สารยับยั้งเชื้อราก่อโรคพืชที่มีพื้นฐานจากเบอร์เบรีน

นางสาววรรณ์จันทร์ นพนิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTI-PHYTOPATHOGENIC FUNGAL AGENTS BASED ON BERBERINE

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Thesis Title	ANTI-PHYTOPATHOGENIC FUNGAL AGENTS BASED ON BERBERINE
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วรรณ์จันทร์ นพนิช : สารยับยั้งเชื้อราก่อโรคพืชที่มีพื้นฐานจากเบอร์เบรีน. (ANTI-PHYTOPATHOGENIC FUNGAL AGENTS BASED ON BERBERINE)
อ. ที่ปรึกษา: ศ.ดร.อุดม ก๊กผล อ.ที่ปรึกษาร่วม: ผศ.ดร. วรินทร ชวศิริ 81 หน้า.

การคัดกรองฤทธิ์ต้านเชื้อราของสารผลิตภัณฑ์ธรรมชาติ 4 ชนิด คือ เอ็นแอเซทิลกลูโคซามีน, กลูโคซามีน, กรดแกลลิก และเบอร์เบรีน ต่อเชื้อราก่อโรคพืช 2 ชนิด *Phytophthora palmivora* และ *P. parasitica* ที่ความเข้มข้น 1000 ppm พบว่าเบอร์เบรีนแสดงฤทธิ์ยับยั้งต่อเชื้อราก่อโรคพืชทั้ง 2 ชนิด ได้อย่างสมบูรณ์ ได้สกัดเบอร์เบรีนจากแห้ม (*C. fenestratum* (Gaertn.) Colebr) และทดสอบฤทธิ์ต้านเชื้อราก่อโรคพืช 6 ชนิด ได้แก่ *Alternaria porri*, *Cercospora* sp., *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *P. parasitica* และ *Pythium deliense* ค่าความเข้มข้นที่สามารถยับยั้งการเจริญของเส้นใยของเชื้อราได้ 50 เปอร์เซ็นต์ของเชื้อทั้ง 6 ชนิด ได้แก่ 101, 653, 1011, 599 48 และ 284 ppm ตามลำดับ ได้สังเคราะห์อนุพันธ์เบอร์เบรีน 7 ชนิด โดยสังเคราะห์หมู่แทนที่ต่างๆที่ตำแหน่ง คาร์บอนที่ 8 และ 13 ของเบอร์เบรีนได้แก่ canadine, 8-methoxy-7,8-dihydroberberine, 8-trichloromethyl-7,8-dihydroberberine, acetonylberberine, 8-cyano-13,14-dehydrocanadine, 13-benzylberberine และ 13-methylberberine ทำการทดสอบฤทธิ์ยับยั้งการเจริญของเส้นใยต่อเชื้อรา *A. porri*, *P. parasitica* และ *P. deliense* พบว่า อนุพันธ์ของเบอร์เบรีน 4 ชนิดได้แก่ acetonylberberine, 8-cyano-13,14-dehydrocanadine, 13-benzylberberine และ 13-methylberberine แสดงฤทธิ์ในการยับยั้งการเจริญเติบโตของเชื้อราได้ดีกว่าเบอร์เบรีน เมื่อทดสอบฤทธิ์ต่อการงอกของสปอร์เชื้อ *P. parasitica* และความเป็นพิษต่อพืช พบว่า 8-cyano-13,14-dehydrocanadine และ 13-benzylberberine แสดงฤทธิ์ยับยั้งการงอกของสปอร์เชื้อ *P. parasitica* ได้อย่างสมบูรณ์ ขณะที่ 13-benzylberberine ไม่แสดงความเป็นพิษต่อพืช

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....

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WANCHAN NOPPANIT : ANTI-PHYTOPATHOGENIC FUNGAL AGENTS BASED ON BERBERINE. THESIS ADVISOR: PROF. UDOM KOKPOL, Ph.D., THESIS COADVISOR : ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., 81 pp.

Screening of antifungal activity against two phytopathogenic fungi; *Phytophthora palmivora* and *P. parasitica*, PDA was incorporated with four natural products: *N*-acetylglucosamine, glucosamine, gallic acid and berberine at concentration of 1,000 ppm. Among them, berberine exhibited the most potent antifungal activity against both fungi by 100 percent inhibition. Berberine was extracted from (*C. fenestratum* (Gaertn.) Colebr) and investigated the antifungal activity against six phytopathogenic fungi; *Alternaria porri*, *Cercospora* sp., *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *P. parasitica* and *Pythium deliense* was further investigated and IC_{50} values were determined as 101, 653, 1011, 599, 48 and 284 ppm, respectively. Seven derivatives of berberine: canadine, 8-methoxy-7,8-dihydroberberine, 8-trichloromethyl-7,8-dihydroberberine, acetonylberberine, 8-cyano-13,14-dehydrocanadine, 13-benzylberberine and 13-methylberberine were manipulated by introducing various groups at C-8 and C-13 of berberine. Four berberine derivatives: acetonylberberine, 8-cyano-13,14-dehydrocanadine, 13-benzylberberine and 13-methylberberine displayed better antifungal activity against selected phytopathogenic fungi than berberine. Spore germination and phytotoxicity bioassay were also investigated. 8-Cyano-13,14-dehydrocanadine and 13-benzylberberine revealed completely inhibition of spore gerimantion of *P. parasitica* while 13-benzylberberine showed relatively no evident on phytotoxic effects.

Field of Study.....Biotechnology.....Student's Signature.....wanchan noppanit.....

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Co-advisor's Signature.....W. Chavasiri.....

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List of Abbreviations

^{13}C NMR	carbon 13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
°C	degree of Celsius
$CDCl_3$	deuterated chloroform
CD_3OD-d_4	deuterated methanol
CH_2Cl_2	dichloromethane, methylene chloride
d	doublet (NMR)
dd	doublet of doublet (NMR)
DMSO	dimethyl sulfoxide
DMSO-d_6	deuterated dimethyl sulfoxide
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
g	gram
h	hour
HCl	hidrochloric
H_2O	water
IC_{50}	inhibitory concentration 50%
J	coupling constant
KCN	potassium cyanide
NMR	nuclear magnetic resonance
m	multiplet
MeOH	methanol
mg	milligram
mL	millilitre
mm	millimetre
mm^2	millimetre square
$NaBH_4$	sodium borohydride
NaOH	sodium hydroxide
NaOMe	sodium methoxide
Na_2SO_4	sodium sulfate
NMR	nuclear magnetic resonance

ppm	part per million
R_f	retardation factor
s	singlet (NMR)
t	triplet (NMR)
TLC	thin layer chromatography
UV	ultraviolet
µL	microlitre

CHAPTER I

INTRODUCTION

Fungal pathogens of plants are still a main problem for the loss of agricultural products in many areas around the world especially agricultural country such as Thailand. Phytopathogenic fungi can cause disease to all plant stages of life (seed, seedling, or mature plant), all parts of plants (root, trunk, leaf, flower, or fruit) or post harvest. Fungicides are the most popular and increasing of using control and against severity of plant disease. However, a frequent use of fungicide can also lead to the formation of resistant pathogen strains. Besides, the fungicides residue on agricultural products is also created problem for exporting the products aboard because of legislation in some countries, damage environmental and had toxicity effect to human. Therefore, the desire for safer agrochemicals with less environmental and human toxicity is a major concern. Natural chemicals from plants have been a promising alternative method to use for plant disease control instead of synthetic fungicides.

Medicinal plants play a key role in human health care. Based on their biosynthetic origins, plant natural products can be divided into three major groups: terpenoids, alkaloids and phenylpropanoids and allied phenolic compounds. These substances, traditionally referred to as secondary metabolites.

Many medicinal plants produce a large variety of biologically active substances representing a vast diversity of fascinating molecule architecture. Sometimes a novel bioactive compound had been found and often been employed as starting material for the preparation of a series of synthetic analog, which may have medicinal or economical values. The plant derived a variety of bioactive compounds such as anticancer, antimalarial, antivirus, antibacterial and antifungal.

The investigation of novel biologically active compounds from herbal medicinal plants is important, especially plants in tropical forest where is plenty of medicinal plants. Thailand is located in a tropical region of the world where a vast biodiversity exists and which possesses a large number of medicinal plants that have been in used traditional treatment in the primary health care system. Therefore natural products research in Thailand is targeted toward the goal of studying the

antiphytopathogenic fungi to contribute to the economic development of Thailand by developing them into new fungicides.

1.1 Plant disease caused by fungi

Fungi are highly variable group of organisms which do not contain chlorophyll. There are about 40,000 different kinds of fungi. Fungi are widely distributed in nature and are frequent pathogens of various species in the plant kingdom. Plant diseases caused by fungi include rusts, smuts, leaf, root, and stem rots, and may cause severe damage to leading cause of agricultural crop loss. Fungi reproduce by releasing spores from a fruiting body into the air and the wind carries the spores off to start the next generation.

In this research, six pathogenic fungi *Alternaria porri*, *Cercospora* sp., *Colletotrichum gloeosporioides, Fusarium oxysporum*, *Phytophthora parasitica* and *Pythium deliense*, causing disease in many Thai crops, were selected as model organisms.

1.1.1 *Alternaria porri*

Purple blotch of onion (*Allium cepa*), shallot *(Allium ascalonicum),* leek *(Allium porrum),* garlic (*Allium sativum* L.) are caused by *A. porri*. Purple blotch, an important disease of *Allium* spp. has caused severe losses in onions in the southern USA, Puerto Rico (Holliday, 1980), especially in warm and humid environments, causing up to 60% damage on garlic in India (Bisht and Agrawal, 1993) and 59% losses in onion bulb yield (Gupta and Pathak, 1988). *A. porri* spores develop in high humidity and can spread to plant foliage and bulbs in the field or storage shed by wind, water splashing, implements and insects or workers.

Early symptoms appear on the older leaves as white flecks. The first symptoms are small, white, leaf lesions. Under suitable environmental conditions (under moist conditions) the white flecks develop expand and produce sunken into elliptical, purplish areas with a yellow to pale-brown border, becoming several cm long the surface of the lesion may be covered by brown to black masses of fungal spores. Sporulation on these lesions results in the formation of dark and light concentric zones. If conditions, after the initial appearance of symptoms, become dry (RH < 70%) then the white fleck does not develop into the purple blotch. After 3-4

weeks the leaves collapse and infection can spread in the bulb causing a deep yellow to reddish water rot. The scales become desiccated and dark; bulbs may be small or fail to develop (Holliday, 1980).

The development of disease (Fig 1.1) caused by *Alternaria* initiates from mycelium and spores infest in plant debris, on seeds and tubers. The mycelium germination is direct penetration or penetration through wound and leaf or stem are attacked and the symptom of disease will be present as collar rot damping off, stem rot, leaf rot and fruit rot.

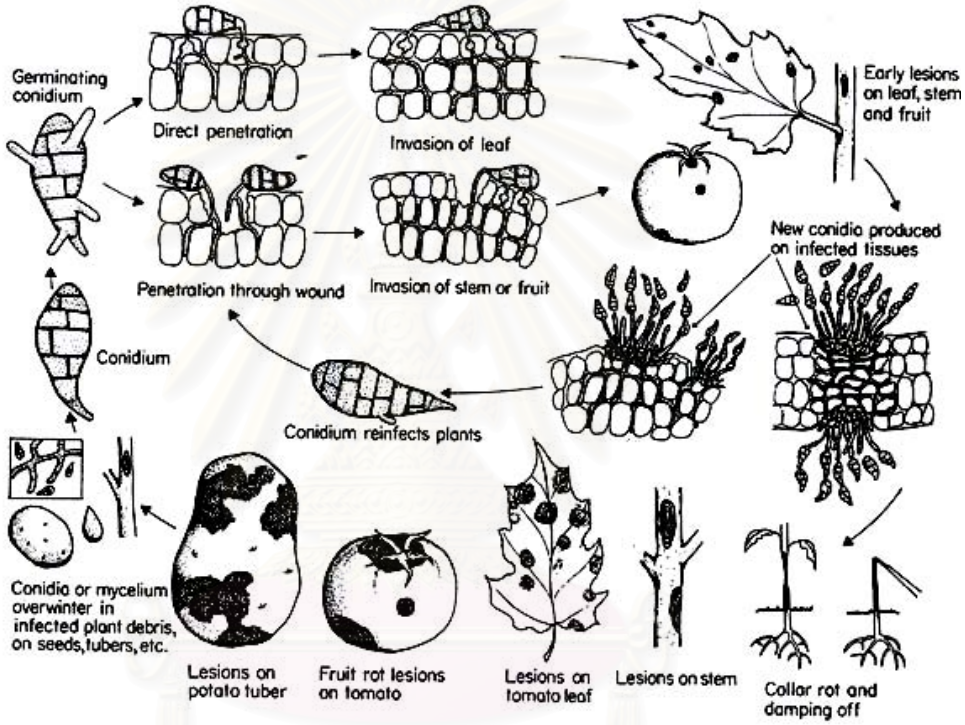


Figure 1.1 Disease cycle of *Alternaria* sp. (Agrios, 1997)

1.1.2 *Cercospora* spp.

Cercospora can cause the diseases on various hosts, most cereals (Agrios, 1997) and grasses, many field crops, vegetables, ornamentals, and trees such as sorghum, soybean carrot, eggplant, pepper, tomato, tobacco, rice, corn, oil palm, cotton and coffee and almost of plant parts such as leaf, stem, seed and fruit. *Cercospora* diseases are almost always leaf spots that either stay relatively small and separate or may enlarge and coalesce, resulting in leaf blights.

The first symptoms of the disease are small reddish dots on leaves. The leaf spots in some plants are brown. These develop into small (3-5 mm) circular grey-

brown lesions with a red-brown margin and roughly circular with reddish-purple borders. Under conditions of high humidity, groups of grey-black conidiophores develop in the centre of lesions. In severe attacks, lesions spread rapidly over the surface of the leaf and leaves may die (Parry, 1990).

On most hosts, the spots are irregularly circular to angular, with or without a distinct border, and often coalesce to form large blighted areas. In monocotyledonous plants the spots are narrow and long, usually 0.5 by 5.0 cm, and may coalesce and kill leaves. In humid weather the affected leaf surface on all hosts is covered with an ashen gray mold barely visible to the naked eye. In severe attacks, all the foliage is destroyed and may fall off. On fleshy plants, similar lesions are produced on stems and leaf petioles.

The fungus produces long, slender, colorless to dark, straight to slightly curved, multicellular conidia on short dark conidiophores arise from the plant surface in clusters through the stomata and form conidia successively on new growing tips. The conidia are easily detached and often blown long distances by the wind. The fungus is favored by high temperatures and therefore is most destructive in the summer months and in warmer climates (Agrios, 1997). Disease cycle of *Cercospora* spp. is depicted in Fig 1.2.

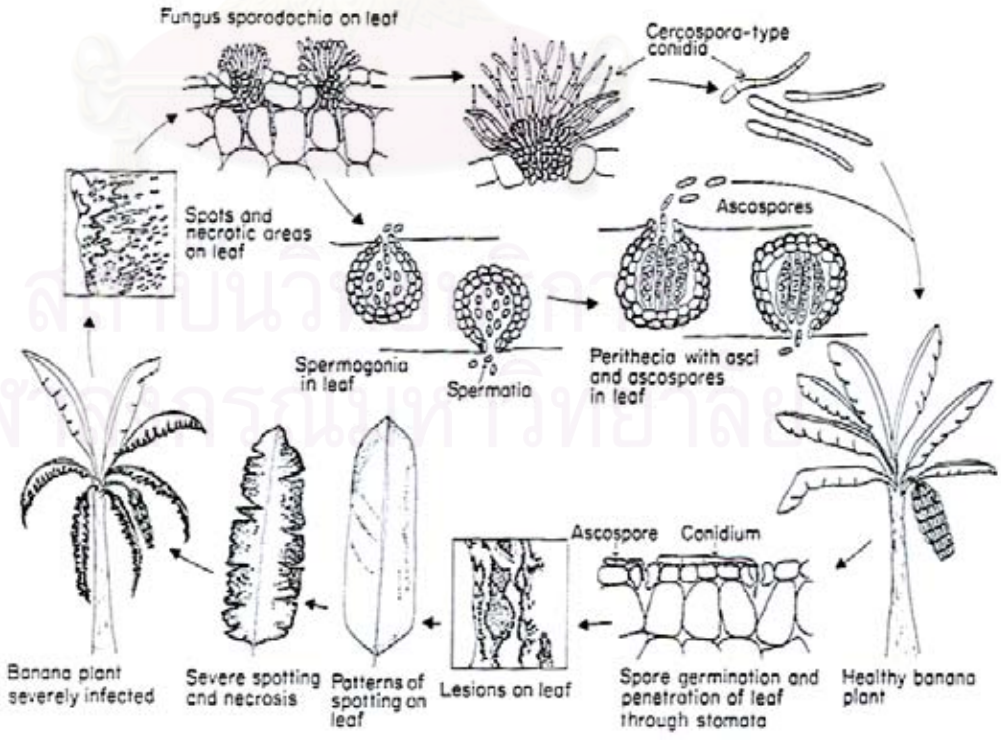


Figure 1.2 Disease cycle of *Cercospora* spp. (Agrios, 1997)

The fungus over winters mainly as mycelium in surface debris. The survival of the fungus is greatly reduced if debris is buried. Long periods of high humidity, together with warm weather, encourage sporulation. Spores are usually rain-dispersed (Parry, 1990).

1.1.3 *Colletotrichum gloeosporioides*

Colletotrichum, causing anthracnose is known to infect a wide variety of hosts such as mango, strawberry, guava, banana, tomato, papaya, and chilli. Symptoms of this disease is regularly seen in the field on ripe or overripe fruits. It is most important on fruits that are refrigerated and exported to overseas markets. Anthracnoses are diseases of the foliage, stems, or fruits that typically appear as dark-colored spots or sunken lesions with a slightly raised rim. Some cause twig or branch dieback. In some fruit crops, the spots are raised and have corky surfaces often result in fruit drop and fruit rot.

At the beginning the hyphae grow rapidly, intercellularly and intracellularly, but cause little or no visible discoloration or other symptoms. Then, more or less suddenly, especially when fruits begin to ripen, the fungus becomes more aggressive and symptoms appear. In many hosts the fungus reaches the seed and is either carried on the seeds or may even invade a small number of seeds without causing any apparent injury to them.

Symptoms may also appear as irregular to circular spots 1 to 10 mm in diameter, sharply defined, occasionally slightly depressed and reddish-brown in color. These lesions are referred to as "chocolate spots." As the fruit ripens, these spots rapidly enlarge (up to 20 mm in diameter), to form the characteristic circular sunken lesions.

The fungus is often present in or on the seed produced in infected pods or bolls. Infected seed may show yellowish to brown sunken lesions. When infected seeds are planted, many of the germinating seedlings are killed before emergence. Dark-brown, sunken lesions with pink masses of spores in the center are often present on the cotyledons of young seedlings. The fungus may destroy one or both of the cotyledons. The spores spread and infect the stem, producing more lesions. The

fungus also attacks the petioles and the veins of the underside of the leaves, on which it causes long, dark-colored lesions (Agrios, 1997).

Biology of *C. gloeosporioides*, a facultative parasite belongs to the order Melanconiales. The fungus produces hyaline, one-belled, ovoid to oblong, slightly curved or dumbbell shaped conidia, 10-15 µm in length and 5-7 µm in width. Masses of conidia appear in pink or salmon colored. The waxy acervuli produced in infected tissue are subepidermal, typically with setae, and simple, short, erect conidiophores (Holliday, 1980). Disease cycle of *C. gloeosporioides* is depicted in Fig 1.3.

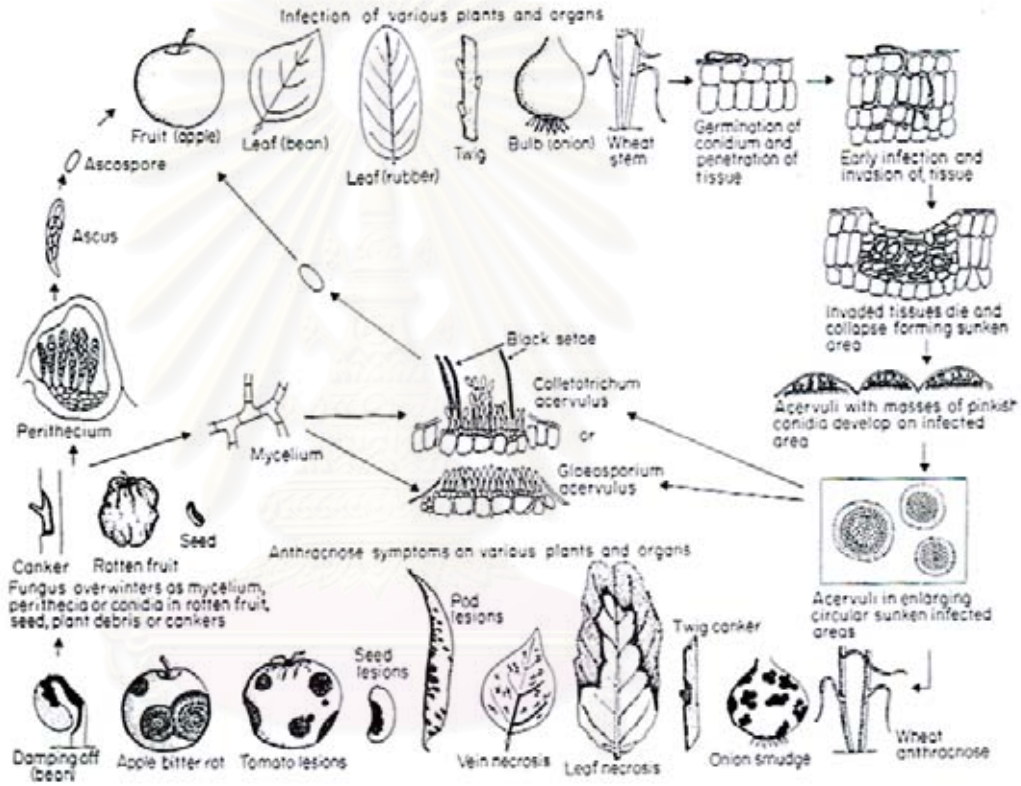


Figure 1.3 Disease cycle of *Colletotrichum* spp. (Agrios, 1997)

The fungus is favored by high temperatures and humid or moist weather. The conidia are released and spread only when the acervuli are wet and are generally spread by splashing and blowing rain or by coming in contact with insects, other animals, tools, and so on. The conidia germinate only in the presence of water and penetrate the host tissues directly (Agrios, 1997).

1.1.4 *Phytophthora parasitica*

Phytophthora cause a variety of diseases on many different types of plants ranging from seedlings of annual vegetables or ornamentals to fully developed fruit and forest trees. The losses caused by such root and stem rots are great, especially on trees and shrubs. Infected plants at first show symptoms of drought and starvation, and the quickly become weakened and susceptible to attack by other pathogens.

The fungus cause damage to their hosts in nearly every part of the world where the soil becomes too wet for good growth of susceptible plants. In many plants, the fungus attacks the plant at or near the soil line where it causes a water soaking and darkening of the bark on the trunk. The infected area enlarges, it may encircle the entire stem, after which the lower leaves drop and eventually the whole plant wilts (Agrios, 1997). Disease cycle of *Phytophthora* sp. is shown in Fig 1.4.

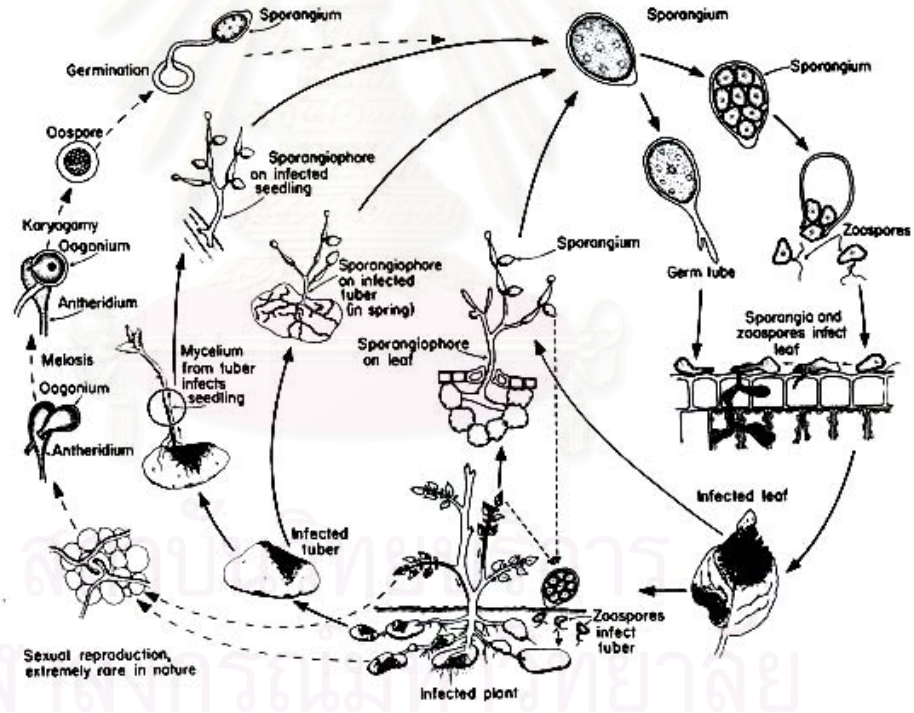


Figure 1.4 Disease cycle of *Phytophthora* sp. (Agrios, 1997)

The pathogen overwinters primarily as oospores in debris and soil. When temperatures rise and abundant moisture becomes available, oospores germinate to form sporangia which then release swimming zoospores, zoospores are attracted to germinating seeds or rootlets where they encyst, germinate, and penetrate plant

tissues. Secondary infections occur when sporangia, produced on rotting rootlets, release zoospores into flooded soils. Leaf and stem infections may also occur if contaminated soil is brown or splashed onto aerial plant parts and humid damp conditions prevail. The disease is most common in heavy, wet clay soils and on farms where minimum cultivation is practised. A temperature range between 25 and 30 ^{o}C favour disease development (Parry, 1990).

1.1.5 *Fusarium oxysporum*

Vascular wilt pathogen such as *F. oxysporum* form special colonies the vascular tissue of many plants and cause a characteristic wilt symptom. The wilting is not only a consequence of the physical blockage of xylem vessels but also a result of toxin production by the fungi (Parry, 1990).

Fusarium causes vascular wilts of vegetables and flowers, herbaceous perennial ornamentals, plantation crops such as banana, coffee, and sugarcane, and a few shade trees. The leaves of infected plants or of parts of infected plants lose turgidity, become flaccid and lighter green to greenish yellow, droop, and finally wilt, turn yellow, then brown, and die. Wilted leaves may be flat or curled. Young, tender shoots also wilt and die.

Mycelium delicate white or peach but usually with a purple tinge, sparse to abundant then floccose becoming felted and sometimes wrinkled in older cultures. Microconidia borne on simple phialides arising laterally on the hyphae or from the short sparsely branched conidiophores. Microconidia generally abundant, variable, oval to ellipsoid cylindrical, straight to curved, 5-12 × 2.2-35 µm.

F. oxysporum has a worldwide distribution as a soil organism. Its survival, in the absence of a suitable substrate is through chlamydospores. Continuous cropping of a susceptible host leads to a build up in inovulum and the creation of what has been termed a wilt sick soil (Holliday, 1980). Healthy plants can be infected by *F. oxysporum* if the soil in which they are growing is contaminated with the fungus. The fungus can invade a plant either with its sporangial germ tube or mycelium by invading the plant's roots. The roots can be infected directly through the root tips, through wounds in the roots, or at the formation point of lateral roots. Once inside the plant, the mycelium grows through the root cortex intercellulary. When the mycelium reaches the xylem, it invades the vessels through the xylem's pits. At this point, the

mycelium remains in the vessels, where it usually advances upwards toward the stem and crown of the plant.

Disease cycle of *F. oxysporum* is an abundant and active saprophyte in soil and organic matter, with some specific forms that are plant pathogenic as shown in Fig. 1.5. Its saprophytic ability enables it to survive in the soil between crop cycles in infected plant debris. The fungus can survive either as mycelium, or as any of its three different spore types.

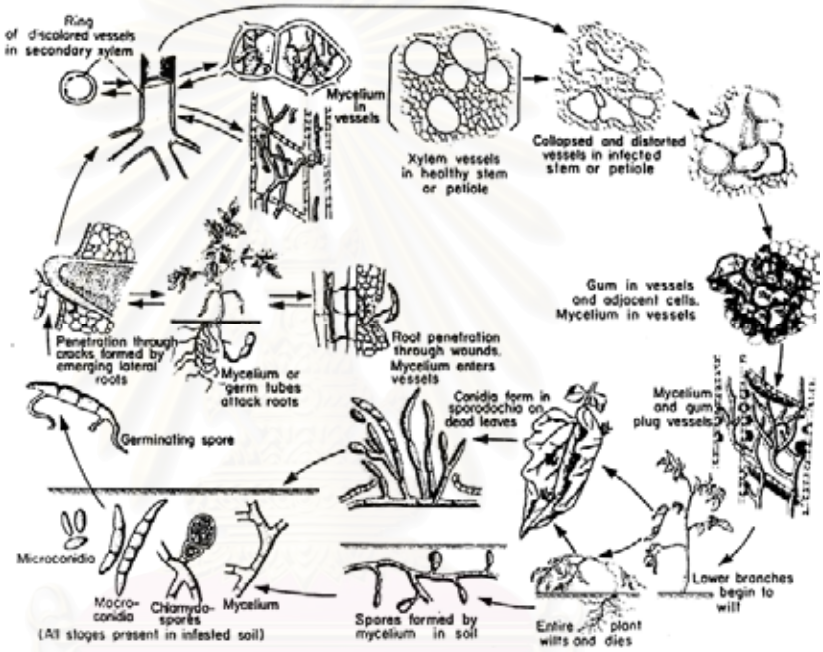


Figure 1.5 Disease cycle of *F. oxysporum* (Agrios, 1997)

1.1.6 *Pythium deliense*

Pythium deliense causing severe damping-off of cucumber seedlings, causes pre- and postemergence damping-off. When seeds of plants are attacked by the fungi, they fail to germinate, become soft and mushy, and then turn brown, shrink, and finally disintegrate. Young seedlings can be attacked before emergence at any point, the invaded cells collapse, and the seedling is overrun by the fungus and dies (preemergence damping-off).

Seedlings that have already emerged are usually attacked at the roots and stems. The invaded areas become water soaked and discolored, and they soon collapse.

In older plants the fungus may kill rootlets or induce lesions on the roots and stem. The lesions cause plants to become stunted and sometimes to wither or die.

Soft, fleshy organs of vegetables in contact with the soil, such as cucurbit fruits, green beans, and potatoes, are sometimes infected by the fungus during extended wet periods. Such infections results in a cottony fungus growth on the surface of the fleshy organ, while the interior turns into a soft, watery, rotten mass, called “leak”.

Pythium produces a white, rapidly growing mycelium. The mycelium gives rise to sporangia, which germinate directly by producing one to several germ tubes or by producing a short hypha at the end of which forms a balloonlike secondary sporangium called a vesicle. In the vesicle, 100 or more zoospores are produced which, when released, swarm about for a few minutes, round off to form a cyst, and then germinate by producing a germ tube. The germ tube usually penetrates the host tissue and starts a new infection, but sometimes it produces another vesicle in which several secondary zoospores are formed, and this maybe repeated.

Pythium species occur in waters and soils throughout the world. They live on dead plant and animal materials as saprophytes or as parasites of fibrous roots of plants. When wet soil is heavily infested with *Pythium*, any seeds or young seedlings in such a soil maybe attacked by the fungus.

1.2 Natural fungicides

There were several methods to protect crop plants from plant diseases. Fungicides are the most popular because of its short-term-expecting products, nevertheless may have severely inverse effect in the long run to both nature and human. Chemical fungicides can pollute all nearby biological system such as soil, water and air and they take a duration of time to expose its threaten. In addition, the accumulation of toxic chemical fungicides in crops makes the crops poisonous to all partaker and can cause cancer and another disease in human in long terms, and if the crops have high poisonous quality it may make death to those consumers.

Natural fungicides are used for thousand years by human throughout the history and by its long time usage. Natural fungicides can degrade easily, so it will not reserve in any crops, safe for consumer, friendly to environment, which is the most important reason for using natural fungicides.

From literature review, several reports involved with natural fungicides. For instance, the chemical composition of essential oil isolated from the floral cone of *Metasequoia glyptostroboides* was tested for anti-fungal activity. The oil and the methanol extract and the derived fractions of methanol showed great potential of anti-fungal activity as a mycelial growth inhibition against the tested phytopathogenic fungi such as *Fusarium oxysporum*, *Fusarium solani*, *Sclerotonia sclerotiorum*, *Rhizoctonia solani*, *Colletotricum capsici*, *Botrytis cinerea*, and *Phytophthora capsici*, in the inhibition range of 49–70% and minimum inhibitory concentration ranging from 500 to 1000 µg/mL (Bajpai *et al*., 2007).

Kava root (*Piper methysticum* L.) powder was reported as a promising material, which might be used as natural herbicide and fungicide in the field to reduce the dependence on synthetic herbicide and fungicide in agricultural production. Kava showed a strong inhibition on the growth of barnyardgrass (*Echinochloa crus-galli*), monochoria (*Monochoria vaginalis*), and knotgrass (*Paspalum distichum* L.), which are among the most harmful paddy weeds. Kava completely controlled emergence of monochoria and barnyardgrass. In addition, Kava significantly inhibited the growth of the five fungi: *Fusarium solani*, *Pyricularia grisea*, *Rhizopus stolonifer*, *Taphrina deformans*, and *Thanatephorus cucumeris* (Xuan *et al*., 2003).

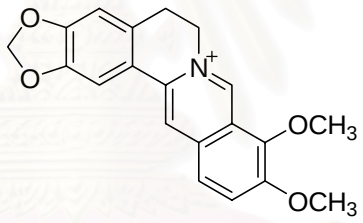
The use of winter wheat (*Triticum aestivum* L.) cultivars displayed resistant activity to four main fungal diseases: septoria tritici blotch (*Mycosphaerella graminicola*), brown rust (*Puccinia triticina*), yellow rust (*Puccinia striiformis*) and powdery mildew (*Erysiphe graminis*), which may make it possible to reduce yield losses without the need to use fungicides which are expensive and may damage the environment (Zhang *et al*., 2007).

Chitosan, a given name to a deacetylated form of chitin, is a natural biodegradable compound derived from crustaceous shells such as crabs and shrimps, has been proven to control numerous pre- and postharvest diseases on various horticultural commodities. Microscopical observations indicate that chitosan has a direct effect on the morphology of the chitosan-treated microorganism reflecting its fungistatic or fungicidal potential. As a nontoxic biodegradable material, as well as an elicitor, chitosan has the potential to become a new class of plant protectant, assisting towards the goal of sustainable agriculture (Bautista-Banos *et al*., 2006).

1.3 Berberine

Berberine-containing plants are used medicinally in virtually all traditional medical systems, and have a history of usage in Ayurvedic and Chinese medicine dating back at least 3,000 years. Berberine's most common clinical uses include: bacterial diarrhea, intestinal parasites, ocular trachoma infections (Timothy *et al.*, 1997), febrifugal, hypotensive, immuno-stimulating and anti-inflammatory (Rosatio *et al.*, 2003).

Berberine has demonstrated significant antimicrobial activity against bacteria, fungi, protozoans, viruses, helminths and chlamydia. In addition, berberine's actions include: antagonism of the effects of cholera and *E. coli* heat-stable enterotoxin, inhibition of intestinal ion secretion, inhibition of smooth muscle contraction, inhibition of ventricular tachyarrhythmias, reduction of inflammation, elevation of platelet count in patients with primary and secondary thrombocytopenia, and stimulation of bile secretion and bilirubin discharge (Timothy *et al.*, 1997).



Berberine

Berberine is a protoberberine alkaloid falling into a group of isoquinoline alkaloids that have phenylalanine as their precursor (Slaninova *et al.*, 2001). They are present in a number of clinically-important medicinal plants, isolated from Papaveraceae, Berberidaceae, and some other families such as *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric) (Timothy *et al.*, 1997). The berberine alkaloid can be found in the roots, rhizomes, stems, and barks of the plants. Its bright yellow color makes it easily seen in most of the herb materials that contain significant amounts of this compound.

In traditional medicine, several Chinese remedies with extracts of various Berberidaceae (*i.e. Berberis aquifolium*, *Berberis vulgaris*, *Berberis aristata*) have been used mainly for rheumatic complains and other types of chronic inflammations.

Antimicrobial activity is also ascribed to *Berberis heterophylla*. The antimicrobial activity of *Berberis heterophylla* leaves, stems and root aqueous extracts was studied *in vitro* on gram-positive and gram-negative bacteria and fungi. The *in vitro* antifungal activity of berberine isolated from the same source against different *Candida* species was also investigated. Berberine displayed a significant antibacterial and antifungal activity against *Staphylococcus aureus* and different *Candida* spp. (Freile *et al*., 2003). The assay of the crude methanolic extract of *Berberis aetnensis* against *Candida* species was investigated and showed good activity against *Candida* species (Lauk *et al*., 2007).

Berberine chloride and the structurally related compounds were assessed for the antiviral activity (Hayashi *et al.*, 2007). Antimalarial activity of berberine was investigated, telomerase activity in synchronized *Plasmodium falciparum* during its erythrocytic cycle was also examined. Berberine, extracted from *Arcangelisia flava* L. Merr., inhibited telomerase activity indicating that *P. falciparum* telomerase might be a potential target for future malaria chemotherapy (Sriwilaijareon *et al.*, 2002).

Berberine demonstrated the therapeutic potential for controlling tumor metastasis based on the observation of its inhibitory effect on invasion and motility of lung cancer cell line A549 (Peng *et al.*, 2006). Berberine obtained from Colombian plants were determined against human tumor cell lines as an indicator of the potential anticancer activity and presented interesting cytotoxicity (Cordero *et al.*, 2004)

From literature review, there are many researches concerning with the antimicrobial activity of protoberberine alkaloids.

Antifungal activities against various human pathogenic fungi were tested, such as *Candida* and *Aspergillus* species. Berberine, isolated from Korean and Chinese medicinal plants, significantly inhibits the growth of various *Candida* species. Berberine derivatives were synthesized by means of the introduction of various aromatic groups at the 13-C to improve the antifungal activity and strongly influenced the activity tested for antimalarial activity *in vitro* against *Plasmodium falciparum* (Iwasa *et al*., 1999). Among them, 13-(4-isopropylbenzyl) berberine exerted the most potent antifungal activities against *Candida* species. The synthesized compounds exhibited more potent antibacterial (Iwasa *et at*., 1996) and antifungal activities than berberine (Park *et al*., 2006). The effect of variations in the length of the alkyl side chain at C-13 of berberine was increased as the length of the alkyl chain increased

(Iwasa *et al*., 1997) and showed good activity against human cancer cell lines (Lee *et al*., 2003). Modified at the alkyl chain at position 8 or 13 strongly influenced the cytotoxic activity. (Iwasa *et al*., 2001).

Berberine dimers were synthesized and exhibited greatly enhanced binding affinities with two double helical oligodeoxynucleotides that have potentially wide applications. For example, in elucidating the action mechanism of antitumor and antivirus drugs and in developing new chemotherapeutic agents. (Chen and Pang, 2005)

As berberinE has been widely possessed a variety of biological activites such as relieve fever, muscle pain, stomach pain, gout, malaria and as antibiotic, antidiarrhea, antitumor, antimicrobial; nevertheless, there was no report concerning antiphytopathogenic fungal activity of this compound and derivatives of this compounds.

1.4 Objective of this research

The main objective of this study was to examine the antiphytopathogenic fungal activity of berberine and derivatives of berberine. The obtained outcome may be used as a new natural fungicide to protect crops instead of the present fungicides.

CHAPTER II

EXPERIMENTAL

2.1 Plant Material

C. fenestratum (Gaertn.) Colebr was collected in October 2000 from Tha Khaek province, Laos. A voucher specimen is deposited in the herbarium of the Royal Foresty Department of Thailand (BKF No. 60811).

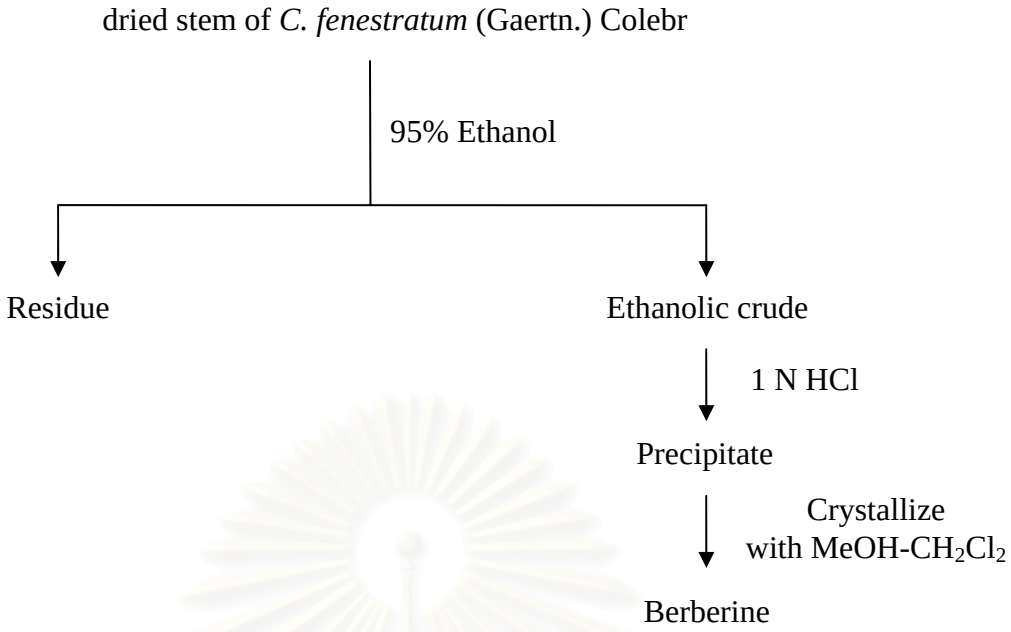
2.2 Chemicals

Merck's TLC was performed on aluminium sheets precoated with silica gel 60 F254 (20 × 20 cm, layer thickness 0.2 mm) for qualitative analysis purpose, spots on the plate were observed under UV light or visualized by spraying with 10% H_2SO_4 in EtOH followed by heating or by dipping in 3% $KMnO_4$ solution and dried to detect spots of some compounds with no UV absorption. Silica gel Merck Kieselgel 60, no. 7734 was used for column chromatography. All organic solvents: hexane, CH_2Cl_2, EtOAc, EtOH and MeOH were commercial grade and were distilled prior to use except for those being analytical grade: DMSO, $CHCl_3$, acetone and acetonitrile. NMR spectra were recorded in deuterated chloroform ($CDCl_3$), deuterated dimethyl sulfoxide (DMSO-d_6) or deuterated methanol (CD_3OD-d_4).

The ^1H and ^{13}C nuclear magnetic resonance including 2D-NMR experiments were carried out with a Jeol 400 MHz JNM-A500 FT-NMR spectrometer.

2.3 Extraction of *C. fenestratum* (Gaertn.) Colebr

The dried stems (205 g) were milled and extracted with 95% EtOH by soxhlet apparatus as presented in Scheme 2.1. The obtained ethanolic crude was treated with 1 N HCl to yield the precipitate which was then recrystallized from MeOH-CH_2Cl_2 for several times to furnish yellow solid crystal.



Scheme 2.1 Extraction of *C. fenestratum* (Gaertn.) Colebr.

2.4 Fungal cultures

Six phytopathogenic fungi, *Alternaria porri, Cercospora* spp., *Colletotrichum gloeosporioides, Fusarium oxysporum, Phytophthora parasitica* and *Pythium deliense,* were used for antifungal assay. All these fungal strains were supplied by the Division of Plant Disease and Microbiology, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. The colony characteristics of six phytopathogenic fungi are shown in Fig 2.1. Fungal cultures were grown on potato dextrose agar (PDA) using the formula presented in appendix A at room temperature (25-30^{o}C). All cultures were maintained and subcultured on PDA every month.

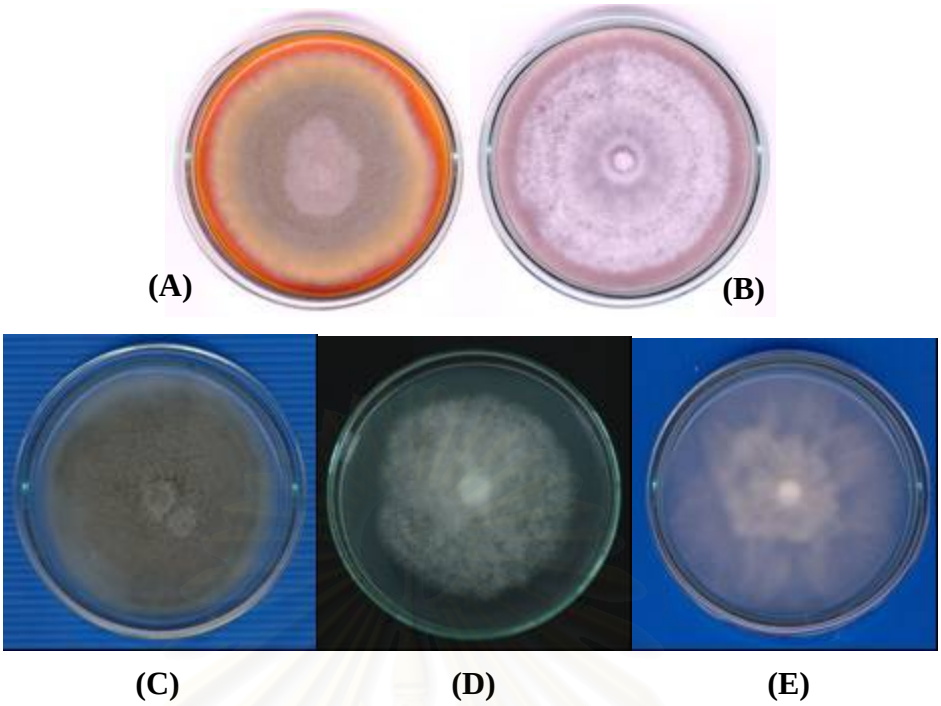


Figure 2.1 The colony characteristics of six phytopathogenic fungi on PDA used in this research: (**A**) *Alternaria porri*; (**B**) *Fusarium oxysporum*; (**C**) *Colletotrichum gloeosporioides*; (**D**) *Phytophthora parasitica;* (**E**) *Pythium deliense*

2.5 Antifungal assay

2.5.1 Preliminary screening test

Four abundant naturally occurring compounds, namely *N*-acetylglucosamine, glucosamine, gallic acid and berberine were preliminary tested for antifungal activity from agar medium assay (Hernandez *et al*., 1999) by adding to PDA at the final concentration of 1000 ppm. The control plates contained only PDA. A 8 mm diameter disc of pure culture of phytopathogenic fungi (*P. parasitica* and *P. palmivora*) was aseptically transferred to the center of the petri plate. The plates were incubated at room temperature. Redial measurements of growth were taken when fungi reached the edge of the control plates, colony diameter was measured in centimeters and calculated for percent inhibition. All treatments were replicated for five times.

$$\text{Percent inhibition} = \frac{(C - T) \times 100}{C}$$

C : colony diameter of control plate (cm)

T : colony diameter of treatment (cm)

2.5.2 Mycelial growth inhibition

The bioassay was conducted using the agar medium assay (Hernandez *et al*., 1999). Berberine was assayed against six phytopathogenic fungal (*A. porri*, *Cercospora* spp., *C. gloeosporioides, F. oxysporum, P. parasitica* and *P. deliense*). IC_{50} values for each fungi was calculated (the concentration that inhibited 50% of the mycelium of fungi growth). Berberine was added to sterile PDA (sterilization at 15 psi and 121 °C) to obtain various experimental concentrations as 10, 100 and 1000 ppm (10, 100, 1000 and 1100 ppm for *C. gloeosporioides*). Sterile medium (20 mL) was added as aseptically to a 90 mm sterile petri dish, and a sterile 8 mm diameter cork borer used to remove plugs of mycelium from stock cultures, which were inverted and placed in the center of each petri dish. Control plates contained only cultural medium. The inoculated plates were incubated at room temperature. Redial measurements of growth were taken when fungi reached the edge of the control plate. Colony diameter was measured in centimeters and calculated for percent inhibition. Each test was replicated for five times, and the data was averaged. The IC_{50} values were calculated by Probit analysis (Finney, 1997).

Tested derivatives of berberine against three phytopathogenic fungi (*A. porri*, *P. parasitica* and *P. deliense*) at IC_{50} value of each fungi were conducted. Derivatives of berberine did not dissolve well in water and thus, for further experiment, DMSO was used as a solvent (final concentration not exceed 0.4%). DMSO solutions of tested compounds were applied to a 13 mm diameter PTFE membrane syringe filter (0.2 µm pore size) before being added to sterile PDA to obtain the experimental concentration. Tested compounds were added to the cooled sterile PDA and poured to a sterile petri dish. The plugs of mycelium from the stock culture were then inoculated to the center of each petri dish. Control plates contained medium cultures plus 0.4% DMSO. Plates were incubated at room temperature. Redial measurements of growth were taken when fungi reached the edge of the control plate. The percent inhibition

was calculated compared with berberine. Metalaxyl and Mancozeb, commercially available fungicides were employed as reference compounds.

2.5.3 Spore germination assays

This test was performed according to Lattanzio *et al.* (1996) with some modification. DMSO solutions of compounds sterilised by filtration through a 13 mm diameter PTFE membrane syringe filter (0.2 µm pore size), were added to cooled sterile PDA (sterilization at 15 psi and 121 °C). Spores obtained from 7-day-old cultures of *P. parasitica* maintained on carrot agar (Appendix A) plates were used to prepare suspensions in sterile distilled water. The concentration of spores per mL was estimated with haemacytometer slide (depth 0.1 mm, 1/400 mm^2) under microscope (Fig 2.2).

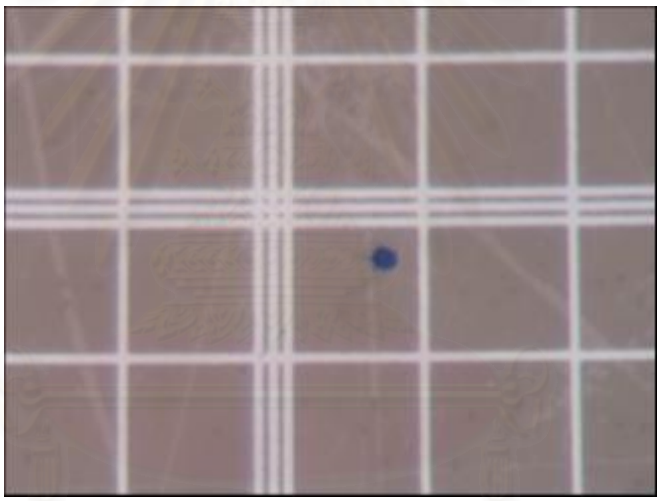


Figure 2.2 Microscopic field of haemocytometer slide with zoospores of *P. parasitica*

To test spore germination streaked aseptically 100 µL of the suspensions (10^3 spore per mL) on PDA plates, 90 mm in diameter, supplemented with and derivatives of berberine compounds at IC_{50} of each fungi concentrations compared with berberine. The plates were incubated at room temperature for 24 h. Fungal colonies originated from germinated spores were enumerated in order to evaluate the %inhibition of spore germination which were calculated as follows:

$$\%\text{inhibition of spore germination} = \frac{(C - T) \times 100}{C}$$

C : mean fungal colony of control plate

T : mean fungal colony of treatment

2.6 Phytotoxicity bioassays

The root growth assay was used as an alternative toxicity test. A petri dish (90 mm diameter) with a sheet of Whatman No.1 filter paper as substratum. Germination and growth were conducted in an aqueous solution. Solutions of the compounds to be assayed were prepared in DMSO and then diluted to reach the tested concentrations (10, 100 and 1000 ppm). Twenty seedlings grown in vertically placed plates containing 5 mL of each compound solution was added to each petri dish compared with control (0.1% DMSO). The plates were incubated at room temperature in the dark. Three replicates were used (60 seeds). Seed germination and root elongation were measured after 4 days. The percentages of relative root growth were calculated as follows:

$$\text{Relative root growth (\%)} = \frac{T}{C} \times 100$$

C : mean root length in control

T : mean root length in solution of treatment

2.7 Synthesis of berberine derivatives and related compounds

2.7.1 Preparation of canadine (Qin *et al*., 2006)

To a solution of berberine 200 mg in refluxing MeOH 15 ml was added $NaBH_4$ powder 20.55 mg for 20 min at 70^{o}C and then at room temperature for another 4 h. The formed pale yellow precipitates were collected and recrystallized from MeOH-CH_2Cl_2 several times to yield pale yellow solid 126 mg (63% yield) of canadine.

2.7.2 Preparation of 8-methoxy-7,8-dihydroberberine (Marek *et al*., 2003)

Berberine 400 mg was stirred in 50 mL of a 20% NaOMe in MeOH under N_2 atmosphere. After 24 h the solvent was evaporated and H_2O was added. The product

was extracted with Et_2O and dried over anhydrous Na_2SO_4. Evaporation of the solvent yielded 203 mg (51% yeild) yellow crystals of 8-methoxy-7,8-dihydroberberine.

2.7.3 Preparation of 8-trichloromethyl-7,8-dihydroberberine (Marek *et al*., 2003)

Berberine 200 mg was dissolved in 25 mL $CHCl_3$ and 1.25 mL of concentrated aqueous ammonia. After 24 h the phases were separated and the organic phase was dried over anhydrous Na_2SO_4. Evaporation of the solvent and the mixture was purified by silica gel column chromatography with 50% hexane-CH_2Cl_2 as an eluent. Monitored by TLC, similar fractions were combined. The desired product was recrystallized from MeOH-CH_2Cl_2 to yield 84 mg (42% yield) of yellow crystals of 8-trichloromethyl-7,8-dihydroberberine.

2.7.4 Preparation of acetonylberberine (Park *et al*., 2006)

Berberine 200 mg was dissolved in 5N NaOH 1 mL and 3 mL of acetone was added dropwise. After sirring for 1 h at room temperature, the reaction mixture was filtered and washed with 80% MeOH to give 155 mg (77% yield) of acetonylberberine.

2.7.5 Preparation of 8-cyano-13,14-dehydrocanadine (Suau *et al*., 2000)

To a suspension of berberine 500 mg in MeOH was added KCN 0.1 g with vigorous stirring. The pale yellow precipitate was filtered to give 366 mg (73% yield) which was identified as 8-cyano-13,14-dehydrocanadine.

2.7.6 Preparation of 13-benzylberberine (Park *et al*., 2006)

Acetonylberberine 300 mg dissolved in acetonitrile was reacted with NaI 0.15 g and benzyl bromide 0.15 mL at 80^{o}C for 4 h. The reaction mixture was concentrated and chromatographed on silica gel using 5% MeOH-CH_2Cl_2 as an eluent. The target product was recrystallized from MeOH-CH_2Cl_2 to give 159 mg (53% yield) of 13-benzylberberine.

2.7.7 Preparation of 13-methylberberine (Park *et al*., 2006)

Iodomethane 1 mL was added into a refluxing acetonitrile containing acetonylberberine 100 mg, then stirred at 80^{o}C for another 6 h. After cooling, the mixture was filtered, washed repeatedly with acetonitrile. Concentrated the mixture

by evaporation and the residue was recrystallized by MeOH-CH_2Cl_2 to give 59 mg (59% yield) of dark yellow solid of 13-methylberberine.

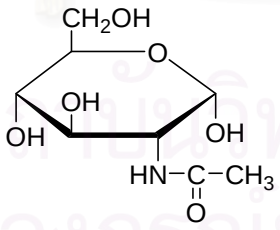
CHAPTER III

RESULTS AND DISCUSSION

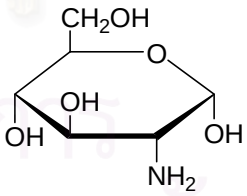
The preliminary screening of four selected natural occurring compounds, *N*-acetylglucosamine, glucosamine, gallic acid and berberine were examined against two phytopathogenic fungi. Berberine was selected to evaluated its derivatives activity indetails and further examination. The potent derivatives on mycelial growth of phytopathogenic fungi have been selected and also explored activities against spore germination and phytotoxicity against seeds germination. The aim of this study was to investigate berberine derivatives could be an interesting and perhaps, unique source of new bioactive compound.

3.1 Preliminary screening of selected natural products against phytopathogenic fungi

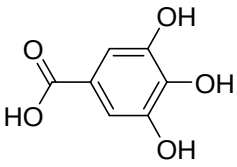
The primary screening of four selected natural products including *N*-acetylglucosamine, glucosamine, gallic acid and berberine against two phytopathogenic fungi, *P. palmivora* and *P. parasitica*, at the final concentration of 1,000 ppm by amended with steriled PDA was conducted. The antifungal percentage was calculated and showed in Table 3.1.



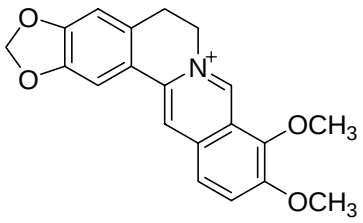
N-acetylglucosamine



glucosamine



gallic acid



berberine

Table 3.1 The percentage of mycelial growth inhibition of four selected natural products at 1000 ppm final concentration on *P. palmivora* and *P. parasitica*.

compounds	% inhibition	
	P. palmivora	*P. parasitica*
N-acetylglucosamine	25.7 ± 0.0	0.0 ± 0.1
Glucosamine	38.4 ± 0.1	6.1 ± 0.1
Gallic acid	84.0 ± 0.0	100.0 ± 0.2
Berberine	100.0 ± 0.0	100.0 ± 0.0

As the results presented in Table 3.1, all four natural products showed antifungal activity with different extents. Berberine exhibited the most potent antifungal activity against both *P. palmivora* and *P. parasitica* by 100 percent inhibition campared with the other selected natural products. Berberine was thus chosen for further investigation. In addition, certain derivatives of berberine were synthesized to explore the structure-antifungal activity relationship.

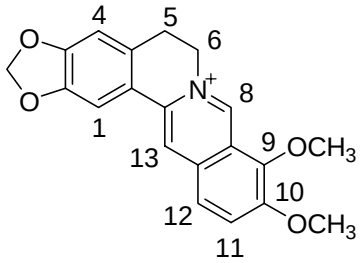
3.2 Extraction of *C. fenestratum* (Gaertn.) Colebr

The stems of *C. fenestratum* (Gaertn.) Colebr 205 g were milled and extracted following the procedures described in Chapter II. The results of extraction indicated that the major constituent of this plant was berberine. The precipitate as dark yellow solid (7.25 g) was obtained when the ethanolic extract was treated with 1 N HCl. Berberine (6.42 g) as a single spot on TLC with R_f 0.34 (10% MeOH-CH_2Cl_2) was obtained as yellow solid after recrystallization for several times from MeOH-CH_2Cl_2. This compound was soluble in various solvents such as DMSO, EtOAc, EtOH and MeOH, but slightly soluble in CH_2Cl_2 and $CHCl_3$. Its identity was confirmed by the aids of ^1H and ^{13}C NMR spectroscopy. The ^1H and ^{13}C NMR spectra are presented in Figs 1-2 in Appendix B.

The ^1H NMR (CD_3OD-d_4) spectrum (Fig 1) exhibits the presence of four singlet aromatic protons at δ_H 7.51 (1H, s), 6.84 (1H, s), 9.65 (1H, s) and 8.55 (1H, s), two doublet aromatic protons at δ_H 7.97 (1H, *d*, *J* = 9.0 Hz) and 7.88 (1H, *d*, *J* = 9.0 Hz), two triplet methylene protons at δ_H 3.15 (2H, *t*, *J* = 6.2 Hz) and 4.82 (2H, *t*, *J* =

6.4 Hz), two singlet methoxy protons at δ_H 4.09 (3H, s) and 3.98 (3H, s) and a singlet of methylenedioxy moiety at δ_H 5.99 (2H, s).

The ^{13}C NMR (CD_3OD-d_4) spectrum (Fig 2) displays 20 carbon signals. Fifteen signals of aromatic carbons were observed at δ_C 150.6, 150.5, 148.4, 144.9, 144.2, 138.1, 133.6, 130.3, 126.5, 123.1, 121.8, 120.3, 120.0, 107.9 and 105.0. A signal of methylenedioxy carbon at δ_C 102.2, two methylene carbon signals at δ_C 55.7 and 26.7, two methoxy carbon signals at δ_C 61.1 and 56.1 were also detected. The spectrum revealed the carbon chemical shifts close to the reported berberine (Janssen *et al*., 1989 and Li *et al*., 2006). The ^1H and ^{13}C NMR chemical shifts of isolated berberine and those reported are presented as shown in Table 3.2.



Berberine

Table 3.2 The comparison of ^{1}H and ^{13}C NMR chemical shifts of isolated berberine (Janssen, 1989)

Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	berberine	Ref.*	berberine
1	7.39 (1H, s)	7.51 (1H, s)	105.1	105.0
2	-	-	148.6	148.4
3	-	-	151.0	150.5
4	6.83 (1H, s)	6.84 (1H, s)	108.5	107.9
4a	-	-	129.8	130.4
5	3.24 (2H, *t*, *J* = 6.0 Hz)	3.15 (2H, *t*, *J* = 6.2 Hz)	27.2	26.7
6	4.88 (2H, *t*, *J* = 6.0 Hz)	4.82 (2H, *t*, *J* = 6.4 Hz)	56.3	55.7
8	9.54 (1H, s)	9.65 (1H, s)	144.1	144.9
8a	-	-	121.8	121.8
9	-	-	144.1	144.2
9-OCH_3	4.19 (3H, s)	4.09 (3H, s)	61.9	61.1
10	-	-	150.5	150.6
10-OCH_3	4.07 (3H, s)	3.98 (3H, s)	56.7	56.1
11	7.90 (1H, *d*, *J* = 9.0 Hz)	7.97 (1H, *d*, *J* = 9.0 Hz)	126.9	126.5
12	7.88 (1H, *d*, *J* = 9.0 Hz)	7.88 (1H, *d*, *J* = 9.0 Hz)	123.1	123.1
12a	-	-	133.5	133.6
13	8.34 (1H, s)	8.55 (1H, s)	120.2	120.0
13a	-	-	138.2	138.1
13b	-	-	119.8	120.3
OCH_2O	6.07 (2H, s)	5.99 (2H, s)	102.3	102.2

*solvent = DMSO-d_6

3.3 Fungal growth inhibition by berberine

As previously reported, the screening for mycelial growth inhibition against *P. palmivora* and *P. parasitica* revealed that berberine was the most effective compound. Berberine was therefore selected for further investigation. Six phytopathogenic fungi (*A. porri*, *Cercospora* spp., *C. gloeosporioides*, *F. oxysporum*, *P. parasitica* and *P. deliense*) were tested with various concenctrtion of berberine: 10, 100 and 1000 ppm (10, 100, 1000 and 1100 ppm for *C. gloeosporioides*) in sterile medium to examine IC_{50} values of each fungi. The percent growth inhibition was calculated and IC_{50} values (ppm) were examined by Probit analysis (Finney, 1971) as shown in Table 3.3 and Fig 3.1. The IC_{50} curve are displayed in Fig 3 (Appendix B).

Table 3.3 Effects of berberine on the mycelial growth of phytopathogenic fungi on a solid culture medium

Fungi tested	Growth inhibition (%)				IC_{50}[a]
	10 ppm	100 ppm	1000 ppm	1100 ppm	(ppm)
A. porri	9.7±0.1	49.7±0.2	100.0±0.0	-	101
Cercospora spp.	6.8±0.0	9.9±0.1	79.0±0.0	-	653
C. gloeosporioides	6.1±0.0	16.5±0.1	46.5±0.0	57.8±0.2	1011
F. oxysporum	0.0±0.0	18.6±0.0	56.4±0.2	-	599
P. parasitica	15.1±0.1	60.2±0.1	100.0±0.1	-	48
P. deliense	2.9±0.0	30.1±0.2	100.0±0.1	-	284

[a] Inhibition percentage of each concentration was calculated as IC_{50} using Probit analysis program

The data from Table 3.3 pointed out that berberine could exhibit mycelial growth of all phytopathogenic fungi and showed good activity against *P. parasitica*, *A. porri*, *P. deliense*, *F. oxysporum*, *Cercospora* spp. and *C. gloeosporioides*, respectively. The IC_{50} values are 48, 101, 284, 599, 653 and 1011, respectively.

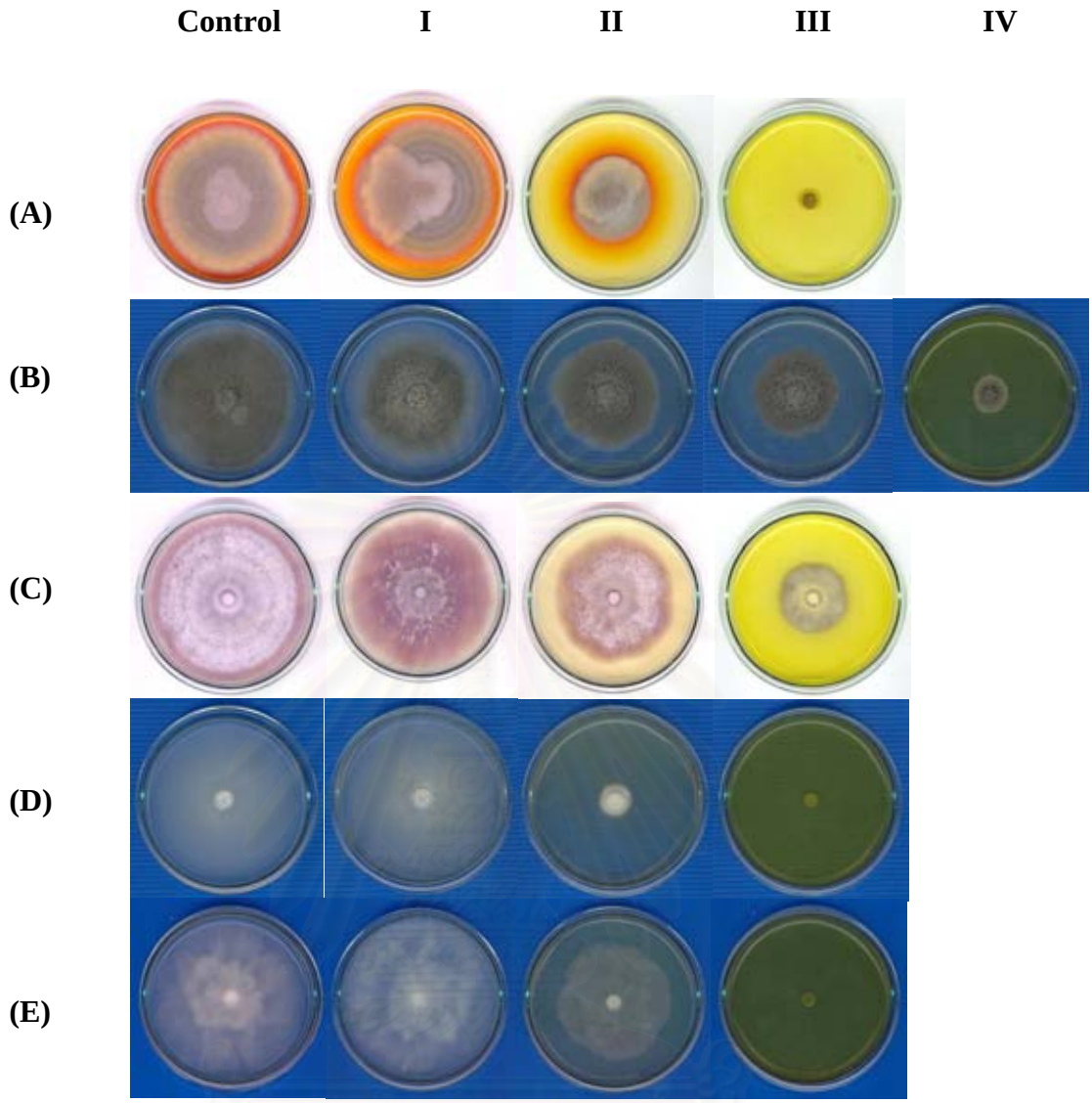


Figure 3.1 The mycelial growth inhibition of berberine concentration (ppm) as 10 (**I**), 100 (**II**), 1000 (**III**)**,** 1100 **(IV); (A)** *A. porri*, **(B)** *C. gloeosporioides*, **(C)** *F. oxysporum*, **(D)** *P. parasitica*, **(E)** *P. deliense*

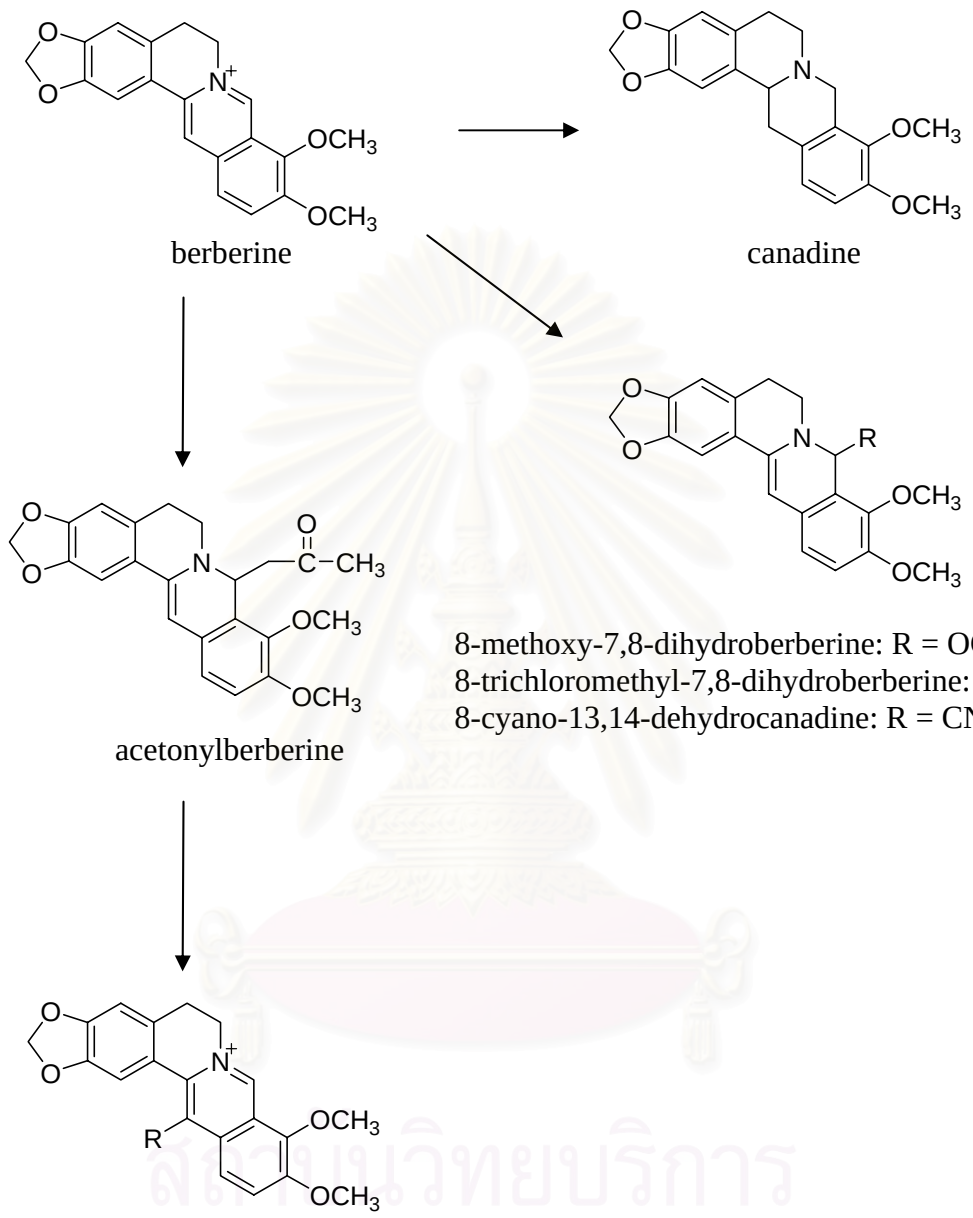
P. parasitica, *A. porri* and *P. deliense* were selected for further study to evaluate the activity of derivatives of berberine at IC_{50} value of each fungi. These selected fungi were also tested with commercially available fungicides, Metalaxyl and Mancozeb as reference compounds. Metalaxyl was tested against *P. parasitica* and *P. deliense*, while Mancozeb was tested against *A. porri* to calculate IC_{50} values. Results are shown in Table 1, Appendix B.

The IC_{50} values of Metalaxyl against *P. parasitica* and *P. deliense* were 0.3 and 0.1 ppm, respectively while that of Mancozeb against *A. porri* was 52.9 ppm.

According to aforementioned results, although berberine displayed higher IC_{50} values against *A. porri*, *P. parasitica* and *P. deliense* compared with synthetic standard compounds, Metalaxyl and Mancozeb, its low cost, naturally available and environmentally friendly compound due to its compatibility to degrade in nature is still advantage point for future concern. Moreover, this compound could be used in higher concentration to achieve the satisfied antifungal activity result. Another alternative to improve the activity of berberine was to synthesize its analogues. Thus, certain derivatives were prepared and subjected to antifungal activity test.

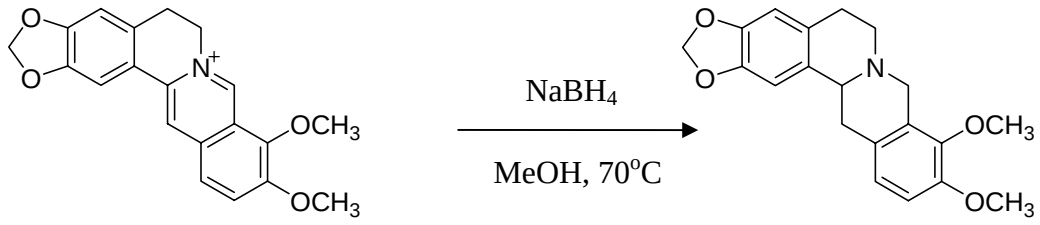
3.4 Synthesis of berberine derivatives

Seven derivatives of berberine were manipulated as overviewed in Scheme 3.1.



Scheme 3.1 Structures of synthesized berberine derivatives

3.4.1 Canadine (Qin *et al*., 2006)



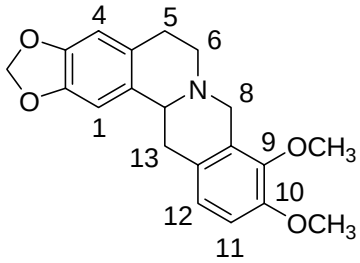
berberine canadine

Reduction of berberine (200 mg) with $NaBH_4$, followed by recrystallization from MeOH-CH_2Cl_2 for several times afforded 126 mg (63% yield) of canadine as pale yellow soild. It exhibited a single spot on TLC (R_f 0.55 5% MeOH-CH_2Cl_2). Canadine was soluble in various solvents such as $CHCl_3$, CH_2Cl_2 and DMSO.

Canadine was confirmed its identity by ^1H and ^{13}C NMR and compared the spectroscopic data with those reported values (Das and Srinivas, 2001; Janssen *et al.*, 1990) as collected in Table 3.4.

The ^1H NMR ($CDCl_3$) spectrum (Fig 4) exhibited two singlets of aromatic protons at δ_H 6.72 (1H, s) and 6.59 (1H, s), two doublets of aromatic protons at δ_H, 6.86 (1H, *d*, *J* = 8.3 Hz) and 6.78 (1H, *d*, *J* = 8.3 Hz), five signals of methylene protons at δ_H 2.64 (2H, *t*, *J* = 15.8 Hz), 3.25-3.09 (3H, m, H-6 and H-13), 4.24 (1H, *d*, *J* = 15.7 Hz), 3.55 (2H, *d*, *J* = 15.0 Hz, H-8 and H-13a), 2.84 (1H, *t*, *J* = 11.8 Hz), 3.25-3.09 (3H, m, H-6 and H-13), two methoxy protons at δ_H 3.84 (6H, s) and a singlet of methylene dioxy moiety at δ_H 5.91 (2H, s).

Twenty carbon signals were detected in the ^{13}C NMR ($CDCl_3$) spectrum. The ^{13}C spectrum (Fig 5) displayed twelve signals of aromatic carbons at δ_C 150.2, 146.1, 145.9, 145.0, 130.6, 128.4, 127.7, 127.5, 123.8, 110.9, 108.3 and 105.5, a signal of methylene dioxy carbon at δ_C 100.7, four signals of methylene carbons at δ_C 53.8, 51.3, 36.3 and 29.4, two signals of methoxy carbons at δ_C 60.1 and 55.8, a signal of methine carbon at δ_C 59.6. The ^1H and ^{13}C NMR spectra are presented in Figs 4-5 in Appendix B.



canadine

Table 3.4 The comparison of ^{1}H and ^{13}C NMR chemical shifts of canadine (Das and Srinivas, 2001; Janssen *et al.*, 1990)

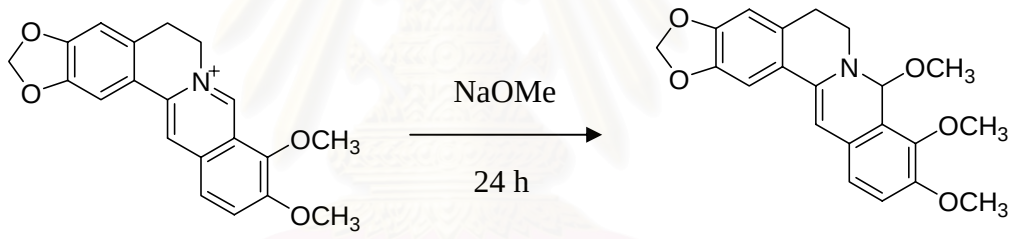
Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	canadine	Ref.*	canadine
1	6.69 (1H, s)	6.72 (1H, s)	105.3	105.5
2	-	-	145.9	145.9
3	-	-	145.7	145.0
4	6.53 (1H, s)	6.59 (1H, s)	108.1	108.3
4a	-	-	127.5	128.4
5	2.88-2.57 (3H, m, H_2-5 and H-13)	2.64 (2H, *t*, *J* = 15.8 Hz)	29.4	36.3
6	3.23-3.02 (3H, m, H_2-6 and H-13)	3.25-3.09 (3H, m, H_2-6 and H-13)	51.2	51.3
8	4.19 (1H, *d*, *J* = 15.0 Hz)	4.24 (1H, *d*, *J* = 15.7 Hz)	53.7	53.8
	3.58-3.42 (2H, m, H-8 and H-13a)	3.55 (2H, *d*, *J* = 15.0 Hz, H-8 and H-13a)		
8a	-	-	128.4	127.5
9	-	-	144.9	146.1
9-OCH_3	3.82 (6H,s)	3.84 (6H,s)	59.9	60.1
10	-	-	150.0	150.2
10-OCH_3	3.82 (6H,s)	3.84 (6H,s)	55.6	55.8
11	6.80 (1H, *d*, *J* = 9.0 Hz)	6.86 (1H, *d*, *J* = 8.3 Hz)	110.8	110.9
12	6.72 (1H, *d*, *J* = 9.0 Hz)	6.78 (1H, *d*, *J* = 8.3 Hz)	123.6	123.8
12a	-	-	127.5	127.7

Table 3.4 (continued)

Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	canadine	Ref.*	canadine
13	2.88-2.57 (3H, m, H_2-5 and H-13)	2.84 (1H, *t*, *J* = 11.8 Hz)	36.2	29.4
	3.23-3.02 (3H, m, H_2-6 and H-13)	3.25-3.09 (3H, m, H_2-6 and H-13)		
13a	3.58-3.42 (2H, m, H-8 and H-13a)	3.55 (2H, *d*, *J* = 15.0 Hz, H-8 and H-13a)	59.4	59.6
13b	-	-	130.6	130.6
OCH_2O	5.88 (2H, s)	5.91 (2H, s)	100.5	100.7

*****solvent = $CDCl_3$

3.4.2 8-Methoxy-7,8-dihydroberberine (Marek *et al*., 2003)

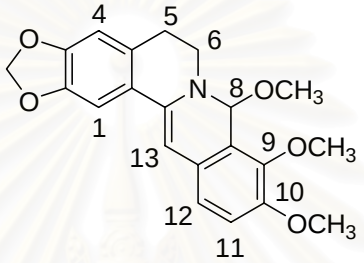


berberine 8-methoxy-7,8-dihydroberberine

8-Methoxy-7,8-dihydroberberine was obtained from the reaction of berberine 400 mg with NaOMe. After recrystallized from MeOH-CH_2Cl_2 for several times, yellow crystals of 8-methoxy-7,8-dihydroberberine 203 mg (51% yield) as a single spot at R_f 0.37 (50% hexane-CH_2Cl_2) was obtained. 8-Methoxy-7,8-dihydroberberine was soluble in various solvents such as $CHCl_3$, CH_2Cl_2 and DMSO.

The ^{1}H and ^{13}C NMR spectral data of synthesized 8-methoxy-7,8-dihydroberberine were compared with those reported values (Marek *et al*., 2003). The ^{1}H NMR ($CDCl_3$) (Fig 6) as presented in Table 3.5 revealed two singlet aromatic protons at δ_H 7.16 (1H, s) and 6.61 (1H, s), two doublet aromatic protons at δ_H 6.97 (1H, *d*, *J* = 8.4 Hz) and 6.87 (1H, *d*, *J* = 8.4 Hz), three multiplet methylene protons at δ_H 2.76-2.69 (1H, m), 3.90-3.84 (1H, m) and 3.73-3.67 (1H, m), three methoxy protons at δ_H 3.38-3.30 (3H, m), 3.94 (3H, s) and 3.87 (3H, s), a singlet olefinic

proton at δ_H 5.64 (1H, s), a singlet methine proton at δ_H 6.10 (1H, s) and a doublet of methylenedioxy moiety at δ_H 5.94 (2H, *d*, *J* = 5.5 Hz). Twenty one carbon signals in the ^{13}C NMR spectrum (Fig 7) were assigned. Twelve signals of aromatic carbons displayed at δ_C 149.6, 147.4, 146.7, 146.3, 129.3, 128.7, 125.0, 118.6, 114.6, 114.5, 108.1 and 105.5. In addition, a signal of methylene dioxy carbon at δ_C 104.1, two signals of methylene carbons at δ_C 51.5 and 30.4, three signals of methoxy carbons at δ_C 73.4, 60.9 and 56.3, two signals of olefinic carbons at δ_C 137.5 and 101.0, a signal of methine carbon δ_C 97.4 were observed.



8-Methoxy-7,8-dihydroberberine

Table 3.5 The comparision of ^{1}H and ^{13}C NMR chemical shifts of 8-methoxy-7,8-dihydroberberine (Marek *et al*., 2003)

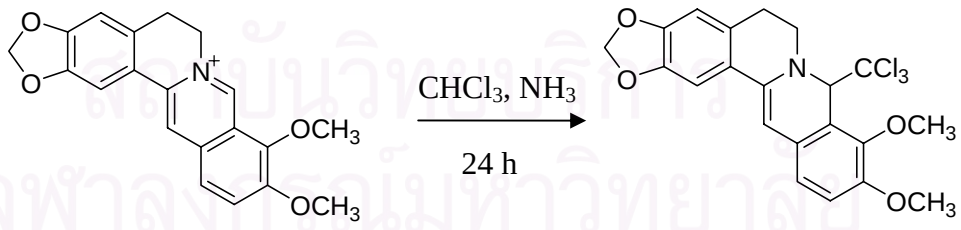
Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	8-methoxy-7,8-dihydroberberine	Ref.*	8-methoxy-7,8-dihydroberberine
1	7.17 (1H, s)	7.16 (1H, s)	104.72	105.5
2	-	-	147.2	147.4
3	-	-	147.9	149.6
4	6.64 (1H, s)	6.61 (1H, s)	108.2	108.1
4a	-	-	129.7	129.3
5	2.88 (1H, m)	2.76-2.69 (1H, m)	30.7	30.4
6	3.64 (1H, m)	3.90-3.84 (1H, m)	47.5	51.5
	3.52 (1H, m)	3.73-3.67 (1H, m)		
8	6.11 (1H, s)	6.10 (1H, s)	85.3	97.4
8a	-	-	119.5	118.6
8-OCH_3	3.03 (3H, s)	3.38-3.30 (3H, m)	52.4	56.3

Table 3.5 (continued)

Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	8-methoxy-7,8-dihydroberberine	Ref.*	8-methoxy-7,8-dihydroberberine
9	-	-	146.1	146.3
9-OCH_3	3.88 (3H, s)	3.94 (3H, s)	61.1	60.9
10	-	-	150.1	146.7
10-OCH_3	3.86 (3H, s)	3.87 (3H, s)	56.5	73.4
11	6.96 (1H, *d*)	6.97 (1H, *d*, *J* = 8.4 Hz)	114.3	114.5
12	6.89 (1H, *d*)	6.87 (1H, *d*, *J* = 8.4 Hz)	119.3	114.6
12[a]	-	-	128.9	128.7
13	6.01 (1H, s)	5.64 (1H, s)	94.7	101.0
13[a]	-	-	137.9	137.5
13b	-	-	125.6	125.0
OCH_2O	5.94 (2H, *d*)	5.94 (2H, *d*, *J* = 5.5 Hz)	101.6	104.1

*solvent = CD_2Cl_2

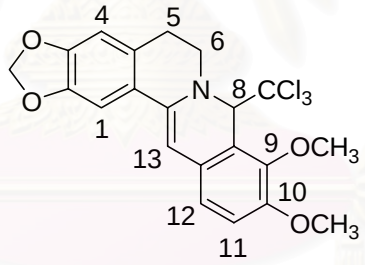
3.4.3 8-Trichloromethyl-7,8-dihydroberberine (Marek *et al*., 2003)



berberine 8-trichloromethyl-7,8-dihydroberberine

8-Trichloromethyl-7,8-dihydroberberine 84 mg (42% yield) was obtained as yellow crystals after crystallization with MeOH-CH_2Cl_2 and showed a single spot on TLC with R_f 0.44 (50% hexane-CH_2Cl_2). 8-Trichloromethyl-7,8-dihydroberberine was soluble in various solvents such as $CHCl_3$, CH_2Cl_2 and DMSO.

The ^{1}H and ^{13}C NMR spectral assignments were compared with those reported values (Marek *et al*., 2003) and tabulated in Table 3.6. The ^{1}H NMR spectrum ($CDCl_3$, Fig 8) displayed two singlet aromatic protons at δ_H 7.16 (1H, s) and 6.61 (1H, s), two doublet aromatic protons at δ_H 6.97 (1H, *d*, *J* = 8.4 Hz) and 6.87 (1H, *d*, *J* = 8.4 Hz), four multiplet methylene protons at δ_H 3.38-3.30 (1H, m), 2.76-2.70 (1H, m), 3.90-3.85 (1H, m) and 3.73-3.67 (1H, m), a singlet olefinic proton at δ_H 6.09 (1H,s), two methoxy protons at δ_H 3.94 (3H, s) and 3.88 (3H, s), a singlet methine proton at δ_H 5.64 (1H, s), and a doublet of methylenedioxy moiety at δ_H 5.94 (2H, *d*, *J* = 3.1 Hz). Twenty one carbon signals in the ^{13}C NMR ($CDCl_3$, Fig 9) spectrum was assigned. Twelve signals of aromatic carbons displayed at δ_C 149.6, 147.6, 146.7, 146.3, 128.7, 124.9, 118.6, 114.8, 114.5, 108.2, 108.1, 104.1, a signal of methylene dioxy carbon at δ_C 101.0, two signals of methylene carbons at δ_C 51.6 and 30.4, two signals of methoxy carbons at δ_C 60.9 and 56.3, two signals of olefinic carbons at δ_C 129.2 and 97.3, a signal of a methine carbon δ_C 73.4 and a signal of trichloromethyl carbon at δ_C 105.4.



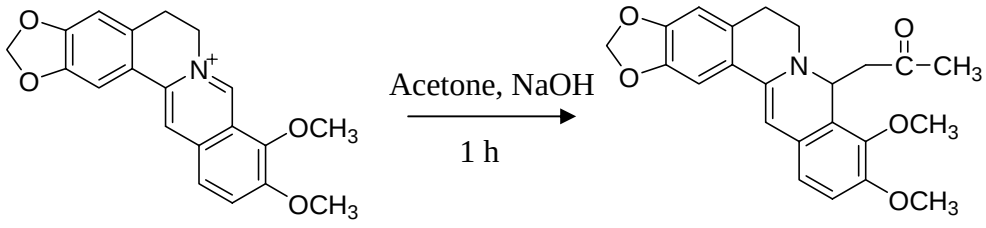
8-trichloromethyl-7,8-dihydroberberine

Table 3.6 The comparision of ^{1}H and ^{13}C NMR chemical shifts of 8-trichloromethyl-7,8-dihydroberberine (Marek *et al.*, 2003)

Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	8-trichloromethyl-7,8-dihydroberberine	Ref.*	8-trichloromethyl-7,8-dihydroberberine
1	7.16 (1H, s)	7.16 (1H, s)	104.3	104.1
2	-	-	147.2	146.7
3	-	-	148.0	147.6
4	6.63 (1H, s)	6.61 (1H, s)	108.4	108.1
4a	-	-	129.3	124.9
5	3.32 (1H, m)	3.38-3.30 (1H, m)	30.8	30.4
	2.72 (1H, m)	2.76-2.70 (1H, m)		
6	3.86 (1H, s)	3.90-3.85 (1H, m)	52.0	51.6
	3.68 (1H, s)	3.73-3.67 (1H, m)		
8	5.65 (1H, s)	5.64 (1H, s)	73.9	73.4
8a	-	-	115.2	114.5
8-CCl_3	-	-	106.1	105.4
9	-	-	146.9	146.3
9-OCH_3	3.91 (3H, s)	3.94 (3H, s)	61.1	60.9
10	-	-	150.1	149.6
10-OCH_3	3.86 (3H, s)	3.88 (3H, s)	56.6	56.3
11	6.99 (1H, *d*)	6.97 (1H, *d*, J = 8.4 Hz)	115.1	108.2
12	6.86 (1H, *d*)	6.87 (1H, *d*, J = 8.4 Hz)	118.8	114.8
12a	-	-	129.8	128.7
13	6.10 (1H, s)	6.09 (1H, s)	97.9	97.3
13a	-	-	137.9	129.2
13b	-	-	125.4	118.6
OCH_2O	5.94 (2H, *d*)	5.94 (2H, *d*, J = 3.1 Hz)	101.7	101.0

*solvent = CD_2Cl_2

3.4.4 Acetonylberberine (Park *et al*., 2006)

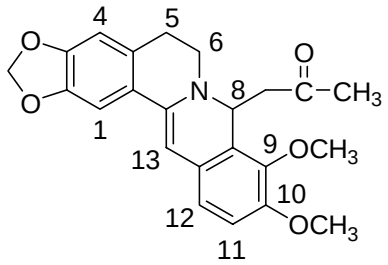


berberine acetonylberberine

Acetonylberberine 155 mg (77% yield) can be readily obtained by condensation of berberine 200 mg with acetone. The blight yellow precipitate was taken by filltration the mixture reaction. It exhibited a single spot on TLC with R_f 0.27 (5% MeOH-CH_2Cl_2). Acetonylberberine was soluble in various solvents such as $CHCl_3$, CH_2Cl_2, DMSO and acetonitrile. The ^1H and ^{13}C NMR chemical shifts are presented as shown in Table 3.7.

The ^1H NMR ($CDCl_3$) spectrum is presented in Fig 10 in Appendix B. Its structure was verified by comparing with reported values (Franceschin *et al*., 2006). Its spectrum displayed the singlet aromatic protons at δ_H 7.12 (1H, s) and 6.57 (1H, s), two doublet aromatic protons at δ_H 6.77 (1H, *d*, *J* = 8.4 Hz) and 6.74 (1H, *d*, *J* = 8.4 Hz), a singlet olefinic proton at δ_H 5.88 (1H, s), two methoxy protons at δ_H 3.88 (3H, s) and 3.83 (3H, s), four methylene protons signals at δ_H 2.88-2.72 (2H, m), 3.33-3.29 (2H, m), 3.06 (1H, *dd*, *J* = 7.0, 7.0 Hz) and 2.42 (1H, *dd*, *J* = 3.9, 4.0 Hz), a doublet methine proton at δ_H 5.32 (1H, *dd*, *J* = 4.0, 4.0 Hz), one doublet of methylenedioxy moiety at δ_H 5.94 (2H, *d*, *J* = 2.5 Hz) and one signal of methyl group at δ_H 2.04 (3H, s).

The ^{13}C NMR ($CDCl_3$) spectrum (Fig 11) showed twenty three carbon signals. Twelve signals belonged to aromatic carbons could be detected at δ_C 150.6, 147.2, 146.5, 143.5, 128.9, 127.6, 123.5, 118.5, 111.9, 107.8 and 104.1, while methylene dioxy carbon at δ_C 101.1, three methylene carbon signals at δ_C 47.5, 46.1 and 30.7, two methoxy carbon signals at δ_C 60.7 and 55.9, a signal of methine carbon at δ_C 54.2, two signals of olefinic carbon at δ_C 138.4 and 95.0, a carbon signal of carbonyl group at δ_C 207.3 and a carbon signal of methyl group connecting with carbonyl at δ_C 30.3.



Acetonylberberine

Table 3.7 The comparison ^{1}H and ^{13}C NMR chemical shifts of acetonylberberine (Franceschin *et al*., 2006)

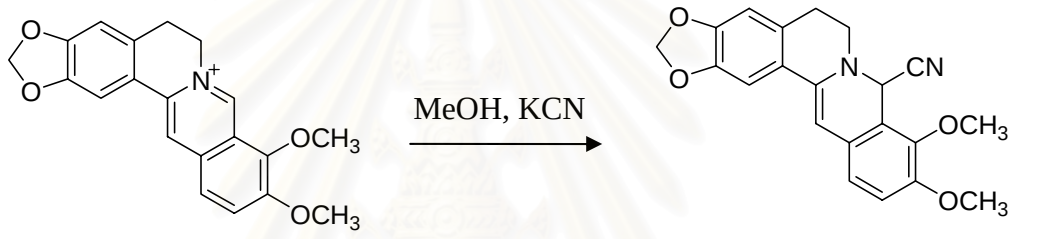
Position	Chemical shift (ppm)		
	^{1}H NMR		^{13}C NMR
	Ref.*	acetonylberberine	
1	7.13 (1H, s)	7.12 (1H, s)	104.1
2	-	-	143.5
3	-	-	146.5
4	6.77 (1H, s)	6.57 (1H, s)	107.8
4a	-	-	128.9
5	2.79 (2H, m)	2.88-2.72 (2H, m)	30.7
6	3.34 (2H, m)	3.33-3.29 (2H, m)	47.5
8	5.32 (1H, *dd*, *J* = 7.0, 4.0 Hz)	5.32 (1H, *dd*, *J* = 4.0, 4.0 Hz)	54.2
8a	-	-	123.5
9	-	-	147.2
9-OCH_3	3.89 (3H, s)	3.88 (3H, s)	60.7
10	-	-	150.6
10-OCH_3	3.84 (3H, s)	3.83 (3H, s)	55.9
11	6.75 (1H, *d*, *J* = 8.0 Hz)	6.77 (1H, *d*, *J* = 8.4 Hz)	111.9
12	6.57 (1H,s)	6.74 (1H, *d*, *J* = 8.4 Hz)	118.5
12a	-	-	127.6
13	5.89 (1H, s)	5.88 (1H, s)	95.0
13a	-	-	138.4
13b	-	-	125.4
OCH_2O	5.94 (2H, m)	5.94 (2H, *d*, *J* = 2.5 Hz)	101.0
-CH_2CO-	3.07 (1H, *dd*, *J* = 7.0, 15.0	3.06 (1H, *dd*, *J* = 7.0, 7.0 Hz)	46.1

Table 3.7 (continued)

Position	Chemical shift (ppm)		
	^{1}H NMR		^{13}C NMR
	Ref.*	acetonylberberine	
	Hz)		
-$COCH_3$	2.41 (1H, *dd*, *J* = 4.0, 15.0 Hz)	2.42 (1H, *dd*, *J* = 3.9, 4.0 Hz)	30.3
	2.05 (3H, s,)	2.04 (3H, s,)	
-CO-	-	-	207.3

*solvent = $CDCl_3$

3.4.5 8-Cyano-13,14-dehydrocanadine (Suau *et al*., 2000)

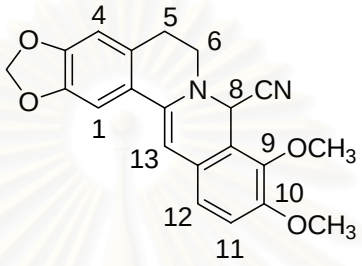


berberine 8-cyano-13,14-dehydrocanadine

The addition of KCN to a methanolic solution of berberine (500 mg) gave the immediately appeared pale yellow precipitate, which as dentified as 8-cyano-13,14-dehydrocanadine (366 mg, 73% yield). It exhibited a single spot on TLC with R_f 0.53 (3% MeOH-CH_2Cl_2). This compound was soluble in various solvents such as $CHCl_3$, CH_2Cl_2 and DMSO. The ^{1}H and ^{13}C NMR spectral data were compared with those reported values (Suau *et al*., 2000) and showed in Table 3.8.

From the ^{1}H NMR (DMSO-d_6) spectroscopic data (Fig 12) of 8-cyano-13,14-dehydrocanadine showed four singlet aromatic protons at δ_H 7.35 (1H, s), 6.78 (1H, s) 5.96 (1H, s) and 6.35 (1H, s), two doublet aromatic protons at δ_H 7.03 (1H, *d*, *J* = 7.0 Hz) and 6.87 (1H, *d*, *J* = 8.4 Hz), three methylene protons at δ_H 3.05 (1H, *dt*, *J* = 10.9, 4.3 Hz) and 3.49-3.46 (1H, m) 2.87-2.80 (2H, m, H-6), a singlet olefinic proton at δ_H 6.35 (1H, s), two methoxy protons at δ_H 3.83 (3H, s) and 3.80 (3H, s), a singlet methine proton at δ_H 5.96 (1H, s) and a doublet of methylenedioxy moiety at δ_H 6.00 (2H, *d*, *J* = 7.4 Hz).

From the ^{13}C NMR (DMSO-d_6) spectroscopic data (Fig 13) showed twenty one carbon signals. Twelve signals of aromatic carbons at δ_C 150.7, 147.8, 147.0, 144.3, 138.5, 129.1, 127.0, 123.9, 120.0, 114.5, 108.3 and 104.3, a signal of methylene dioxy carbon at δ_C 101.6, two signals of methylene carbon at δ_C 47.3 and 29.1, two signals of methoxy carbon at δ_C 61.0 and 56.3, two signals of olefinic carbon at δ_C 117.4 and 98.2, a signal of methine carbon δ_C 49.4 and a signal of nitrile carbon at δ_C 117.0.



8-Cyano-13,14-dehydrocanadine

Table 3.8 The comparision of ^{1}H and ^{13}C NMR chemical shifts of 8-cyano-13,14-dehydrocanadine (Suau *et al*., 2000)

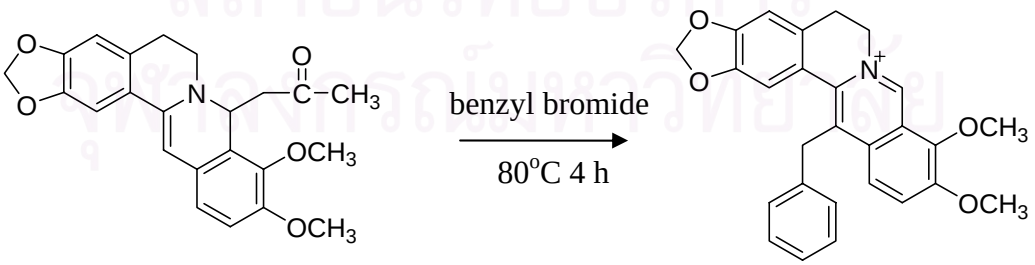
Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	8-cyano-13,14-dehydrocanadine	Ref.*	8-cyano-13,14-dehydrocanadine
1	7.15 (1H, s)	7.35 (1H, s)	119.8	120.0
2	-	-	150.6	150.7
3	-	-	147.7	147.8
4	6.58 (1H, s)	6.78 (1H, s)	113.8	114.5
4a	-	-	138.4	138.5
5	3.25 (1H, *ddd*, *J* = 15.2, 4.0, 3.0 Hz) 2.81 (1H, *ddd*, *J* = 15.2, 3.0, 3.0 Hz)	3.05 (1H, *dt*, *J* = 10.9, 4.3 Hz) 2.87-2.80 (2H, m, H-6)	29.6	29.1
6	2.98 (1H, *ddd*, *J* = 15.2, 11.9, 4.0 Hz) 3.41 (1H, *ddd*, *J* = 15.2, 11.9, 4.0 Hz)	3.49-3.46 (1H, m) 2.87-2.80 (2H, m, H-6)	47.9	47.3

Table 3.8 (continued)

Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	8-cyano-13,14-dehydrocanadine	Ref.*	8-cyano-13,14-dehydrocanadine
8	5.73 (1H, s)	5.96 (1H, s)	49.9	49.4
8a	-	-	128.4	129.1
9	-	-	146.9	147.0
9-OCH_3	3.96 (3H, s)	3.83 (3H, s)	60.9	61.0
10	-	-	144.4	144.3
10-OCH_3	3.85 (3H, s)	3.80 (3H, s)	56.1	56.3
11	6.82 (1H, *d*, *J* = 8.4 Hz)	7.03 (1H, *d*, *J* = 7.0 Hz)	107.8	108.3
12	6.88 (1H, *d*, *J* = 8.4 Hz)	6.87 (1H, *d*, *J* = 8.4 Hz)	104.1	104.3
12a	-	-	126.9	127.0
13	6.13 (1H, s)	6.35 (1H, s)	98.1	98.2
13a	-	-	117.0	117.4
13b	-	-	124.1	123.9
OCH_2O	5.95 (2H, *d*, *J* = 2.1 Hz)	6.00 (2H, *d*, *J* = 7.4 Hz)	101.2	101.6
CN	-	-	116.5	117.0

*solvent = $CDCl_3$

3.4.6 13-Benzylberberine (Park *et al*., 2006)

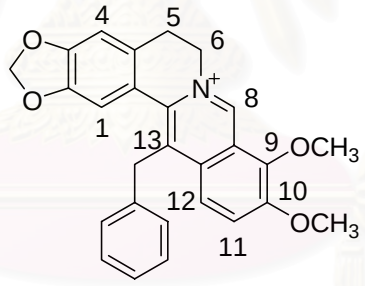


acetonylberberine 13-benzylberberine

Treatment of acetonylberberine 300 mg with benzylbromide directly afforded 13-benzylberberine 159 mg (53% yield). It showed a single spot on TLC with R_f 0.56

(10% MeOH-CH_2Cl_2). After stirring at 80°C, the mixture were crystallized from MeOH-CH_2Cl_2 to give dark yellow crystals. 13-Benzylberberine was soluble in various solvents such as $CHCl_3$, CH_2Cl_2 and DMSO. The ^1H and ^{13}C NMR spectral data were collected in Table 3.9 and elucidated compared with those reported values (Samosorn and Bremner, 2003).

The ^1H NMR (DMSO-d_6) spectrum (Fig 14) displayed three singlet aromatic protons at δ_H 6.94 (1H, s), 4.84 (2H, s) and 7.41 (3H, s), three doublet aromatic protons at δ_H 7.27 (1H, *d*, *J* = 6.6), 8.03 (1H, d, J = 9.4) and 7.75 (1H, d, J = 9.4), a triplet aromatic proton at δ_H 7.34 (2H, *t*, *J* = 6.6), three methylene protons at δ_H 3.14 (2H, s), 4.84 (2H, s) and 4.72 (2H, s), two methoxy protons at δ_H 4.09 (3H, s) and 3.99 (3H, s), and a doublet of methylenedioxy moiety at δ_H 6.05 (2H, s). The ^{13}C NMR (DMSO-d_6) spectrum (Fig 15) displayed 25 carbon signals. Nineteen signals of aromatic carbons at δ_C 150.8, 149.8, 147.0, 146.0, 139.7, 137.8, 134.6, 133.4, 130.6, 129.7, 128.6, 127.4, 126.7, 122.3, 121.9, 120.6, 109.1, 108.7, a signal of methylenedioxy carbon at δ_C 102.7, three methylene carbon signals at δ_C 57.6, 36.1 and 27.9, two methoxy carbon signals at δ_C 62.7 and 57.5 were also detected.



13-benzylberberine

Table 3.9 The comparision of ^1H and ^{13}C NMR chemical shifts of 13-benzylberberine (Samosorn and Bremner, 2003)

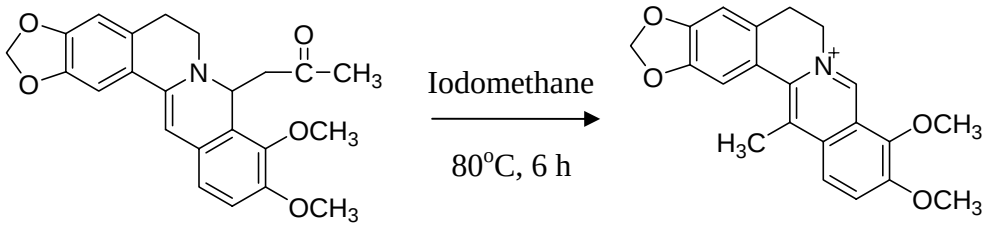
Position	Chemical shift (ppm)			
	^1H NMR		^{13}C NMR	
	Ref.*	13-benzylberberine	Ref.*	13-benzylberberine
1	6.95 (1H, s)	7.27 (1H, *d*, *J* = 6.6 Hz)	108.6	109.1
2	-	-	147.0	147.0

Table 3.9 (continued)

Position	Chemical shift (ppm)			
	^{1}H NMR		^{13}C NMR	
	Ref.*	13-benzylberberine	Ref.*	13-benzylberberine
3	-	-	149.8	149.8
4	6.88 (1H, s)	6.94 (1H, s)	108.4	108.7
4a	-	-	119.7	120.6
5	3.27 (2H, *t*, *J* = 5.4 Hz)	3.14 (2H, s)	28.3	27.9
6	5.35-5.20 (2H, m)	4.84 (2H, s)	56.8	57.6
8	10.61 (1H, s)	9.99 (1H, s)	145.6	146.0
8a	-	-	121.5	122.3
9	-	-	145.5	146.0
9-OCH_3	4.38 (3H, s)	4.09 (3H, s)	62.6	62.7
10	-	-	150.1	150.8
10-OCH_3	4.02 (3H, s)	3.99 (3H, s)	56.7	57.5
11	7.71 (1H, *d*, *J* = 9.6 Hz)	8.03 (1H, *d*, *J* = 9.4)	125.6	126.7
12	7.62 (1H, *d*, *J* = 9.4 Hz)	7.75 (1H, *d*, *J* = 9.4)	120.9	121.9
12a	-	-	133.5	134.6
13	-	-	133.3	133.4
13a	-	-	137.4	137.8
13b	6.00 (2H, s)	6.05 (2H, s)	130.3	130.6
OCH_2O	4.68 (2H, s)	4.72 (2H, s)	101.9	102.7
CH_2Ph	7.11 (2H, *d*, *J* = 7.2 Hz)	7.34 (2H, *t*, *J* = 6.6)	36.4	36.1
aromatic	7.42-7.26 (3H, m)	7.14 (3H, s)	127.0	129.7
aromatic			137.9	128.6
aromatic			129.2	139.7
aromatic			127.6	127.4

*solvent = $CDCl_3$

3.4.7 13-Methylberberine (Park *et al*., 2006)

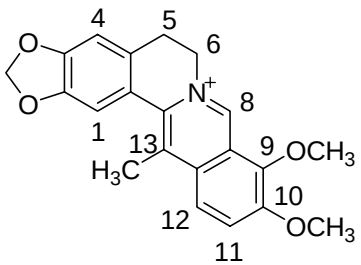


acetonylberberine 13-methylberberine

Acetonylberberine was transformed to 13-methylberberine by displacement of the acetonyl group with iodomethane. The heating of acetonylberberine 100 mg with iodomethane at 80°C, the 13-substitued was obtained in moderate yield (59 mg, 59% yield). It exhibited a single spot on TLC with R_f 0.29 (5% MeOH- CH_2Cl_2). Dark yellow solid were taken by crystallization from MeOH-CH_2Cl_2. The ^1H and ^{13}C NMR chemical shifts of 13-benzylberberine were presented in Table 3.10, compared with those reported values (Miyuji *et al*, 1989).

The ^1H NMR ($CDCl_3$) spectrum (Fig 16) displayed three singlet aromatic protons at δ_H 7.09 (1H, s), 6.88 (1H, s) and 10.22 (1H, s), two doublet aromatic protons at δ_H 7.89 (1H, *d*, *J* = 9.3) and 7.91 (1H, *d*, *J* = 9.3), two triplet methylene protons at δ_H 3.21 (2H, *t*, *J* = 5.8) and 5.09 (2H, *t*, *J* = 4.4), two singlet methoxy protons at δ_H 4.33 (3H, s) and 4.06 (3H, s), one doublet of methylenedioxy moiety at δ_H 6.08 (2H, s) and one singlet methyl group at δ_H 2.91 (3H, s).

The ^{13}C NMR ($CDCl_3$) spectrum (Fig 17) showed twenty one carbon signals. Fifteen signals of aromatic carbons at δ_C 150.4, 149.6, 146.9, 145.4, 144.5, 136.2, 133.6, 133.1, 129.8, 125.7, 121.2, 120.5, 120.2, 110.4 and 108.4, a signal of methylenedioxy carbon at δ_C 102.1, two methylene carbon signals at δ_C 57.7 and 28.3, two methoxy carbon signals at δ_C 62.9 and 57.1 and a methyl carbon signal at δ_C 18.3.



13-methylberberine

Table 3.10 The comparison of ^{1}H and ^{13}C NMR chemical shifts of 13-methylberberine (Miyuji *et al*, 1989)

Position	Chemical shift (ppm)		
	^{1}H NMR		^{13}C NMR
	Ref.*	13-methylberberine	
1	7.47 (1H, s)	7.09 (1H, s)	108.4
2	-	-	146.9
3	-	-	149.6
4	7.15 (1H, s)	6.88 (1H, s)	110.4
4a	-	-	129.8
5	3.13 (2H, *t*, *J* = 5.0 Hz)	3.21 (2H, *t*, *J* = 5.8 Hz)	28.3
6	4.85 (2H, *t*, *J* = 5.0, 2.0 Hz)	5.09 (2H, *t*, *J* = 4.4 Hz)	57.7
8	9.94 (1H, s)	10.22 (1H, s)	144.5
8a	-	-	120.2
9	-	-	145.4
9-OCH_3	4.11 (3H, s)	4.33 (3H, s)	62.9
10	-	-	150.4
10-OCH_3	4.10 (3H, s)	4.06 (3H, s)	57.1
11	8.20 (2H, s, H-11 and H-12)	7.89 (1H, *d*, *J* = 9.3 Hz)	120.5
12	8.20 (2H, s, H-11 and H-12)	7.91 (1H, *d*, *J* = 9.3 Hz)	125.7
12a	-	-	133.1
13	-	-	133.6
13a	-	-	136.2
13b	-	-	121.2
13-CH_3	2.94 (3H, s)	2.91 (3H, s)	18.3
OCH_2O	6.18 (2H, s)	6.08 (2H, s)	102.1

*solvent = DMSO-*d*6

3.5 The antiphytopathogenic fungal activity of berberine derivatives

Based on previous results that berberine displayed good antifungal activity against *A. porri*, *P. parasitica* and *P. deliense* with IC_{50} 101, 48 and 284 ppm, respectively. Seven derivatives were thus synthesized to examine the structure-*in vitro* antifungal activity against various phythopathogenic fungi (*A. porri*, *P. parasitica* and *P. deliense*) and to compare with berberine using the agar medium assay. Each compound was added to sterile PDA to obtain the tested concentrations (IC_{50} of each fungus). The antifungal activity was determined. The results are summarized in Table 3.11 and Fig 3.2.

Table 3.11 Effects of berberine derivatives on the mycelial growth of phytopathogenic fungi on a solid culture medium

Derivatives of berberine	Growth inhibition (%)		
	A. porri[a]	*P. parasitica*[b]	*P. deliense*[c]
berberine	52.2 ± 0.1	55.3 ± 01	58.2 ± 0.2
canadine	27.6 ± 0.2	23.1 ± 0.1	62.1 ± 0.1
8-methoxy-7,8dihydroberberine	16.9 ± 0.2	47.1 ± 0.1	22.0 ± 0.2
8-trichloromethyl-7,8-dihydroberberine	19.6 ± 0.2	53.5 ± 0.2	12.4 ± 0.2
acetonylberberine	40.8 ± 0.2	63.0 ± 0.1	77.8 ± 0.1
8-cyano-13,14-dehydrocanadine	49.9 ± 0.1	56.6 ± 0.2	71.6 ± 0.1
13-benzylberberine	42.2 ± 0.2	44.6 ± 0.2	89.7 ± 0.1
13-methylberberine	17.0 ± 0.2	62.3 ± 0.2	66.1 ± 0.3

a: concentration of testing sample at 101 ppm

b: concentration of testing sample at 48 ppm

c: concentration of testing sample at 284 ppm

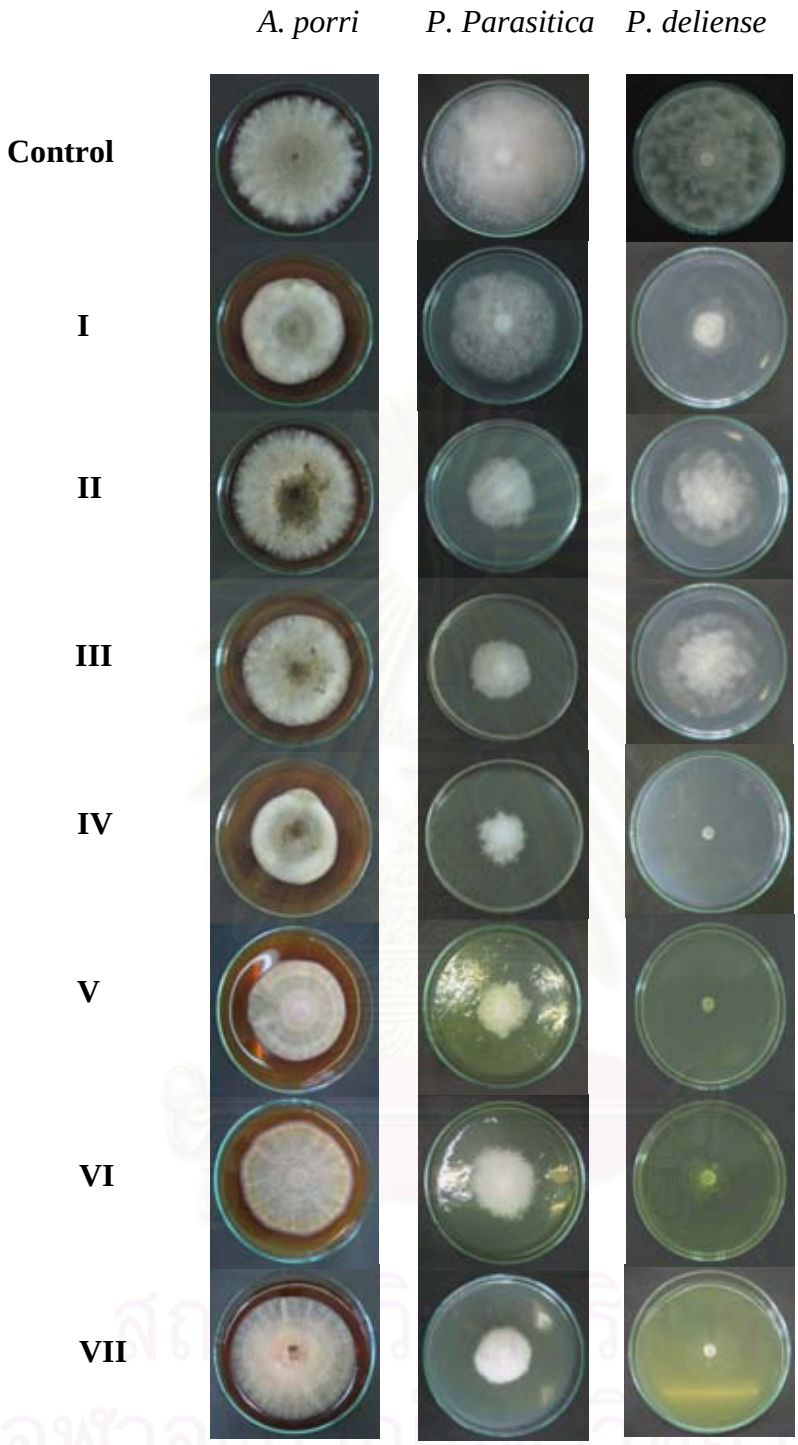


Figure 3.2 Effects of berberine derivatives on the mycelial growth of phytopathogenic fungi on a solid culture medium, canadine **(I)**, 8-methoxy-7,8-dihydroberberine **(II)**, 8-trichloromethyl-7,8-dihydroberberine **(III)**, acetonylberberine **(IV)**, 8-cyano-13,14-dehydrocanadine **(V)**, 13-benzylberberine **(VI)** and 13-methylberberine **(VII)**

The results in Table 3.11 and Fig 3.12 exhibited that all seven synthesized compounds could inhibit the mycelial growth of *A. porri*, *P. parasitica* and *P. deliense*. 8-Cyano-13,14-dehydrocanadine showed the most effective antifungal activity against the mycelial growth of *A. porri* comparable to that of berberine. Other derivatives including 13-benzylberberine, acetonylberberine, canadine, 8-trichloromethyl-7,8-dihydroberberine, 13-methylberberine and 8-methoxy-7,8-dihydroberberine displayed lower activity than the parent compound, berberine.

In the case of *P. parasitica*, acetonylberberine and 13-methylberberine exerted the most potent antifungal activity with % inhibition of 63 and 62, respectively. 8-Cyano-13,14-dehydrocanadine and 8-trichloromethyl-7,8-dihydroberberine exhibited the same level of activity as that observed for berberine. The rest compounds: 8-methoxy-7,8-dihydroberberine, 13-benzylberberine and canadine revealed the activities less potent than the starting compound, berberine.

For *P. deliense*, four from seven derivatives including 13-benzylberberine, acetonylberberine, 8-cyano-13,14-dehydrocanadine and 13-methylberberine showed more potent antifungal activity. Canadine revealed the same level of activity as that of berberine whereas 8-methoxy-7,8-dihydroberberine and 8-trichloromethyl-7,8-dihydroberberine expressed very low antifungal activity to this fungus.

According to the antifungal activity study, four derivatives: acetonylberberine, 8-cyano-13,14-dehydrocanadine, 13-methylberberine and 13-benzylberberine displayed more potent activity than berberine against *P. parasitica* and *P. deliense.* Thus they were selected for further investigation.

It was observed that the reduction and the modifications of the structure of berberine at C-8 with trichloromethyl or methoxy groups rendered the antifungal activity against *P. parasitica* and *P. deliense*. On the other hand, the substitution of methyl and benzyl groups at C-13, acetonyl and cyano groups at C-8 increased antifungal activity against *P. parasitica* and *P. deliense.* In addition, the substitution of hydrogen at the C-8 position of berberine with acetonyl and cyano groups raised the activity higher than the substitution of methyl and benzyl groups at C-13.

3.6 Spore germination assays

Four selected derivatives of berberine: acetonylberberine, 8-cyano-13,14-dehydrocanadine, 13-benzylberberine and 13-methylberberine were incorporated into PDA medium. To test spore germination, 100 µL of the suspensions (10^3 spore per mL) were streaked aseptically on PDA plates, supplemented with different berberine and berberine derivatives at 48 ppm (IC_{50} of *P. parasitica*). The plates were incubated at room temperature. Following incubation, fungal colonies originated from germinated spores were enumerated in order to evaluate the percentage of inhibition of spore germination. The results are tabulated in Table 3.13.

Table 3.13 Effect of berberine and derivatives of berberine on spore germination of *P. parasitica* at 48 ppm

Berberine and derivatives	% inhibition of spore germination
berberine	90.7 ± 0.1
acetonylberberine	53.7 ± 0.0
8-cyano-13,14-dehydrocanadine	100.0 ± 0.0
13-benzylberberine	100.0 ± 0.0
13-methylberberine	36.4 ± 0.2

The inhibition of spore germination of these compounds were examined and it was found that the most effective compound was 8-cyano-13,14-dehydrocanadine and 13-benzylberberine with 100% of spore germination inhibition on *P. parasitica* at 48 ppm. This value was better than that observed for berberine, acetonylberberine and 13-methylberberine.

In summary, two derivatives as 8-cyano-13,14-dehydrocanadine and 13-benzylberberine exhibited good activity *in vitro* both spore germination and mycelial growth of *P. parasitica*.

3.7 Phytotoxicity bioassays

Chinese cabbage (*Brassica campestris* var. pekinensis) seeds were used for phytotoxicity bioassays (according to the procedure described in Chapter II). The effects of berberine and its derivatives including acetonylberberine, 8-cyano-13,14-

dehydrocanadine, 13-benzylberberine and 13-methylberberine on relative root growth were analyzed and the results are displayed in Fig 3.3.

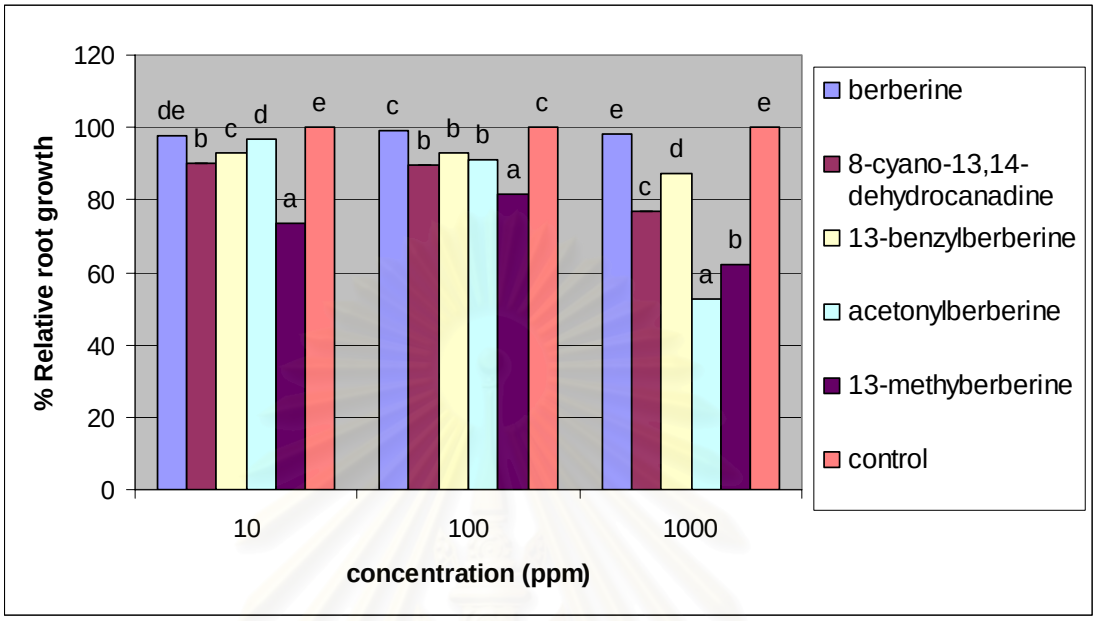


Figure 3.3 % Relative root growth for Chinese mustard. Different letters (a, b, c, d, e) are significantly different at the level of *P*=0.05 according to the Duncan's test.

As shown in Fig 3.3, in all cases the percentage of germinated seeds with respect to the control was 100%. Conducting the seedlings growth in the solutions of berberine (10, 100 and 1000 ppm) for four days did not significantly alter the total root length of the seedlings compared with the controlled experiment. This indicated that berberine did not display phytotoxic activity to the tested plants. At 10 and 100 ppm, acetonylberberine, 8-cyano-13,14-dehydrocanadine and 13-benzylberberine did not show significantly growth effect to Chinese mustard seeds while 13-methylberberine revealed relatively phytotoxic activity. However, at 1000 ppm acetonylberberine, 8-cyano-13,14-dehydrocanadine and 13-methylberberine displayed phytotoxixity. On the other hand, 13-benzylberberine did not display any significant effect on plant growth compared with berberine.

Therefore, it could be concluded that reduction and replacement of trichloromethyl or methoxy groups at C-8 not displayed better antifungal activity than berberine. Four derivatives of berberine, 8-cyano-13,14-dehydrocanadine, 13-benzylberberine, acetonylberberine and 13-methylberberine enhanced activities than

the mother compound. From the structure relationship showed that synthesized derivatives of berberine by manipulated cyano group at C-8 and benzyl group at C-13 (8-cyano-13,14-dehydrocanadine and 13-benzylberberine) displayed potent antifungal effects against mycelial growth and spore germination while 13-benzylberberine showed relatively no evident on phytotoxic effects.

CHAPTER IV

CONCLUSION

During the course of this research, four naturally occurring compounds were screened for their antifungal activity. Berberine was found to reveal its prominent activity against six phytopathogenic fungi studied. In order to explore what parts of berberine responsible for the activity, seven derivatives were synthesized by means of the introduction of various groups at C-8 and C-13. The results clearly indicated that the chemical modifications of berberine can remarkably improve the antifungal activity. The presence of acetonyl and cyano groups at C-8 position, benzyl and methyl groups at C-13 position are perceived to be important for enhancing antifungal activity. Especially the modification of berberine by introducing a cyano group at C-8 and a benzyl group at C-13 revealed completely inhibition of spore germination of *P. parasitica* while a modification of benzyl group at C-13 in deed showed relatively no evident on phytotoxic effects. On the other hand, the reduction and the modifications of trichloromethyl or methoxy groups at C-8 showed not better antifungal activity than the mother compound. From seven berberine derivatives, 13-benzylberberine showed good activities against mycelial and spore germination of plant pathogenic fungi including no phytotoxic effects which are interesting for developing continually. This finding was definitely informative to show that berberine could be a lead compound for developing for the future potential natural fungicide instead of synthetic fungicide. A part of the modification performed is likely to make the starting material, berberine more lipophilic, which may increase the permeability of the cell membrane.

In summary, this research is a good example to display the necessity of studying the modification of natural occurring compounds. The derivatives modified at C-8 and C-13 of berberine can enhance the antifungal activity of the mother natural compound which is interesting to developing in the future to gain the antifungal activity by introducing other groups at C-8 and C-13 and its still interesting for further examination on other antimicrobial activity.

Proposal for the future work

This research demonstrated the examination on phytopathogenic fungal activity of berberine and its derivatives. Therefore, the possible future work is the synthesis of a series of berberine containing different substituents at C-8 and C-13 position by more lipophilic and study structure activity relationship against many biological assays. Moreover, other active sites in the structure of berberine should be further considered. Further studies should also be concentrated, for instance, the mode of action of active compounds to fungi and toxicity to human.

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APPENDICES

APPENDIX A

Fungal Culture Media

Potato Dextrose Agar

Potato	200 g
Dextrose	20 g
Agar	15 g
Distilled water add to	1000 ml

Carrot Agar

Carrot	200 g
Agar	15 g
Distilled water add to	1000 ml

APPENDIX B

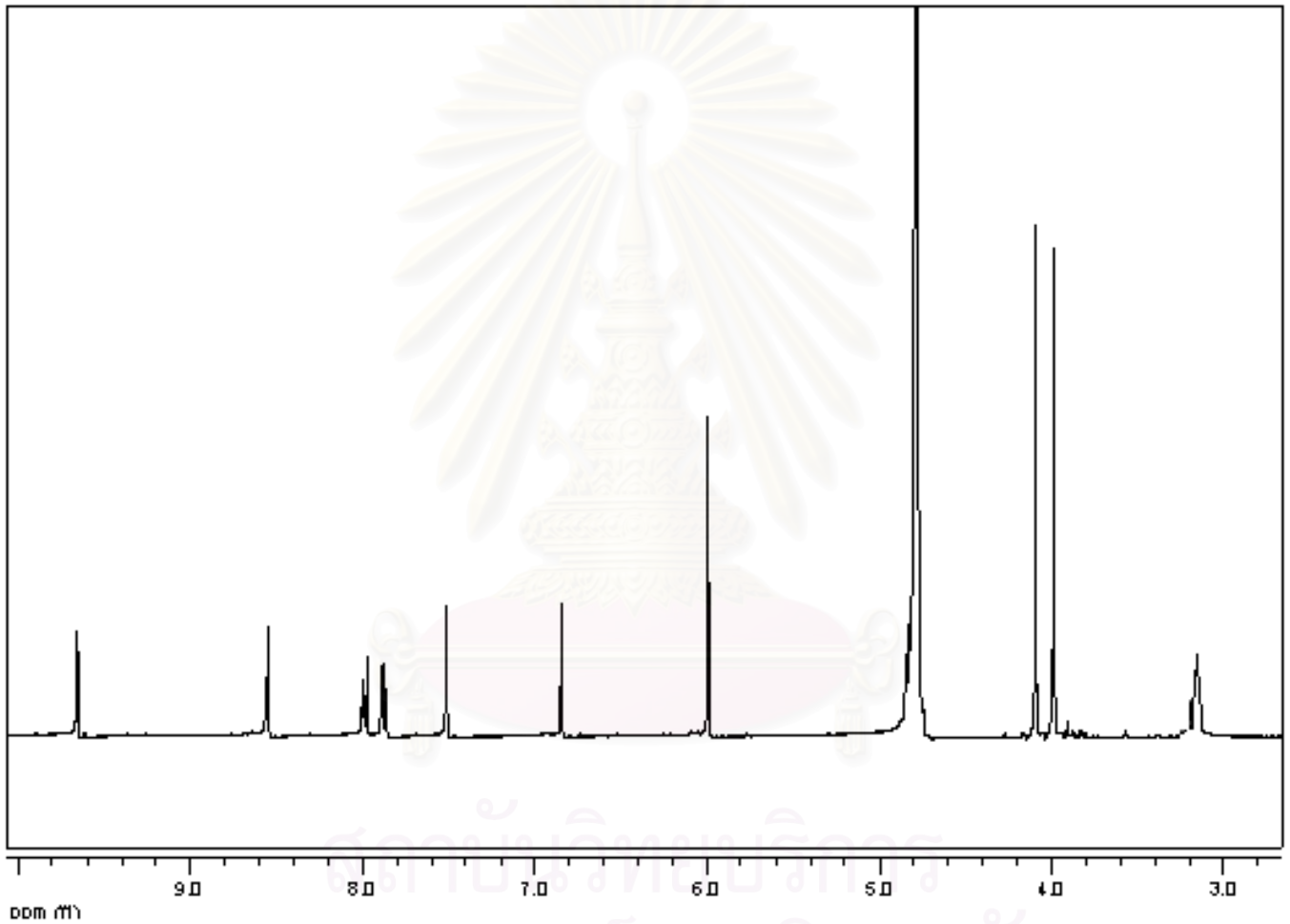

Figure 1 ^{1}H NMR spectrum of berberine

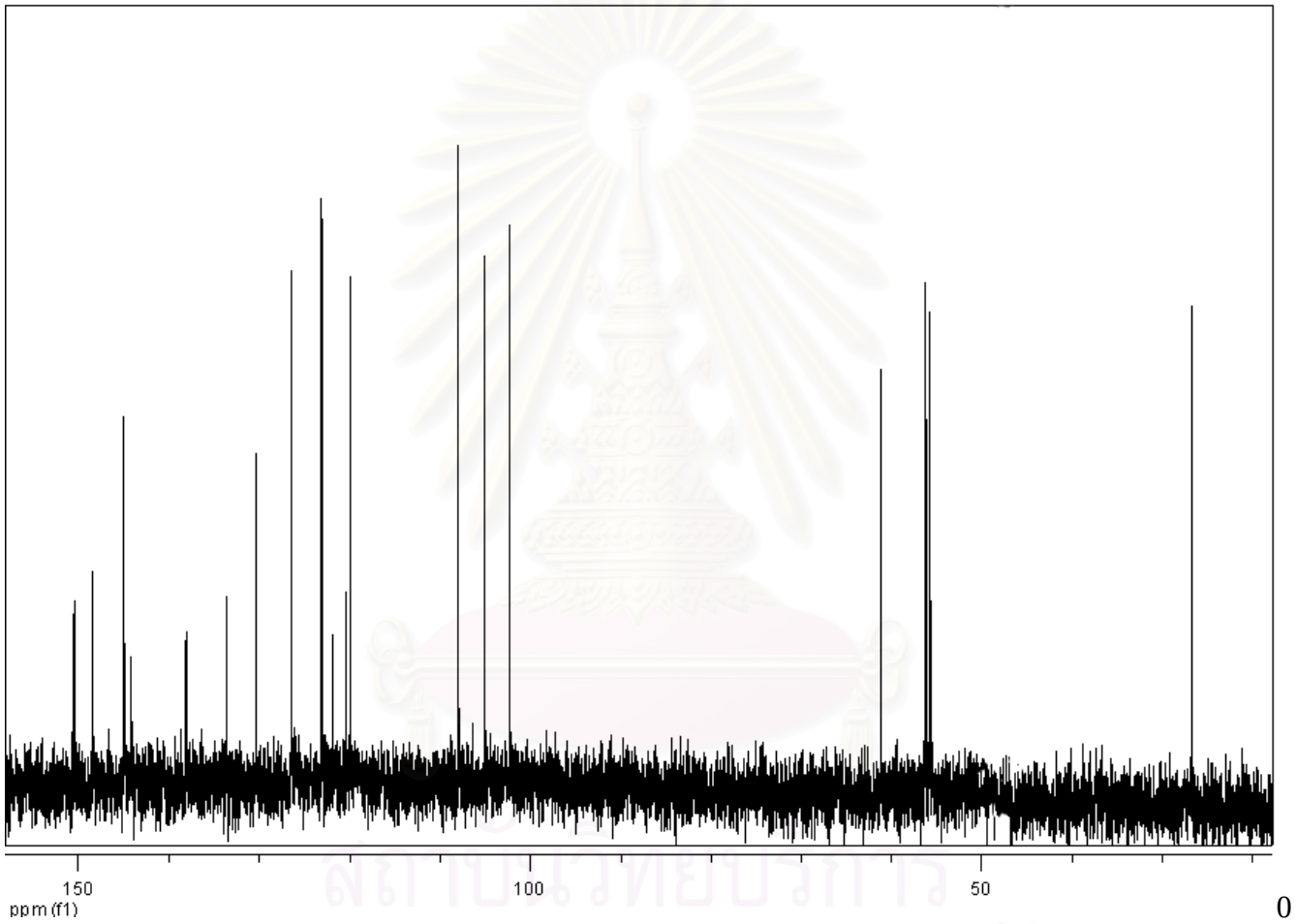

Figure 2 ^{13}C NMR spectrum of berberine

64

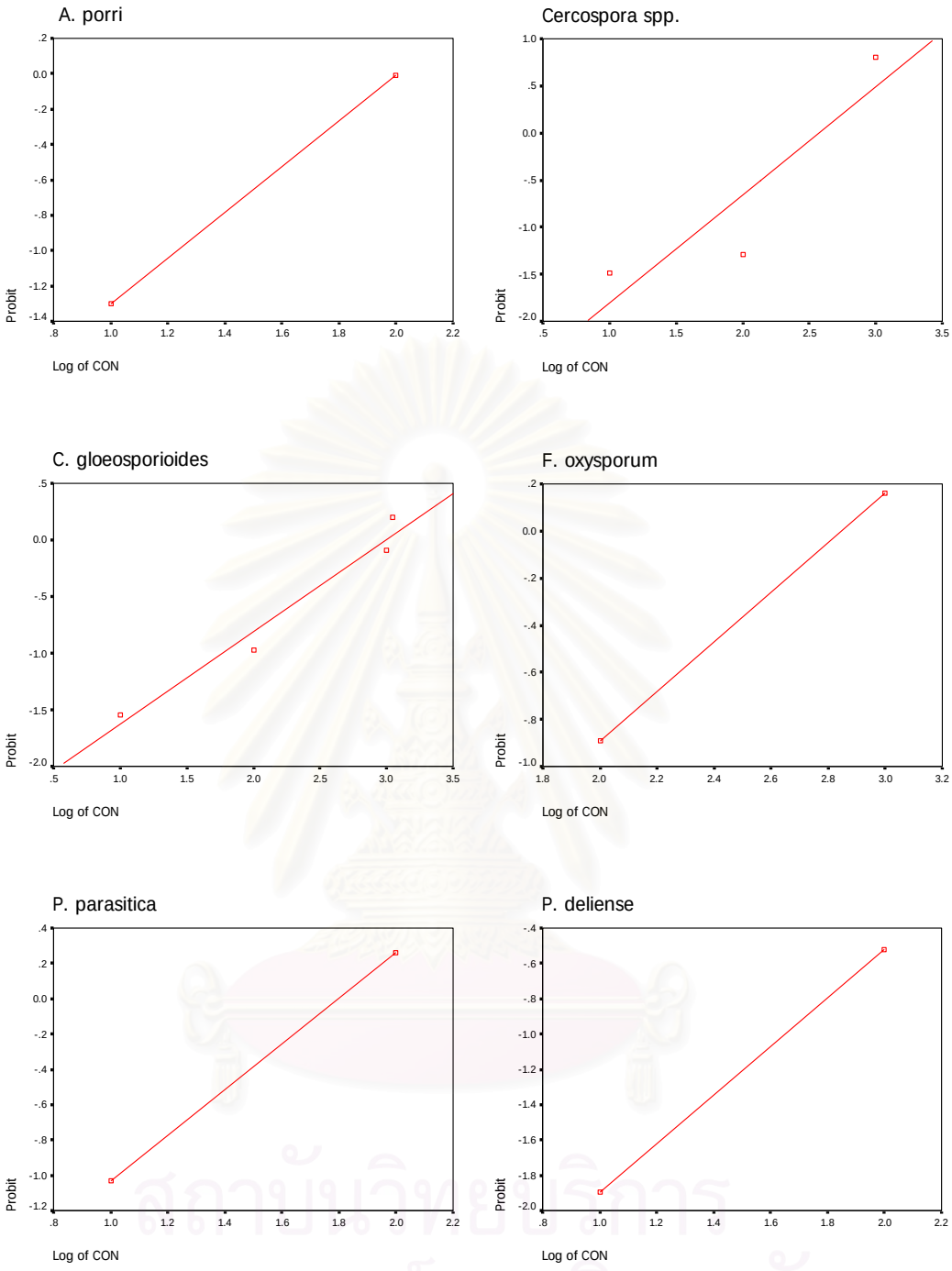

Figure 3 Regression curve of inhibition concentration at 50 percentage of berberine against phytopathogenic fungi.

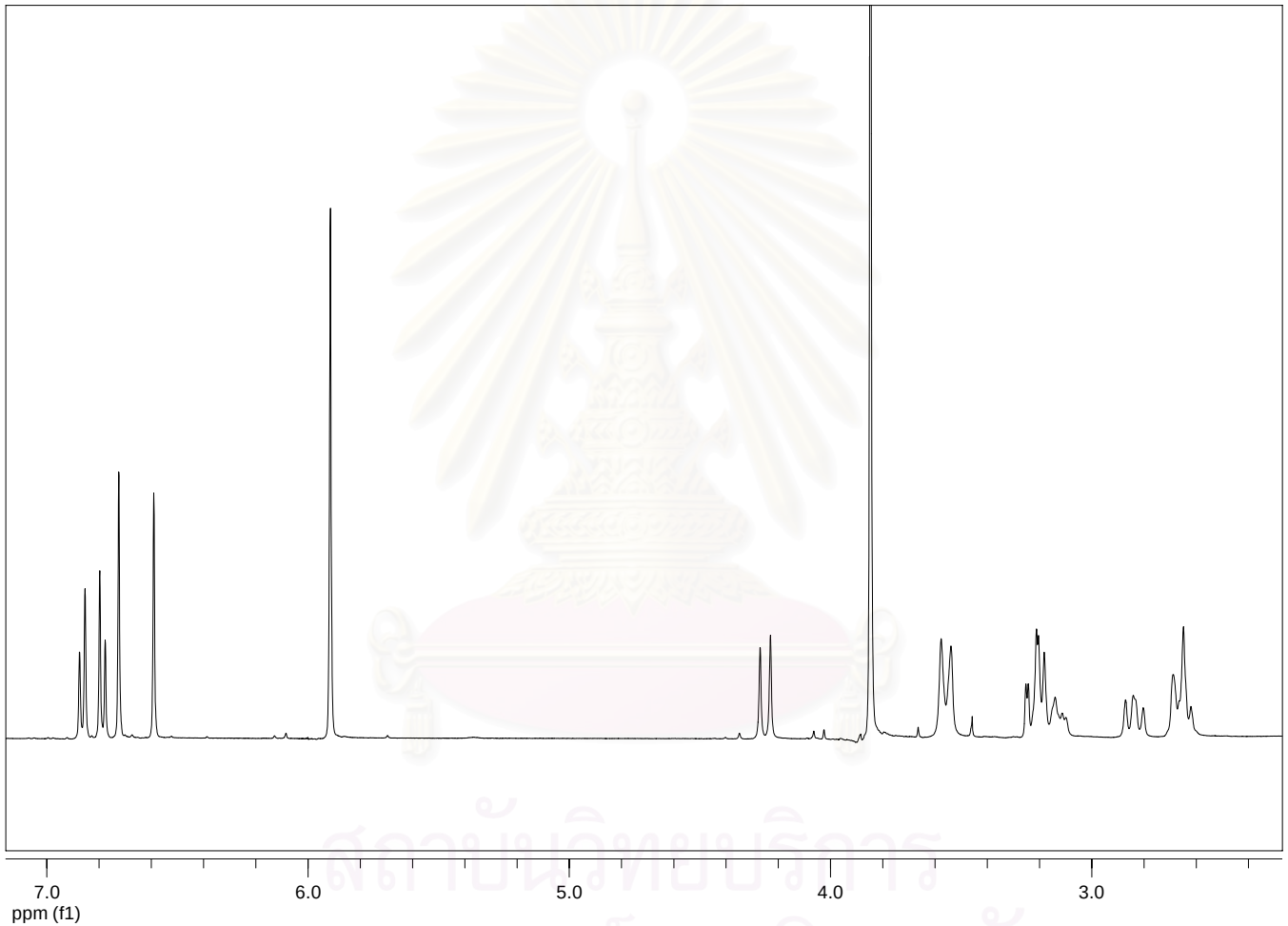

Figure 4 ^{1}H NMR spectrum of canadine

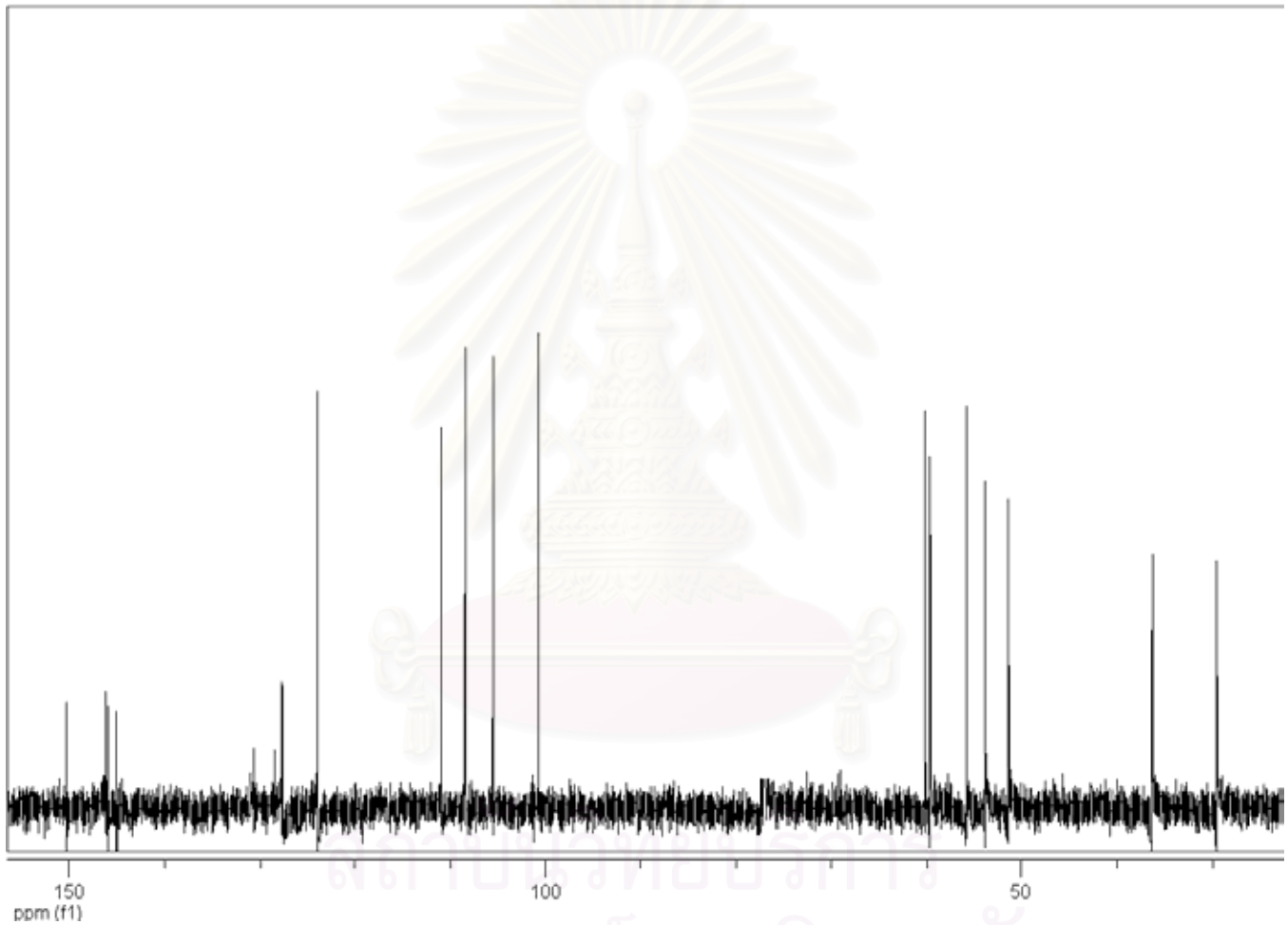

Figure 5 ^{13}C NMR spectrum of canadine

67

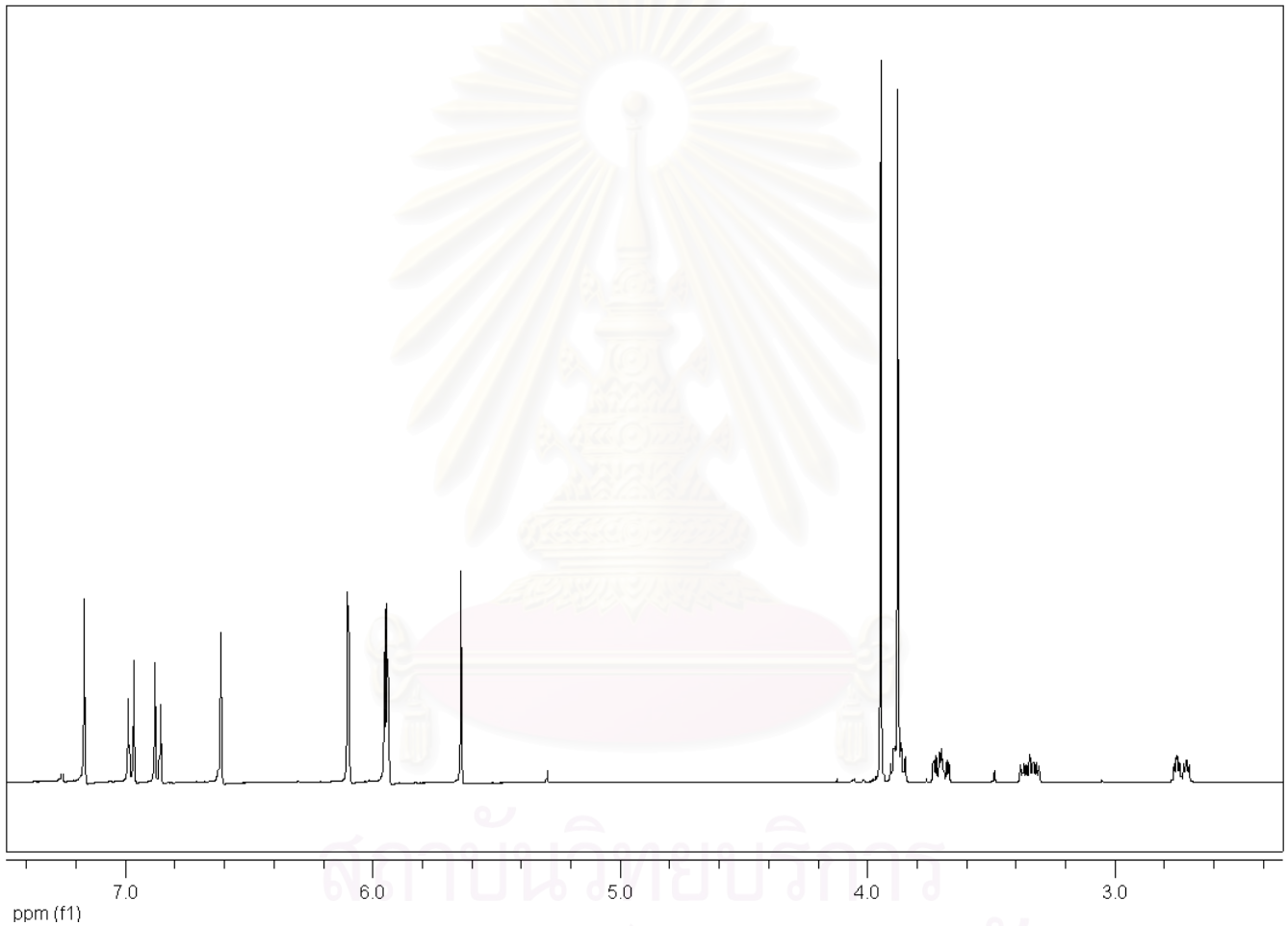

Figure 6 ^{1}H NMR spectrum of 8-methoxy-7,8-dihydroberberine

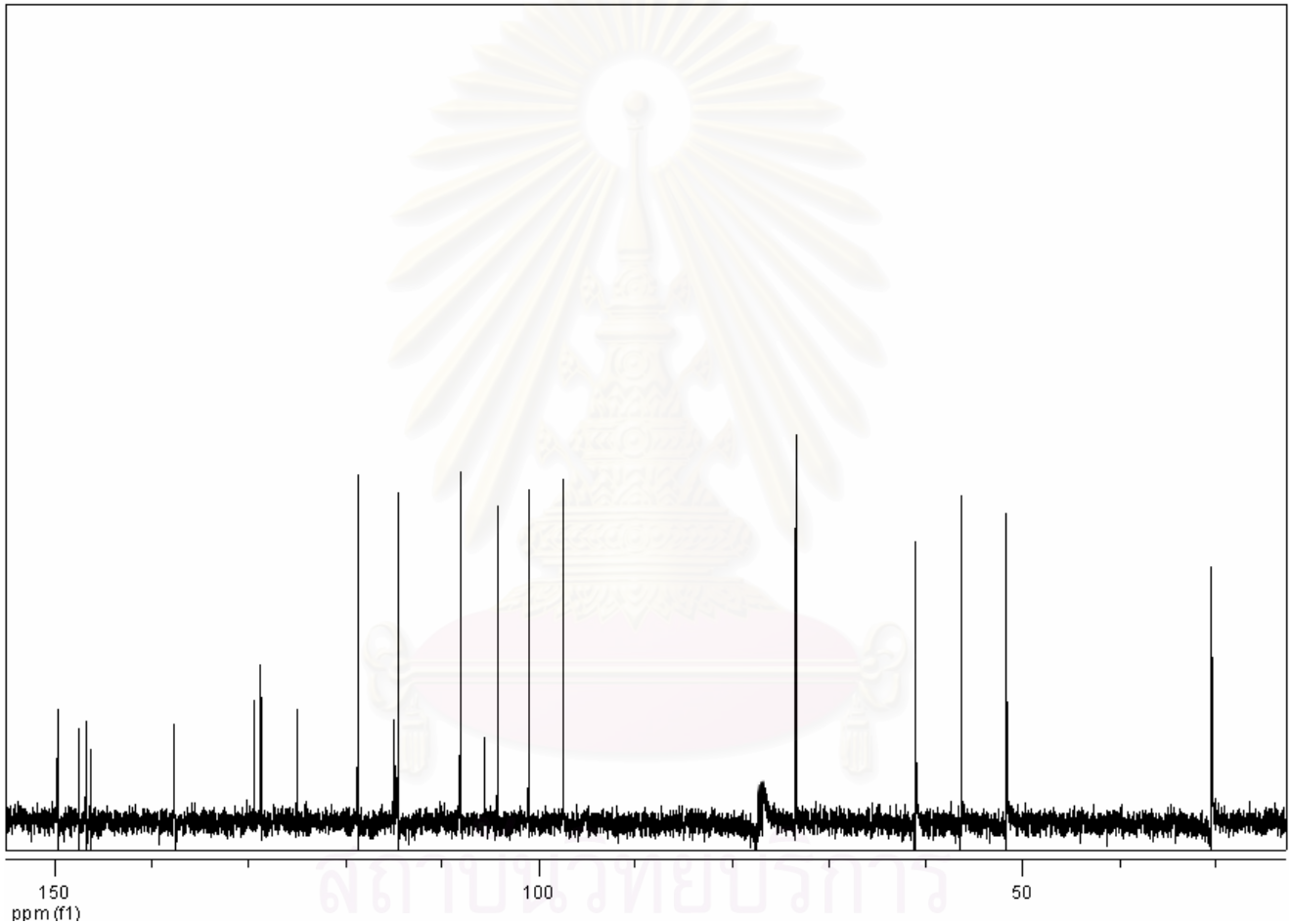

Figure 7 ^{13}C NMR spectrum of 8-methoxy-7,8-dihydroberberine

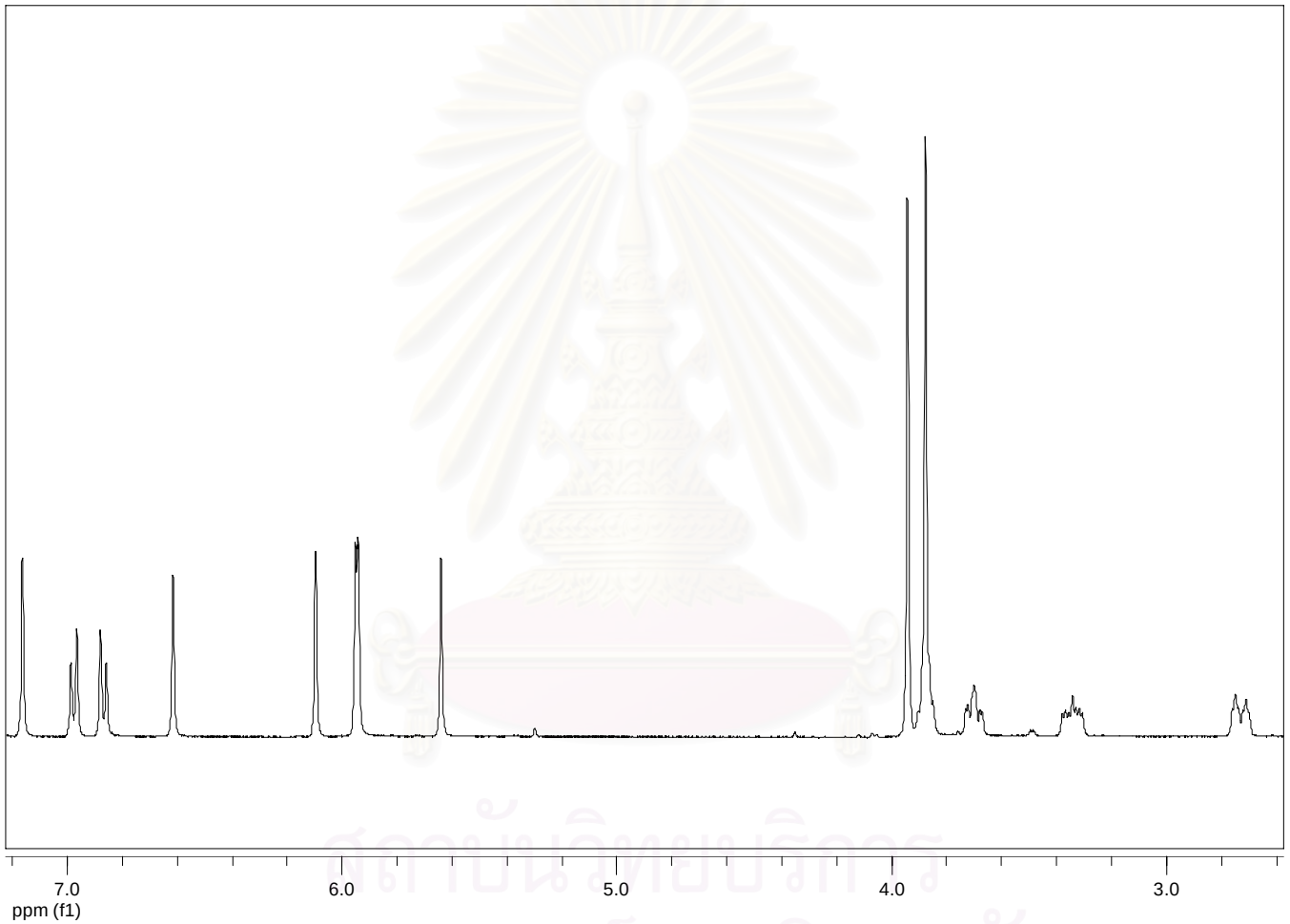

Figure 8 ^{1}H NMR spectrum of 8-trichloromethyl-7,8-dihydroberberine

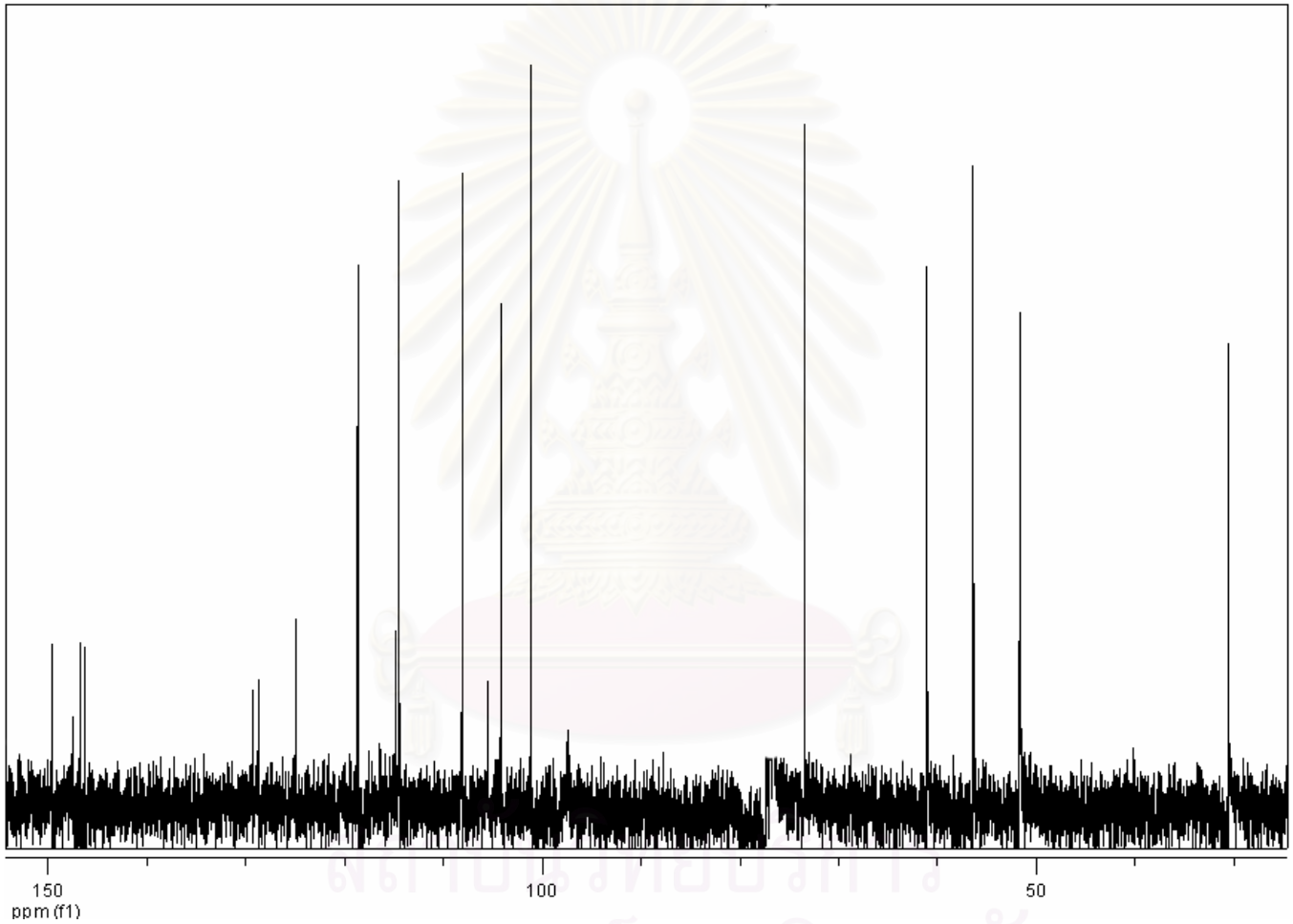

Figure 9 ^{13}C NMR spectrum of 8-trichloromethyl-7,8-dihydroberberine

71

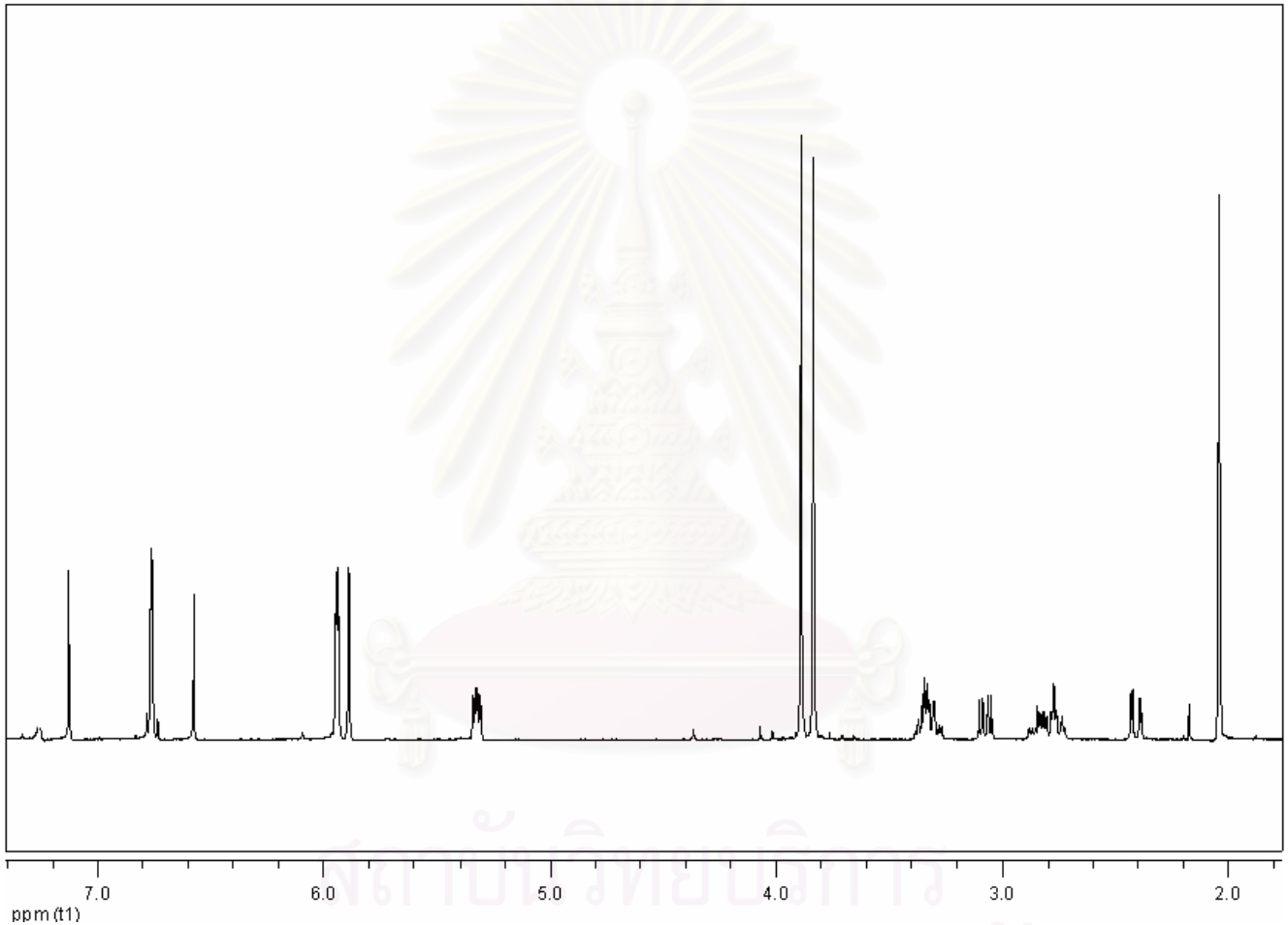

Figure 10 ^{1}H NMR spectrum of acetonylberberine

72

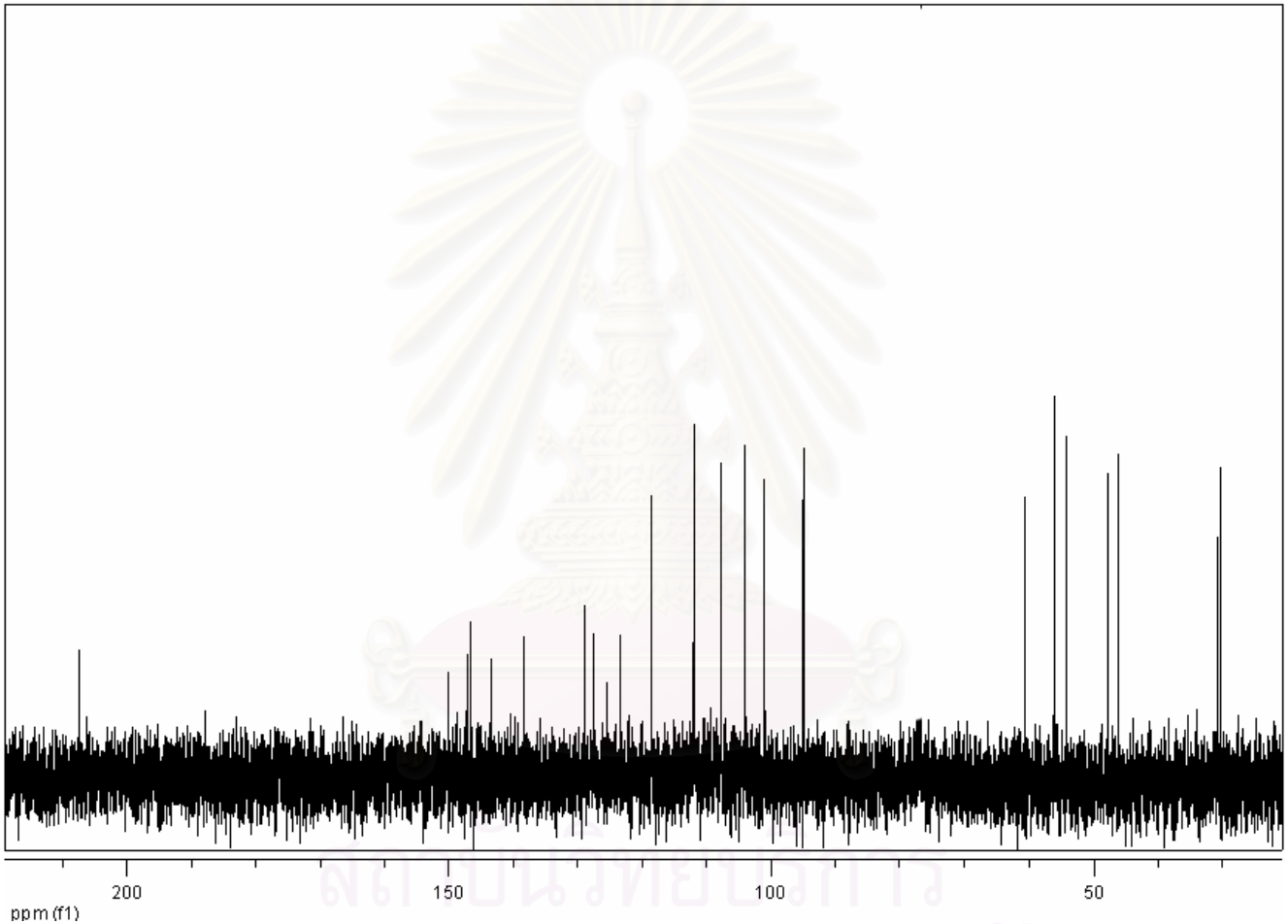

Figure 11 ^{13}C NMR spectrum of acetonylberberine

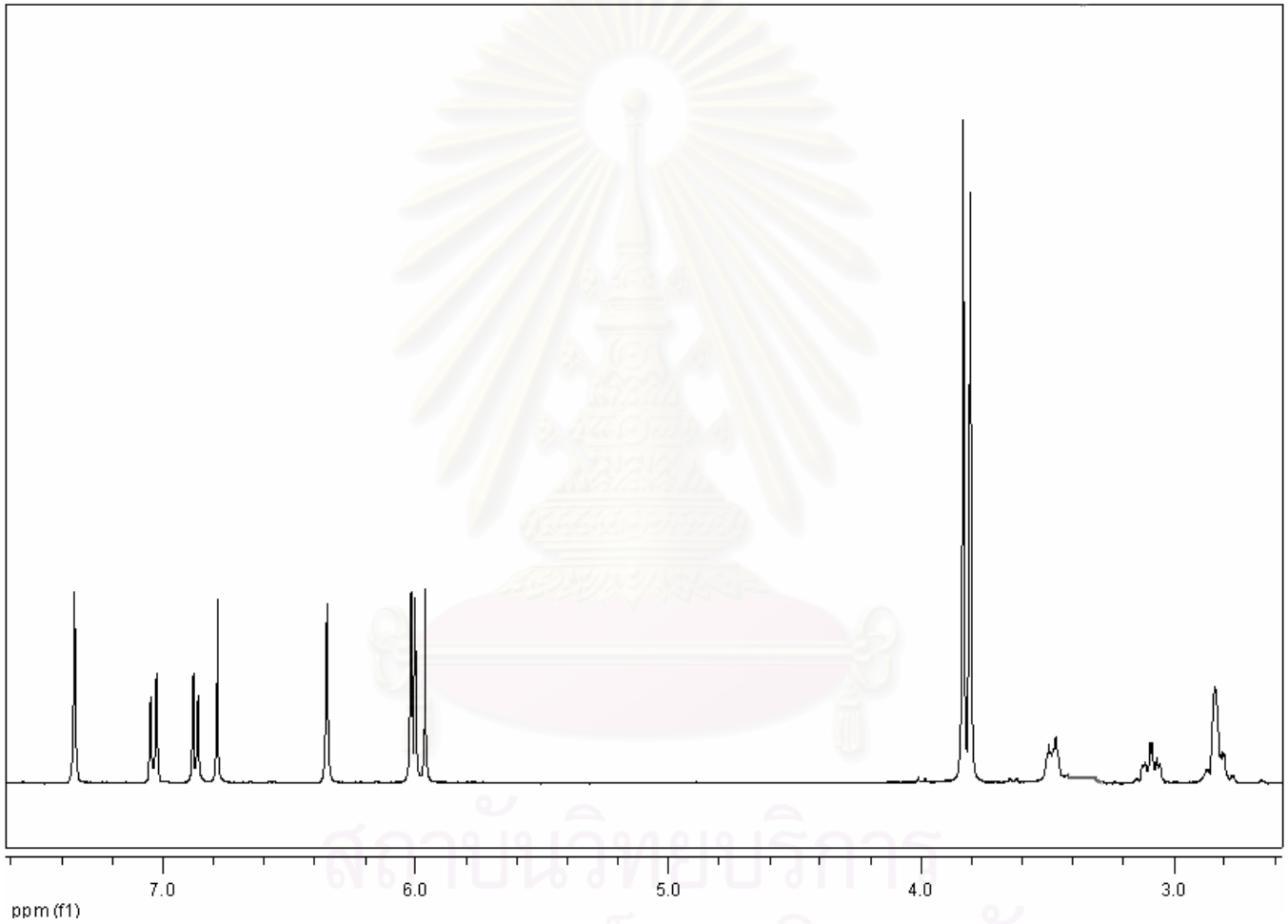

Figure 12 ^{1}H NMR spectrum of 8-cyano-13,14-dehydrocanadine

74

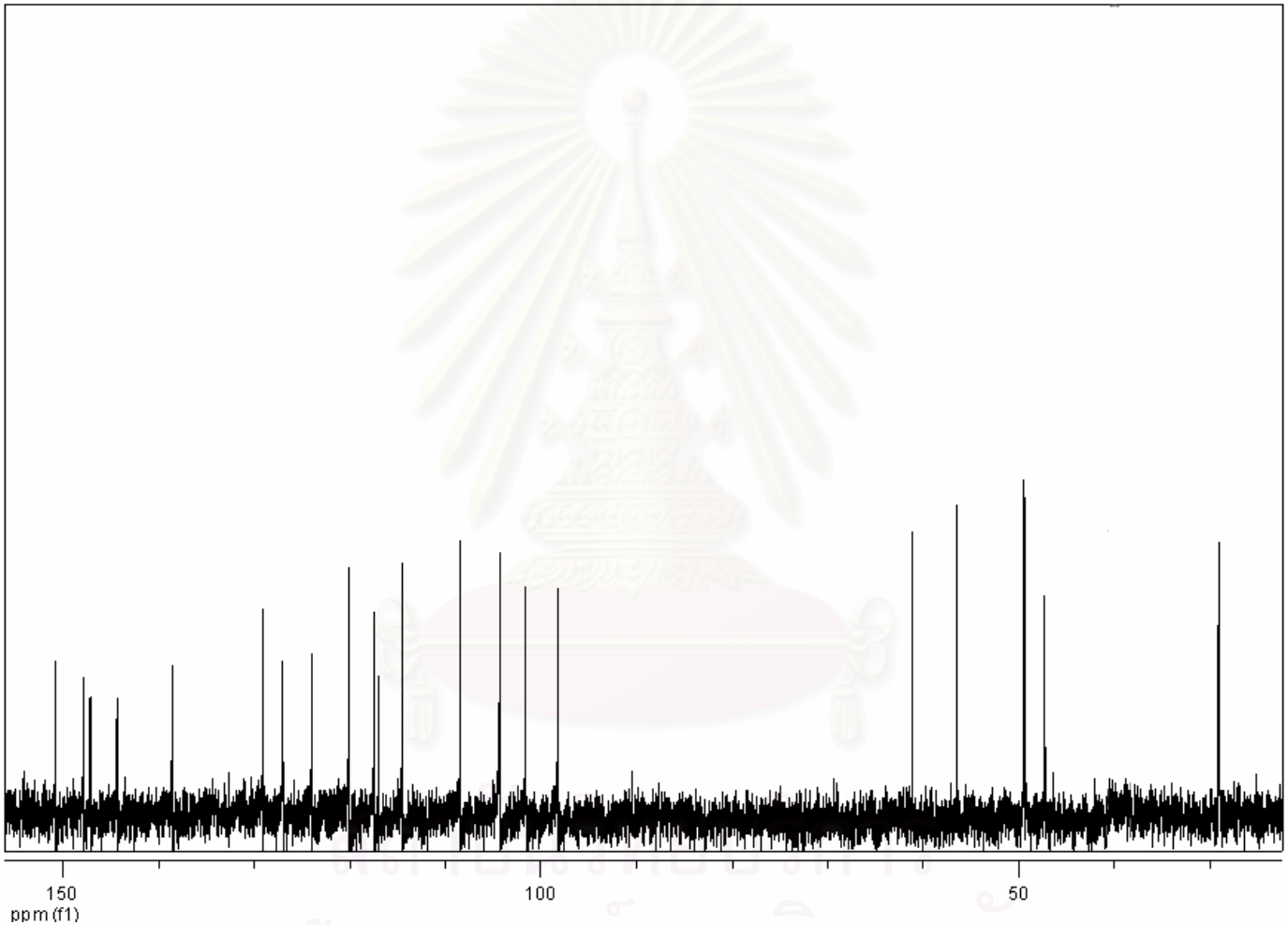

Figure 13 ^{13}C NMR spectrum of 8-cyano-13,14-dehydrocanadine

75

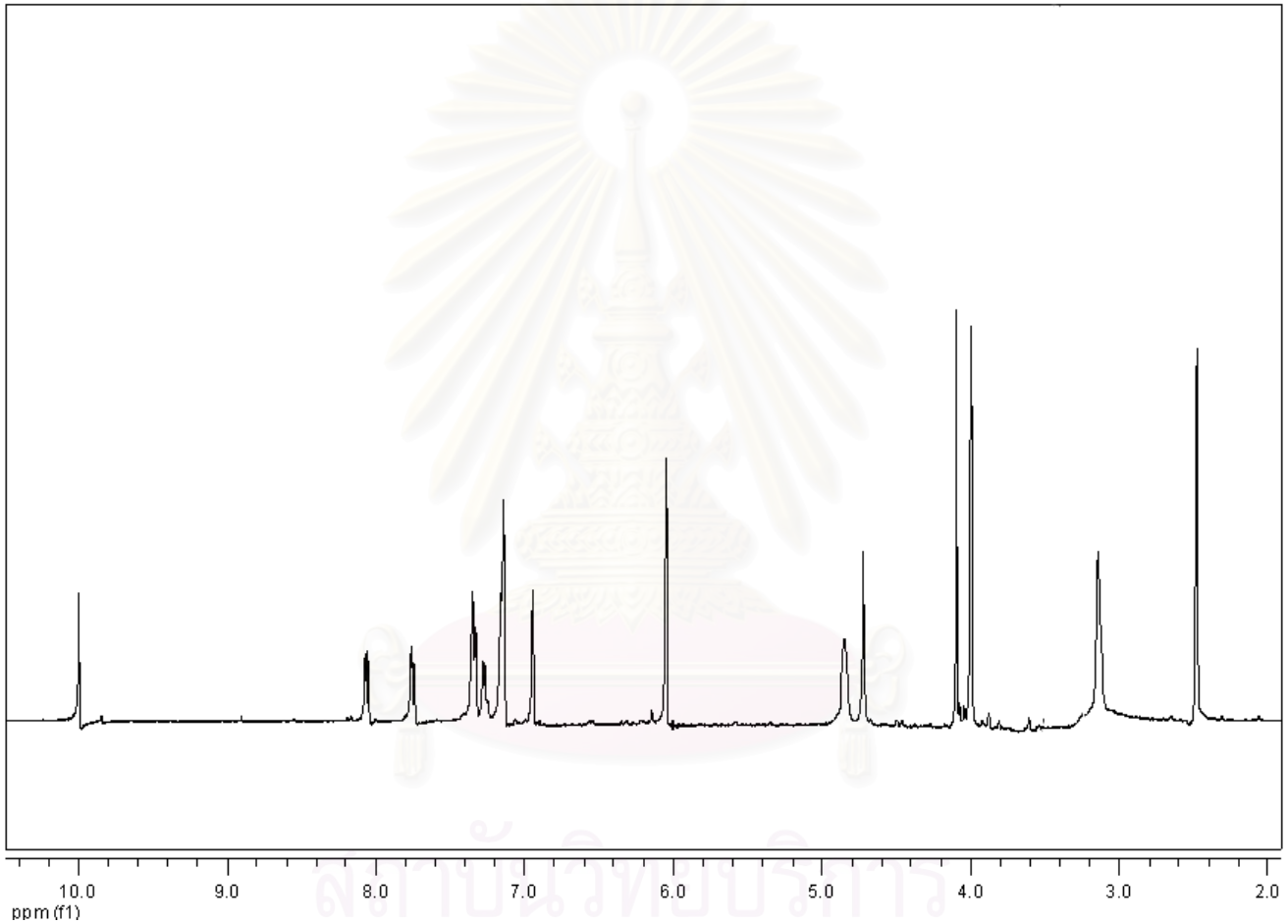

Figure 14 ^{1}H NMR spectrum of 13-benzylberberine

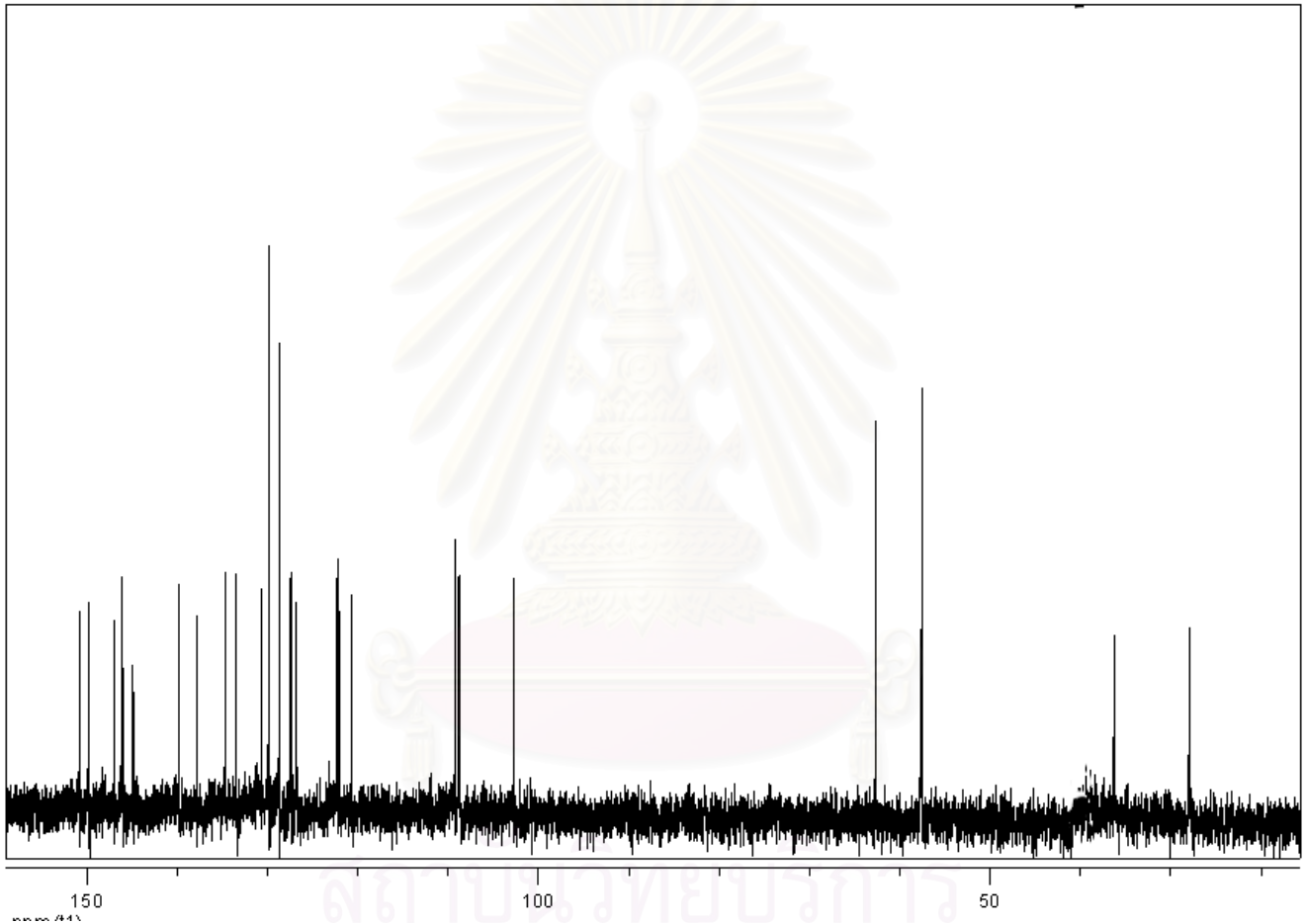

Figure 15 ^{13}C NMR spectrum of 13-benzylberberine

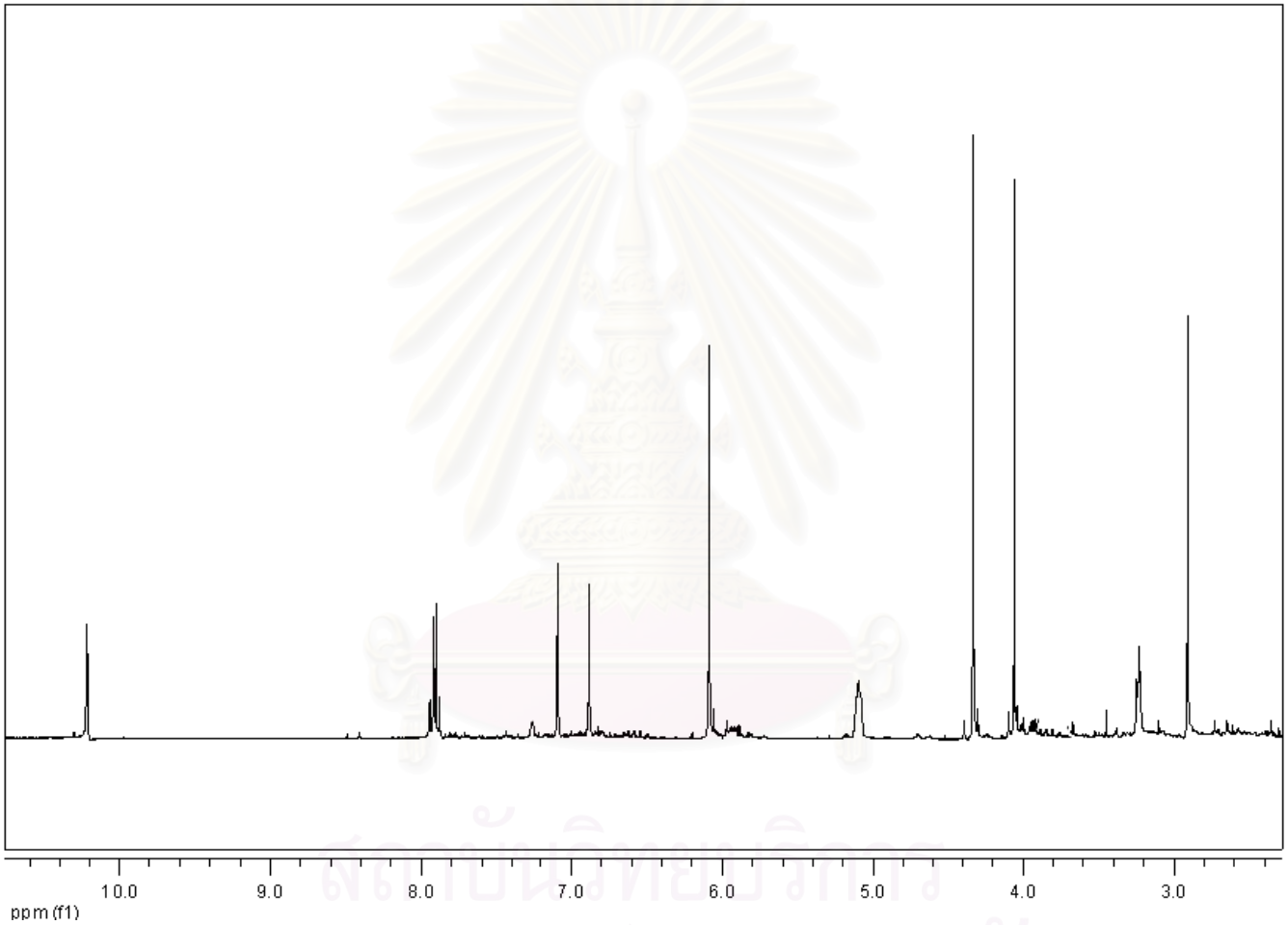

Figure 16 ^{1}H NMR spectrum of 13-methylberberine

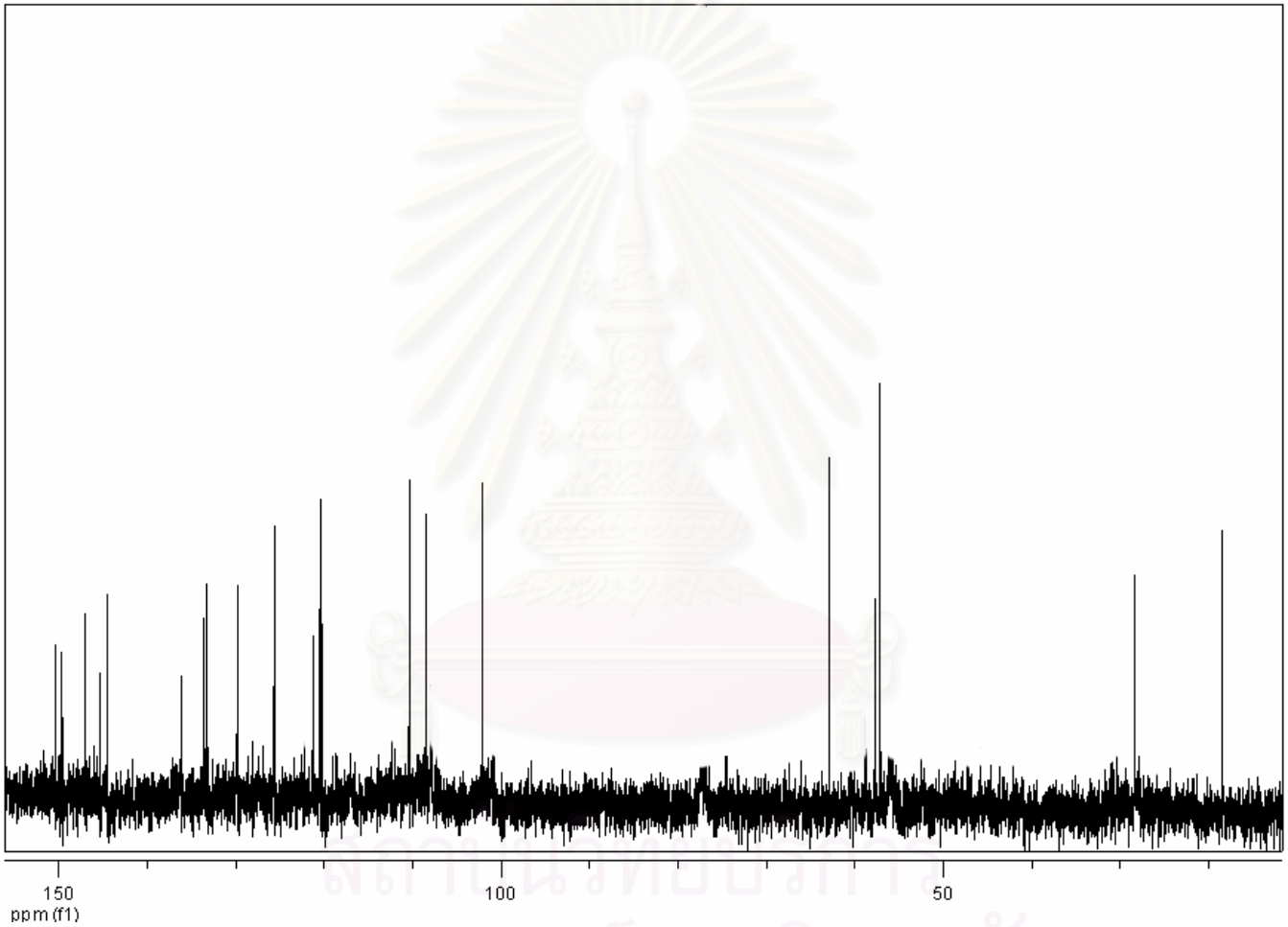

Figure 17 ^{13}C NMR spectrum of 13-methylberberine

Table 1 Effects of Metalaxyl and Mancozeb as a function of concentration on the mycelial growth of phytopathogenic fungi on a solid culture medium

Fungi tested	Growth inhibition (%)								IC_{50}[a] (ppm)
	Metalaxyl				Mancozeb				
	1 ppm	5 ppm	10 ppm	100 ppm	1 ppm	5 ppm	10 ppm	100 ppm	
A. porri	-	-	-	-	12.6±0.1	21.3±0.4	27.1±0.4	60.0±0.2	52.9
P. parasitica	52.2±0.1	64.5±0.1	65.8±0.2	71.6±0.3	-	-	-	-	0.3
P. deliense	52.7±0.0	66.3±0.1	78.7±0.1	100.0±0.1	-	-	-	-	0.1

[a] Inhibition percentage of each concentration was calculated as IC_{50} using Probit analysis program

VITA

Miss Wanchan Noppanit was born on January 1, 1979 in Yala Province, Thailand. She graduated with Bachelor Degree of Science in Botany from Faculty of Science, Chulalongkorn University, in 2001. During she was studying in Master Degree in Biotechnology program, she received a research fund for her thesis from Graduate School, Chulalongkorn University and TRF-master research grants for science and technology 2005.