* DC and corn silk *of Zea mays* L. The use of corn silk in urological disorder is well established and being used since the old days (Grieve, 1971). Though the leaves of *Blumea balsamifera* are used as a folk medicine in kidney stone diseases in South-East Asia (Burkill, 1966), but there are no studies to supports its medical uses. In view of these, the present work was aimed to isolate the bioactive compounds from the leaves of *Blumea balsamifera* and corn silk of *Zea mays*.

Among the numerous substances identified in medicinal plants, flavonoids represent one of the most interesting groups biologically active compound. Due to their presence both in edible plants and foods and beverages derived from plants, flavonoids are important constituents of the non-energetic part of the human diet (Manach *et al.*, 1996). It has been found that they possess antioxidant and free radical scavenging activity in foods (Shahidi & Wanasundara, 1992) and several studies have indicated that their consumption is associated with a reduced risk of cancer (Wattenburg, 1985, 1990; Wei *et al.*, 1990; Hertog *et al.*, 1994; Verma *et al.*, 1998). Flavonoid preparations have long been used in medical practice to treat disorders of peripheral circulation, to lower blood pressure and to improve aquaresis (Jaeger *et al.*, 1988). Many of these alleged effects of pharmacological doses of flavonoids are linked to their known functions as strong antioxidants, free radical scavengers, metal chelators (Kaur & Perkins, 1991;

Shahidi & Wanasundara, 1992; van Acker *et al.*, 1996a) and their interaction with enzymes and biomembranes (Esterbauer *et al.*, 1992; Formica & Regelson, 1995; Saija *et al.*, 1995; Ji *et al.*, 1996). The study on these chemical and biochemical properties of flavonoids can be employed to evaluate the medicinal value of the flavonoids and flavonoids rich extracts of the leaves of *Blumea balsamifera.*

The antioxidant activities of plant extracts cannot be evaluated by only a single method due to the complex nature of phytochemicals present in plants. Therefore, commonly accepted assays, including lipid peroxidation inhibitory activity, free radical scavenging activity, the xanthine oxidase inhibitory and superoxide scavenging activities using the enzymatic and non-enzymatic methods, can be employed to evaluate the total antioxidant effects of *Blumea balsamifera* leaves and its flavonoids. The chemical structure of flavonoids is a large determinant for the antioxidant activity. Establishment of structure activity relationship for flavonoids will allow for their selection as antioxidants based on structure alone, without having to screen every possible compound for activity.

The polyphenolic structure of flavonoids confers them with the ability to scavenge free radicals and chelate metal ions such as iron, copper and calcium (Afanas'ev *et al.*, 1989; Raihan, 1992; Morel *et al.*, 1994; van Acker *et al.*, 1996a; Ismail *et al.*, 1999). The antioxidant and metal chelating properties of these compounds can be employed to determine the effect of flavonoids on the *in-vitro* growth rate of calcium oxalate crystals, as it is a most important constituent of kidney stone. In addition, the test can be carried out in the presence of human urine. The positive inhibitory effect of flavonoids and flavonoids rich extracts as well as their potential antioxidant properties will allow the selection for flavonoids and extracts as antiurolithic agents.

The urinary tract infections by bacteria affect the structure and function of the kidneys and can chemically affect the urine itself leading to stone formation, known as "struvite" or infection stones (Savitz & Leslie, 2003). Therefore, the antibacterial studies on the extracts and flavonoids of the leaves of *Blumea balsamifera* and corn silk of *Zea mays*, can be carried out to strengthen their medicinal use in urolithiasis.

Upon evaluation of medicinal potential of flavonoid rich extracts, a sensitive and accurate reversed-phase high-performance liquid-chromatographic (RP-HPLC) method need to be developed to determine these flavonoids present in extracts of the leaves of *Blumea balsamifera* for standardization of the crude drug. The developed method can be applied for quantification of flavonoids in rat plasma following oral administration of the crude drugs in rats in order to support pre-clinical pharmacokinetic studies.

1.2 OBJECTIVES OF THIS RESEARCH

- The present work was undertaken with the following objectives:

1. To study on the chemical constituents of the leaves of *Blumea balsamifera* and corn silk of *Zea mays* and to establish a fairly comprehensive data on the compounds isolated from these plants.
2. To evaluate the medicinal value of the extracts and flavonoids of the leaves of *Blumea balsamifera* and corn silk of *Zea may* by means of several studies related to antioxidant, antiurolithic and antibacterial activities, and to establish the structure-activity relationship for the studied flavonoids.
3. To develop an accurate and sensitive RP-HPLC method for the quantification of flavonoids of crude drugs and to evaluate the pharmacokinetic profiles for the extracts of the leaves of *Blumea balsamifera* to promote the development of standardized phytomedicine.

Chapter 2.0: LITERATURE REVIEW

2.1 KIDNEY STONES DISEASE

The medical terminology for kidney stone is nephrolithiasis or urolithiasis. A kidney stone is a solid lump made up of crystals that separate from urine and build up on the inner surfaces of the kidney. Kidney stone diseases are one of the most common disorders of the urinary tract. It is estimated that one to five per cent of the population will develop stones at some point in life.

There are geographical, temporal and ethnic correlations of stone formation that reinforce the notion that stones are symptoms arising from multiple causes (King, 1971), like from malnutrition and vitamin deficiency (Bergmann & Nagel, 1982), more industrialized country (Li *et al.*, 1985) and high standard of living (Borghi *et al.*, 1990; Savitz & Leslie, 2003). In less well-developed countries in Asia, Anderson (1973) reported dietary factors are the principle factor for kidney stone diseases such as a deficiency in protein, less fat in food, and dependence on vegetable as the main source of protein may be the principal dietary factors.

A high incidence of renal stone, were also observed in people who lived in the tropical countries for a sufficient long period. Incidence of stones in Peninsular Malaysia appears to show the same trend as in other industrialized countries. This rise is due to the rapid physical development and change in the food habits of the people. In 1980, the incidence of urinary calculi in Malaysia was estimated at 34.9 per 1,00,000 population (Sreenevasan, 1990). Recurrence of stone disease occurs in 60% of adults within 9 years of the initial episode (Sreenevasan, 1990). It is a challenge to reduce the high recurrence rate of this painful condition. Therefore, identification and treatment of predisposing causes are important, to prevent recurrence of kidney stone.

2.1.1 TYPES OF KIDNEY STONES

2.1.1.1 Calcium oxalate stone

The most common kidney stone is the calcium oxalate stone. When the body's *calcium*, an abundant mineral throughout the system and a compound called *oxalate* combine in the kidneys, they form an insoluble salt that can easily turn into a stone. Some 85% of all kidney stones are calcium oxalate stones (Coe, 1978; Savitz & Leslie, 2003).

2.1.1.2 Calcium phosphate stone

Calcium phosphate stones are less common. Both calcium and phosphate are very abundant in the body, and calcium phosphate crystals stiffen the bones and make them rigid. The body eliminates large amounts of phosphate through the urine every day, but if one suffers from a condition that increases calcium output in the urine, which makes the urine more alkaline, the calcium combines with the abundant phosphate in the urine and produces stones. Most calcium oxalate stones have a certain amount of calcium phosphate material in them (Badenoch, 1974; Savitz & Leslie, 2003).

2.1.1.3 Uric acid stones

Uric acid stones are much less common than calcium oxalate stones. Sometimes they are made up of pure uric acid, and sometimes they form as a mixture of uric acid with calcium oxalate. The body makes uric acid when it breaks down RNA and DNA as a normal body function. Uric acid can crystallize into stones when the urine is persistently acid. About 10% of stones are made of uric acid (Badenoch, 1974; Savitz & Leslie, 2003).

2.1.1.4 Struvite

Struvite stones are made up of magnesium, ammonium and phosphate. They always result from infection. During a urinary infection, certain bacteria can break down urea, which is a component of urine, into ammonia. This makes the urine surrounding the bacteria extremely alkaline, so that the magnesium and phosphorus, which normally present in the urine form crystals with the ammonia. About 9% to 17% of stones are struvite (Savitz & Leslie, 2003).

2.1.1.5 Cystine stone

This type of stone is much less common. It is made up of the amino acid, cystine, and occurs only with patients suffering from a genetic disease called cystinuria. Children who get kidney stones may get cystine stones, since the condition is hereditary. About 1% of stones are made of cystine (Savitz & Leslie, 2003).

2.1.2 CAUSES OF CALCIUM OXALATE STONE

Two third of all kidney stones are made of calcium oxalate or mixed with calcium phosphate (Wardle, 1979; Sidabutar, 1981; Savitz & Leslie, 2003). The majority of patients, 85 percent, formed calcium oxalate stones were not due to specific diseases but to idiopathic hypercalciuria, hyperuricosuria, mild hyperoxaluria, or hypocitraturia (Solomon, 1978; Savitz & Leslie, 2003). The most common cause of calcium stone production is excess calcium in the urine termed as hypercalciuria. Excess calcium is normally removed from the blood by the kidneys and excreted in the urine. In hypercalciuria, excess calcium builds up in the kidneys and urine, where it combines with other waste products to form stones (Dent & Watson, 1965; Nakada *et al.*, 1988). Low levels of citrate, high levels of oxalate and uric acid, and inadequate

urinary volume may also cause calcium stone formation (Robertson & Peacock, 1980; Kok *et al.*, 1986; Embon *et al.*, 1990).

Increased intestinal absorption of calcium termed as absorptive hypercalciuria, excessive hormone levels as in hyperparathyroidism, and renal calcium leak results from kidney defect that causes excessive calcium to enter the urine, can cause hypercalciuria and therefore may cause calcium stones (Badenoch, 1974; Solomon, 1978; Schwille, 1979; Savitz & Leslie, 2003).

Diet plays an important role in the development of calcium oxalate stones, especially in patients who are predisposed to the condition (Robertson, 1987; Trinchieri *et al.*, 1991; Heller *et al.*, 2003). A diet high in sodium, fats, meat, and sugar, and low in fiber, vegetable protein, and unrefined carbohydrates increases the risk for renal stone disease (Breslau *et al.*, 1982; Brockis *et al.*, 1982). Recurrent kidney stones may form in patients who are sensitive to the chemical byproducts of animal protein and who consume large amounts of meat (Robertson *et al.*, 1979; Kok *et al.*, 1990a).

Hyperoxaiuria, a condition of high levels of oxalate in urine also responsible for calcium stones formation. High doses of vitamin C (i.e., more than 500 mg per day) can result in high levels of oxalate in the urine and increase the risk for calcium oxalate stones. Food containing high levels of oxalate, such as berries, vegetables (e.g., green beans, beets, spinach, squash, tomatoes), nuts, chocolate, and tea are also responsible for calcium oxalate stone (Brinkley *et al.*, 1981; Trinchieri *et al.*, 1991).

Hyperuricosuria, causes increased urinary uric acid excretion is also associated with calcium oxalate stone. The mechanism may be the deposition of calcium oxalate crystals upon uric acid crystals. Hyperuricosuria may either be isolated or associated with hypercalciuria. Treatment is with allopurinol, an agent that reduces uric acid

excretion, and with restriction of excess purine and animal protein intake (Solomon, 1978; Coe, 1983).

Hypercalciuria condition is generally treated by both counseling against certain dietary excesses, and by drug administration. Patients are advised against a calcium intake of more than 1 g per day, and a high sodium intake. The most commonly used drug treatment consists of thiazide diuretics, which reduce renal calcium excretion (Wardle, 1979).

Ebisuno *et al.* (1986) reported that rice bran effectively reduces urinary calcium excretion by reducing the intestinal absorption of calcium and may be effective in preventive urinary calcium stone. Proper hydration prevents the urine from becoming concentrated with crystals, which leads to stone formation; and it reduces the risk of urinary tract infections, which reduces the risk of formation of stones (Wardle, 1979; Embon *et al.*, 1990).

Human urine is normally supersaturated with respect to calcium oxalate hydrates, octacalcium phosphate [$Ca_4H(PO_4)_3$, OCP], hydroxyapatite [$Ca_5(PO_4)OH$, HAP] and sometimes dicalcium phosphate dihydrate [$Ca_2HPO_4.2H_2O$, DCPD]. The degree of supersaturation is usually higher in stone former patients mainly because they tend to have higher urinary calcium (Nancollas, 1983). Many non-stone formers have a higher than normal urinary calcium excretion, and their urine are saturated with respect to calcium oxalate and calcium phosphate, yet crystallization does not occur. Along with saturation of urine, other factors are also involved in stone formation (Dent & Sutor, 1971).

Randall (1937) suggested that renal cell injury is an important factor that facilitates the retention and subsequently the formation of renal calculi. Experimental evidence suggested that oxalate induced membrane injury was mediated by lipid

peroxidation (LPO) reaction through the generation of oxygen free radicals (Scheid *et al.*, 1996; Thamilselvan *et al.*, 1997; Thamilselvan *et al.*, 2000). In urolithic rat kidney or oxalate exposed cultured cells, both superoxide anion and hydroxyl radicals were generated in excess, causing cellular injury (Bijikurien & Selvam, 1989). In hyperoxaluric rat kidney, both superoxide and H_2O_2 generating enzymes such as xanthine oxidase were increased (Ravichandran & Selvam, 1990; Selvam & Ravichandran, 1991; Selvam & Bijikurien , 1991, 1992a; Adhirai & Selvam, 1998) and hydroxyl radicals and transitional metal ions, iron and copper were accumulated (Ravichandran & Selvam, 1990, 1991; Selvam & Ravichandran, 1991). The lipid peroxidation products were excessively released in tissues of urolithic rats and in plasma of rats as well as in stone patients (Scheid *et al.*, 1996; Thamilselvan *et al.*, 2000). The accumulation of these products was concomitant with the decrease in the antioxidant enzymes such as superoxide dismutase (SOD) as well as radical scavengers, vitamin E and ascorbic acid (Ravichandran & Selvam, 1990, 1991; Selvam & Bijikurien, 1991, 1992a). All these parameters were decreased in the urolithic condition, irrespective of the agents used for the induction of urolithiasis.

Slater (1984) reported that antioxidant therapy to urolithic rats prevented cell damage by preventing free radical mediated diseases in urolithiasis. Citrate or magnesium citrate was advocated to stone patients, and the reduction of excretion of stone risk factors was attributed to its complex formation with calcium, thereby preventing calcium oxalate crystal formation (Lee *et al.*, 1999). However, citrate treatment had no effect on free radical-mediated reactions in experimental urolithiatic rats, on either reducing lipid peroxidation (LPO) or improving antioxidant levels, even though it prevented stone formation (Selvam & Bijikurien, 1992b).

2.1.3 THEORIES OF STONE FORMATION

The initial step in the formation of stone- is the development of a nidus. A stone can form only when urine is supersaturated with respect to its constituent crystals. Supersaturation means that the concentration of a stone forming salt, such as calcium oxalate, exceeds its solubility in urine. Urine of most non-stone formers is supersaturated with respect to calcium oxalate, so in principle all non-stone formers can form such stones (Robertson, 1977; Savitz & Leslie, 2003). Normal urine is not supersaturated with respect to uric acid, cystine or struvite. Conditions that raise calcium oxalate supersaturation raise the risk of calcium oxalate stones. Several mechanisms have been postulated for the formation of stones in the urinary tract, such as supersaturation of stone-forming ionic species, extacellular matrix nucleation, absence of inhibitors and cell injury leading to attachment of crystals followed by retentions of the crystals by the renal cells.

Increased concentration of crystal forming substances occur if the volume of urine is significantly reduced or there are abnormally higher amounts crystal forming substances, such as calcium, oxalate, uric acid, cystine or xanthine, being excreted in the urine. Fifty percent of all calcium stone former have increased urinary excretion of calcium or oxalate salts (Dent & Sutor, 1971; Robertson & Peacock, 1972; Drach *et al.*, 1980).

The inhibitor-absence theory indicates that normal urine contains inhibitors of crystal formation. Inhibitors of nucleation may include certain peptides, magnesium, citrate, pyrophosphate, and other substances that prevent or inhibit stone-building substances from forming crystals. Low levels of these inhibitors can contribute to the formation of kidney stones. Of these, citrate is thought to be the most important. Changes of the pH of the urine that causes acid or alkaline imbalances can also affect

stone precipitation (Soloman, 1978; Nancollas, 1983; Kok *et al.*, 1990a; Fuselier *et al.*, 1998).

The extacellular matrix nucleation theory implies that a urinary substance such as mucoprotein forms the initial matrix around which the crystalloid is deposited. The factors predisposes to matrix formation is unknown but some data suggest that nucleation of calcium phosphate monohydrate (brushite) is the initial step in the formation of stones containing calcium phosphate alone or mixed with calcium oxalate (Sallis, 1987; Baumann, 1990).

Cell injury is the primary event for crystal binding. In order for the renal cell to retain calcium oxalate crystals, the crystals should bind to the cell primarily. In support of this, Randall (1937) was the first to show initiation of renal calculi to occur as subepithelial calcified plaques in the renal papilla, both in the interstitium and within the nephronic duct. Carr (1953) had observed renal calculi formation following the obstructions of the lymphatic system in the form of small radiographic opacities (Carr bodies) that were found in all the kidneys from stone-forming patients. He observed that the presence of renal cavities with low urodynamic efficacy retain urine for long periods of time, favoring calculus formation. In these studies, it is evident that both molecular adhesion and stagnation of crystals in an anatomically constrained region play a vital role for the pathogenic mechanism in the growth of renal stones.

Free calcium oxalate crystals formed within the renal tubule cannot grow rapidly enough to block a collecting duct at the rate of normal urinary flow and become a kidney stone, because the time needed for a crystal to grow to a diameter of 200 μm and block the nephron is calculated to be from 90 min to 1500 years (Finlayson, 1974). So Finlayson & Reid (1978) concluded that, in order to form a stone, the crystals should be attached to the epithelium, and they suggested the fixed particle hypothesis. In support

of this, Vermuelen *et al.* (1967) demonstrated crystal retention in the rat renal papilla in experimentally induced crystalluric rat. The renal papilla was found to be the primary nucleation site because of the existence of oxalate and calcium gradient (Hautmann *et al.*, 1981). The evidence for crystal attachment to the epithelial basal lamina in crytalluric rat kidney was presented by the studies of Khan *et al.* (1982). Mandel & Riese (1991) observed more crystals attachment to cells that have lost partial or complete intercellular junctional integrity. They suggested that membrane injury exposes the basolateral or basement membrane crystal-binding molecules, facilitating crystal attachment. Further studies using chemically injured urothelium (Gill *et al.*, 1979; Khan *et al.*, 1982), gentamicin-pretreated rat kidney (Sigmon *et al.*, 1991), or damaged epithelium with reduced glycosaminoglycan layer (Grases *et al.*, 1998) were in support of membrane injury as the cause of the enhanced crystal retention. These findings have suggested that membrane injury plays a significant role as a predisposing factor for crystal binding and retention reaction.

Much attention was focused on oxalate-induced cell injury for the facilitation of crystal adherence (De Water *et al.*, 1999; Scheid *et al.*, 2000). Hackett *et al.* (1994) reported that calcium oxalate crystals injured the membrane by interacting with the cell membrane and released the cellular contents. Similarly, in hyperoxaluria, renal tubular membrane injury was observed with excretion of enzymes of epithelial membrane origin. Further crystal deposition led to the detachment of the basement membrane, and membranous cellular degradation products were found to promote crystal formation and aggregation (Khan *et al.*, 1990) and facilitate its retention. Bijikurien & Selvam (1989) supported that oxalate damaged the membrane by promoting lipid peroxidation (LPO) and suggested that increased LPO formation by oxalate was probably associated with the generation of oxygen free radicals such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl

radicals ($^{\bullet}OH$), lipid peroxidation (LPO) products and hydrogen peroxide (H_2O_2). Free radicals induced the cell injury and subsequently damaged cell layers favored the adherence of calcium oxalate crystals and intra-tubular stone formation (Selvam & Bijikurien, 1991; Muthukumar & Selvam, 1997; Selvam & Adhirai, 1997; Thamilselvan & Selvam, 1997; Grases *et al.*, 1998; De Water *et al.*, 1999; Scheid *et al.*, 2000; Selvam & Kalaiselvi, 2001) as shown in Figure 2.1.

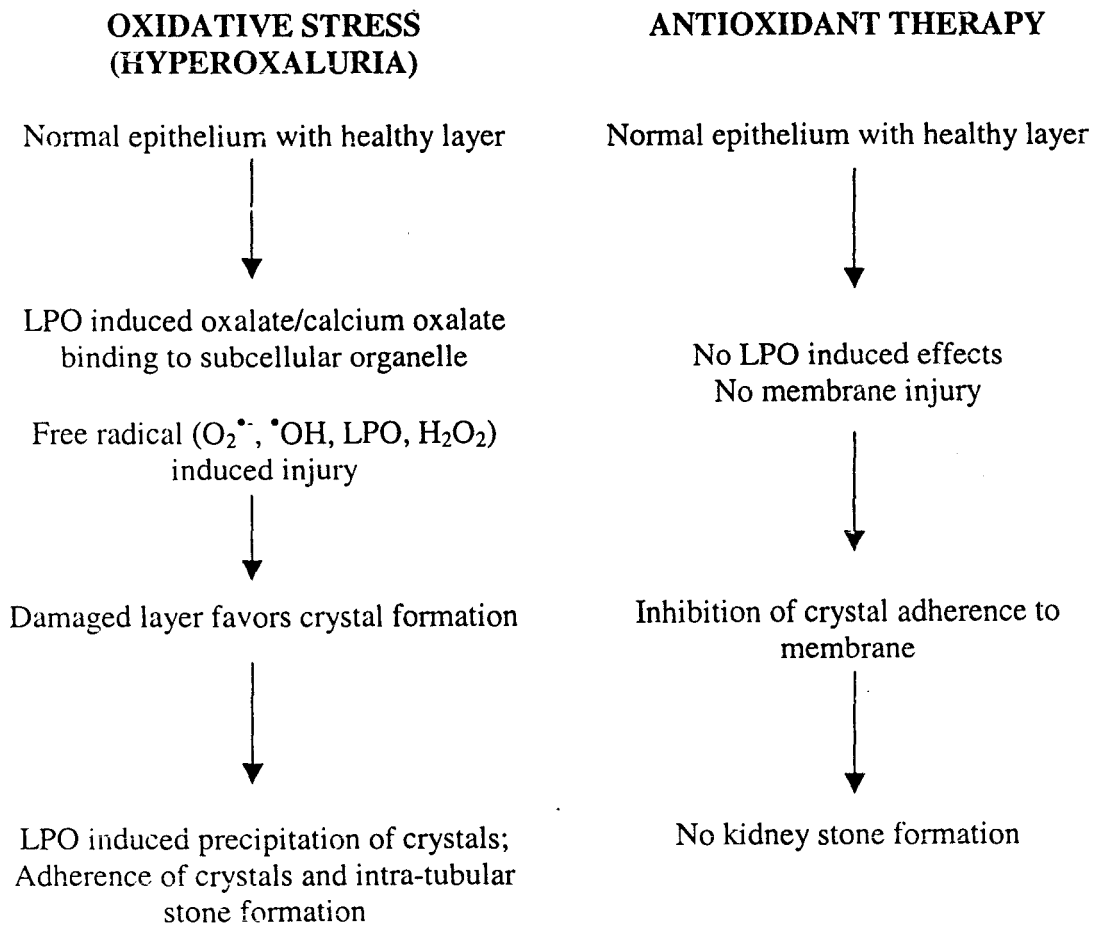


Figure 2.1: Renal cell injury facilitates the adherence of calcium oxalate crystals and the preventive role of antioxidant therapy.

Grases *et al.* (1998) described the effect of free radicals on the development of calcium oxalate crystals on urothelium. They demonstrated that free radical-damaged cells produce a favorable environment for crystal development. At low free radical concentrations, crystals develop on calcium-enriched zones, whereas at higher concentrations, crystals develop on areas with a destroyed monolayer of superficial cells. Antioxidants, such as ascorbic acid and mannitol, exerted the most remarkable effects in avoiding calcium oxalate crystal development, whereas crystal inhibitors, such as citric acid, did not produce any remarkable reduction in calcium oxalate crystallization. Phytic acid notably decreased calcium oxalate crystal development. The ability of phytic acid to diminish calcium oxalate crystallization was attributed to the combination of its inhibitory capacity of calcium oxalate crystallization and its preventive antioxidant action (Grases *et al.*, 1998).

Neprolithiasis, which was readily produced in control animals, was prevented in the experimental animal by pretreatment with fish oil and urine calcium was significantly reduced. The urinary calcium and oxalate excretion in the recurrent hypercalciuric stone formers was significantly reduced with fish oil treatment. All these observations implicated the radical-mediated membrane changes, predisposing a favorable environment for subsequent crystal disposition and retention. Antioxidants can protect the membrane from injury and prevent adherence or retention of the crystals (Bigelow *et al.*, 1997).

2.1.4 NATURAL INHIBITORS IN URINE

Human urine normally contains substances that can prevent the formation of calcium oxalate crystals and hence prevent the formation of stone (Nakagawa *et al.*, 1983). On the basis of molecular weight, inhibitors of calcium oxalate stone formation

have been classified into two groups. The low molecular weight material (10,000 Daltons) probably accounts for approximately 80% of the total inhibition activity in urine (Sallis, 1987). It includes organic substances such as urea [1], citrate [2] or inorganic ionic substances such as magnesium, pyrophosphate [3] (Figure 2.2). It is believed that these substances act as inhibitors of stone formation by forming a soluble complex with the crystalline ions and/or by adsorption onto the crystal surfaces with subsequent blockage of further growth of crystals (Throne & Resnick, 1983; Fuselier *et al.*, 1998).

The high molecular weight substances includes glycosaminoglycans (710,000 Daltons), acid peptides, RNA-like substances, acid glycoproteins (17,000 Daltons), nephrocalcin (NC) (14,000 Daltons), Tamm-Horsfall glycoprotein (THP) (80,000 Daltons), glycoproteins or protoglycoproteins (Rose & Sulaiman, 1984; Thorne & Resnick, 1983; Asplin *et al.*, 1991). There are several publications reported and suggested that these high molecular weight urinary components are strong inhibitors of calcium oxalate crystal growth and their aggregation (Nakagawa *et al.*, 1983; Nishio *et al.*, 1985; Koide *et al.*, 1990; Hess, 1992; Chang *et al.*, 2001a). It is also reported that the inhibitory activity of an inhibitor on crystal aggregation depends on the urinary pH and its ionic strength (IS). At high pH and low IS, THP is a powerful inhibitor of crystal aggregation and in contrary, it acts as promoter of calcium oxalate crystal aggregation at low pH and high IS (Hess, 1992). Chen *et al.* (2001) reported that THP showed uncertain inhibitory action on the crystallization of calcium oxalate monohydrate. This inhibitory action was partly related to sialic acid.

The interesting role of glycosaminoglycans (GAGS) in calcium stone crystallization leading to the identification of a number of GAGS, which includes chondroitin sulphate, hyluronic acid, dermatan sulphate, heparin and heparan sulphate

(Nicolla, 1963; Goldberg & Cotlier, 1972; Robertson *et al.*, 1973). Their mechanism of action are generally considered to be a result of adsorption of highly charged polysaccharide to crystal nidus and the surface of the growing crystal, thus retarding nucleation and growth (Sallis, 1987).

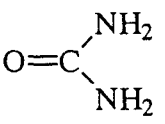
Recenltly, Webber *et al.* (2002) purified urinary prothrombin fragment 1 (UPTF1) and Hoyer *et al.* (2001) isolated osteopontin from human urine [uropontin (uOPN)], both exhibited potent inhibitory activity on calcium oxalate crystallization. Laube *et al.* (2001) reported that hippuric acid (HA) is a physiological component of human urine showed inhibitory activity on calcium oxalate growth and considerably enhances the calcium oxalate solubility in artificial urine. They concluded that HA was a major modifier of calcium oxalate formation.

2.1.5 CHEMICAL INHIBITORS

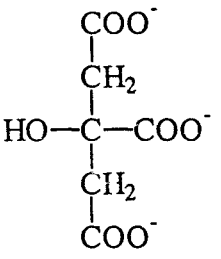
There are some chemical compounds found to have markedly inhibitory effects on growth, nucleation or aggregation of calcium oxalate crystals such as allopurinol [4], methylene blue [5] and EDTA [6] as shown in Figure 2.2. Some are clinically useful compounds, such as sodium cellulose phosphate, disodium and dipotassium phosphates (Lepage & Tawashi, 1982; Tanagho & McAninch, 1988).

Allopurinol is beneficial in prevention of calcium oxalate or uric acid stone (Charles & Pak, 1981; Emmerson, 1993). Methylene blue has been suggested as urolithiatic inhibitor, was found to be effective in the treatment of new stone formation in patients with calcium oxalate renal calculi (Boyce *et al.*, 1967; Ahmed & Tawashi, 1978), but it gives adverse effect on uric acid calculi (Ismail *et al.*, 1985). Ethylenediaminetetraacetic acid (EDTA), a chelating agent has also been used in a number of patients to dissolve the calcium containing calculi. However dissolution

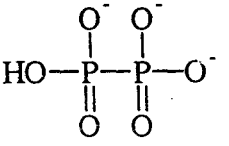
often required thousands of irrigation, and a long hospitalization required to keep this treatment from achieving clinical popularity (Timmermann & Kallistrators, 1966). Oosterlinck *et al.* (1992) reported that dipotassium ethylenediaminetetraacetic acid is toxic to urothelium. The clinical use of calcium ligands is therefore unsafe.



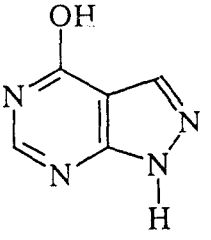
[1] Urea



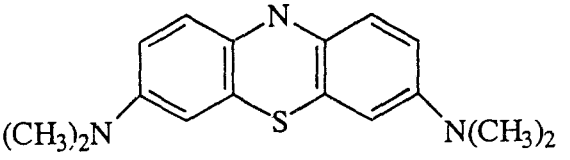
[2] Citrate



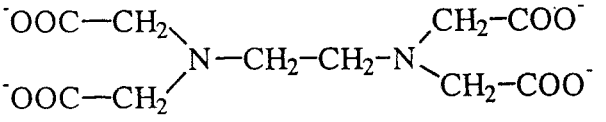
[3] Pyrophosphate



[4] Allopurinol



[5] Methylene blue



[6] EDTA

Figure 2.2: Structural formula of low molecular weight urinary inhibitors (urea, citrate and pyrophosphate) and chemical inhibitors (allopurinol, methylene blue and EDTA).

2.1.6 NATURAL INHIBITORS FROM PLANTS (HERBAL MEDICINE)

Herbal medicines rarely have significant side effects when used appropriately and at suggested doses. Occasionally, an herb at the prescribed dose causes stomach upset or headache. This may reflect the purity of the preparation or added ingredients, such as synthetic binders or fillers (Standard of Asean Herbal Medicine, 1993). For this reason, it is recommended that only high-quality products be used. Some plants traditionally found acceptable in treating diseases in the human body system in conditions related to kidney disorders are listed in Table 2.1.

Table 2.1: Plants used for kidney stone and related diseases

Plant name	Family	Constituent	Reference
Blumea balsamifera DC (Capa)	*Compositae*	Flavonoids, phytosterols, hydrocarbons	Burkill & Haniff, 1930; Burkill, 1966; Zhari *et al.*, 1999
Zea mays L (Corn silk) (Benang sari)	*Gramineae*	Flavones, saponins, tannin, minerals, phenolic acid	Perry, 1985 Seno, 1988
Orthosiphon stamineus (Misai kuching)	*Labiatae*	Flavonoids, terpenoids, phytosterols, phenolic acid	Burkill, 1966
Orthosiphon grandiflorus (Kumis kuching)	*Labiatae*	Flavonoids, minerals	Perry, 1985 Seno, 1988
Sonchus arvensis (L) (Tempuyung)	*Compositae*	Flavonoids, ceryl alcohol, choline	Perry, 1985 Seno, 1988
Raphanus sativus (Lobak putih)	*Cruciferae*	Flavonoids, carbohydrate, amino acids	Capta, 1987
Shima noronhae (Huru batu)	*Lauraceae*	Saponin and tannin	Perry, 1985
Malpighia coccigera (Teh hutan)	*Malpighiaceae*	Flavonoids, triterpene, phytosterol	Burkill, 1966

2.1.7 CRYSTALLIZATION INHIBITION STUDIES

Stone formation is a biological process that involves a physicochemical element, crystallization. Two major aspects of crystallization are a thermodynamic one which includes supersaturation leading to nucleation, and a kinetic one which comprises the rate of nucleation, crystal growth and crystal agglomeration respectively (Kok *et al.*, 1990b).

Baumann (1990) reported that chelators, inhibitors and promoters could disturb the crystallization process. In principle chelators trap the free ions and form soluble complex. Low molecular weight substances, such as magnesium, citrate, act as chelators as well inhibitors of growth and nucleation, are supposed to block growth sites on crystal niduses, on preformed crystal and also on some promoters.

Macromolecules substances can convert an inhibitor to a promoter by polymerization (Embon *et al.*, 1990) or immobilization on crystal surface (Hess, 1992). In this situation, they probably bind with a series of calcium ions, in a constellation that is ideal of epitaxy growth of calcium oxalate or calcium phosphate (Baumann, 1990). It seems that more than one factor are always involved in the formation and prevention of stone nucleus (King, 1967).

Crystallization inhibition is a phenomena that clearly affects crystal growth and aggregation and related to the adsorption of a particular substance on the crystal surface. A number of methods on calcium oxalate crystallization inhibition studies have been published using inhibitors either in urine, from patients with urolithiasis or with normal subjects. It appears to be of fundamental importance to get information on the inhibiting properties of the urine.

A number of methods have been used for crystallization inhibition studies such as U-tube and polarized light (Bisaillon & Tawashi 1976), coulter counter technique

(Ahmed & Tawashi 1978), mixed suspension mixed product removal (Drach *et al.*, 1980), titrimetric method (Baumann & Wacker 1980), radioactivity technique (Tiselius & Fornander 1981), photometry technique (Schneider *et al.* 1983), vertical light path photometry technique (Achilles, 1985), nephelometry and optical microscopy technique (Grases *et al.* 1988), i*n vitro* calcium oxalate crystallization system using [^{45}Ca]calcium chloride (Atmani & Khan 1995), scanning electron microscopy and molecular modeling (Wierzbicki *et al.* 1995), turbidimetric method (Hennequin *et al.* 1997), immunofluorescence photomicrography coupled with scanning electron microscopy (Yamate *et al.* 1998), atomic force microscopy (Shirane *et al.* 1999) and spectrophotometric time-course measurements of optical density (Hess *et al.* 2000).

All of the above methods measured the inhibitory activity of an inhibitor on a group of crystals. None of the above methods measures the inhibition activity on individual crystal formed. It is therefore necessary to develop a simple but accurate standardized method for estimating the inhibitory activity on individual crystals and direct measurements of calcium oxalate crystal growth.

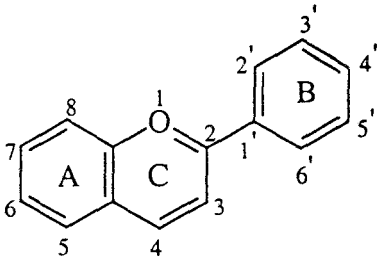
2.2 FLAVONOIDS

2.2.1 GENERAL STRUCTURE AND MAJOR CLASSIFICATIONS

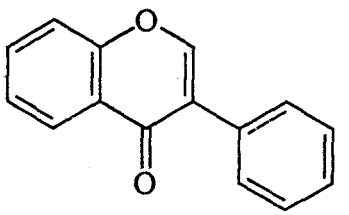
Flavonoids are an ubiquitous group of polyphenolic substances, which are present in most plants, concentrating in leaves, seeds, fruit skin or peel, bark, and flowers (Herrmann, 1976, 1993; White & Xing, 1997). They are characterized by carbon skeleton C_6-C_3-C_6 [7] as shown in Figure 2.3. The basic structure of these compounds consists of two aromatic rings linked by a three carbon aliphatic chain, which normally has been condensed to form a pyran or, less commonly a furan ring (Hollman *et al.*, 1996).

There is an array of substitution patterns on A, B, and C rings, as well as differences in where the B ring is bonded to the C ring, providing possibility for the existence of numerous flavonoids, differing in their biological characteristics and chemical structures (Middleton & Kandaswami, 1994). Examples of different flavonoid families are isoflavones [8], flavones [9], flavonols [10], flavanones [11], dihydroflavonols [12] and anthocyanidins [13] (Figure 2.3).

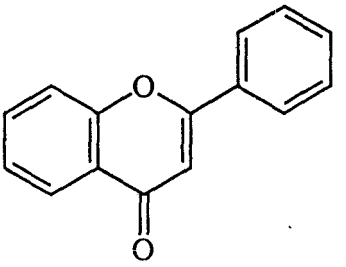
As with other flavonoids, the most frequently found flavonols and flavones are those with B-ring hydroxylation in the C-3′ and 4′ positions (Herrmann, 1976). Flavones lack the hydroxyl group at C-3 in the middle ring that characterizes the flavonols. Quercetin and kaempferol are typical flavonols, the corresponding flavones being luteolin and epigenin respectively. Approximately 90% of the flavonoids in plants occur as glycosides (Macheix *et al.*, 1990). The preferred binding site for the sugar residues is C-3 and less, frequently, in the A-ring at the C-7 position (Herrmann, 1976). The sugar free part of the flavonoid molecule is called the aglycone.



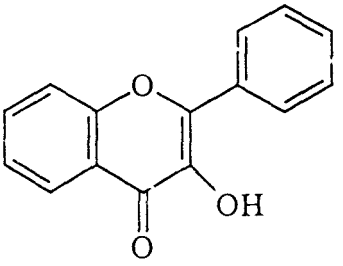
[7] C_6-C_3-C_6 configuration



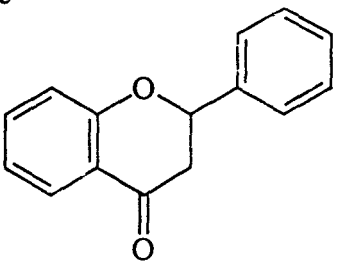
[8] Isoflavone



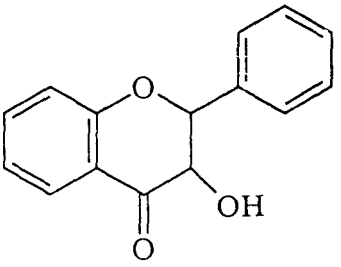
[9] Flavone



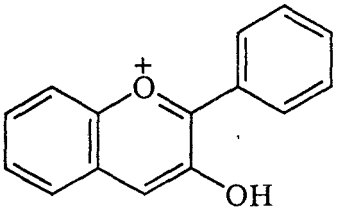
[10] Flavonol



[11] Flavanone



[12] Dihydroflavonol



[13] Anthocyanidin

Figure 2.3: Structures of some flavonoid families.