CHAPTER II
LITEATURE REVIEW

2.1 Plant Cytochrome P450

Cytochrome P450 (CYP or P450) is a heme thiolate protein for a class of hemoprotein in which a thiolate group (typically from a cysteine) is a axiol ligand of heme iron and exhibit a characteristic UV absorption maximum upon binding of a carbon monoxide by reducing enzyme (Guengerich, 2002a; Guengerich, 2002b; Guengerich et al., 2002; Yamazaki et al., 2002). P450 is an intermediated biocatalyst enzyme in the metabolic pathways of various primary and secondary metabolites which are important in pharmaceutical applications. Its catalytic reactions occurring in a wide variety of hydroxylations require one molecule of oxygen and are dependent on electron transfer from NADPH via NADPH-P450 reductase (CPR) (Figure 1) (Durst and Nelson, 1995; Jensen et al., 2011; Laursen et al., 2011)

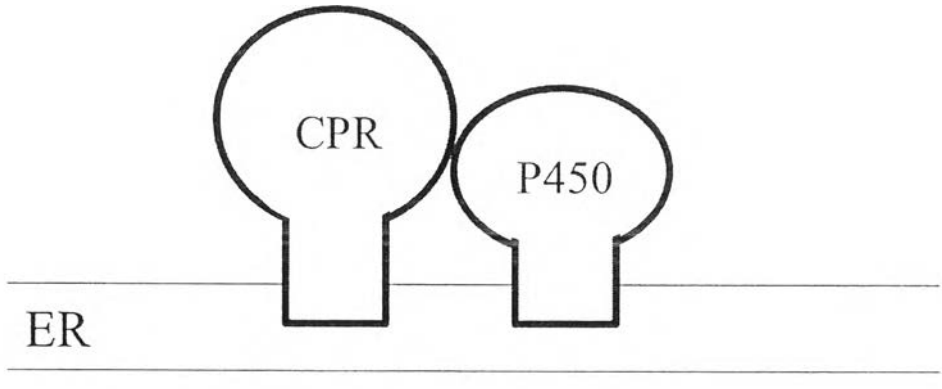

Figure 1 Two molecules of cytochrome P450 reductase and cytochrome P450

P450s belong to the heme-containing enzyme superfamily, which are membrane bound proteins anchoring on the endoplasmic reticulum (ER) as microsome-P450 type and presenting the hydrophobic signal at the N-terminal. Each P450 contains a molecule of metalloporphyrin which has a primary function of hydroxylation or oxygen binding and also many other functions overlapping among plant P450s (Bolwell et al., 1994).

2.2 Nomenclature, classification

Plant P450s are classified into families and subfamilies according to amino acid identities using the "40% rule" and phylogenetic criteria (Nebert and Nelson, 1991; Nebert et al., 1989; Nelson, 1998; Nelson, 1999; Nelson, 2006; Nelson, 2009). The same family is set if the amino acid homology is higher than 40%. Within the same family, normally, P450 members share at least 55% identity (Nebert and Nelson, 1991; Nebert et al., 1989; Nelson, 1998; Nelson, 1999; Nelson, 2006; Nelson, 2009). Genes encoding P450 enzymes, and the enzymes themselves, are designated with the abbreviation CYP, followed by a number indicating the gene family, a capital letter indicating the subfamily, and another numeral for the individual gene. The convention is to *italicise* the name when referring to the gene. For example, *CYP97C1* is the gene that encodes the enzyme CYP97C1 (Figure 2) (Nelson, 2006; Werck-Reichhart et al., 2002). The names of plant P450s have so far been assigned from CYP71A1 to CYP99xy (Werck-Reichhart et al., 2002).

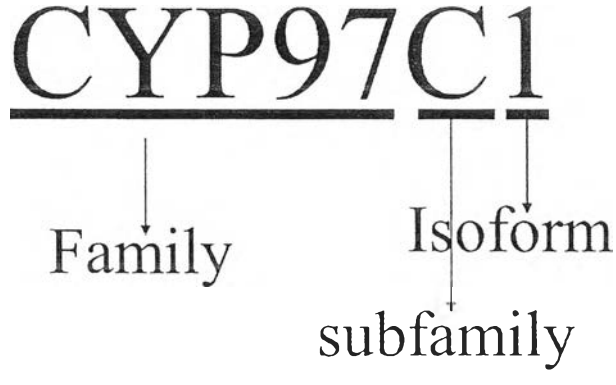

Figure 2 Nomenclature of cytochrome P450 symbol.

2.3 Cytochrome P450 clans

Depending on the phylogenetic analysis, plant cytochrome P450 can be divided into two main clades: the A-type and non-A type (Mizutani and Ohta, 2010). The A-type clade is specific to plants, and its P450s are involved in the secondary metabolism. The non-A type clade is more divergence in sequences and found in both plant and non-plant P450s. The non-A type P450s are primarily involved in the primary metabolism (Werck-Reichhart et al., 2002).

Clan is defined as a group of the CYPs closely related in the phylogenetic tree. Among plant orders, plant CYPs can be divided into ten clans with seven clans of identified chemical reactions and three clans of unknown reactions (Nelson, 1998; Poulos, 2005; Schuler and Werck-Reichhart, 2003). The seven clans consist of P450 clans 51, 71, 72, 74, 85, 86, and 97, and the three clans consist of clans 710, 711, and 713. Within each clan, plant CYP families are distributed across the plant orders from moss to gymnosperms and angiosperms (Mansuy, 1998; Nebert and Nelson, 1991; Nebert et al., 1989; Nelson and Werck-Reichhart, 2011). Presently, complete genomes of six higher plant species are known, including *Arabidopsis thaliana*, rice, papaya, grave vine, soybean, and tomato. The full-length sequences are in the range from 246 CYP in *Arabidopsis thaliana*, 312 in *Populus tricocarp*a (Popular), to 356 CYP in *Oryza sativa* (Rice), and 457 CYP in *Vitis vinifera* (Grape). Among 1317 full-length sequences there are consisted of 1317 putative functional genes and exists only 73 families as defined in standardized nomenclature development for P450 sequences (Durst and Nelson, 1995; Hamberger and Bohlmann, 2006; Nelson et al., 1996; Nelson et al., 2004).

2.4 P450 Structure Features

Plant P450 amino acid sequence usually consists of 450-550 amino acid residues with 50-60 kDa (Chapple, 1998). There are four consecutive conserve regions in the molecules (Figure 3) (Werck-Reichhart et al., 2002; Werck-Reichhart and Feyereisen, 2000) as follows:

2.4.1 Heme-binding motif

Phe-x-x-Gly-x-x-x-Csy-x-Gly. This motif is essential for the catalysis and give the P450 ability to bind with carbon monoxide (CO) (Guengerich, 2002b). The important characteristic is the occurrence of the Seret peak in the 380-460 nm region which can be confirmed by UV-VIS spectroscopy (Coon, 2003; Guengerich, 2002b). The heme-posthetic group in the protein can alternate between the ferrous and oxide (Ferric, Fe^{3+}) states during the electron transport of the reduce (Ferrous, Fe^{2+}) form with carbon monoxide binding that give an absorbance at 450 nm.

2.4.2 Glu-x-x-Arg motif

This motif is needed to solubilize the core structure and the proximal side of heme.

2.4.3 A proline-rich motif

This motif is located in the N-terminal hydrophobic helix. Most of the eukaryote P450s are associated with microsomal membrane and frequently have a cluster of prolines (Pro)-Pro-Gly-Pro-x-Gly/Pro to form a hinge proceeded by a cluster of basic residues (the half-transfer signal) between the hydrophobic amino-terminal membrane anchoring segment and the globular part of the protein.

2.4.4 Proton transfer motif

This motif: Ala/Gly-Gly-s-Asp/Glu-Thr-Thr/Ser, corresponds to the proton transfer groove on the side of the heme.

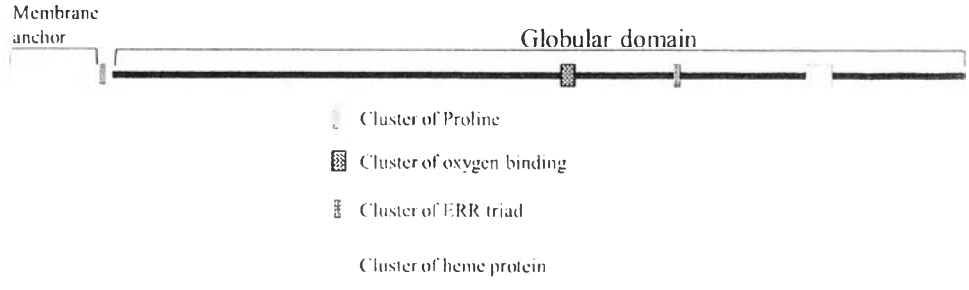

Figure 3 Primary structure of cytochrome P450 protein.

2.5 Localization

P450s are generally membrane-bound proteins that contain heme groups and carry out electron transport or catalyze reductive/oxidative or redox reactions. They are found in the mitochondria inner membrane and endoplasmic recticulum of eukaryotes, and in the chloroplast of plastids. In Arabidopsis, 16 CYP families have been reported that 1) proteins containing potential chloroplast-targeting sequences may be targeted to the chloroplast at the N-terminal region, 2) CYP74A1 located in the inner part of the chloroplast membrane facing the stoma, 3) the other three CYP families consisting of CYP74B2 are located at the outer part of the chloroplast membrane facing the inter-membrane space, and 4) CYP86B1 and CYP701A3 are located at the outer part of the chloroplast membrane facing cytosol (Table 1).

Table 1 Plant cytochrome P450 localization

CYP family	Activity	Localization	Reference
CYP74A1	alpha olefin sulfonate (AOS)	Inner chloroplast membrane facing the stroma	(Froehlich et al., 2001)
CYP74B2	Hydroperoxide Lyase (HPL)	Outer chloroplast membrane facing the inter-membrane space	(Froehlich et al., 2001)
CYP86B1	Not defined	Outer chloroplast membrane facing cytosol	(Watson et al., 2001)
CYP701A3	Kaurene oxidase	Outer chloroplast membrane facing cytosol	(Watson et al., 2001)
CYP71B2,CYP72A14, CYP76A2,CYP76C4, CYP77A3,CYP78A2, CYP78A6,CYP79B2, CYP79B3,CYP86B2, CYP93D1,CYP94B3, CYP97A3,CYP707A2, CYP724A1,and CYP97C1	*Arabidopsis* P450 protein containing potential chloroplast-targeting sequences may be targeted to the chloroplast at the N-terminal region (Nelson, 2002; Nelson, 2006; Nelson, 2009)		
References?			

2.6 Mechanism of enzyme catalytic reaction

Cytochrome P450 enzyme catalysis involves the electron donor NADPH-O_2 hydroxylation. The reaction occurs in a coordinated manner of the reductase cycle and P450 cycle as shown in Figure 4.

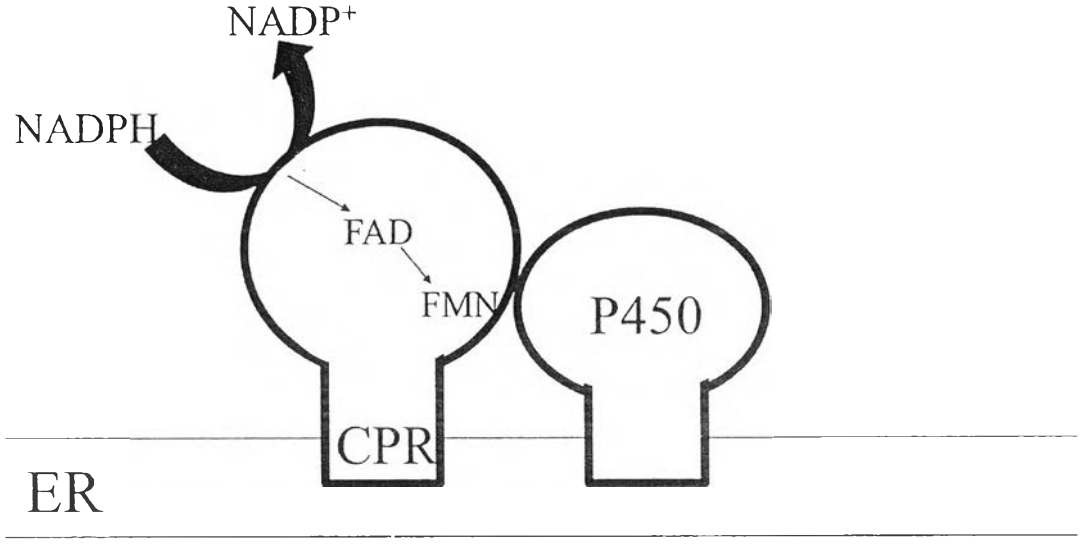

Figure 4 A diagram showing the coordination of the electron flow from cytochrome P450 reductase (CPR) to the P450 globule.

Plant P450s catalyze the oxidative reactions of non-activated hydrocarbons with regiospecific and stereospecific type at physiological temperatures (Coon, 2002; Coon, 2003; Coon, 2005a; Coon et al., 1992; Coon et al., 1996). In the most common P450 oxygenase reactions, two electrons from P450 are donated to O_2, then one oxygen atom combines (hydroxylates) with the substrate and the second oxygen atom is used to form a molecule of water as follow: $RH + O_2 + NADPH^+ \rightarrow ROH + H_2O + NADP^+$ (Laursen et al., 2011; Urlacher and Eiben, 2006; Urlacher and Girhard, 2012). The characteristic sequence of the catalytic reaction is involved in six steps (Coon, 2003; Coon, 2005b; Coon et al., 1992; Coon et al., 1996; Guengerich, 2002b; Newcomb et al., 2003a; Newcomb et al., 2003b) as shown in Figure 5.

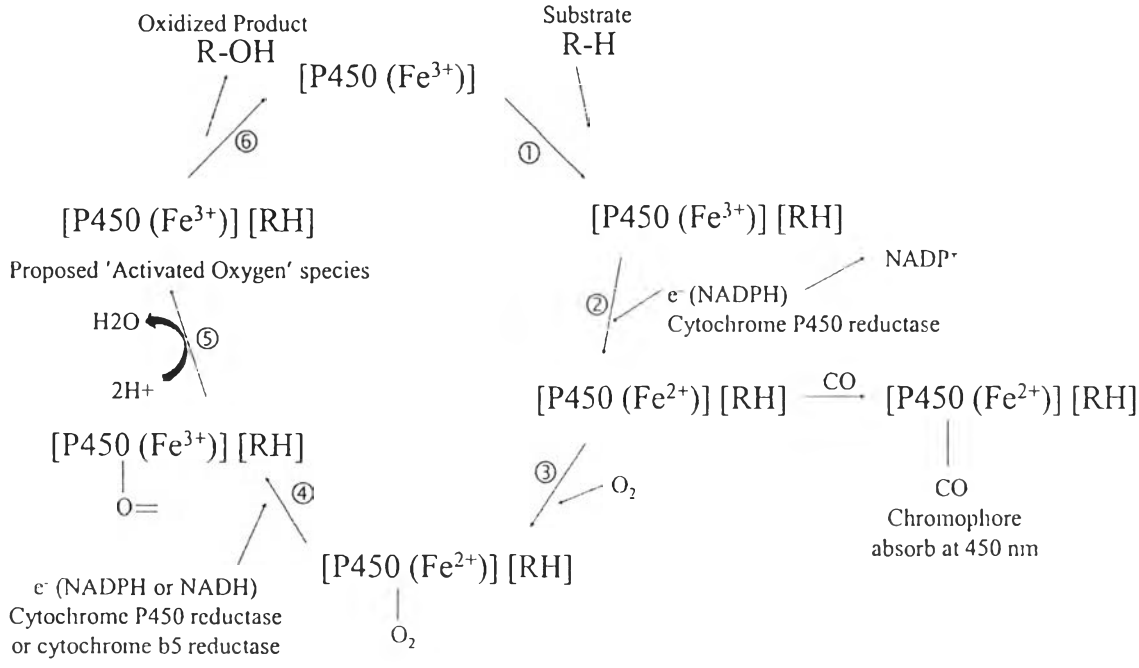

Figure 5 Catalytic mechanism of P450 enzyme

Step 1 The substrate binds to the active site of the enzyme, in close proximity to the heme group, on the side opposite to the peptide chain. The bound substrate induces a change in the conformation of the active site, often displacing a water molecule from the distal axial coordination position of the heme iron, and sometimes changing the state of the heme iron from low-spin to high-spin. This gives rise to a change in the spectral properties of the enzyme, with an increase in absorbance at 390 nm and a decrease at 420 nm.

Step 2 The change in the electronic state of the active site favors the transfer of an electron from NADPH via cytochrome P450 reductase or another associated reductase. This takes place by way of the electron transfer chain, as described above, reducing the ferric heme iron to the ferrous state. If carbon monoxide (CO) binds to reduced P450, the catalytic cycle is interrupted. This reaction yields the classic CO difference spectrum with a maximum at 450 nm. P450 contains a heam as prosthetic group which plays an essential role in the electron transfer as part of the respiratory chain. Letters 'P' recognizes their coloration in the visible range due to a Seret band around 420 nm. Thus, carbon monoxide forms with dithionite-redued cytochrome P450 complex which absorbs maximum at 450 nm.

Step 3 Molecular oxygen binds covalently to the distal axial coordination position of the heme iron. The cysteine ligand is a better electron donor than histidine, which is

normally found in heme-containing proteins. As a consequence, the oxygen is activated to a greater extent than in other heme proteins. However, this sometimes allows the iron-oxygen bond to dissociate, causing the so-called "uncoupling reaction", which releases a reactive superoxide radical and interrupts the catalytic cycle.

Step 4 A second electron is transferred via the electron-transport system, from either cytochrome P450 reductase, ferredoxins, or cytochrome b5, reducing the dioxygen adduct to a negatively charged peroxo group. This is a short-lived intermediate state.

Step 5 The peroxo group formed in step 4 is rapidly protonated twice by local transfer from water or from surrounding amino-acid side-chains, releasing one water molecule, and forming a highly reactive species commonly referred to as P450 Compound 1 (or Compound I). This highly reactive intermediate was not "seen in action" until 2010, although it had been studied theoretically for many years. P450 Compound 1 is most likely a iron (IV) oxo (or ferryl) species with an additional oxidizing equivalent delocalized over theporphyrin and thiolate ligands. Evidence for the alternative perferryl iron (V)-oxo is lacking.

Step 6 Depending on the substrate and enzyme involved, P450 enzymes can catalyze any of a wide variety of reactions. A hypothetical hydroxylation is shown in this illustration. After the product has been released from the active site, the enzyme returns to its original state, with a water molecule returning to occupy the distal coordination position of the iron nucleus.

The main function of plant P450s is correlated with the clans of P450s (families and subfamilies). The P450s within the same family can catalyze the same pathway (Nelson et al., 2004; Schuler and Werck-Reichhart, 2003) or same reaction (Table 2) (Mansuy, 1998). For example, CYP97 family enzyme can catalyze the reactions of the carotenoid pathway with different substrates in the pathway (Stigliani et al., 2011) (Table 2). The P450s families are also categorized into four classes of metabolic reactions/pathways as follows:

-Essential reactions conserved in the plant kingdom.

-Core reactions/pathways conserved in all land plants.

-Essential reactions/pathways that emerged during flowering plant evolution (e.g., plant hormones homeostasis).

-Specialized reactions/pathways unique to plant species. Taxol, diterpenoid Resin acid

Table 2 Cytochrome P450 clans which catalyze specific reactions

CYP-Clan	Function
CYP51	Sterol
CYP71	Shikimate production intermediates
CYP72	Catabolism of isoprenoid hormones
CYP74	Allene oxide sythase/Hydroperoxide lyase
CYP85	Sterol & acyclic terpenes in bassinosteroid and absisic acid in GA pathway
CYP86	Hydroxylation of fatty acid
CYP97	Hydroxylation of carotenoid
CYP710	Not identify
CYP711	Not identify
CYP713	Not identify

Because the analytical methods for assaying and screening of P450 actively can be approached by various means it is initially important to understand how these enzymes perform their reaction. While the details of catalytic cycle can still subject if ongoing reaction, the hydroxylation mechanism of P450 enzymes is well understand and can be described as shown in Figure 5.

2.7 Cytochrome P450 Reductase (CPR)

CPR is a membrane bound protein located on the endoplasmic reticulum (ER) belonging to ferredoxin superfamily protein (Jensen and Moller, 2010). CPR was first isolated from yeast by its ability to reduce the cytochrome c (Hamann and Moller, 2007; Urban et al., 1994). By now, even the CPR isolated directly from yeast, plant, or from the heterologous expressoion (yeast or *Escherichia coli* or insect cell) as the nature restricted on the ER or the soluble as a microsome fraction or by the detergent solubilization form, it still permits transfer of the electron to the P450 (Murataliev and Feyereisen, 1999). Plant CPRs have been isolated and characterized from various taxa of both dicotyledon and monocotyledon. The cDNA sequences encode for the plant CPR genes show highly identity among plant domain (68% to 91%) and molecular mass was ~79 kDa. The recombinant CPR expression was high

level found in yeast. The pattern and level of expression were indicated by the distribution of mRNA, *Catharanthus roseus* shown high level expression in flower and lower in leaf (Meijer et al., 1993).

2.7.1 Associated with cytochrome P450

In terms of the metabolic pathways and gene regulations of secondary metabolism for pharmaceutical applications, plant P450s have been viewed as "metabolic intermediate enzymes". Particularly, the chemical group of terpenoids which represent the largest class of characterized natural plant compounds, are often substrates for the P450s hydroxylation reactions. Plant P450s are closely associated with the gene called cytochrome P450 reductase (CPR) which plays an important role as a partner of the P450 in catalyzing various oxidation reactions, including hydroxylation. CRPs catalyze the reactions by transferring the electron sequentially from NADPH to FAD, to FMN binding domain, and then to the heme binding domain in P450 (Nadler and Strobel, 1988). The ratio between CPR and P450 in the microsomal fraction has been estimated as 1:15, and the reactions can be catalyzed into many compounds (Shephard et al., 1983).

2.7.2 Phylogenetic analysis

In terms of phylogenetic relationship analysis, putative CPR sequences from the database (http://www.p450.kvl.dk) can be divided into 2 distinct groups (Paquette et al., 2009). One is CPR class I which contains sequences from the eudicotyledons and the other is CPR class II which contains sequences form the monocotyledons and a specific group of eudicotyledons (Ro et al., 2002). The isoform (homolog) of the CPRs is based on species in which most vascular plants have been shown to have 1 to 3 isoforms, such as three isoforms in *P. tricocarpa* and *A. thaliana*, two isoforms in *V. vinifera*, and one isoform in *R. communis* (Benveniste et al., 1991; Hull and Celenza, 2000; Koopmann and Hahlbrock, 1997; Meijer et al., 1993; Rana et al., 2013; Ro et al., 2002; Scherbak et al., 2011; Yang et al., 2010). Regarding to the sequences, the database shows that some species contain paralog of the gene but display a high degree of conservation at the N-terminal and functional domains by multiple alignment. The paralog of the enzyme might reflect that one CPR can catalyze various reactions with P450 leading to various compounds.

2.8 Biotechnological aspects of P450

The isolation of the native system of P450s to be used in an industrial process is limited due to the stability, complexity and low catalytic turnover of the P450s. The natural environment-inside the whole cell is essential for the enzyme to be active as membrane associated protein. Genetic engineering is one way to be used to identify the P450 enzyme to alter the protein backbone for a specific biotechnological process (Zelasko et al., 2013).

2.8.1 Genetic engineering and isolation of P450

The metabolic engineering if terpenoids in microbes is rising by now because of the advantage of the DNA technology in manipulating the products with the objectives to obtain high yields and environment concerns. High values of terpenoid products are found in low abundant of the natural sources. For example, paclitaxel is found only 0.01% from bark of the Pacific yew, *Taxus brevifolia* (Mizutani, 2012). The issues of large raw material requirement and sustained supplied have raised the problems of both the economical effect and the environmental concerns. Plant P450s, which is the largest in terms of both of protein family and available collection, has been used for comparative studies and biotechnological applications (Bohlmann et al., 1998). As mentioned earlier, P450 genes which play an important role in plant natural product biosynthetic pathway can be functionally expressed using the heterologous system (Kirby and Keasling, 2009), including the bacteria, yeast, and insect. The purpose of using a variety of heterologous systems is to increase in producing plant deriving compounds (Kirby and Keasling, 2009; Misawa, 2011). The yield of P450 proteins depends on the limited of the translation in the heterologous expression systems. Plant P450 functional expression in bacteria needs both purified and reconstituted with their electron transfer partner. The successful expression of P450 protein in bacteria has been done in *Escherichia coli* (Ajikumar et al., 2008; Zelasko et al., 2013).

2.8.2 Activity assay of cytochrome P450

2.8.2.1 Frequently used substrates

Cytochrome P450 enzyme can frequently catalyzed wide range of reactions, and thus their activities can be monitored by varying types of substrates. The analysis can be performed using different techniques. The list of substrates used for the detection of P450 reactivity has been summarized by Delaporte *et al.*, 2001. However,

confirmation of the reaction assay is still required to elucidate the structural details of products by i.e. LC-MS or NMR spectroscopy (Chang et al., 2007).

2.8.2.2 Cleavage products

Besides using various potential substrates by itself, appropriate design of substrate molecules may also allow one to generate a cleavage product that can be detected in a high-throughput manner. One example is the hydroxylation of p-nitrophenoxycarboxylic acids (p-NCA, 8a), which is specifically catalyzed by P450BM-3. Another one is the hydroxylation at the ω-position of fatty acids leads to cleavage of the acetal and, thus, to the formation of the ω-oxycarboxylate and the release of the chromophore p-nitrophenolate at pH 8. The formation of p-nitrophenolate can be directly monitored with a spectrophotometer at a wavelength of 410 nm. In addition, it is also possible to set up systems in which a cleavage product reacts with a second substance to form a dye. As shown by Peters et al. [78], the conversion of the substrate by P450BM-3 led to production of formaldehyde which then reacts with Purpald to yield an intermediate, which after oxidation in air gives rise to a purple product, conveniently detectable spectrophotometrically at 550 nm.

2.8.2.3 Substrate-independent screening by measuring NADPH consumption

To avoid problems of limited substrate suitability, alternative methods have been developed for the detection of P450 activity in which the consumption of NADPH is followed instead of substrate conversion. The advantage is that the detection is independent of the P450 substrate, which means that the detection setup does not need to be adjusted particularly to each individual substrate (Ansede and Thakker, 2004). Most P450 enzymes known so far require a NADPH-dependent reductase to supply electrons (Fig. 2). Therefore, the rate of NADPH consumption can be used as an indication of P450 reactivity within a purified system. The NADPH depletion can conveniently be followed in a multiwell plate reader at 340.

2.9 Terpenoid P450 intermediated-enzymes

P450s involved in many biochemical pathways synthesize the important pharmaceutical compounds such as terpenoids and indole alkaloids (Hamberger and Bak, 2013). Terpenoids form a group of major natural chemical products found in plants. The terpenoids are volatile substrates found in higher plants such as conifer,

citrus, and eucalyptus. The volatile terpenoids give the plant fragrance. The terpenoids have been applied in health and societal issues, and have extensively been applied to pharmaceuticals (e.g., artemisinin, sesquiter- pene; paclitaxel (Taxol); diterpene), herbal medicines (e.g., glycyrrhizin and ginsenosides; triterpenes), nutraceu- ticals (e.g., astaxanthin and lycopene; carotenoids), flavors (e.g., limonene and linalool; monoterpenes), fragrances (e.g., citronellol and geraniol; monoterpenes), cosmetics (e.g., astaxanthin), colorants (e.g., β-carotene; carotenoid), or agrichemicals (e.g., gibberellins; diterpenes) (Croteau et al., 2006; Wen et al., 2007).

2.9.1 Terpenoid biosynthetic pathways

Terpenoids are derived from the C5 carbon skeleton of isoprene. The production of terpenoids in higher plant is both form the classical acetate mevalonate (MVA) pathway and the non-mevalonate pathway called 1-deoxy-D-xylulose-5-phosphate (DXP) or the 2C-methy-D-erythriol-4-phosphate (MEP) pathway (Chang et al., 2007). The MEP is taken place in the plastid-related compartments and the MVA pathway is located in cytosol. Triterpenes are known to be produced in cytosol via MVA pathway and a vast variety of mono- and diterpenoids are produced in plastid compartment via MEP pathway. Diversity of terpenoids is derived from the precursors in the pathway, essentially, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The diversity of the terpenoids generated in the biosynthetic pathways arises from the reactions of cyclization, elongation, and hydroxylation by so many enzymes involved in the pathways (Chang et al., 2007). In the tepenoid biosynthetic pathway, the two building blocks IPP and DMAPP with the catalysis by specific prenyltransferases, various linear prenyl diphosphate molecules with different carbon number, such as 10-carbon geranyl diphosphat (GDP), 15-carbon fenesyl diphosphate (FDP) and 20-carbon geranylgeranyl diphosphate (GGDP) can be generated as shown in Figure 6.

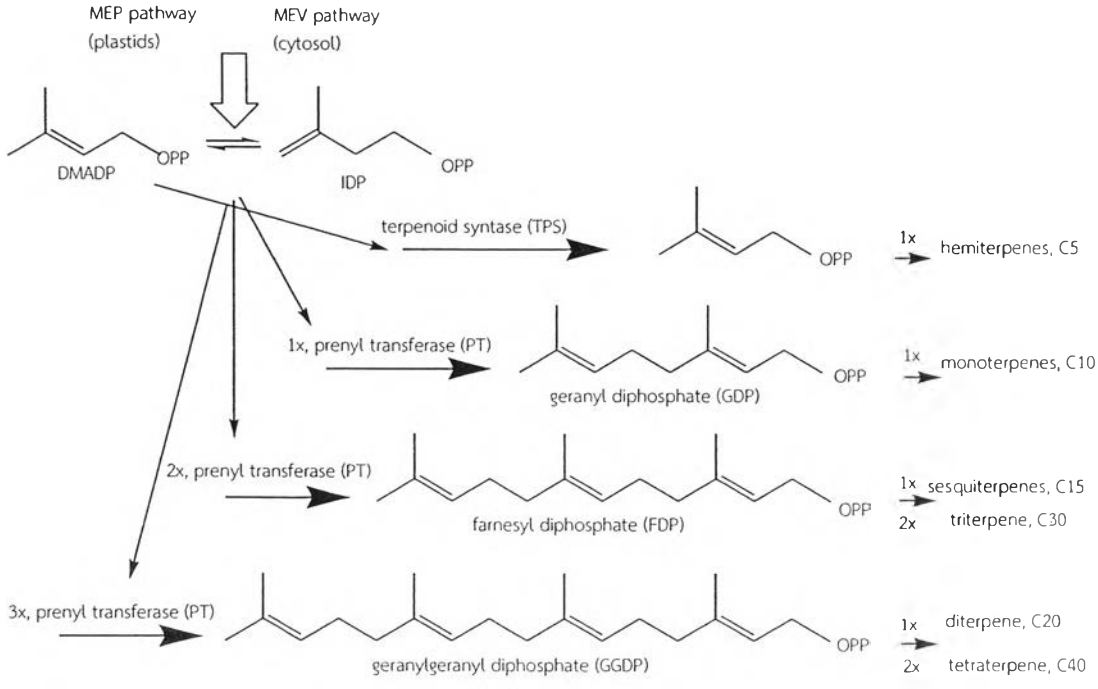

Figure 6 General of plant terpenoid pathway

During the terpenoid biosynthesis, the active isoprene unit IPP is repeatedly added to DMAPP of a prenyl diphosphate in a manner of sequential head-to-tail condensation (Figure 7).

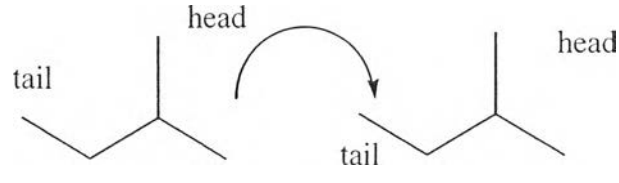

Figure 7 2-methylbuta-1,3-diene, isoprene unit head-to-tail join to produce mono-terpenoid backbond.

A rational classification of the terpenes has been established based upon the number of isoprene (or isopentane) units incorporated into the basic molecular skeleton. Mono-, sesqui-, di-, and sesterterpenes contain the isoprene units linked in a head to tail fashion, whereas the triterpenes and carotenoids (tetraterpenes) contain two C15 and C20 units respectively linked by a head to head fashion (Harada and Misawa, 2009; Harada et al., 2009; Kakinuma et al., 2001; Misawa, 2011).

Most natural terpenoid hydrocarbons have the general formula of $(C_5H_8)_n$ (Table 3). They can be classified on the basis of the n value or number of carbon atoms

present in the structure. Many terpenes are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes or ketones are also found (Harada et al., 2009). These derivatives are frequently named terpenoids. Mono- and sesquiterpenes are the chief constituents of the essential oils while the other terpenes are constituents of balsams, resins, waxes, and rubber (Harada and Misawa, 2009; Harada et al., 2009; Kakinuma et al., 2001; Misawa, 2011).

Table 3 Classification of Terpenoids

Name	No. of isoprene unit	No. of carbon atoms
Hemiterpenoids	1	5
Monoterpenoids	2	10
Sesquiterpenoids	3	15
Diterpenoids	4	20
Sesterterpenoids	5	25
Triterpenoids	6	30
Tetraterpenoids	8	40
Polyisopenoids	>8	>40
Steroids triterpenoid which produce Diels's hydrocarbon when distilled from zinc dust		
Carotenoids	8	40

2.9.2 Example of the terpenoids used in the pharmaceutical applications

Monoterpene, sesquiterpene, and diterpene are all substrates for cytochrome P450s (Figure 8). Some of these reactions amongst the easiest diagnosed as being cytochrome P450 dependent. The 10-hydroxylation of geraniol and niral has long been known to be a P450 function and was one of the first forms of plant P450 to be partially purified (Collu et al., 2001; Hofer et al., 2013).

Microsomal P450s catalyze numerous reactions with countless substrates. The terpenoids with therapeutic properties, including anti-cancer, anti-parasitic, anti-microbial, anti-allergenic, and in food industrial as food ingredients, natural food

colorants, nutraceuticals is a major advantage of the compounds for genetic engineering (Chang et al., 2007; Hull and Celenza, 2000).

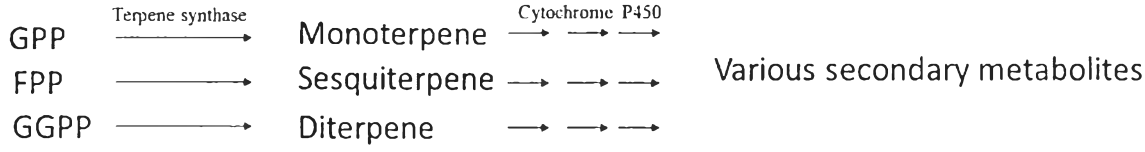

Figure 8 Down regulated terpenoid biosynthetic pathway which cytochrome P450 involve as an intermediate enzyme.

2.10 *Croton stellatopilosus* Ohba

2.10.1 Taxonomy

Plau-noi or *C. stellatopilosus* Ohba is a Thai medicinal plant which refers to the specific distribution in Thailand. *C. stellatopilosus* Ohba which the new name of the formerly *C. sublylatus* Kurz that have been approved that restricted as an endemic species in Thailand (Esser and Chayamarit, 2001). The *C. stellatopilosus*, a tropical plant, was classified in the order Malpighiaales and family Euphorbiaceae in the taxonomic level. *C. stellatopilosus* plant is shrub to six meters tall, and eight centrimeters branching from base; younger parts distinctly pubescent; flowering with mature leaves (Figure 9). *C. stellatopilosus* are distributed throughout Thailand especially in Central and Southern parts.

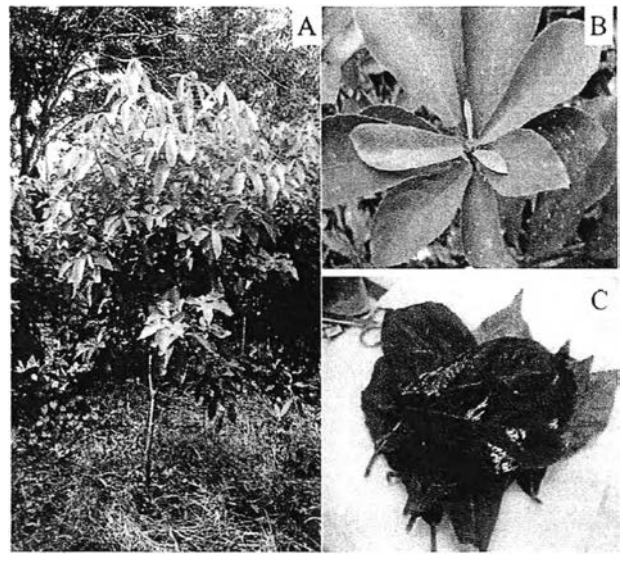

Figure 9 *Croton stellatopilosus* Ohba (A) plant of the *C. stellatopilosus* (B) leaves shub and (C) leaves which used for the experiment after washing.

2.10.2 Natural product, the plaunotol

Plauotol is a major product which found in various parts of the *C. stellatopilosus* including leaves, roots, tiggars, fruits, and flowers. Plaunotol is a diterprenoid classification consisted of the 4 isoprene units $(C_5H_8)_4$ as a backbone attached with the hydroxyl group at C18 (Figure 10) (Tansakul and De-Eknamkul, 1998).

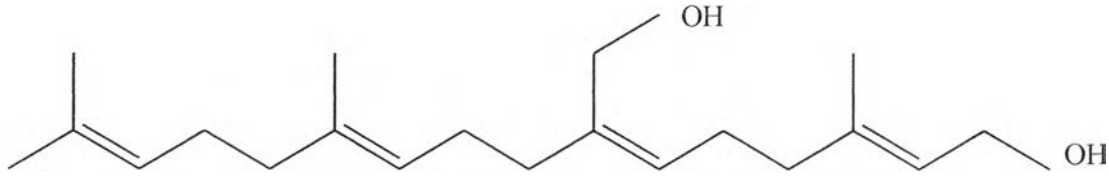

Figure 10 Plaunotol, a diterpenoid structure

Plaunotol is a generic name for (2E,6Z,10E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14- hexadecatetraen-1-ol or 18-hydroxygeranylgeraniol and have $C_{20}H_{34}O_2$ is a molecular formula. The plaunotol has molecular mass is 306.255 (Table 4).

Table 4 Plaunotol chemical properties

Chemical properties	
Generic name	Plaunotol (CS-684)
Chemical name	(2E,6Z,10E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14- hexadecatetraen-1-ol or 18-hydroxygeranylgeraniol
Molecular formula	$C_{20}H_{34}O_2$
Molecular mass	306.255

2.10.3 Plaunotol biosynthesis

The biosynthesis of plaunotol has been found to occur via the deoxyxylulose phosphate pathway with the last two steps catalized by the two enzymes, geranylgeraniol diphosphate phosphatase (GGPPp) and geranylgeraniol-18-hydroxylase (GG18H) (Figure 11). The reactions are using the substrate as geranylgeraniol diphosphate (GGPP) and geranylgeraniol (GGOH), repectively (Tansakul and De-Eknamkul, 1998).

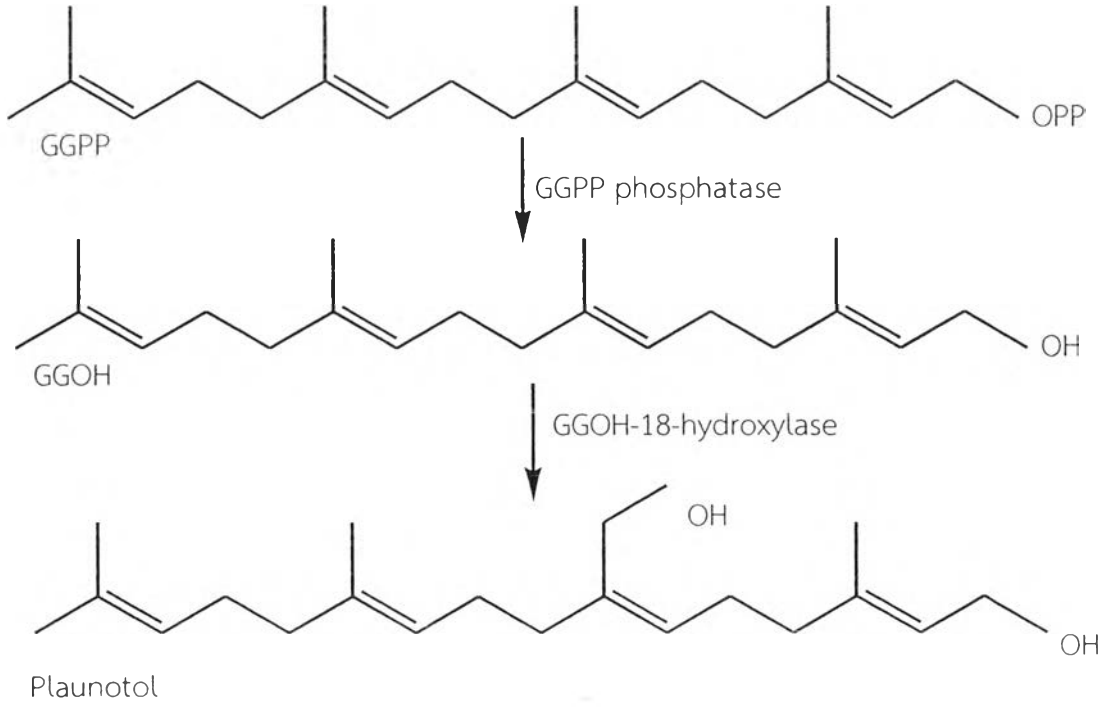

Figure 11 Proposed biosynthetic pathway for the formation of plaunotol.

2.10.4 Gene in pluanotol biosynthesis pathway

Novel issues involved the gene had continuously concentrated. The GGPP and MEPP gene have been studies and got the full length. The genes involving in both the up-stream and down-stream regulated pathway consist of, 1-Deoxy-D-xylulose 5-phosphatesynthase (DXS), 2C-methyl-D-erythritol-4-phosphate (MEPS), geranylgeranyl diphosphate synthase (GPPPs), geranylgeranyl diphosphate phosphatases (GGPPp) (Figure 12). The last step of the pathway gene is a geranlygeraiol-18-hydroxylase (GG18H) gene is report as a cytochrome P450 in the inhibitor reaction enzyme (Nualkaew et al., 2006; Sitthithaworn et al., 2009; Sitthithaworn et al., 2010; Wungsintaweekul et al., 2008; Wungsintaweekul et al., 2007)

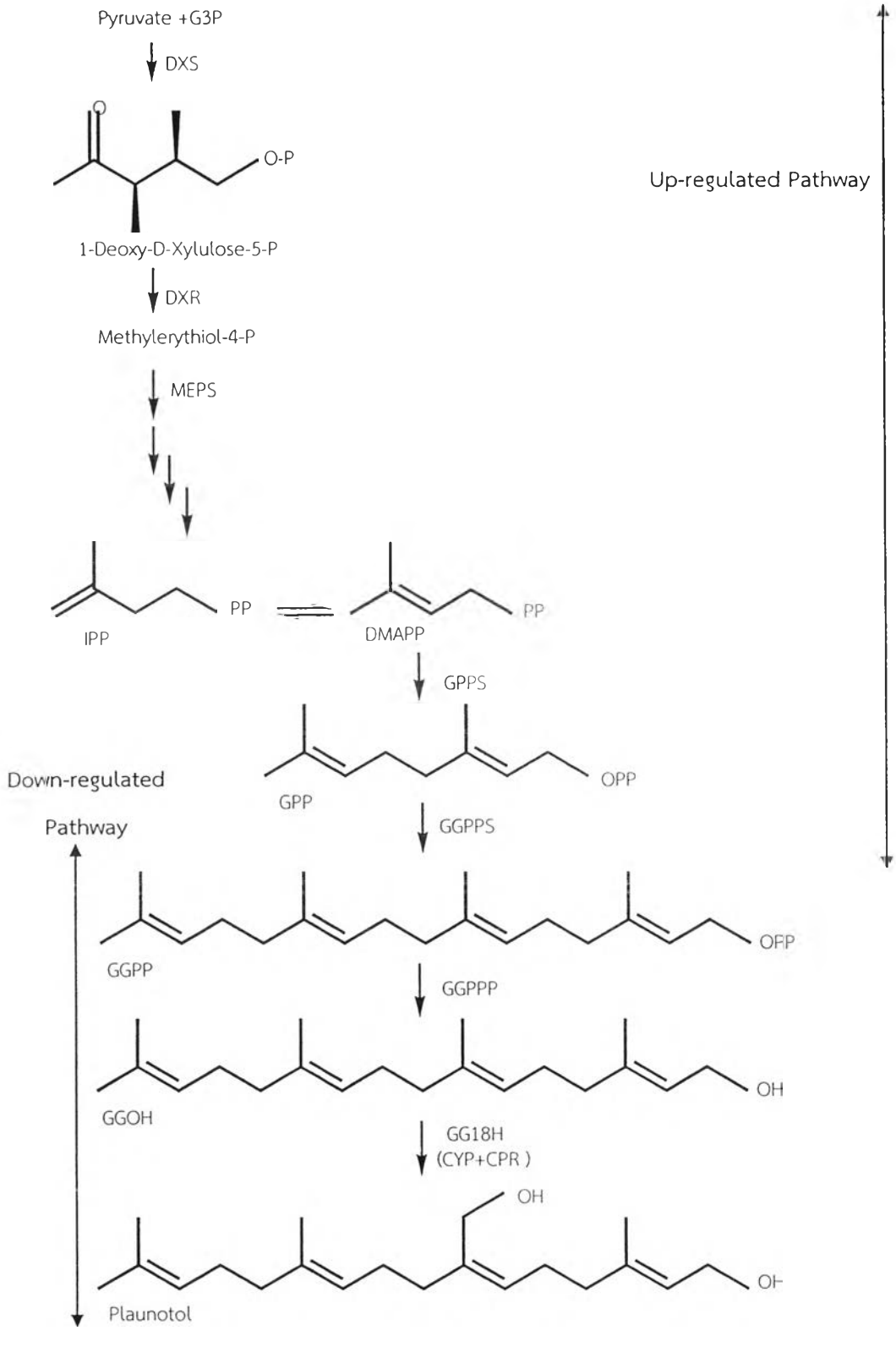

Figure 12 Genes in the pathway both up- and down-regulated of plaunotol biosynthetic pathway.

2.10.5 Plaunotol accumulation and detection

2.10.5.1 Plaunotol accumulation

Normally the plaunotol was extracted and isolated from the *C. stellatopilosus* parts, including in leaf, root, fruit, and stigma. According to the previous studies (Sitthithaworn et al., 2010; Tansakul and De-Eknamkul, 1998) the plaunotol had been accumulated as an oil glubules which found in the leaf more than others parts. Within the leaf the plaunotol oil grublues were distributed among the dorsal part of the leaf in the parisade cell layer (Tansakul and De-Eknamkul, 1998).

2.10.5.2 Plaunotol detection

According to the principle of this method, the crude extraction of sample could be used in the process to separate the compounds depended on the Rf. The advantage of the method is the small volume of the sample can be apply to fingered out the active ingredient in the sample. Both of the contents and the activities of the enzyme in reaction, plaunotol can be done on the TLC plate. The TLC densitometry chromatogram (the area peck) was used to estimate the amount of the plaunotol versus the authentic plaunotol standard (Rinthong et al., 2009; Vongchareonsathit and De-Eknamkul, 1998; Wungsintaweekul et al., 2007).

2.10.6 Plaunotol application

Among the secondary metabolites from *C. stellatopilosus* they have no reported to use as a medicinal except the plaunotol. Plaunotol as a phamarceutical used, and therefore often cultivated. This species is currently under active research in Thailand as a source of a drug against peptic ulcer (Plaunatol), and to stop stomach bleeding (in particular the leaves). Stem and bark are antidiarrheal and normalize menstruation. The flowers are antihelmintic. A tonic helps women who cannot eat, and after giving birth.

2.10.6.1 Anti-microbial

Plaunotol, in purified form, has been used specifically to treat peptic ulcer. In 1996, Koga and colleges have been reported that the plaunotol could be show the antibacterial activity against Helicobacter pyroli, which the plaunotol might interact with cell surface component, but the mechanism of its anti-bacterial effect is still unclear (Koga et al., 1996a; Koga et al., 1996b).

2.10.6.2 Anti-cancer

Moreover, effect of Plaunotol has been shown to involve with the regeneration process of soft tissue lining in the gastric system, thus facilitating the recovery of ulcer wounds used as a material source to anti-cancer (apoptosis) (Yoshikawa et al., 2009)