CHAPTER II

HISTORICAL

1. BOTANICAL ASPECT OF IMPATIENS BALSAMINA L.

Impatiens balsamina Linn. (Fig. 1) is in the family of Balsaminaceae. It has a number of synnonyms, including *Balsamina hortensis* DC., *B. impatiens* Hort. and *I. coccinea* Sims. Its local names in various countries are Garden Balsam (English); Kamantigi (Iloko, Tagalog, Pampangan); Saungga (Sulu); Suranga (Bikol, Bisaya), Tien-Ban (Thai).

The name "Impatiens" is from the Latin. It refers to the pods, which when ripe, on slight pressure burst open and scattering the seeds.

I. balsamina is native of India, and is now cultivated in all warm countries. The plant is an erect, much-branched, half-succulent annual. The height is about 2 feet. Their leaves are either narrowly or broadly lanceolate and acuminate, deeply serrate and have the glandular petiole. The flowers are clustered in the axils of the leaves and on very short stalks. They have various colors from white to purple. The sepals and petals are similar in clour and not easily distinguished. One of the sepals seems to be three and long-spurred. The petals are apparently three, but two of them probably represent two unit petals, thus making five petals. The pod is explosive. When the capsules are ripe, a pinch will cause the valves to separate and contract. The seeds are then being thrown with considerable force.

I. balsamina is a very easy culture. The seeds are large and germinate quickly. The plants prefer a rich, sandy loam and must not suffer from moisture.

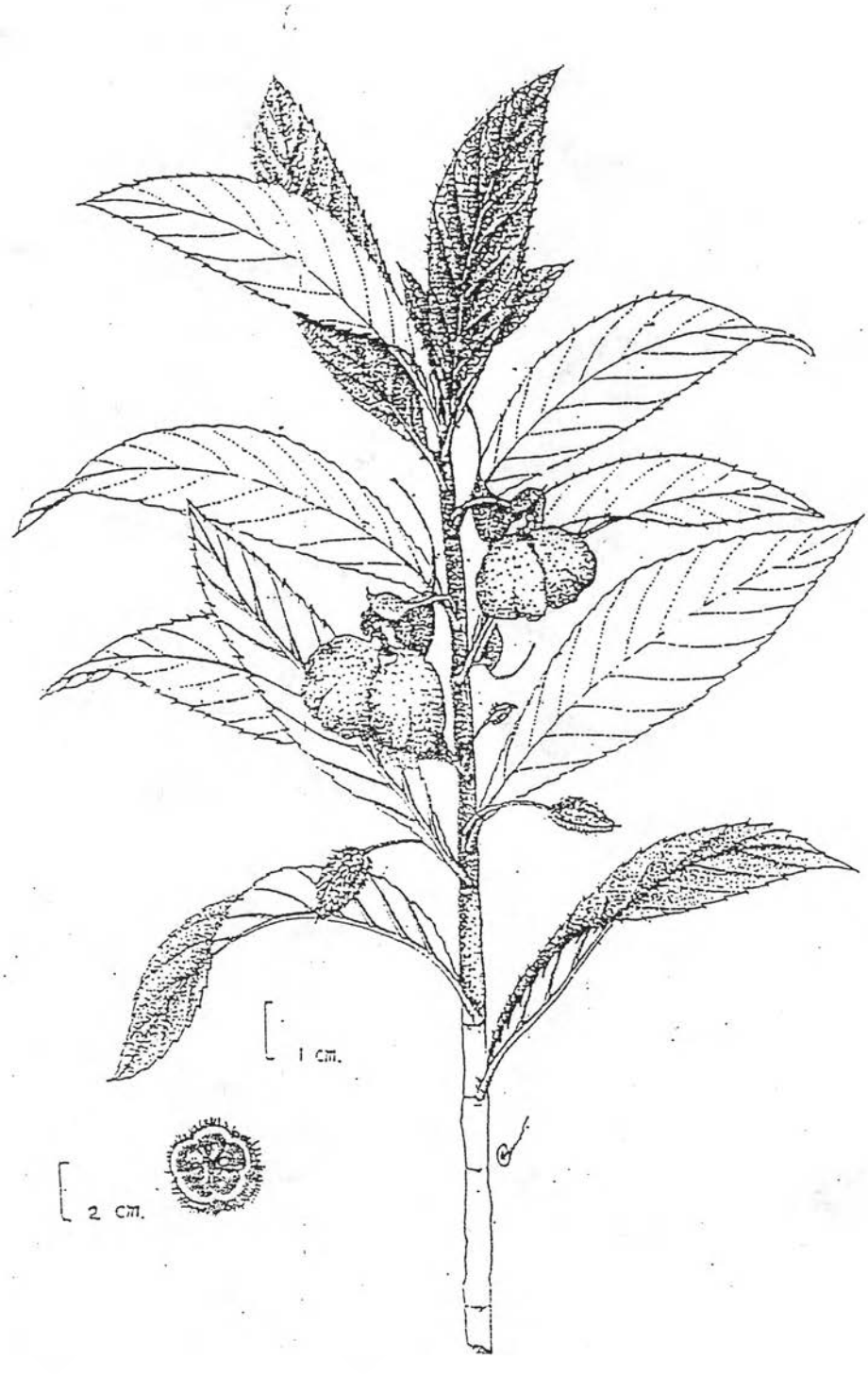


Fig. 1 *Impatiens balsamina* L. (Balsaminaceae)

2. CHEMICAL CONSTITUENTS OF IMPATIENS BALSAMINA

Since 1948, when Little *et al.* isolated and identified 2-methoxy-1,4-naphthoquinone from the dried flowers of *I. balsamina* L.(Little *et al.*, 1948), the search for constituents from different parts of *I. balsamina* has continued and has been intensified in recent years. The groups of compounds commonly found in *I. balsamina* are naphthoquinones, phenolic acids, flavonoids, anthocyanidins, and coumarin derivatives. List of the compounds found in various parts of *I. balsamina* is shown in Table 1.

3. THE USES OF IMPATIENS BALSAMINA

I. balsamina has long been used as folkloric medicine in Thailand. The leaves and stems are usually pounded and used in poultices to treat abscesses, nail ingrown and dermatophytosis (ชัยโย ชัยชาญทิพยุทธ และ คณะ, 1979 ; ศูนย์ข้อมูลสมุนไพร , 2530) .

In China , the old Chinese use the powdered seeds of *I. balsamina* to treat difficult labor, to suppress puerperal pain, to act as an emmenagogue and expectorant. The seeds are also used to soften fish bone while cooking. They are also used to stop hiccups. When mixed the powdered seeds with arsenious acid and applied to dental caries, the teeth can be removed easily. The lower part of the stem pounded to juice and added to rice liquor is used as a cure for contusions. It may also be used to wash abscesses and to reduce swellings. The dried stem is used for improved circulation and to relieve pain. It is prescribed for the treatment of hard labor, leg cramps, and rheumatism. The flowers are mucilaginous and cooling, and are used for the treament of snake bite, lumbago and intercostal neuralgia. They are thought to improve the circulation and to relieve stasis (Lily and Metzger, 1980).

In the Philippines, the leaves are pounded and used in poultices to dissolved felongs. Similarly, the leaves are used in Malaya for poulticing broken and torn nails. In Bali, the leaves are eaten as food, and in Asia, the flowers and leaves are used as a substitute for Henna tor dyeing (Quisumbing, 1951).

Table 1. Chemical constituents of various parts of *I. balsamina*

Plant part	Category	Chemical substance	Reference
Leaves	Naphthoquinone	lawsone	Bohm *et al.*, 1962
		2-methoxy-1,4-naphthoquinone	Bohm *et al.*
	Phenolic acid	caffeic acid	Bohm *et al.*
		p-coumaric acid	Bohm *et al.*
		ferulic acid	Bohm *et al.*
		gentisic acid	Bohm *et al.*
		p-hydroxybenzoic acid	Bohm *et al.*
		sinapic acid	Bohm *et al.*
	Flavonoid	kaempferol	Weissenboeck *et al.*, 1971
	Coumarin derivative	scopoletin	Bohm *et al.*
Flowers	Naphthoquinone	lawsone	Clevenger, 1958
		2-methoxy-1,4-naphthoquinone	Little *et al.*, 1948
	Phenolic acid	p-coumaric acid	Mansel *et al.*, 1970
		ferulic acid	Mansel *et al.*
		hydroxycinnamic acid	Mansel *et al.*
	Flavonoid	kaempferol	Clevenger, 1958
		myristin	Clevenger
		quercetin	Clevenger
	Anthocyanidin	pelargonidin	Clevenger
		cyanidin	Clevenger
		peonidin	Clevenger
		malvidin	Clevenger
	Enzyme	o-methyltransferase	Mansell *et al.*, 1971
		galactosidase	Boylen *et al.*, 1969
Seeds	Flavonoid	quercetin	Wellmann, 1975
	Fat and Fatty acid	palmitic	Sharkar and Charkabarty,1955-1956
		stearic	Sharkar and Charkabarty
		arachidic	Sharkar and Charkabarty
		oleic	Sharkar and Charkabarty
		linoleic	Sharkar and Charkabarty
		linolenic	Sharkar and Charkabarty
		parinaric	Sharkar and Charkabarty
	Steroid	β-sitosterol	Dikshit *et al.*, 1973

In Indo-China, a decoction of the leaves is used to wash the hair and supposedly to promote its growth (Lily and Metzger, 1980). The oil from *I. balsamina* seed may be used for cooking and for burning lamps. It is also suitable for the surface-coating industry (Sastri *et al.*, 1959).

In Thailand, it has been found recently that the crude chloroform and alcoholic extracts of the leaves contain antifungal and antibacterial activities (สันติ ถุงสุวรรณ และคณะฯ 1985). Alcoholic extracts of the flowers also possess marked antibiotic activity (Sastri et al,1959).

4. CHEMISTRY, DISTRIBUTION AND DETECTION OF NAPHTHOQUINONES

4.1 Chemistry and distribution

A large number of naphthoquinones (Fig. 2) are found in nature as plant pigments. Most of them are oil or crystalline materials ranging from yellow to red color. They are soluble easily in organic solvents such as benzene. Some of them are toxic and antimicrobial and, therefore, the plants containing these naphthoquinones have been used as drugs and poisons. For example, several plant species of Ericaceae which contain chimaphilin [I] and the plants in Plumbaginaceae which contain plumbagin [II] (Thomson, 1971).

In addition, some naphthoquinones have been used as coloring agents. For example, alkannin [VI] and its optical isomer shikonin from some species in Boraginaceae (Thomson,1971).

Naphthoquinones are commonly hydroxylated with phenolic properties and occur either in combined form with sugar as glycoside or in reduced aglycone form (Harborne, 1983). Although widely distributed, the naphthoquinones make relatively little contribution to color in higher plants. They are frequently present in the bark,

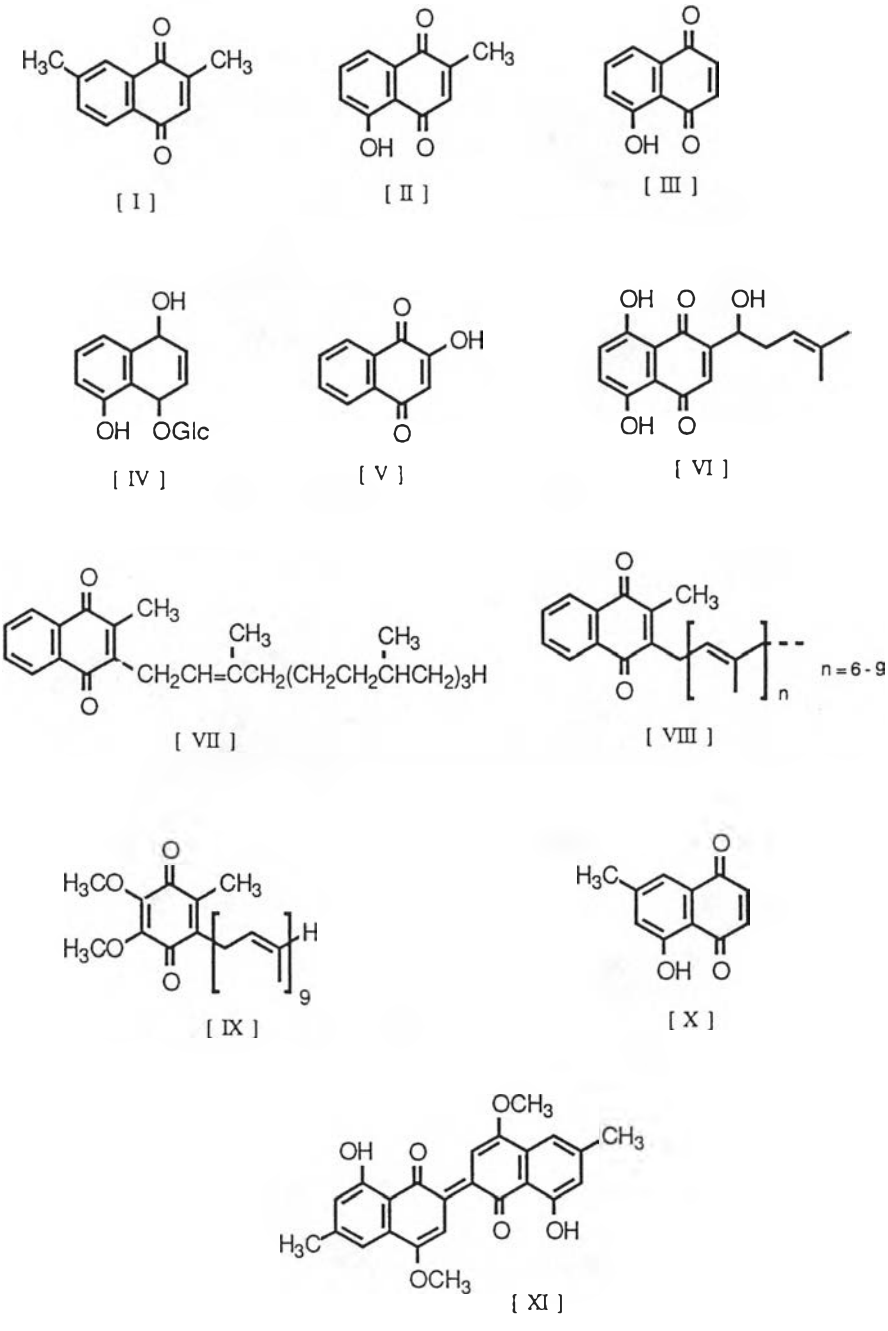


Fig.2 Some naphthoquinones and benzoquinones in higher plants.

heartwood or root. They are also present in the tissues of leaves where their colors are masked by other pigments. When present in living tissue, the naphthoquinones are usually colorless. Upon extraction of the compounds under acidic conditions, however, color is produced. This is due to the hydrolysis of sugar linkages and oxidation of quinol to quinone (Bell and Charlwood, 1980). This phenomenon is found in both juglone (5-hydroxynaphthoquinone) [III] of walnut (*Juglans nigra*) and plumbagin of *Plumbago* roots. Juglone is widely present in the leaves and roots of the members of Juglandaceae. It commonly occurs as 4-glucoside of the corresponding 1,4.5,- trihydroxynaphthalene [IV]. Similarly plumbagin, an orange pigment, is also present in bound form in plants. It is more widespread than juglone and has been detected in many plants in Plumbaginaceae, Droseraceae, and Ebenaceae and also one species in Euphorbiaceae (*Pera ferrugnea*). Another closely related naphthoquinone is lawsone [V]. This quinone has been found in either Balsaminaceae (e.g. *Impatiens* spp.) or Lythraceae (e.g. *Lawsonia inermis*). It is believed that lawsone present in these leaves occurs, at least in part, in a colorless reduced form. For shikonin and its enantiomer alkannin, both have long been known to be coloring constituents of some plants in Boraginaceae. Shikonin has been found in the genus *Lithospermum*, whereas alkannin in the root of *Alkanna tinctoria.* Alkannin is thought to occur in the plant as an ester joining between angelic acid and the hydroxyl group of the side chain.

The most important naturally occurring naphthoquinones belong to the group of K-vitamins. Vitamin K1 (phylloquinone) [VII] and vitamin K2 [VIII] are especially widespread. Similar to the ubiquinones [IX] they have isoprenoid side-chains and are thought to play some roles in the electron transport of plant photosynthesis (Goodwin and Mercer, 1983). When present in the heart wood of trees, naphthoquinones usually occur as free forms and a wide range of structures may be present together in any one given source. *Diospyros* (Ebenaceae) heartwoods, for example, are particularly rich. These heartwoods contain many derivatives of 7- methyljuglone [X] in

which its molecules are linked together to form dimers, trimers, and tetramers. The dimers is the blue diosindigo [XI] obtained from *Diospyros* (Goodwin and Mercer,1983)

4.2 Dectection, Separation and Identification of Naphthoquinones

4.2.1 Detection

Generally, preliminary information on the presence of naphthoquinones in crude extracts or even in natural tissues can be obtained by color reaction methods. These color reactions are relatively sensitive and only little material is required. The reactions may also be carried out by spraying on chromatographic plates or papers. However, these rough tests should preferably be confirmed by spectrophotometric measurments on the purified material. The most useful diagnostic test depends upon the redox properties of naphthoquinones and the presence of hydroxyl groups. Leucomethylene blue is a useful spray for the detection of naphthoquinones on paper or thin layer chromatography (Thomson, 1971). After spraying, naphthoquinones will appear as blue spots on a white background. For hydroxylated naphthoquinones, the color changes are more striking in alkaline solution and re-oxidation (by air) is more rapid. The characteristic color given by hydroxylated naphthoquinones in alkaline solution are very useful in helping their structure determination. Various hydroxylated naphthoquinones and their colors obtained in the presence of excess sodium hydroxide are shown in Table 2 (Thomson, 1971).

Table 2. The colors of hydroxylated naphthoquinones in alkaline solution.

Naphthoquinone	Color	λ_{max} (EtOH) (log ε)
2-hydroxy-	Orange	459
5-hydroxy-	Violet	538
6-hydroxy-	Violet-red	520
2,3-dihydroxy-	Blue	650
2,5-dihydroxy-	Violet-red	490
3,5-dihydroxy-	Red	435
5,6-dihydroxy-	Blue	571
5,7-dihydroxy-	Violet	542
5,8-dihydroxy-	Blue	655

4.2.2 Separation

The general procedure for separation of naphthoquinones is thin layer chromatography on silica gel. Naphthoquinones are very lipid-soluble and they may be separated in pure benzene, chloroform, petroleum ether or simple mixtures of these solvents. On the other hand, highly hydroxylated naphthoquinones are very polar and complex solvent mixtures are required to make the mobile phases. Since most naphthoquinones are colored, there is no difficulty in detecting them in visible light on TLC plates. However, examination in UV light may be useful and provide a more sensitive means of detection (Harborne, 1983).

4.2.3 Identification

For the identification, UV spectral measurments are essential since the UV and visible spectrum indicates the types of naphthoquinone present. The spectra of numerous simple 1,4-naphthoquinones have been measured and analysed. Illustrative spectra for some natural quinones are given in Table 3 (Thomson, 1971).

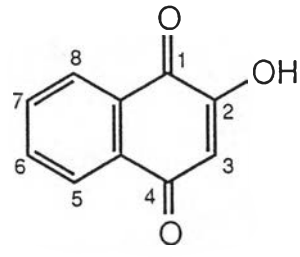
Table 3. Ultraviolet-visible absorption of some naturally occurring naphthoquinones.

Naphthoquinone	λ_{max} (EtOH) (log ε)		
Lawsone	242 (4.41) 248 (4.12)	274 (4.41)	3.34 (3.04)
2-Methoxy- 1,4-naphthoquinone	243 (4.22) 248 (4.24)	277 (4.16)	330 (3.36)
Juglone	249 (4.09)		345 (3.08) 422 (3.56)
Chimaphilin	248 (4.19) 254.5 (4.19)	265 (4.02)	338 (3.19)
Plumbagin		266 (4.12)	418 (3.61)
Alkannin		280 (3.84)	480 (3.74) 510 (3.78) 546 (3.60)

5. THE CHEMISTRY AND BIOLOGICAL ACTIVITIES OF LAWSONE AND 2-METHOXY-1,4-NAPHTHOQUINONE

5.1 Chemistry of Lawsone

Lawsone (2-hydroxy-1,4-naphthoquinone ; $C_{10}H_6O_3$; MW 174.15)



Lawsone was first isolated from *Lawsonia alba* Lam. (*L. inermis* Lam.) (Tommasi, 1920) and has also been found in *L. spinosa* (Latif, 1959) which belong to the family Lythraceae. It has also been found in the family of Balsaminaceae, particularly the *Impatiens* species, including *I. balsamina* L., *I. capensis* Meerb. and *I. pallida* Nutt.(Bohm and Towers, 1962). Lawsone belongs to the chemical group of 1,4-naphthoquinone with a hydroxy functional group attached to C-2 of the molecule. It is relatively polar and soluble well in alcohol. It has been reported that lawsone is a degradative and autooxidative product of its primary glycosides, hennosides A, B, and C (Kapadia *et al.*, 1969).

For the physical properties, lawsone has yellow needle form of crystal with the melting point of 192°C (dec.). Its UV spectrum shows λ_{max} (EtOH) at 242.5, 248, 274, 334 nm (log ε 4.17, 4.21, 4.14, 3.40). For IR spectrum, lawsone shows υ_{max} (KBr) at 3150, 1674, 1640 cm^{-1} and NMR spectrum shows δ at 8.0, 7.6, 6.27 ppm (Thomson, 1971).

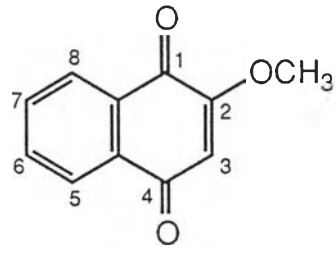
5.2 Biological Activities of Lawsone

Lawsone has been reported to exert various biological activities against microorganisms. It shows antifungal activities against *Alternaria*, *Aspergilus*, *Absidia*,

Penicillium and *Cladosporium* at the concentration of 0.1% (Steffen and Peschel, 1975 ; Tripathi, Srivastava and Dixit, 1978 ; Farnsworth and Cordell, 1983). It also has antibacrial activities against *Bacillus*, *Staphylococcus*, *Salmonella*, *Pasteurella*, *Escherichia coli*, *Brucella* and *Neisseria* with a concentration range between 0.005 and 0.025% (Karawya *et al.*, 1969 ; Kulkarni *et al.*, 1983 ; Kelkar *et al.*, 1986). In addition, lawsone has been found to have antitumor activities, for example, against sarcoma 180 in mice and Walker 256 carcinosarcoma in rat (Goncalves, 1971) ; and antispasmodic properties as well as weak vitamin K activity (Leung, 1980).

5.3 Chemistry of 2-Methoxy-1,4-naphthoquinone

2-Methoxy-1,4-naphthoquinone ($C_{11}H_8O_3$; MW=188.2)



2-Methoxy-1,4-naphthoquinone was first isolated from the dried flowers of *I. balsamina* L. (Little, Sproston and Foote, 1948). It has also been found in the leaves of *I. pallida* Nutt., *I. herzogii* K. Schum, *I. parviflora* DC.(Bohm and Towers, 1962), and *I. glandulifera* Royle (Chapelle, 1974). 2-Methoxy-1,4-naphthoquinone is the methyl ether of lawsone in which a methoxy group attached to C-2 of the molecule and thus has less polarity than lawsone. It is extremely soluble in chloroform and benzene, and moderately soluble in alcohol and ether. It is almost insoluble in petroleum ether and water but slighly soluble in boiling water. It has been reported that in alkali solution it will be hydrolysed to lawsone (Jutima Boonleang, 1991).

For the physical properties, 2-Methoxy-1,4-naphthoquinone has yellow needle form of crystal with melting point of 183°C. Its UV spectrum shows λ_{max} (EtOH)

at 243, 248, 277 and 330 nm (log ε 4.22, 4.24, 4.16 and 3.36). For IR spectrum, It shows υ_{max} (KBr) at 1680, 1645, 1600 and 1240 cm^{-1}, and NMR spectrum shows δ at 8.10, 7.75, 6.19 and 3.93 ppm (Chapelle, 1974).

5.4 Biological Activities of 2-Methoxy-1,4-naphthoquinone

Similar to lawsone, 2-methoxy-1,4-naphthoquinone also exhibits potent antifungal activities. It activities against *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum gypseum*, *Epidermophyton floccosum* and *Candida albicans* have been reported (Thatree Phadungcharoen *et al.*, 1988). The values of both minimal inhibitory concentrantion (MIC) and minimal fungicidal concentration (MFC) of this compound against *Trichophyton* and *Microsporum* have been found to be 2.50 μg/ml whereas both values for *Epidermophyton* and *Candida* found to be 1.25 μg/ml (Thatree Phadungcharoen *et al.*, 1988). 2-Methoxy-1,4-naphthoquinone also shows antibacterial activities against *Staphylococcus aureus* (Thatree Phadungcharoen *et al.*, 1988). Because of these antimicrobial activities, attempts were made at the Faculty of Pharmaceutical Scienes, Chulalongkorn University to formulate antifungal preparations for the treatment of dermatophytosis. The composition of the solution and cream which were found to give satisfactory results are described below :

2-Methoxy-1,4-naphthoquinone solution

(Pranom Pothiyanont *et al.*, 1992) :

2-Methoxy-1,4-naphthoquinone	0.25	g
Alcohol USP	50	ml
PEG 400	30	ml
Purified water to	100	ml

The solution contained sodium metabisulfite, sodium bisulfite and citric acid 0.01-1 % as antioxidant.

2-Methoxy-1,4-naphthoquinone in cream base

(Pranom Pothiyanont *et al.*, 1992):

2-Methoxy-1,4-naphthoquinone	0.5	g
Cutina MD	10.0	g
Stearic acid	3.0	g
Cutina AGS	3.0	g
Emulgin C 700	1.5	g
Emulgin C 1000	1.5	g
Myristol 318	4.0	ml
Propylene glycol	5.0	ml
Paraben concentrate	1.0	ml
Purified water to	100.0	g

6. THE PURPOSED BIOSYNTHETIC PATHWAY OF NAPHTHOQUINONES

There have been at least four biosynthetic pathways of naphthoquinones purposed in higher plants. Among these, three pathways involve a direct incorporation of shikimic acid into the benzene ring of the naphthoquinone and one pathway involves acetate-malonate condensation to form the naphthoquinone skeleton.

6.1 The Shikimate-Derived Pathways

The three shikimate derived pathways which lead to different types of naphthoquinones branch from the main shikimate pathway at three different points. Each of these branches forms its own unique intermediate for synthesizing a particular type of the naphthoquinone. The key intermediates of the three different pathways are 4-hydroxybenzoic acid, homogentisic acid and 2-succinylbenzoic acid.

In the next section, these so-called "4-hydroxybenzoic acid pathway", "homogentisic acid pathway", and "2-succinylbenzoic acid pathway" will be described in more details.

6.1.1 The 4-Hydroxybenzoic Acid Pathway

This pathway has been discovered in the plants of Boraginaceae (Schmid and Zenk, 1971), a family containing the naphthoquinone alkannin. Results from feeding experiments have led to this novel alkannin pathway which involves p-hydroxybenzoic acid and two molecules of mevalonic acid as precursors. In this route, the aromatic precursor is incorporated into the benzene ring with loss of the carboxy group and the quinoid nucleus being formed from mevalonic acid. This conclusion has been verified by degradative experiments (Brockmann, 1936). It has been proposed that alkannin is biosynthesized probably by addition of geranyl pyrophosphate to p-hydroxybenzoic acid and subsequent decarboxylation, forming geranylhydroquinone followed by ring closure analogous to that observed in chimaphilin biosynthesis (Fig.3) (Schmid and Zenk, 1971 ; Inovye *et al.*, 1979)

6.1.2. The Homogentisic Acid Pathway

This route is involved in the biosynthesis of the dimethylnaphthoquinone "chimaphilin" (Bolkart and Zenk, 1969). In this pathway, a single prenyl unit is added *para* to the α-carbon atoms of homogentisic acid followed by its cyclization to form chimaphilin (Fig. 4).

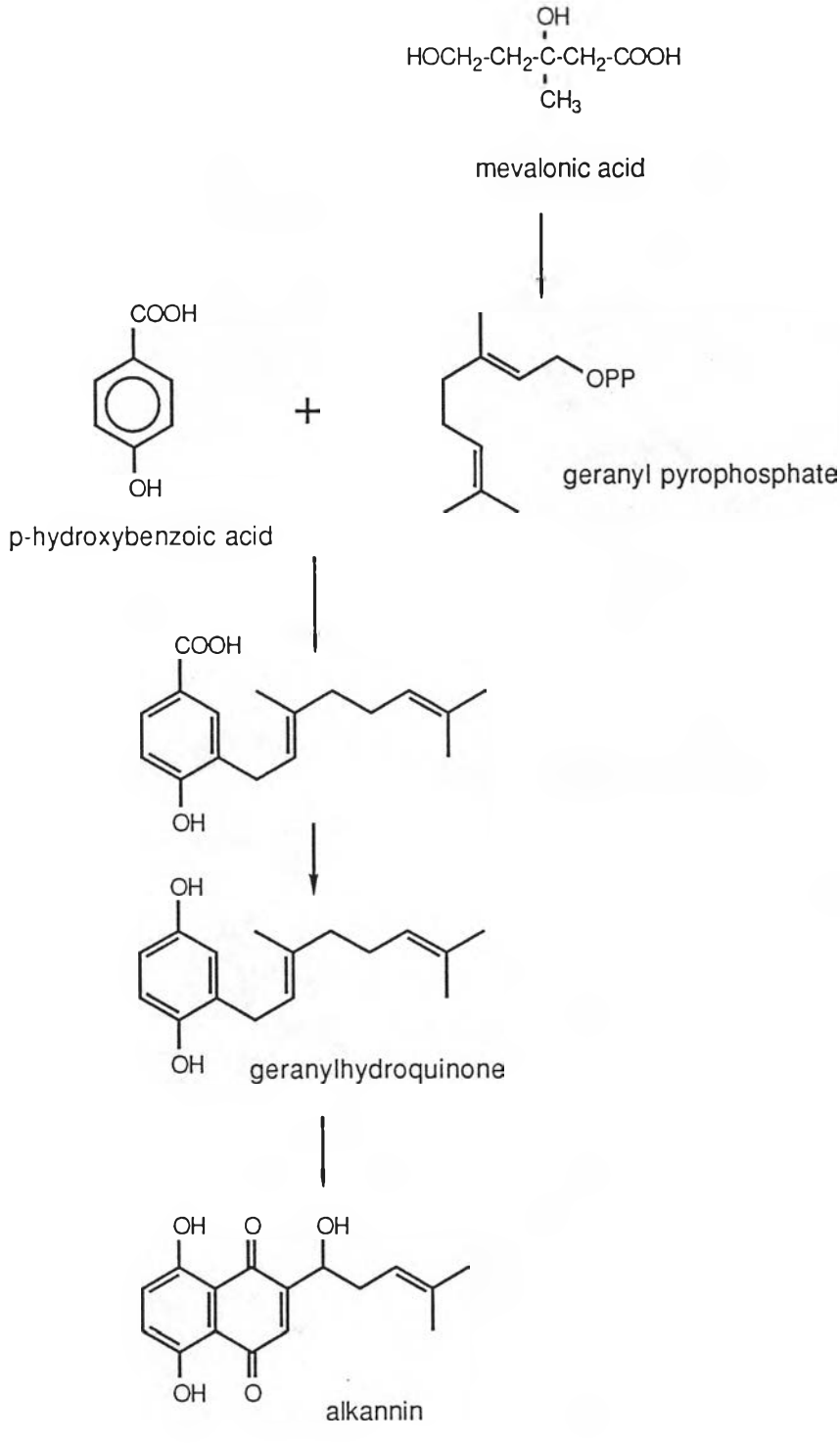


Fig. 3 The purposed biosynthetic pathway of alkannin.

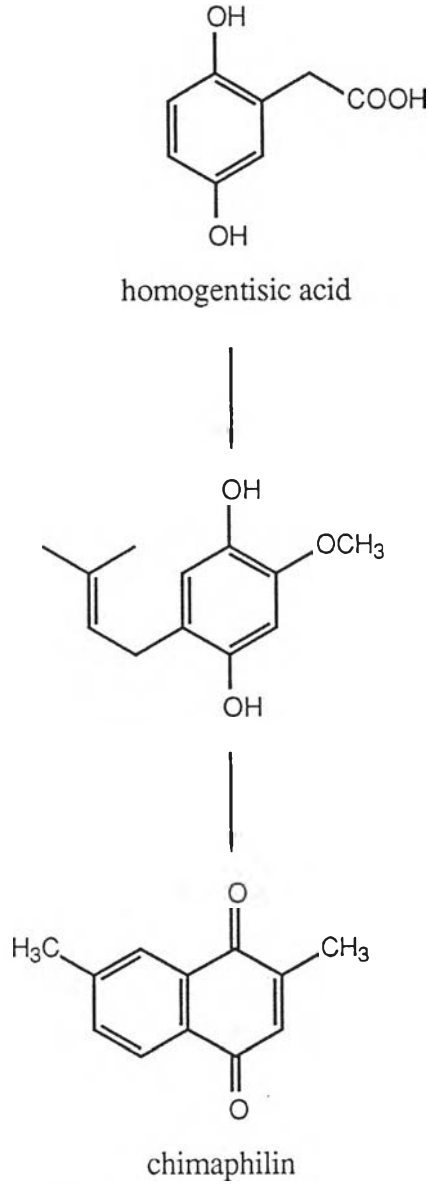


Fig. 4 The purposed biosynthetic pathway of chimaphilin.

6.1.3 The 2-Succinylbenzoic Acid Pathway

This is one of the best established pathways to naphthoquinones in higher plants. It involves incorporation of shikimate and the intermediacy of 2-succinylbenzoic acid [4-(2-carboxyphenyl)-4-oxobutyric acid] into lawsone (Grotzinger and Campbell, 1974), a naphthoquinone found in *I. balsamina* (see section 6.3).

6.2 The Acetate-Malonate Pathway

The fourth route leading to naphthoquinones is *via* acetate-malonate, a pathway which is well established in microorganisms. This route has been discovered for plumbagin (5-hydroxy-2- methylnaphthoquinone) (Bolkart and Zenk, 1969), which is produced by *Drosera* and *Plumbago* species (Fig.5). In young shoots of *Plumbago europea* , the labelled acetate and [2-^{14}C] malonic acid have been shown to be incorporated into plumbagin (Durand and Zenk, 1971).

6.3 The Biosynthesis of Lawsone

The naphthoquinone lawsone found in *I. balsamina* is thought to be biosynthesized by the so-called "2- succinylbenzoic acid pathway". This pathway involves shikimic acid as a precursor and unsymmetrical aromatic intermediate, 2-succinylbenzoic acid as a key intermediate. According to this pathway, the naphthalene nucleus of lawsone is derived from shikimic acid with C-1 and C-2 appearing at the naphthoquinone ring junction. The carboxy-group of shikimic acid is retained during this naphthoquinone formation. The remaining C 3 unit has been identified as having its origins from glutamic acid or its transamination product, 2-ketoglutaric acid (Fig. 6) (Chen and Bohm, 1966 ; Grotzinger and Campbell, 1972).

The compound 2-succinylbenzoic acid has been shown to be an important intermediate of this pathway (Grotzing and Campbell, 1972). This is consistent with the original proposal that the initial step in the construction of ring B of the nucleus is the condensation of shikimic acid with the thiamine pyrophosphate complex of succinyl semialdehyde . The latter complex can be formed from 2- ketoglutaric acid by the action of the Krebs cycle enzyme, 2- ketoglutarate dehydrogenase (Fig. 6). It appears that diversion from the shikimate pathway to naphthoquinone biosynthesis may occur at chorismic acid.

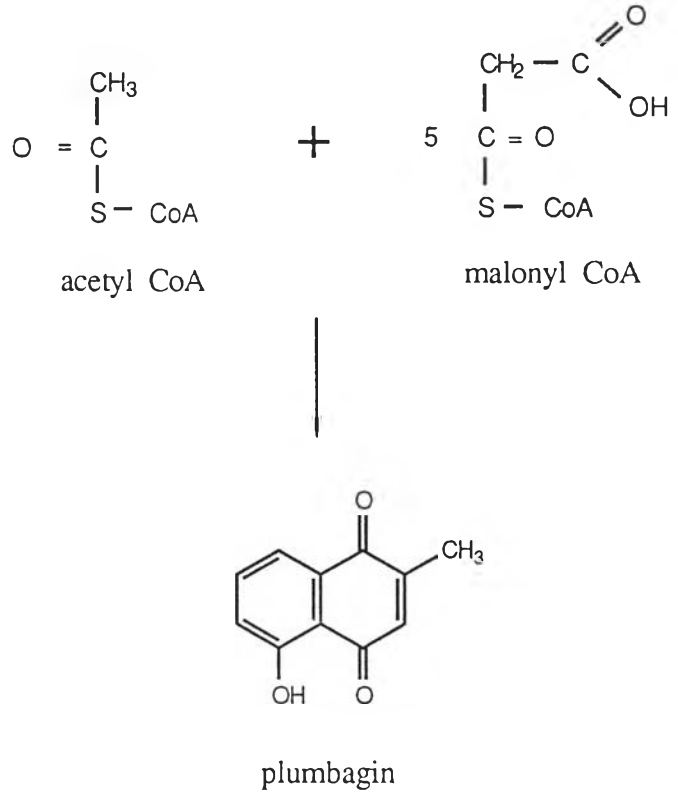


Fig.5 The purposed biosynthetic pathway of plumbagin.

Since it has been clearly shown that [2-^{14}C] acetic acid preferentially labels C-2 of lawsone (Grotzinger and Campbell, 1972), we can deduce that the hydroxyl group found in lawsone is attached to that carbon atom which was C-2 in 2-succinylbenzoic acid. This fact sheds light on the mechanism by which the latter stages of lawsone biosynthesis might proceed. Two pathways seem feasible (Fig. 6). The first involves cyclization of 2-succinylbenzoic acid to the 1,4-dihydroxy-2-naphthoic acid and subsequent oxidative decarboxylation. Although there were not successful in using this naphthoic acid to swamp out incorporation of activity from 2-succinylbenzoate into lawsone, and although there were unable to detect this acid in *I. balsamina* extracts by combined GLC-MS, the involvement of this compound still has to be excluded positively.

An alternative pathway of lawsone biosynthesis could involve conversion of 2-succinylbenzoic acid to the α-ketoacid by successive dehydrogenation, hydration and oxidation in the fashion of fatty acid biosyhthesis. Decarboxylative closing of the ketoacid in a manner analogous to the coupling of shikimic acid with the glutamate derived unit would yield lawsone. While this route seems to involve many steps, it would account for the problems with the naphthoic acid derivative.

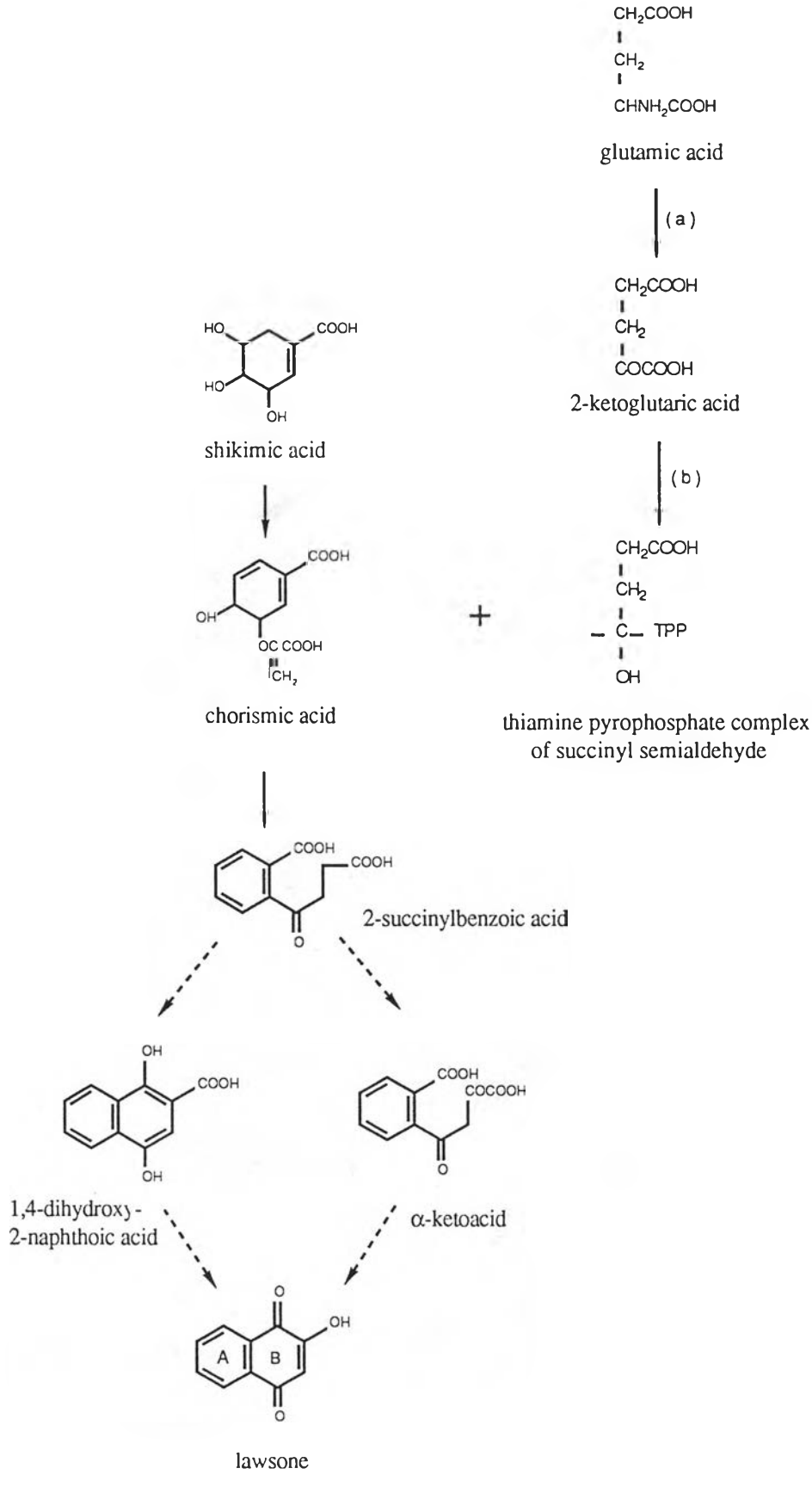


Fig.6 The purposed biosynthetic pathway of lawsone. (a) Transaminase ; (b) A modified 2-ketoglutarate dehydrogenase.

7. IN VITRO CULTURE OF HIGHER PLANTS

7.1 Sources and Types of Cultures

The art of growing, aseptically and heterotrophically, isolated plant as explants on appropriate media is presenlty known as plant tissue culture techniques. These techniques can be considered to be extended from the nutritional methods of microbiology to higher plants.

Now the term " tissue culture" embraces procedures and practices which may be applied to plant materials from source that may cover the entire morphological range. This range may be from young leaves, immature seeds, embryos, buds, apical shoots or roots and protoplasts. Since these plant parts can be grown without the requirement of the essential organization that supports life, the term " *in vitro* " culture was introduced. As there are many different building materials within a plant , there are many different types of *in vitro* cultures (Fig. 7).

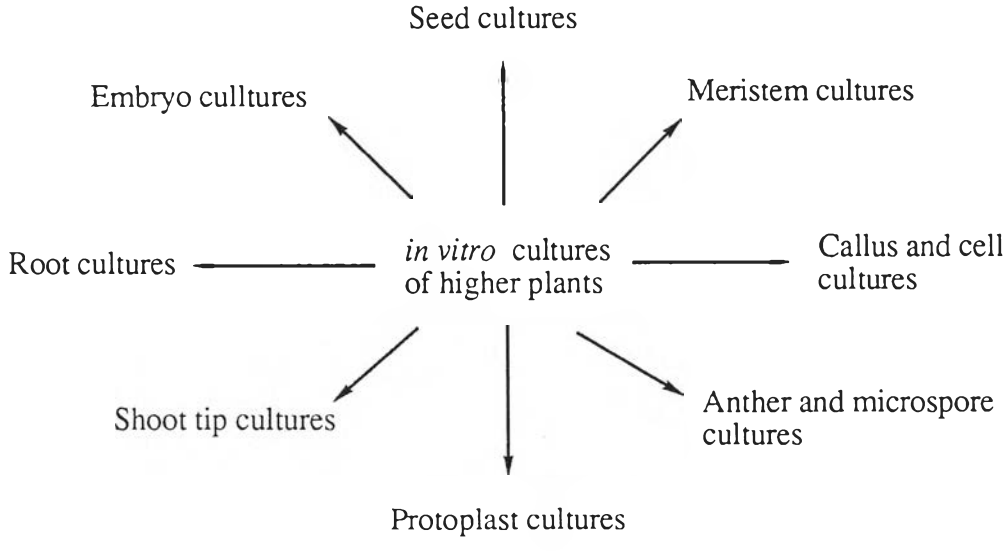


Fig. 7 Schematic representation of *in vitro* cultures of higher plants.

7.2 Callus Cultures

Callus cultures can be defined as tissues which proliferate continuously in a disorganized fashion giving rise to an apparently amorphous mass with no regular form on the solid medium. Before attempting to initiate a callus culture, it is first necessary to sterilize the plant organ from which an explant is to be taken. If taken from a seedling, it is usually more convenient to sterilize the seed before imbibition and allow it to germinate in aseptic conditions. Then, at the suitable stage, the appropriate organ can be excised and transferred to solid media.

Depending on the nature of an explant and medium composition, callus proliferation may arise from the cambium, cortex, pith, secondary phloem or even xylem parenchyma. The callus normally takes 3 to 8 weeks to reach a size when it may be subcultured by transferring small pieces of tissue (about 100 mg) to fresh medium. Once well established, most callus cultures will require a regular subculture at approximately 4-week interval.

The calli usually have a large number of morphological types. They vary according to external appearance, texture and cellular composition. Some calli consist of hard compact tissues with small closely packed cells, while others consist of soft tissues with minimal cellular contact. The pigmentation of callus is also variable, even among isolated from the same species. Many calli lack pigmentation while others are pale green (chlorophyll) or yellow (carotenoids or flavonoids).

7.3 Cell Suspension Cultures

Cell suspension cultures consist of isolated cells and very small cell aggregates remaining dispersed as they grow in agitated liquid media. They represent a lower of organization than callus cultures. Most suspension cultures are obtained by transfer of friable callus to agitated liquid medium. For each cell culture there is a minimum inoculum size below which the culture will not grow. The lag phase of the culture increase in length

as the inoculum size decreases towards the minimum level. Agitation rate of shakers should be in the range of 60-150 r.p.m. At the first subculture into fresh medium, remove large clumps of initial inoculum either by transfering material with a pipette or syringe suitable orifice diameter to exclude large cell aggregates or alternatively by allowing the culture to settle for a short time and then transfering the cells from only the upper part of the culture. Some callus cultures grow as compact, non-friable lumps and do not readily break up to from suspension. In this case, however, may be improved by modifying the culture medium, such as, increasing the concentrations of auxin, altering the ratio of auxin to cytokinin or adding low concentration of cell-wall degrading enzymes such as cellulase and pectinase are all effective.

In general, the media suitable for growing callus cultures for a particular species are also suitable for growing suspension cultures, providing that agar is omitted. However, in some cases suspension are more exacting in their requirements, for example, the concentration of auxins and cytokinins are often more critical.

Cell suspension cultures provide a relatively homogeneous population of cells, readily accessible to exogenously applied chemical and growing under defined, aseptic conditions. Cell suspension cultures are widely used as model system for studying pathways of secondary metabolism, enzyme induction and gene expression, degradation of xenobiotics and as a source of material for enzyme purification. The lack of chlorophyll and carotenoid pigments in most plant cell suspension cultures is of great benifit for work involving isolation of enzymes or secondary products.

7.4 Root Cultures

Root cultures are always derived from a single initial root tip or callus culture or suspension culture. Root formation generally takes place in a medium with a relatively high auxin and low cytokinin concentration (Pierik, 1987). In 1934, the first root culture established from tomato was reported. The root culture showed to be capable of unlimited growth in the medium with yeast extract. This original root clones were grown for at least

25 years in chemically defined liquid medium (Butcher and Street, 1964). Since then, root cultures of numerous plant species have been established and used for studies on effect of nutrients and growth regulators on root development.

In addition to the normal root cultures, it is possible to induce a special root formation using the bacterium *Agrobacterium rhizogenes* , which is the causal agent of the plant disorder known as "hairy root" disease. The expression of the diseased phenotype is preceded by the stable integration of a portion of the Ri (root-inducing) plasmid into the plant genome (Chilton *et al.*, 1982). The gene encoded in the RiT-DNA apparently regulate, among other things, the balance of endogenous hormones produced by the transformed cells in a way that results in the proliferation of fast-growing adventitious roots at the host wound site. Hairy roots can be established as aseptic cultures upon treatment with antibiotics such as carbenicillin, which eliminate excess bacteria. The "hairy root" phenotype is stable in culture and these roots grow much faster than normal roots.

7.5 Plant Cell Cultures as Metabolic Model Systems

In the last two decades, plant cell culture techniques have been developed to a level where their application in the field of natural product chemistry can be recommended. Plant cell cultures offer a number of advantages over intact plants for studies of metabolism. They are relatively easy to establish and maintain under strictly controled nutritional and environmental conditions. They can be grown in either small containers (e.g. 250 ml Erlenmeyer flasks) or large fermentors and chemostats, and thus the amount of biomass are sufficiently available as needed. As cell suspension cultures often consist of a relatively homogenous population of cells, they can be rapidly and uniformly exposed to exogenously added chemical agents, often a difficult task when dealing with the intact plants. Finally, because of their limited degree of differentiation cell cultures generally display simpler metabolic patterns.

In spite of these advantages, the use of tissue cultures for metabolic studies has so far been limited, mainly because of the special metabolic characteristic impose by the usual

culture environment. In the case of secondary metabolism, the cultures of many species do not produce significant amount of the compound characteristic of intact plants. This may sometimes be due to the loss of genetic information during prolong culture, but in many cases it has been shown that even long-term cultures remain totipotent (Chaleff, 1983 Davey, 1983). It is more likely that the failure of cultured cell to prduce the pattern or level of secondary metabolites typical of the source plant is a consequence of the specific physiological and morphological state of cultured tissue.

In numerous plant cell cultures the biosynthetic pathways for secondary metabolites can be expressed at a level and a rate much higher than in intact plant. Such cell cultures are therefore valuable experimental system to elucidate the biosynthesis and enzymology of their secondary metabolites, for example, the biosynthetic studies of rosmarinic acid in cell suspension culturs of *Anchusa officinalis* (Wanchai De- Eknamkul and Ellis,1984).

8. SECONDARY PRODUCT FORMATION IN PLANT TISSUE AND CELL CULTURES.

Aside from the primary metabolic pathways common to all life forms, some reactions lead to the formation of compounds unique to a few species or even to a single cultivar. These reactions are classified under the term "secondary metabolism" and their products known as "secondary metabolites" (Luckner and Nover, 1977). These substances include alkaloids, antibiotic, volatile oil, glycoside, resin, tannin, and saponins. These products appear to be important in the interactions between the plant and its environment (Harborne, 1982). Their functions may be in protecting the plant from predators, pathogens, environmental stress or may be related to the reproductive machinery of the plant in promoting pollination (Rhodes *et al.*, 1987 ; Charlwood and Rhodes, 1990). Many of these unique plant secondary products have found economic importance as medicines, fragrances, insecticides, food coloring and flavours. Because of their short

supply some of these compounds are expensive and there is considerable interest in finding alternative source of supply which avoid dependence on the whole plant.

Since plant cells isolated from field grown plants and cultivated *in vitro* have the potential to produce and accumulate chemicals similar to those produced by the whole plant, plant cell cutures have been considered as alternative sources to agricultural production. In the last decade numerous studies with callus and suspension cultures from various plants have been published describing the formation, and sometimes accumulation in rather high concentration, of many different secondary metabolites. The accumulation of these products in cell culture has often been compared to the concentration of these compounds in the intact plants (Table 4) and many cases have been reported in which the accumulation in cell cultures was considerably higher than in the intact plants (Vasil, 1987).

Table 4. Examples of Secondary Metabolite Production by Plant Tissue Culture (Constabel, 1987)

Plant	Metabloite	Culture method	Content (%dwt)	Content in plant (%dwt)	Ratio of content (cell culture/plant)	Reporter
Papaver somniferum	Sanguinarine	Liquid	2.9	-	-	Eilert *et al.*, 1985
Dioscorea deltoidea	Diosgenin	Liquid	2	2	1	Kaul and Staba, 1968
Coffea arabica	Caffeine	Agar	1.6	1.6	1	Frischknecht *et al.*, 1977
Coptis japonica	Berberine	Agar	7.4	7	1	Fukui *et al.*, 1982
Macleaya cordata	Protopin	Agar	0.4	0.32	1.25	Koblitz *et al.*, 1975
Coptis japonica	Berberine	Liquid	13	-	2	Sato and Yamada, 1984
Catharanthus roseus	Ajmalicine	Liquid	1	0.3	3	Zenk *et al.*, 1977
Coleus blumei	Rosmarinic acid	Liquid	15	3.6	5	Razzaque and Ellis, 1977
Panax ginseng	Ginsengoside	Agar	27	4.5	6	Furuya *et al.*, 1983
Lithospermum erythrorhizon	Shikonin	Agar	12	1.5	8	Tabata *et al.*, 1978
Morinda citrifolia	Anthraquinones	Liquid	18	2.5	8	Zenk *et al.*, 1975
Lithospermum erythrorhizon	Shikonin	Liquid	14	1.5	9.3	Fujita and Tabata, 1986
Cassia tora	Anthraquinone	Agar	6	0.6	10	Tabata *et al.*, 1975
Nicotiana tabacum	Ubiquinone-10	Liquid	0.18	0.003	60	Matsumoto *et al.*, 1981
Catharanthus roseus	Catharanthine	Liquid	0.24	0.002	77	Smith *et al.*, 1987

Although more than 30 years of research has been devoted to achieving the feasibility of cell cultures as source of plant constituents, only a few cases have been successful in term of economic benefits. One of the major problems is the low production

of secondary metabolites under *in vitro* conditions (Narong Chomchalow and Oradee Sahavacharin, 1981). This is expectable since such constituents are present in very small amount even in intact plants. Another problem includes a decline in the amount in succeeding subcultures as reported in *Nicotiana* callus (Tabata and Noboru, 1976). The nicotine contents in the cultures were rapidly decreased to trace amounts in succeeding subculture in association with the decline of the root regenerating activity. Thus the lack of specialised cell structures in some cultures may be another reason for the absence of accumulated secondary metabolites. In fact, decreased secondary metabolite yields have sometimes been reported for cultures as a consequence of organogenesis. For example undifferentiated cultures of *Dioscores deltoidea* and *Agave wightii* yield 1-2 % dry weight steroidal sapogenins, but when cultures differentiate to produce roots or bulbils, only trace amounts of sapogenine are produced (Kaul and Staba, 1986 ; Sharma and Khanna, 1980).