Ph.D. Thesis Proposal

BIOLOGICAL CONTROL OF FRUIT FLIES (*Bactrocera correcta* Bezzi) BY PLANT EXTRACTS

การควบคุมแมลงวันผลไม้ (*Bactrocera correcta* Bezzi) โดยชีววิธีด้วยสารสกัดจากพืช

By

Miss Parichat Kruainok

School of Biology

Institute of Science

Suranaree University of Technology

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Miss Parichat Kruainok **ID. No.** M4810209

School of Biology **Institute of Science**

Thesis advisor: Assoc. Prof. Dr. Korakod Indrapichate

1. Thesis title

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2. Introduction

2.1 Background / Problem

Varieties of fruits grown in Thailand, including guava, mango, litchi, longan, peach, rose-apple, sapodilla, have great economic value and are important exports. Fruits are vulnerable to pests and germs. Insect is the most damaging pest of fruit crops affecting the valuable export trade of agricultural products of Thailand. Fruit fly is considered as a major pest causing damage to a wide variety of fruits and vegetable crops throughout the tropics and subtropics of the world, including Thailand. With increasing emphasis on quality of fruit and vegetable produce and with the possibility of expansion of trade in horticultural commodities, the countries importing as well as exporting are giving attention to fruit fly management at preharvest and postharvest levels (Drew, 1992). The guava fruit fly,

Bactrocera correcta (Bezzi), is one of examples of economically important pests of fresh fruits of Thailand.

However, the quarantine restrictions imposed by the presence of fruit fly hinder the development of export markets. Fruit flies attract host plants during their fruits are developing. Fruit flies feed and breed in their host plants. Adult fruit flies mostly lay their eggs in fresh fruits. The eggs hatch into larvae (maggots) which feed on the fruit pulp, causing the fruit soft and mushy mess (Dekker and Messing, On-line, 1999). The secondary infections by bacteria or fungi sometimes follow the egg-laying and lead to further badly marking on the surface of the fruit (Cantrell et al., On-line, 2002). Infested fruits quickly become rotten and inedible, this can induce fruits drop prior to harvest, thus causing considerable losses in production or if harvested, the result of damage makes the fruit unsaleable both to export market and domestic market (Collins, 1998). Other major losses result from quarantine restrictions that are imposed by importing countries to prevent the entry or establishment of unwanted fruit fly species. Considerable financial burdens are imposed on governments, farmers and exporters, who have choice but to implement quarantine surveillance systems, quality assurance schemes and acceptable post-harvest quarantine treatment if they wish to export fruit fly host product (Allwood and Drew, 1997).

Fruit flies management involves application of chemically synthetic insecticides. Although they are effective but there are reported that their use for several decades had disrupted biological control system of natural enemies and led to outbreaks of insect pests including widespread of resistant development, undesirable effects on non-target organisms, and environmental and human health concerns (Kim et al., 2003). It is difficult to design chemicals which act specifically towards a given group of target insects (Wells et al., 1993). Besides hazardous effects on natural enemies, the limited availability, dangers

and cost associated with the use of synthetic insecticides, there are problems regarding the resistance of the pest insect against these products (Boeke et al., 2004). The accumulation of insecticides in plants and animals can lead to long term human health problems as well.

A simple and safe technology has been developed to protect various fruits from the flies. There is a growing interest in the use of botanical insecticides to reduce the use of chemically synthetic insecticides and also to avoid problems of insecticide resistance (Thomas and Callaghan, 1999). The plant products that are traditionally used and produced by farmers in developing countries appear to be quite safe and promising for consumer health (Rajapakse and Van Emden, 1997). Many plants may provide potential alternative to currently used insect control agents because they constitute a rich source of bioactive chemicals (Kim et al., 2003). The interaction between plants and insects is chemically mediated by secondary metabolites (Pascual-Villalobos and Robledo, 1999). Since these are active against a limited number of species including specific target insect, biodegradable to non toxic products, and potentially suitable for use in integrated pest management, thus, they could lead to the development of new classes of safer insect control agents (Kim et al., 2003).

This research focuses on some plant extracts for potentially useful products as guava fruit fly (*Bactrocera correcta* Bezzi) control agents.

2.2 Literature review

Guava fruit fly (Diptera: Tephritidae)

Scientific name: *Bactrocera correcta* (Bezzi)

Synonym: *Chaetodacus correctus* (Bezzi), *Dacus* (*Strumeta*) *correctus* (Bezzi), *Batrocera zonata* (Bezzi) (Weems and Fasulo, 2001)

Common name: Guava fruit fly

Life cycle of the guava fruit fly

The fruit fly has complete metamorphosis life cycle composed of four stages. Fruit fly life cycle depends on temperature. Cool temperature slows the developmental cycle and warm temperatures speeds it up. The development from egg to adult is under summer conditions which requires about 16 days. Eggs are barely visible, they are white and elongate. They are laid on a food source of fermenting fruit or other moist organic materials and hatch into larvae approximately 24 hours after being laid. Larvae are pale white, feed constantly, and reach full size in 5 up to 6 days (Lind, 1999). They are very difficult to be seen until they feed for a while and get larger. While feeding, they tunnel throughout the fruit, destroy the pulp and allow an entry of secondary infestation of bacteria and fungi (Vossen et al., 2004). Larvae feed on fungi and yeast organisms and grow in their food sources. Their feeding effort turns their food into a semi-liquid mess. When they are fully grown, the larvae move to a drier area to pupate. Pupae are straw-colored and shaped like small wheat grains. Emergence of the adult takes place after a few days when the adult fruit fly forces its way through the anterior end of the pupa. Shortly after emerging, the adult fly darkens in color, its abdomen expands, and it extends its wings. The adult is 1/8 – 1/4 inch long with clear wings, a dull brown body (the female's abdomen is crossed by dark lines), and distinctive red compound eyes. Adult females can begin laying eggs within 48 hours after emerging from the pupae and begin mating within 12 hours. Adult fruit flies can live from a few days up to 30 days and the females lay approximately 500 eggs in their life span (Lind, 1999).

The control of fruit fly

The fruit fly activity and population vary throughout the year and widespread in Thailand. Because of the potential losses from fruit fly infestations, control is typically carried out on routine basis, especially in commercial plantings. Fruit fly control involves

application of synthetic insecticides, although the removal of infested or fallen fruit can reduce fly populations to some extend and practice of bagging can lessen damage from individual fruit (Collins, 1998). The most common method for controlling fruit fly is synthetic insecticide spray. Because this method has a potential to drop down fruit fly population in a minimum (Allwood and Drew, 1997). However, there are several disadvantages of synthetic insecticide spray. An indirect lost from the use of synthetic insecticide spray in the impact on other insect species which are beneficial to production. These species include pollinators and natural parasites and predators of other fruit pests. The intensive use of synthetic insecticide spray can also elevate grower risk of exposure and the potential for long term health problems (Collins, 1998).

The alternative methods for fruit fly control has been increasing for replace synthetic insecticide spray in recent years. The natural products have also proved effective fruit fly control. Some plants are especially rich in chemicals that can be extracted and used for fruit fly control. These products are known as botanical insecticides (Cranshaw, On-line, 2006). The extracted of mugwort (*Artemisia vulgaris*), mangosteen peel (*Garcinia mangostana*), croton (*Croton tiglium*), tobacco (*Nicotina tabacum*), Japanese poinsettia (*Pedilanthus tithymaloides*), pencil tree (*Euphorbia tirucalli*) and ginger (*Alpinia officinarum*) are reported to kill adult fruit flies (*Bactrocera dorsalis* Hendel) (รุจนี เล้ารัตนบูรพา, 2523). The efficacy to repellent adult fruit flies was found in extracts from lipsticktree (*Bixa orellana* Linn.), neem (*Azadirachta indica* var. *siamensis* Veleton), Kaffir Lime (*Citrus hystrix*), melon (*Cucumis melo* Linn.), lemongrass (*Cymbopogon citraius* Stapf.), heliotrope (*Heliotropium indicum* R. Br.), shrubby basil (*Ocimum gratissimum* Linn.), orange gingerlily (*Hedychium occineum* var.), and Castor-oil plant (*Ricinus communis* L.) (Areekul et al., 1978). Chuenwong (2006) showed that the potential

of the extracts from neem (*Azadirachta indica* Juss.), sugar apple (*Anona squamosa* L.) and mintweed (*Hyptis suaveolens* L.) can control egg, larva, pupa and adult fruit flies (*Bactrocera dorsalis* Hendel).

Plants for insect pest control

Marigold (*Tagetes erecta* Linn), a common garden plant grown throughout Thailand is an herb of ancient medicinal repute (Cetkovic et al., 2004). In traditional and homeopathic medicine it has been used for skin complaints, wounds and burns, conjunctivitis and poor eyesight, menstrual irregularities, varicose veins, hemorrhoids, duodenal ulcers, etc. The yellow or golden-orange flowers of marigold are used as spice, tea and medicine. The pharmacological activity of marigold is related to the content of several classes of secondary metabolites such as essential oils, flavonoids, sterols, carotenoids, tannins, saponins, triterpene alcohols, polysaccharides and resin (Cetkovic et al., 2004). Marigold is typically planted as intercrops or in rotation with crops to control nematodes. Natarajan et al. (2006) found that population of tomato root knot nematode, *Meloidogyne incognita*, were reduced by cold aqueous extracts of marigold. Broussalis et al. (1999) demonstrated that the activity of marigold extracts by maceration with dichloromethane and methanol to against rice weevils (*Sitophilus oryzae*). Sain (2004) showed the potentiality of callus cultures of marigold to produce ascorbic acid as well as insecticidal pyrethrins. The pyrethrins extracted from the callus cultures can be safely used as an insecticide on flour beetles (*Tribolium* spp). In Thailand, marigold used locally as insecticides. The plants have a history of usage as folk remedies and are still used to kill or repel insect such as white flies, common cutworms and cabbage moths (ไพบูลย์ บุญชัย, On-line, 2546).

Siam weed (*Chromolaena odorata* (Linn) King & Robinson, *Eupatorium odoratum* Linn), a perennial, is a diffuse, scrambling shrub that is mainly a weed of plantation crops

and pastures in Asia and Western Africa (Thang et al., 2001). Due to its fast growth rate, and prolific, wind-dispersed seed production, the plant can spread very easily (McFadyen and Skarratt, 1996). Traditionally, fresh leaves or a decoction of Siam weed have been used throughout Vietnam for many years as well as in other tropical countries for the treatment of leech bite, soft tissue wound, burn wound, skin infection and dento-alveolitis (Thang et al., 2001). Bouda et al. (2001) reported that essential oil extracts from leaves of Siam weed has an insecticidal effect on the adult of maize grain weevils (*Sitophilus zeamais*). Leaf powder of Siam weed has been found to reduce infestation and damage caused by rice moth (*Corcyra cephalonica*). The powder showed a high efficacy against rice moth egg hatch into adult (Allotey and Azalekor, 2000). Siam weed extracts was used as domestic insecticide in Thailand. Leaves of Siam weed can used to control common cutworms, cabbage moths, aphids and beetles (อำนวย อิศรางกูร ณ อยุธยา, 2535).

Hedge flower (*Lantana camara*) is commonly found in Thailand. It is also an important weed that infested farm and land around house. Sometime hedge flower found as an ornamental garden plant. In Africa, hedge flower play the role as medicinal plant. Leaves of hedge flower are used to treat skin itch, ulcers, hepatitis and rheumatism (Bouda et al., 2001). Moreover, essential oil extracts from leaves of hedge flower has an insecticidal effect on maize grain weevils (*Sitophilus zeamais*). Insecticidal activity of the essential oil can exploit for maize grain weevils (*S. zeamais*) control in stored products (Bouda et al., 2001). Hedge flower leaves extracted was found an effective against termites (*Microcerotermes beesoni*). The extracts exhibit excellent termites mortality (Verma, 2006). In Thailand, hedge flower has been used as folk insecticide. Leaves extracts of hedge flower has a strongly insecticidal activities against aphids and beetles (อำนวย อิศรางกูร ณ อยุธยา, 2535).

3. Research objectives

1) To observe the cytotoxicity of marigold (*Tagetes erecta* Linn), Siam weed (*Chromolaena odorata* Linn) and hedge flower (*Lantana camara*).
2) To control guava fruit fly (*Batrocera correcta*) by the extracts of marigold (*Tagetes erecta* Linn), Siam weed (*Chromolaena odorata* Linn) and hedge flower (*Lantana camara*) on eggs, larvae, pupae and adults of guava fruit flies.
3) To investigate the mortality effect of plant extracts on insect cytochrome c.

4. Research hypothesis

Marigold, Siam weed and hedge flower extracts can control guava fruit fly eggs, larvae, pupae and adults.

5. Scope and limitation of the study

4.1 Plants will be collected on SUT campus.

4.2 Plants will be collected at one time through out the study.

4.3 The cytotoxicity of plant extracts will be tested on brine shrimps.

4.4 Fruit fly pupae will be obtained from the Office of Atomic Energy for Peace, Thailand.

6. Research methodology

6.1 Plants

Leaves of marigold (*Tagetes erecta* Linn.), Siam weed (*Chromolaena odorata* (Linn) King & Robinson, *Eupatorium odoratum* Linn) and hedge flower (*Lantana camara*) will be collected at SUT campus and then dried by sun light for 2 days before extraction.

6.2 Plant extracts preparation

Dried plant samples will be extracted in distilled water or 70% ethanol by universal extraction. The extracts will be evaporated, dried by lyophilizer and stored at –20 °C for further studies. The dried extracts will be dissolved in its original solvent during study.

6.3 Determination of some phytochemical properties

6.3.1 Determination of total phenolic compounds availability.

Phenolic compound will be determined by the Folin-ciocalteau colorimetric method using gallic acid as a standard phenolic compound (Huang et al., 2004). Sample will be dissolved in solvent (distilled water), added Folin reagent and incubated 30 minute. The absorbance will be measured at 760 nm.

6.3.2 Thin layer chromatography fingerprintings of plant extracts

Thin layer chromatography (TLC) is a standard technique for separate compound mixture. Its sensitivity is high which allows separation of less than microgram amounts of material. Silica gel on a support material such as glass or aluminum is most widely employed (Harborne, 1998). TLC will be used to obtain the fingerprinting of plant extracts in order to figure out the differences of their components.

6.3.3 Determination of cytotoxicity of plant extracts by Brine Shrimp Lethality Assay (BSLA).

The brine shrimp lethality assay, modified from Solis et al. (1993), will be used for preliminary assessment of cytotoxicity. Brine shrimp (*Arthemia salina*) will be cultured in artificial seawater. After eggs hatching (24 hours), the first instar of brine shrimp will be

transferred to a 24-well plate and plant extracts will be added. The number of mortality will be observed and counted after 6, 12 and 24 hours. The LC_{50} values will be calculated.

The determination of some phytochemical properties treatments are designed as the following figure 1.

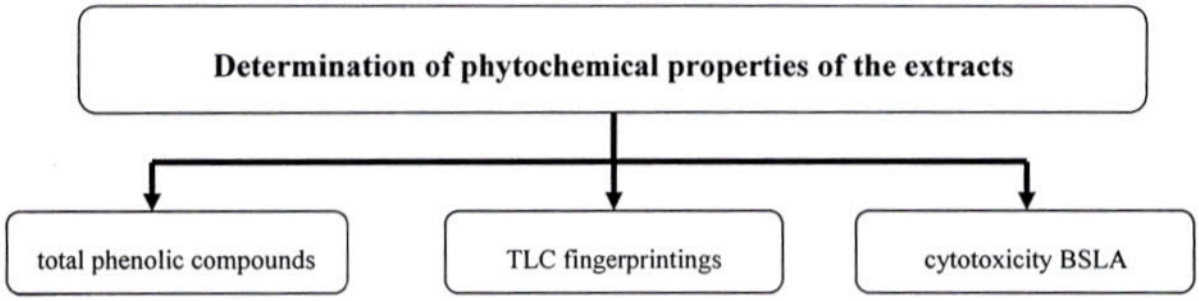

Figure 1. The determination of some phytochemical properties of the extracts of marigold, Siam weed and hedge flower

6.4 Guava fruit fly culture

Pupae of guava fruit fly (*Bactrocera correcta*) will be obtained from the Office of Atomic Energy for Peace, Thailand. Adult flies emerge from the pupa cases in 7 days. Fruit flies will be cultured in wire-net cages, fed with artificial food (Walker et al., 1997), and allowed to mate. The females will be allowed to lay eggs in the egg dome (contain guava juice). After hatching, the larvae feed on artificial food and allow moving to pupa stage in wood chip trays.

6.5 Repellent or attractant tests

The repellent or attractant properties of the individual extracts or the combination (1:1, 3:1, 1:3) of the three extracts will be tested in an olfactometer (Fig.2), consisting of a-75 cm long plastic tube and 4 cm in diameter, with a-29 mm diameter hole in the middle (Boeke et al., 2004).

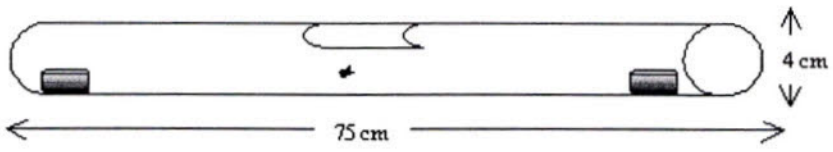

Figure 2. Olfactometer set up for repellent and attractant tests.

At one end of the tube, a 10-ml beaker containing 0-10 g/ml of individual extracts or combination (1:1, 3:1, 1:3) of the three extracts. At another end of the tube, a 10-ml beaker containing 1 ml distilled water without plant extracts or 2 g of finely chopped ripe guava. The treatment is designed as the following table 1.

Table 1 Repellent or attractant tests

Tube End 1 Positive control # 1	Tube End 1 Negative control # 2	Tube End 2
Guava (g)	dH_2O (ml)	Plant Extracts (g/ml)
2	1	0
2	1	2
2	1	4
2	1	6
2	1	8
2	1	10

The hole in the middle will be covered with gauze, whereas the ends of the tube will be covered. Female and male of fruit flies will be introduced through the hole at the middle of the tube. The fly's behavior will be observed for 1-6 hours. All repellent and attractant tests will be repeated for 30 flies (15 females and 15 males).

6.6 Insecticidal activity to adult fruit flies

Thirty adults guava fruit fly will be place into a plastic boxes (100×100×60 mm^3) 10 fly/ box, which the lid is punched to make a hole and covered with steal gauze. The extracts of three plants at the concentrations of 0-10 mg/ml individual or combination (1:1, 3:1, 1:3) will be spray directly on guava fruit fly (รุจนี เล้ารัตนบูรพา, 2523). Mortality of guava fruit flies will be counted after 6, 12, 24 and 48 hours.

6.7 Antibiosis to egg hatching

The antibiosis to egg hatching assay was modified from previous method (วรนาฎ คงตระกูล, 2544). Thirty eggs will be placed on Whatman filter paper disc #3 (42.5 mm in diameter), which will be damped with 0-10 mg/ml individual extracts or combination (1:1, 3:1, 1:3) of the three extracts. The paper will be placed in the artificial food. The eggs will be allowed to hatch into larvae. The number of larvae will be counted within 24 hours.

6.8 Antibiosis to larval growth

6.8.1 Feeding

The antibiosis to larval growth by feeding assay was modified from previous methods (Chuenwong, 2006 and พรพิมล เตชะวัฒนเศรษฐ์, 2542). and Thirty second-instar larvae of guava fruit fly will be cultured in artificial food mixed with individual extracts or combination (1:1, 3:1, 1:3) of the three extracts at the concentrations of 0-10 mg/ml. Mortality of larvae will be counted after 12, 24 and 48 hours.

6.8.2 Dipping

The antibiosis to larval growth by dipping assay was modified from previous methods (Chuenwong, 2006, พรพิมล เตชะวัฒนเศรษฐ์, 2542 and สมบูรณ์ แสงมณีเดช และ

คณะ, 2548). Thirty second-instar larvae of guava fruit fly will be dipped into individual extracts or combination (1:1, 3:1, 1:3) of the three extracts at the concentrations of 0-10 mg/ml for 3 seconds. Treated larvae will be put in a 100-ml bottle, which the lid with a small hole is covered. Mortality of larvae will be counted after 12, 24 and 48 hours.

6.9 Antibiosis to pupation and adult emergence

Thirty third-instar larvae of guava fruit fly will be cultured in artificial food mixed with individual extracts or combination (1:1, 3:1, 1:3) of the three extracts at the concentrations of 1-10 mg/ml. The larvae will be allowed to develop into pupae in the food. Pupae will be counted and then allowed to further molt to adult. The number of molted adult flies will be counted.

The effects of plant extracts on biosis of fruit fly treatments are designed as the following figure 3.

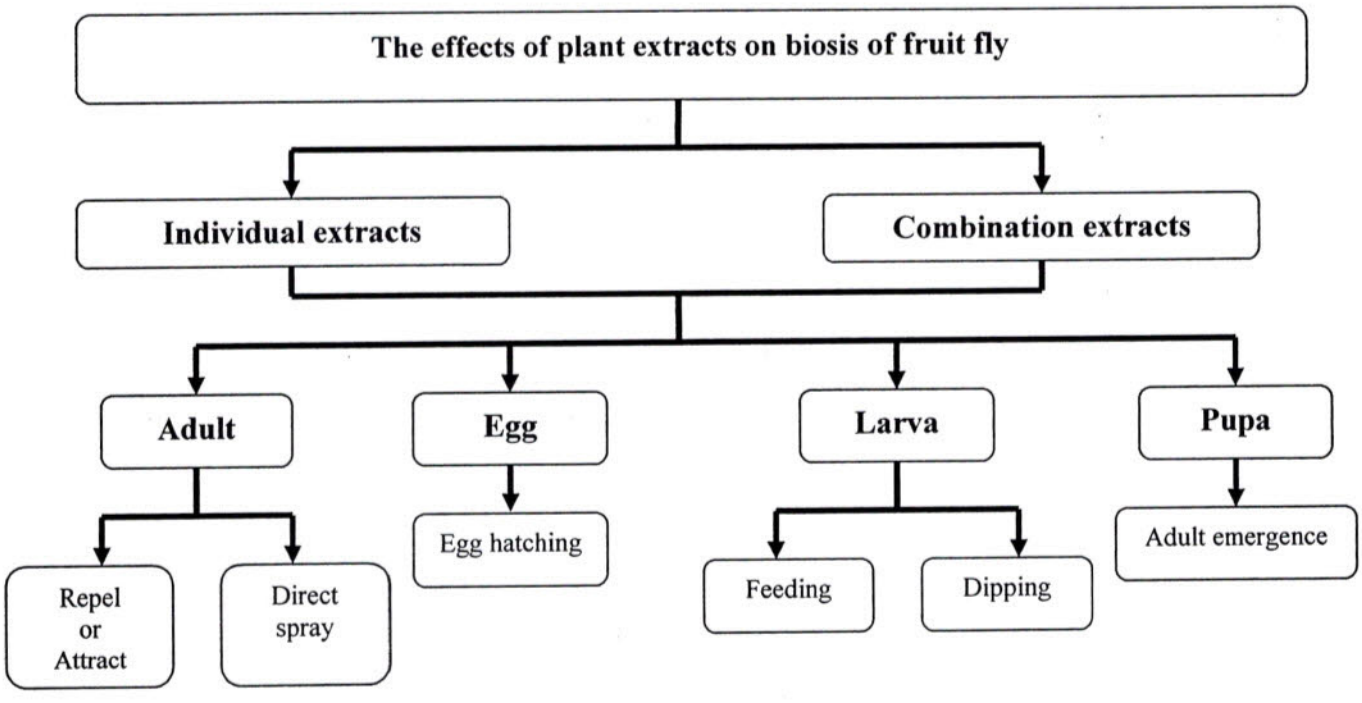

Figure 3. The effects of plant extracts on biosis of fruit fly

6.10 Determination of cytochrome c oxidase

Cytochrome c oxidase activities of guava fruit flies will be measure using the spectrophotometric methods base on the method of Haritos and Dojchinov (2003). The guava fruit flies from the insecticidal test and untreated insects will be used for cytochrome c oxidase activity test. Briefly, mitochondria of guava fruit fly will be isolated. Guava fruit flies will be homogenized on ice cold medium containing bovine serum albumin. The homogenate will be centrifuged, the supernatant will be spreated. The pellet will be resuspended in specify reaction buffer. The cytochrome c will be added. The mitochondria suspension will be added in phosphate buffer and lauryl meltoside. Cytochrome c oxidase from crude extracts will be measured the absorbance at 550/565 nm by spectrophotometric.

6.11 Statistical analysis

The mortality percentage of each treatment will be evaluated by using program Statistical Package for the Social Science (SPSS) for analysis of variance (ANOVA) in type of Completely Randomized Design (CRD) comparing the average by using Least Significant Difference (LSD).

6.12 Instrument

1) Universal extraction apparatus (Buchi: model B-811)
2) Rotary evaporator (Buchi: model R-205)
3) Freezer and lyophilizer
4) Stereomicroscope
5) Fruit fly cages
6) Olfactometer
7) Plastic boxes ($100\times100\times60$ mm^3)
8) 24-well culture plates (cell well Ø 16 mm)

9) UV-VIS-Spectrophotometer

6.13 Location of research

This research will conduct at the Cell and Molecular Laboratory, Building 9, the Center for Scientific and Technological Equipment (CSTE) and Animal building, Suranaree University of Technology.

7. Expected result

This study will develop the biological control of guava fruit flies by the leaves of marigold, Siam weed and hedge flower to which the value is added. The plant-based insecticides can be used to replace synthetic insecticides which is beneficial to human health and sustain the environment.

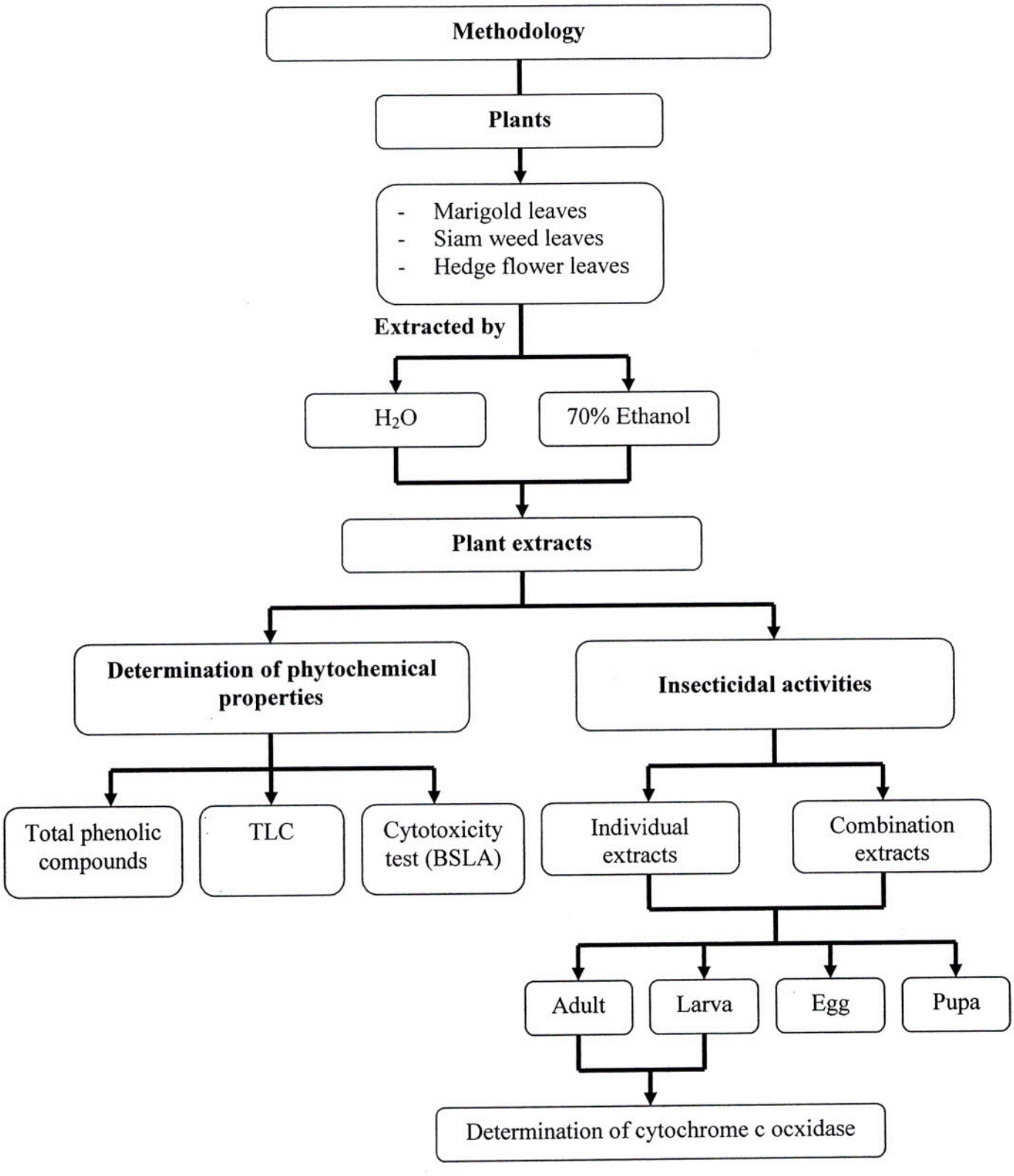

Figure 4. Flow diagram showing research plan

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9. Research plan

	Activities	Period																			
		2009												2010							
		1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8
1	Plant collection																				
2	Plant extract preparation																				
3	Determination of total phenolic compounds availability																				
4	Determination of cytotoxicity of plant extracts																				
5	Thin layer chromatography fingerprintings of plant extracts																				
6	Fruit fly culture																				
7	Repellent or attractant tests																				
8	Insecticidal activity to adult																				
9	Antibiosis to egg laying, larval growth, adult emergence																				
10	Determination of cytochrome c oxidase																				
11	Data analysis and thesis writing																				

Student signature....Parichat Kruainok.....

(Miss Parichat Kruainok)

.....20 / February / 2009.....

Thesis advisor signature.....K. Indrapichate.....

(Assoc.Prof.Dr.Korakod Indrapichate)

.....20 / February / 2009.....

Ph.D. Thesis Proposal

AFFILIATION OF GREENHOUSE GAS FLUX DYNAMIC AND MICROBIAL DISSEMINATION IN WASTEWATER TREATMENT CONSTRUCTED WETLAND MICROCOSM.

ความสัมพันธ์ระหว่างการปล่อยก๊าซเรือนกระจกและจุลินทรีย์ในบึงประดิษฐ์ที่ใช้บำบัดน้ำเสียชุมชน

By
Mrs. Sukanda Chuersuwan

School of Biology
Institute of Science,
Suranaree University of Technology
March 2009

Thesis Proposal

Mrs. Sukanda Chuersuwan **I.D. No. D4910459**

School of Biology **Institute of Science**

Thesis advisors

A) Dr.Pongthep Suwanwaree **Advisor**

1. Thesis title :

AFFILIATION OF GREENHOUSE GAS FLUX DYNAMIC AND MICROBIAL DISSEMINATION IN WASTEWATER TREATMENT CONSTRUCTED WETLAND MICROCOSM

ความสัมพันธ์ระหว่างการปล่อยก๊าซเรือนกระจกและจุลินทรีย์ในบึงประดิษฐ์ที่ใช้บำบัดน้ำเสียชุมชน

2. Introduction

Due in large part to the expectation that climate changes will follow upon an increase in atmospheric concentration of greenhouse gases (e.g. CO_2, CH_4, N_2O, etc.), there is intense interest in the sources and sinks of these gases, and in the strength of their respective emission and consumption (Houghton *et al.,* 2001). Natural sources are investigated to reveal natural fluctuations and magnitudes, while anthropogenic sources are intensively targeted in efforts to cut their emissions and mitigate climate change (Houghton *et al.,* 2001). Natural wetlands, as an significant greenhouse gases sources, contribute to the global balance of the key greenhouse gases. They act as sinks for CO_2 by photosynthetic assimilation from the atmosphere and sequestration of the organic matter produced in the wetland soil. In contrast, wetlands are sources of CH_4 and N_2O (Brix *et al.,* 2001).

Constructed wetlands (CWs) systems are combinations of natural wetlands and conventional wastewater treatment plants and are constructed in order to reduce input of nutrients and organic pollutants to water bodies. Constructed wetland systems, a cost-effective alternative, apply various technological designs, using natural wetland processes, associated with wetland hydrology, soils, microbes and plants. When wetlands are used for purification of wastewater, microbial processes and gas dynamics are likely to be altered.

With increased inputs of nutrients and organic pollutants, the productivity of the ecosystem could increase as well as the production of greenhouse gases, which are by- or end-products of microbial decomposition processes. Constructed wetlands, therefore, can be sources of important greenhouse gases. (Kadlec and Knight, 1996; Mitsch and Gosselink, 2000).

However, there are relatively few studies consider CO_2, N_2O and CH_4 fluxes from wetlands constructed for water quality controlling purposes. Since total area of CWs worldwide is negligible as compare to all natural wetlands and agricultural areas. But the worldwide increase in the development of CWs necessitates an understanding of their potential atmospheric impact in light of the trend that natural wetlands in many countries are decreasing (e.g., Thailand) while environmental regulatory agencies are trying to stimulate an increase in CW acreage. So comprehensive knowledge to clarify the atmospheric impact of such wetlands is an urgent need.

In constructed wetland microcosm, soil–plant is a highly complex environmental system that acts as a reservoir for microorganisms with their activity varying over space and time. Plants release root exudation, which easily decomposable and preferentially used by microorganisms, increased carbon input into the system (Tanner, 2001). Microbial growth in wetlands soil has been believed to depend upon the plant species and substrate. Furthermore, many species of emergent macrophytes in CWs possess a convective flow mechanism; oxygen is transported to the roots and gaseous microbial by-products are emitted from plant roots to the atmosphere (Brix, 1989; Brix *et al.,* 1996). The transport of gases by the convective mechanism is faster than diffusion through water. The presence of plants in constructed wetland system may increase gas emissions from the soil. Therefore, plant species affect on microbial ecology and gas emission from treatment processes.

Wetland gas dynamics are also greatly affected by climatic and weather conditions, especially by temperature and moisture (MacDonald *et al.,* 1998). Rate of photosynthesis (the source of energy and carbon in ecosystems) and microbial activities producing greenhouse gases increase with increasing temperature. Both denitrification and methane formation depend on the oxygen status of the soil or sediment and decomposition rates of organic matter. As a result, the temporal and seasonal variability of fluxes of CO_2 (Liikanen *et al.,* 2006) N_2O and CH_4 (Inamori *et al.,* 2007) are extremely high resulting from variation in the environmental factors regulating the microbial processes behind the gas fluxes. In some seasons wetland can act as a source or sink for C and there can be great differences in the CH_4

(Nyk¨anen *et al.*, 1995) and N_2O fluxes (Huttunen *et al.*, 2002). Therefore, prospective studies are needed to obtain a holistic picture of the gas dynamics of constructed wetlands.

Although constructed wetlands can be beneficial for wastewater treatment they may have an unfavorable environmental impact by increasing the fluxes of greenhouse gases to the atmosphere. Further understanding to cut emission from constructed wetland, magnitude and variation of gas fluxes including influential factors should be extremely explored to provide essential knowledge associated major greenhouse gas emission and the benefit of wastewater treatment.

3. Literature review

3.1 Global warming and greenhouse gases

3.1.1 Global warming and expected effect

Global warming is the increase in the average measured temperature of the Earth's near-surface air and oceans. The average global air temperature near the Earth's surface increased 0.74 ± 0.18 °C during the 100 years ending in 2005 (IPCC, 2007). The Intergovernmental Panel on Climate Change (IPCC) concludes that most of the observed increase in globally averaged temperatures since the mid-twentieth century is very likely due to the observed increase in anthropogenic greenhouse gas concentrations via an enhanced greenhouse effect (IPCC, 2007). Although most studies focus on the period up to 2100, warming is expected to continue for more than a thousand years even if greenhouse gas levels are stabilized.

Increasing global temperature is expected to cause sea levels to rise, an increase in the frequency and intensity of extreme weather events, and significant changes to the amount and pattern of precipitation and increased pace of desertification. Other expected effects of global warming include changes in agricultural yields, glacier retreat, reduced summer streamflows, mass species extinctions and increases in the ranges of disease vectors (IPCC, 2007).

3.1.2 Cause of global warming : greenhouse gases

The detailed causes of the recent warming remain an active field of research, but the scientific consensus is that the increase in atmospheric greenhouse gases due to human activity caused most of the warming observed since the start of the industrial era.

Greenhouse gases are gaseous constituents of the atmosphere, both natural and anthropogenic, that absorb and emit radiation at specific wavelengths within the spectrum of

thermal infrared radiation emitted by the Earth's surface, the atmosphere itself, and by clouds. This property causes the greenhouse effect (IPCC, 2007). Greenhouse gases are essential to maintaining the temperature of the earth; without them the planet would be so cold as to be uninhabitable (Karl *et al.,* 2003). However, an excess of greenhouse gases can raise the temperature of a planet to lethal levels. The most abundant greenhouse gases are, in order of relative abundance: water vapor , carbon dioxide, methane, nitrous oxide, ozone and CFCs. This study focuses on carbon dioxide (CO_2) methane (CH_4) and nitrous oxide (N_2O) since ozone and CFCs are irrelevant to the use of constructed wetland.

Carbon Dioxide

Carbon dioxide (CO_2) is a colorless, odorless non-flammable gas and is the most prominent greenhouse gas in the earth's atmosphere. It is recycled through the atmosphere by photosynthesis, which makes human life possible. Photosynthesis is the process of green plants and other organisms transforming light energy into chemical energy. Light Energy is trapped and used to convert carbon dioxide, water, and other minerals into oxygen and energy rich organic compounds. Carbon dioxide is emitted into the air as humans exhale, burn fossil fuels for energy, and deforest the planet. Since the beginning of the industrial revolution, the concentrations of many of the greenhouse gases have increased. The concentration of CO_2 has increased by about 100 ppm (i.e., from 280 ppm to 380 ppm). The first 50 ppm increase took place in about 200 years, from the start of the industrial revolution to around 1973; the next 50 ppm increase took place in about 33 years, from 1973 to 2006 (Karl, 2003).

Methane

Methane (CH_4) is a colorless, odorless, flammable gas. It is formed when plants decay and where there is very little air. It is often called swamp gas because it is abundant around water and swamps. Bacteria that breakdown organic matter in wetlands and bacteria that are found in cows, sheep, goats, buffalo, termites, and camels produce methane naturally. Since 1750, methane has doubled, and could double again by 2050. Each year anthropogenic sources add 350-500 million tons of methane to the air. It stays in the atmosphere for only 12-17 years, but traps 23 times more heat than carbon dioxide.

Nitrous Oxide

Nitrous oxide is another colorless greenhouse gas; however, it has a sweet odor. It is primarily used as an anesthetic because it deadens pain. Nitrous oxide is emitted

by bacteria in soils and oceans. Agriculture is the main source of human-produced nitrous oxide: cultivating soil, the use of nitrogen fertilizers, and animal waste handling can all stimulate naturally occurring bacteria to produce more nitrous oxide. Nitrous oxide is the main naturally occurring regulator of stratospheric ozone. Considered lifetime over a 100 year period, it has 296 times more impact per unit weight than carbon dioxide. Thus, despite its low concentration, nitrous oxide is a largest contributor to these greenhouse gases. It ranks behind carbon dioxide, and methane. Control of nitrous oxide is part of efforts to curb greenhouse gas emissions. Comparation of GHG is in table 1.

Table 1 Compare characteristics of three major greenhouse gases

Characteristics	**CO_2**	**CH_4**	**N_2O**
Natural source	Respiration	Wetland	Soil, Tropical forest
Anthropogenic source	Deforestation, Fossil fuel combustion	Paddy field, Live stock, Biomass burning	Soil fertilization, Land use activity
Lifetime[a]	50 – 200 yrs.	12 – 17 yrs.	120 yrs.
Concentration[a]	365 ppm	1,750 ppb	310 ppb
GWP [a]	1	23	296
Cause Greenhouse Effect	49% [b]	25% [c]	5% [a]

[a] = *IPCC,2001* [b] = *Lyman ,1990* [c] = *Mosier,1998*

3.2 Global carbon budget

Since approximately 1980, researchers have estimatesd the uptake of carbon by the world's oceans and terrestrial ecosystems at the global level, with an emphasis on terrestrial ecosystems. The world's terrestrial ecosystems were a net source of 40 PgC to the atmosphere over the period 1850–2000. Total emissions to the atmosphere were, thus, 315 PgC (275 from fossil fuels and cement production plus 40 from land), and the airborne fraction, defined relative to total emissions. The flux of carbon from changes in land use depends on the area of land affected, the carbon stocks before and after change, and the rates of decay and recovery following disturbance or management. Over the past 300 years, forests have been replaced with agricultural lands and, thus, the amount of carbon on land has decreased. Although carbon has accumulated on land in some regions (Houghton *et al.,* 1999), the change resulting from direct human activity over the 150-year period from 1850 to 2000 is estimated to have been a release of 156 PgC (Houghton 2003).

Table 2 The global carbon budget for 1850-2000 (units are PgC)

Carbon budget	**1850-2000**
Emissions from fossil fuels and cement production	275
Atmospheric increase	-175
Oceanic uptake	-140
Net terrestrial flux	40
Land-use change	156
Residual terrestrial flux	-116

Source: Houghton, 2007.

3.3 Global methane budget

Wetlands are the most important sources of atmospheric methane as listed in Table 3. Although the major source terms of atmospheric CH_4 have been identified, many of the source strengths are still uncertain due to the difficulty in assessing the global emission rates of the biospheric sources, whose strengths are highly variable in space and time: e.g., local emissions from most types of natural wetland can vary by a few orders of magnitude over a few metres. Nevertheless, new approaches have led to improved estimates of the global emissions rates from some source types. Attempts have been made to deduce emission rates from observed spatial and temporal distributions of atmospheric CH_4 through inverse modelling (e.g., Hein *et al.,* 1997; Houweling *et al.,* 1999). The emissions derived depend on the precise knowledge of the mean global loss rate and represent a relative attribution into aggregated sources of similar properties. The results of some of these studies have been included in Table 3.

The mean global loss rate of atmospheric CH_4 is dominated by its reaction with OH in the troposphere.

$$OH + CH_4 \rightarrow CH_3 + H_2O$$

This loss term can be quantified based on the mean global OH concentration derived from the methyl chloroform (CH_3CCL_3) budget. In addition there are other minor removal processes for atmospheric CH_4. IPCC 2nd assessment report estimates a soil sink of 30 Tg/yr. Minor amounts of CH_4 are also destroyed in the stratosphere by reactions with OH, Cl, and

$O(^1D)$, resulting in a combined loss rate of 40 $Tgyr^{-1}$. Summing these, estimate of the current global loss rate of atmospheric CH_4 totals 576 $Tgyr^{-1}$ (see Table 3).

Table 3 Estimates of the global methane budget (in $Tg(CH_4)/yr$) from different sources.

Reference:	Fung *et al.*, 1991	Hein *et al..*, 1997	Lelieveld *et al.*, 1998	Houweling *et al.*, 1999	Mosier *et al.*, 1998a	Olivier *et al.*, 1999	Cao *et al.*, 1998	IPCC 2nd Assessment report	IPCC 3th Assessment report
Base year:	1980s	-	1992	-	1994	1990	-	1980s	1998
Natural sources									
Wetlands	115	237	225[a]	145			92		
Termites	20	-	20	20					
Ocean	10	-	15	15					
Hydrates	5	-	10	-					
Anthropogenic sources									
Energy	75	97	110	89		109			
Landfills	40	35	40	73		36			
Ruminants	80	90[b]	115	93	80	93[b]			
Waste treatment			25	-	14				
Rice agriculture	100	88		-	25-54	60	53		
Biomass burning	55	40	40	40	34	23			
Other	-	-	-	20	15				
Total source	**500**	**587**	**600**					**597**	**598**
Imbalance (trend)	+40	+52	+20					+37	+22
Sinks									
Soils	10	-	30	30	44			30	30
Tropospheric - OH	450	489	510					490	506
Stratospheric loss	-	46	40					40	40
Total sink	**460**	**535**	**580**					**560**	**576**

IPCC third assessment report budget based on 1,745 ppb, 2.78 Tg/ppb, lifetime of 8.4 yr, and an imbalance of +8 ppb/yr.

[a] *Rice included under wetlands.*

[b] *Waste treatment included under ruminants.*

3.3.1 Methane emission from wetland

Methane is produced microbiologically in anaerobic environments where oxygen and sulfate are scare such as natural wetlands, rice fields, enteric fermentation in animals, termites and landfills. The biogenic methane is mostly produced by methanogenic archaea (methanogens) in anaerobic environments. Microorganism can also remove methane from the environment through aerobic (Rudd and Taylor, 1980) and anaerobic (Alperin and Reeburgh, 1984) oxidation by methanotrophs.

Production of methane

In anaerobic habitats, organic carbon is converted to CH_4 and CO_2 by an anaerobic microbial food chain that includes fermentative, acetogenic, and methanogenic bacteria. The methanogens are the terminal bacteria in this food chain. In anaerobic condition, methane diffusion to the atmosphere is slow because a significant fraction may be lost through aerobic and anaerobic oxidation before it leaves the sediment. Although the anaerobic oxidation of methane has been well documented in sulfate-containing sediments and anoxic waters (Cicerone and Oremland, 1988), little is known about organisms that carry out this process (Rogers and Whitman, 19991). In contrast, much is known about the organisms that oxidize methane in aerobic environments. A substantial part of the gas that diffuses into the aerobic zone is metabolized by organisms, such as the methanotrophic bacteria, that are typically present in large numbers in or at the periphery of anaerobic zones. Methanotrophs can obtain all of their carbon and energy from CH_4 under aerobic conditions. For example, 85% of the methane produced in deep sediments of freshwater lakes may be consumed by methanothrophs in the overlying water column before it reaches the surface. This following is an overview of the underlying microbial basis for production, oxidation and emission of methane in natural wetland. (Characterization of methanogens and methanotrophs are also described).

Methanogens

Methanogens are strictly anaerobic unicellular organisms originally thought to be bacteria but now recognized as belonging to a separate phylogenetic domain, the *Archae* (Garcia, 1990). Phenotypic characteristics of methanogenic bacteria are listed in Table 4.

Table 4 Characteristics of methanogenic and methanotrophic bacteria

Characteristics	Methanogens	Methanotrophs
Cell form	rods, cocci, spirilla, filamentous, sarcina	rods, cocci, vibrios
Gram stain reaction	Gram +/–	Gram –
Classification	Archaebacteria	Eubacteria
Metabolism	Anaerobic	Aerobic
Energy and carbon source	H_2+CO_2; H_2+methanol; formate; methylamines; methanol, acetate	methane; methanol; dimethylether, methylformate, dimethylcarbonate
Catabolic products	CH_4 or CH_4+CO_2	CO_2
Typical species	*Methanobacterium bryanthii* *Methanobrevibacter smithii* *Methanomicrobium mobile* *Methanogenium cariaci*	*Methylosinus trichosporium* *Methylomonas methanica* *Methylocystis minimus* *Methylobacter albus*

Source: Dubey, 2005.

Methanogens can be categorized under three groups. Group I comprises of *Methanobacterium* and *Methanobrevibacter,* Group II contains *Methanococcus,* and Group III comprises of the genera including *Methanospirillum* and *Methanosarcina* (Garcia, 1990). They proliferate in anaerobic fresh water environments, such as sediments and the digestive tract of animals (Topp and Pattey, 1997). In these habitats, methanogens play an important role in the degradation of complex organic compounds. Methanogens mainly use acetate (contributes about 80% to CH_4 production) as a carbon substrate but other substrate like H_2/CO_2 and formats also contribute 10-30% to CH_4 production (Dubey, 2005).

Methanogenesis

Methane is produced in the anaerobic layers of soil by bacterial decomposition of organic matter. The organic matter converted to CH_4 is derived mainly from plant-borne material, and organic manure. The anaerobic degradation of organic matter involves four main steps: a) hydrolysis of polymers by hydrolytic organisms, b) acid formation from simple organic compound by fermentative bacteria, c) acetate formation from metabolites of fermentations by homoacetogenic or syntrophic bacteria, and d) CH_4 formation from H_2/CO_2, acetate, simple methylated compounds or alcohols and CO_2 as shown in Figure 1 and Table 5.

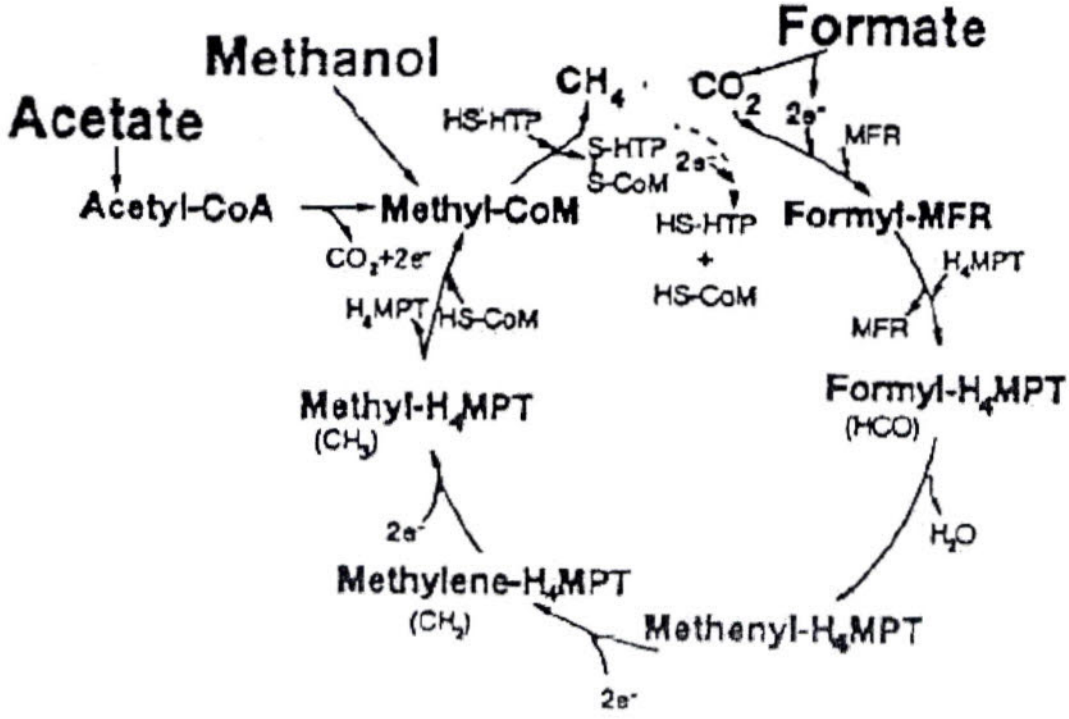

Figure 1 Generalized pathway for methane production from CO_2, acetate, methanol, and formate. Abbreviations: CoM, coenzyme M; H4MPT, tetrahydromethanopterin; MFR, methanofuran; HS-HTP, 7-mercaptoheptanoyltheronine phosphate (Jones,1991).

Table 5 Substrates and energetics of methane production

Reactions		ΔG_0' (kJ/mol of methane)[a]
Hydrogenotrophic reactions		
$4H_2 + CO_2$ ⟶	$CH_4 + 2H_2O$	-135.6
4 Formate ⟶	$CH_4 + 3CO_2 + 2H_2O$	-130.1
4(2-proponal)+CO_2 ⟶	CH_4 + 4 acetone + H_2O	-36.5
Aceticlastic reaction		
Acetate ⟶	$CH_4 + CO_2$	-31.0
Disproportionation reactions		
4-Methanol ⟶	$3CH_4 + CO_2 + 2H_2O$	-104.9
4 Methylamine +$3H_2O$ ⟶	$3CH_4 + CO_2 + 4NH_4^+$	-75.0
2 Dimethyl sulfide + $2H_2O$ ⟶	$3CH_4 + CO_2 + H_2S$	-73.8

Source: Jones, 1991.

Methanotrophs

Methanotrophs (gram negative, aerobic bacteria belonging to the subset of a physiological group of bacteria known as methylotrophs) oxidize CH_4 via methanemono oxygenase (MMO) enzyme. These bacteria are classified into three groups: Type-I, Type-II and Type-X. According to Conrad (1999) all the methanotrophs that have been isolated and described belong to the Proteobacteria, of the γ sub-class (Type I) or α sub-class (Type II). The Type I group is represented by the *Methylomonas, Methylocaldum, Methylosphaera, Methylomicrobium* and *Methylobacter*. The Type-II comprises of *Methylosystis* and *Methylosinus*. The members of the genus *Methylococcus* occupy an intermediate position and have been kept into a separate group Type-X (Hanson and Hanson, 1996). By using molecular ecology techniques, it has become clear that methanotrophs are ubiquitous in nature and well adopted to high or low temperature, pH and salanity. Methanotrophic bacteria are present in the aerobic soil layer, rhizosphere and on the roots and stem bases of flooded plants (Watanabe *et al.*, 1997).

3.3.2 Pathways of methane emission

The net amount of CH_4 emitted from soil to the atmosphere is the balance of two opposite processes - production and oxidation. Methane, the product of methanogenesis, escapes to the atmosphere from soil via aerobic interfaces where CH_4 oxidation takes place. There are three pathways of CH_4-transport into the atmosphere – molecular diffusion, ebullition and plant transport (Fig. 2). Diffusion is not the only mechanism for release of trace gases from anaerobic environments to the atmosphere. Ebullition is also the common and significant mechanism of CH_4 flux in natural wetlands (Wassmann and Martius, 1997). Aquatic plants also can provide and important pathway for the transfer of gases between anaerobic environments and the atmosphere. Gas can move from the root zone up through the stems into the atmosphere or from the atmosphere into the root zone .

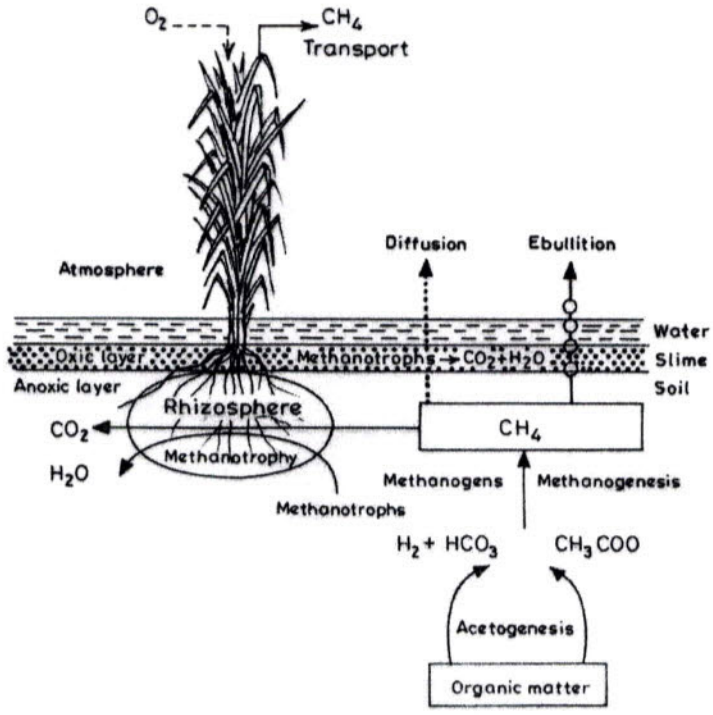

Figure 2 Primary modes of gas transfer to the atmosphere from aquatic environments (Dubey, 2005).

3.4 Global nitrous oxide budget

Agricultural soils and wet forest are important sources of N_2O as listed in Table 6 with estimates of their emission rates and ranges. As with CH_4, N_2O remains difficult to assess global emission rates from individual sources that vary greatly over small spatial and temporal scales. The study calculated values for agricultural N_2O emissions that include the full impact of agriculture on the global nitrogen cycle and showed that N_2O emissions from soils are the largest term in the budget. Emissions from other anthropogenic and natural sources to calculate a total emission is 17.7 $TgNyr^{-1}$ for 1994 (Table 6).

The identified sinks for N_2O are photodissociation (90%) and reaction with electronically excited oxygen atoms ($O(^1D)$); they occur in the stratosphere and lead to an atmospheric lifetime of 120 years. The small uptake of N_2O by soils is not included in this lifetime, but is rather incorporated into the net emission of N_2O from soils because it is coupled to the overall N-partitioning (IPCC, 2001).

Table 6 Estimates of the global nitrous oxide budget (in TgN/yr) from different sources.

Reference:	Mosier *et al.*, 1998b Kroeze *et al.*, 1999		Olivier *et al.*, 1998		SAR	TAR
Base year:	1994	range	1990	range	1980s	1990s
Sources						
Ocean	3.0	1-5	3.6	2.8-5.7	3	
Atmosphere (NH_3 oxidation)	0.6	0.3 -1.2	0.6	0.3 -1.2		
Tropical soils						
Wet forest	3.0	2.2 -3.7			3	
Dry savannas	1.0	0.5 -2.0			1	
Temperate soils						
Forests	1.0	0.1 -2.0			1	
Grasslands	1.0	0.5 -2.0			1	
All soils			6.6	3.3 -9.9		
Natural sub-total	9.6	4.6 -15.9	10.8	6.4 -16.8	9	
Agricultural soils	4.2	0.6 -14.8	1.9	0.7 -4.3	3.5	
Biomass burning	0.5	0.2 -1.0	0.5	0.2 -0.8	0.5	
Industrial sources	1.3	0.7 -1.8	0.7	0.2 -1.1	1.3	
Cattle and feedlots	2.1	0.6 -3.1	1.0	0.2 -2.0	0.4	
Anthropogenic Sub-total	8.1	2.1 -20.7	4.1	1.3 -7.7	5.7	6.9[a]
Total sources	**17.7**	**6.7 -36.6**	**14.9**	**7.7 -24.5**	**14.7**[b]	
Imbalance (trend)	3.9	3.1 -4.7			3.9	3.8
Total sinks (stratospheric)	**12.3**	**9 -16**			**12.3**	**12.6**
Implied total source	16.2				16.2	16.4

[a] *SRES 2000 anthropogenic N_2O emissions.*

[b] *N.B. total sources do not equal sink + imbalance.*

3.4.1 Nitrous oxide emission

The formation of nitrous oxide results from the inefficient conversion of ammonium ion to nitrate or nitrate to molecular nitrogen. Denitrification has been considered the principal source of nitrous oxide to the atmosphere. Nitrification, however, also contributes a significant amount of nitrous oxide to the atmosphere.

Denitirification

Denitrification is a microbially facilitated process of dissimilatory nitrate reduction that may ultimately produce molecular nitrogen (N_2) through a series of intermediate gaseous nitrogen oxide products. This respiratory process reduces oxidized forms of nitrogen in response to the oxidation of an electron donor such as organic matter. The preferred nitrogen electron acceptors in order of most to least thermodynamically favourable include: nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O). In terms of the general nitrogen cycle, denitrification performs the opposite function of nitrogen fixation, which converts gaseous nitrogen into a more oxidised and biologically available form. The process is performed primarily by heterotrophic bacteria (such as *Paracoccus denitrificans* and various pseudomonads), although autotrophic denitrifiers have also been identified (e.g.,*Thiobacillus denitrificans*). Denitrifiers are represented in all main proteolytic groups. Generally several species of bacteria are involved in the complete reduction of nitrate to molecular nitrogen, and more than one enzymatic pathway have been identified in the reduction process.

Denitrification takes place under special conditions in both terrestrial and marine ecosystems. In general, it occurs where oxygen, a more energetically favourable electron acceptor, is depleted, and bacteria respire nitrate as a substitute terminal electron acceptor. Due to the high concentration of oxygen in our atmosphere, denitrification only takes place in environments where oxygen consumption exceeds the rate of oxygen supply, such as in some soils and groundwater, wetlands, poorly ventilated corners of the ocean, and in seafloor sediments. Denitrification generally proceeds through some combination of the following intermediate forms:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \text{ gas}$$

Denitrification is the second step in the nitrification-denitrification process, the conventional way to remove nitrogen from sewage and municipal wastewater. It is also an instrumental process in wetlands and riparian zones for the removal of excess nitrate from groundwater with excess nitrate levels, commonly by extensive agricultural or residential fertilizer usage.

Nitirification

Nitrification is the biological oxidation of ammonia with oxygen into nitrite followed by the oxidation of these nitrites into nitrates. The nitrification process is primarily

accomplished by two groups of autotrophic nitrifying bacteria that can build organic molecules using energy obtained from inorganic sources, in this case ammonia or nitrite.

In the first step of nitrification, ammonia-oxidizing bacteria oxidize ammonia to nitrite according to equation (1).

$$NH_3 \rightarrow NH_2OH \xrightarrow{N_2O} NO_2^- \qquad (1)$$

The oxidation of ammonia into nitrite is performed by two groups of organisms, ammonia oxidizing bacteria and ammonia oxidizing archaea. Ammonia oxidizing bacteria can be found among the β- and γ-proteobacteria (Purkhold *et al.,* 2000). In soils the most studied ammonia oxidizing bacteria belong to the genera *Nitrosomonas* and *Nitrosococcus*. Although in soils ammonia oxidation occurs by both bacteria and archaea in harsher environments like oceans ammonia oxidation is dominated by *Archaea* (Treusch *et al.,* 2005). Recent works have shown that certain *Archaea* can also oxidize ammonium to nitrite with a metabolism similar to that of bacterial ammonium (Konneke *et al.,* 2005).

The second stage is nitrite oxidation to nitrate, with nitric oxide acting as an intermediate and possible precursor of N_2O, according to equation (2).

$$NO_2^- \xrightarrow{N_2O} NO_3^- \qquad (2)$$

Nitrobacter is the most frequently identified genus associated with this second step, although other genera, including *Nitrospina, Nitrococcus*, and *Nitrospira* can also autotrophically oxidize nitrite (Watson *et al.,*1981).

Both steps are producing energy to be coupled to ATP synthesis. Nitrifying organisms are chemoautotrophs, and use carbon dioxide as their carbon source for growth. Nitrification also plays an important role in the removal of nitrogen from municipal wastewater. For many years, denitrification was thought to be the only source of N_2O. However, it is now well recognized that N_2O can be produced during nitrification. Production of $NO_2^-$and NO_3^- from NH_4^+ via nitrification can result from a number of different pathways (Firestone and Davidson, 1989). Chemoautotrophic nitrifying bacteria can obtain energy from the oxidation of NH_4^+ to NO_2^- and NO_3^-. The most thoroughly investigated of these bacteria are the genera *Nitrosomonas* and *Nitrobacter*. A second important group are the heterotrophic nitrifying bacteria that oxidize ammonium ion at the expense of a carbon substrate. Various groups of heterotrophic bacteria and fungi can also carry out nitrification, although at as lower

rate than autotrophic organisms (Watson *et al.,* 1981). Chemoautotrophic nitrifying bacteria have specific activities 10^2 to 10^3 greater than heterotrophic nitrifying bacteria; however, heterotrophs vastly outnumber chemo- autotrophs in the environment.

3.5 Diurnal and seasonal variations of greenhouse gas fluxes

3.5.1 Diurnal variations

Diurnal variation of greenhouse gases are different pattern governed by several factors as described below.

CH_4 emission

Emission rates of CH_4 generally increase after sunrise, reach a peak in the early afternoon then decline at night. Grünfeld and Brix (1999) measured methane emission from *Phragmite australis* in Denmark. They found that rates of CH_4 emission from *P. australis* peaked at midday and were 50–150% higher than the relatively constant rates observed in the morning and during the night. Peaks in emission rates were associated with high solar illumination, high air temperatures and low humidity, factors that are known to stimulate pressurized convective flow in *P. australis* (Brix *et al.,* 1996). Whereas, Yang and Chang (1999) investigated diurnal methane emission from paddy field in Taiwan and found that methane emission rate was high from 12 a.m. to 3 p.m., and low from 2 to 5 a.m. Methane emission showed high correlation coefficient with air temperature.

Whiting and Chanton (1996) studied diurnal pattern of methane emission from swamp in the coastal plain of southeastern Virginia, United States. The wetland was covered by two plants are *Typha lafifolia* and *Peltandra virginica*. The study showed methane emission from *T. lafifolia* displayed a transient peak between 10.00 and 11.00 h. This peak was over 50% greater than emission rates determined on either side of this peak period and associated with rising light levels. This pattern of emission was similar to *T. latifolia and T. domingensis* emissions measured in the Everglades of Florida where emissions peaked about 400% above adjacent base emissions (Chanton *et al.,* 1993). While emission from *P. virginica* revealed a gradual rise throughout the daytime with a peak of emissions during the mid-afternoon (15.00 h). This pattern corresponded to rising air temperatures throughout the morning and midday with a maximum in the mid-afternoon. Corresponding to Wang and Han (2005) studied diurnal variation in methane emissions from marshes of the Xilin River basin in the eastern Inner Mongolia Plateau. Riparian marshes mainly covered with *Carex* spp., *Juncus*

spp., *Glyceria* spp., and *Scirpus* spp. CH_4 emissions increased with sunrise and decreased with sunset. The highest flux rates appeared in late afternoon about 12–15 h after sunrise.

N_2O and CO_2 emission

Du *et al.,* (2006) studied diurnal variation of N_2O fluxes from semi-arid temperate native *Leymus chinensis* grassland of inner Mongolia. The peak of N_2O flux commonly appeared during daytime, whereas fluxes were low at night, and the timing of the peak flux varied in different growing periods. In addition, Maljanen *et al.,* (2002) reported variation of N_2O fluxes from cultivated and forest soil in Eastern Finland. The dominant plant comprise of grass (a mixture of *Phelum pretense, Festuca pratensis* and *Trifolium pretense*) and barley. They found a strong diurnal variation in N_2O and CO_2 fluxes from cultivated and forest soil. The maximum N_2O emission from agricultural soil took place during daytime 10:00-16:00 but, in forest soil, the maximum N_2O emission occurred in early morning. Whereas, the maximum CO_2 emission from agricultural and forest soil took place during daytime 10:00-16:00.

Jun *et al.,* (2008) studied CO_2 efflux on subalpine meadows of Shangri-La, Northwest Yunnan Province, China. The dominant grass species were *Blysmus sinocompressus* and *Kobresia setchwanensis*. They revealed that, in summer, highest rates of both ecosystem respiration and soil respiration occurred at 14:00 while the lowest rates occurred at 6:00 and 8:00. In winter, the highest rates also occurred at 14:00 while the lowest rates occurred at 2:00 and 6:00. The highest values were more than twice the lowest.

Bolpagni *et al.,* (2007) investigated CO_2 fluxes across the water–atmosphere interface in a shallow oxbow lake colonized by the water chestnut (*Trapa natans* L.) (Lanca di Po, Northern Italy). They found that the water chestnut stand was a net sink of CO_2 during the day-light period but it was a net source at night

3.5.2 Seasonal variation

Several studies indicated that greenhouse gases in constructed wetland ecosystems showed seasonal variation. As Sovik *et al.,* (2006) studied emission of the nitrous oxide and methane from constructed wetlands in Estonia, Finland, Norway, and Poland during winter and summer in horizontal and vertical subsurface flow (HSSF and VSSF), free surface water (FSW) wetlands. They reported that emissions of N_2O, CO_2 and CH_4 were significantly higher during summer season than during winter season. Contrast to Sovik and Klove (2007) investigated emission of N_2O and CH_4 from a free surface water wetland polishing chemically

treated municipal wastewater in southeastern Norway and consists of three ponds as well as trickling, unsaturated filters with light weight aggregates. They revealed that flux of N_2O has a significant difference between the summer, winter and autumn, with the highest emissions occurring during the autumn. The fluxes of CH_4 were, on the other hand, not significantly different with regard to seasons. Both the emissions of N_2O and CH_4 were positively influenced by the amount of total organic carbon (TOC).

Inamori *et al.,* (2008) studied seasonal N_2O emission from sub-surface flow constructed wetland treating artificial domestic wastewater, established in the National Institute for Environmental Studies of Tsukuba, Japan. The treatment cells (monoculture) were planted to *Phragmites australis*, *Typha latifolia* and *Zizania latifolia*. They revealed that N_2O fluxes showed significant differences with seasonal fluctuations. The emission peak appeared in growth seasons (July–September) and the N_2O amount was much higher with variation of years.

Liikanen *et al.,* (2006) investigated CO_2, CH_4, and N_2O from constructed surface and subsurface flow wetland treating peat mining area situated in Northern Finland. The dominant vegetation species were *Menyanthes trifoliata, Carex lasio carpa* and *Potentilla palustris*. They found that CH_4 fluxes were smallest in winter and highest in autumn. The N_2O fluxes were high in spring and summer, but negligible in autumn and winter. For CO_2, release of CO_2 varied from a wintertime minimum to summertime maximum.

3.6 Factors affecting greenhouse gases emission

Greenhouse gas emission from constructed wetland is controlled by a complex set of parameters such as temperature, plant, soil redox state etc. The emission of CH_4, CO_2, and N_2O from wetland systems could be explained more precisely by analyzing the methanogenic and methanotrophic microbial populations. On the other hand, the activities of these microbial populations were reported to be influenced by many factors such as temperature, water quality, pH, water table level, and redox state of the rhizosphere (Kang *et al.,* 1998; Le Mer and Roger, 2001; Yang and Chang, 1998).

Soil pH, redox potential and texture

Methane production in flooded soils is very sensitive to pH with an optimum range between 6.7 and 7.1 (Wang *et al.,* 1993). Yagi and Minami (1990) reported that values of redox potential (Eh) varied from -100 to -200 mV for the initiation of CH_4 production in paddy soils. All researches found the range of oxidation reduction potential (ORP), that favored both N_2O and CH_4 generation, were lower than -100 mV. Some suggested that soils

containing greater amounts of readily decomposable organic substrates (acetate, formate, methanol, methylated amines, etc.) and low amounts of electron acceptors ($Fe3^+$, $Mn4^+$, $NO3^-$, SO_4^{2-}) are likely to show high production of CH_4. According to sequential oxidation - reduction order, molecular O_2 is the first to be reduced at an Eh of about +30 mV followed by NO_3^- and Mn_4^+ at +250 mV, Fe_3^+ at +125 mV and SO_4^{2-} at -150 mV (Patrick, 1981). Subsequent to SO_4^{2-} reduction, methanogens will start producing methane. As texture determines various physico-chemical properties of soil, it could influence CH_4 production indirectly. Jackel *et al.,* (2001) found that rates of CH_4 production increased when the aggregate size of the soil increased.

Temperature

Methane emission is much more responsive to temperature. Seiler (1984) reported that methane production increased twice when temperature rose from 20° to 25°C. Sass *et al.,* (1991) found that methane production peaks when soil temperature reaches at 37° C. Temperature not only has an effect on methane production itself but also has an effect on the decomposition of organic materials from which the methanogenic substrates are produced. The influence of temperature on CH_4 production rates has been reported for several ecosystems. In constructed wetland, seasonal shift strongly related to changes in the surface soil, sediment and water temperature (Johansson, 2004; Picek *et al.,* 2007). Air temperature is another parameter that affects net CH_4 flux, by influencing the CH_4-oxidizing and CH_4-producing microbial community and its level of activity (Moore, 1993). Activity of methanogens has commonly been found to fluctuate in response to temperature (Schultz *et al.,* 1989). Grunfeld and Brix (1999) recorded the highest rates of gas exchange through the plant component during hot and dry summer days, which was related to the effect of solar radiation on the convective gas flow. The flow rates through the efflux culms were significantly correlated with solar radiation. However, the degree of variation explained by this relationship was fairly limited (Picek *et al.,* 2007). For N_2O, nitrification reaction rate depends on temperature and the optimum temperature for nitrifiers' activity is approximately 30 °C (Thornley, 1998). Liiakanen *et al.,* (1984) reported that CO_2 emission from constructed wetland had a strong correlation with soil and air temperatures, but CH_4 and N_2O fluxes had weak correlations with surface soil or air temperatures.

Water pollutant

Both the emissions of N_2O and CH_4 were positively influenced by the amount of total organic carbon (TOC) (Sovik and Klove, 2007) and BOD concentration (Sovik *et al.,* 2006; Inamori *et al.,* 2007)). Since, Supply of organic matter is essential for CH_4 production, even though only a small portion of the organic substrate pool is directly utilized by the methanogens, mainly acetate and CO_2 (Oremland, 1988). Comparable to Wang *et al.,* (2008) revealed that difference in emission intensity varied with influent pollutants concentrations. Thus, substrate supply and degree of oxidation are the principal controls on CH_4 and N_2O fluxes from all soils. For N_2O, Sovik *et al.,* (2006) indicated that wetlands receiving water with high concentrations of total N (i.e., the wetlands receiving municipal wastewater) are also the wetlands with highest emissions of N_2O. Probably both the nitrification and the denitrification processes are causing the high emission rates from constructed wetlands. Nitrification has been found to release N_2O to a large degree in microaerobic conditions, whereas high loading rates of wastewater may give partial anaerobic conditions where nitrate may be reduced to N_2O and N_2 gases.

Plant

The variations among the methane flux rates depend on plant type 31–88% (Johansson, 2004). As Wang *et al.,* (2008) showed that CH_4 flux properties, activities of methanogens and methanotrophs and the relationship between CH_4 flux rate and some environmental parameters were greatly different in different plant species within constructed wetland systems. It is assumed that aquatic plant oxygen release enhanced CH_4 generation. The capabilities of oxygen transportation and carbon accumulation were affected by different aquatic plant species. In addition, rhizosphere structure of wetland aquatic plants had a large effect on the microbial ecology. Inamori *et al.,* (2007) found that there was very different rhizosphere structure for the *Zizania latifolia* versus *Phragmites australis* systems. The root of *Z. latifolia* is shallow, and 90% of the root biomass is concentrated in the upper 10 cm of the experimental unit. Conversely, the root of *P. australis* is deeper and the root biomass more evenly distributed from near the soil surface to the bottom of the rhizosphere. The shallow root of *Z. latifolia* confines oxygen's availability and the activity of methanotrophs in the upper portion of the soil, while the root of *P. australis* is deeper and can oxidize methane to a greater depth resulting higher CH_4 emission from *Z. latifolia.*

For N_2O, Inamori *et al.,* (2008) revealed that different aquatic plants resulted in different N_2O emission. Since plant root exudates provide a source of reduced carbon,

nitrogen and other nutrients for microorganisms. Acting as a conduit for oxygen transportation into and out of the substratum, the areas of active root growth of different plants species have played important roles in N_2O conversion (Tanner, 2001).

The growth state of aquatic plants rhizosphere may be of importance to control greenhouse gases emission. Nouchi *et al.,* (1994) indicated that the maximum CH_4 emission occurred at the maximum growth phase of plants due to stimulation of methanogenic bacteria by root exudation. Comparable to Wang *et al.,* (2008) revealed that fluxes of N_2O in the growth season were 2-6 fold higher than those of the senescence period. During the maximum growth stage of the vegetation, N mineralization by the roots was accelerated as a result of increased NH_4^+-N and NO_3^-- N concentration in the soil (Li, 1999). Such an increase in mineral N might promote denitrification in addition to autotrophic and heterotrophic nitrification processes (Marzluf, 1997)

Additionally, methane emission was significantly influenced by plant harvest. High methane emission was recorded immediately after harvesting in both wetlands (Zhu *et al.,* 2007). As in wetlands inhabited by plants, vascular transport is most important for the flux of CH_4 (Schütz *et al.,* 1991). The increase in CH_4 flux immediately after plants were cut may have been due to the rapid release of CH_4 retained inside the vascular systems of the stalks

3.7 Constructed wetland

3.7.1 Definition

A constructed wetland is an artificial marsh or swamp, created for anthropogenic discharge such as wastewater, storm water runoff or sewage treatment, and as habitat for wildlife. They have four key components:

- Soil and drainage materials (such as pipes and gravel)
- Water
- Plants (both above and below the water)
- Micro-organisms

Constructed wetlands purify the water that flows through them. Compared to conventional treatment methods, they tend to be simple, inexpensive, and environmentally friendly. Constructed wetlands may be used to treat water from many different sources:

- Sewage (from small communities, individual homes, and businesses)
- Stormwater

- Agricultural wastewater (including livestock waste, runoff, and drainage water)
- Landfill leachate
- Partially treated industrial wastewater
- Drainage water from mines
- Runoff from highways

3.7.2 Constructed wetland types

Constructed wetlands are of two basic types: free water surface-flow and subsurface-flow wetlands. Free water surface-flow constructed wetlands (FWS) move effluent above the soil in a planted marsh or swamp whereas subsurface-flow constructed wetlands (SSF) move effluent through a medium on which plants are rooted. SSF can be further classified as horizontal sub-surface flow (HSSF) and vertical sub-surface flow (VSSF) constructed wetlands which the effluent may move either horizontally, parallel to the surface, or vertically, from the planted layer down through the substrate and out, respectively. These systems admit variations in the construction criteria and may be operated differently according to several specific designs (Table 7).

Table 7 Summary of the characteristics of different constructed wetland types.

Type	Advantages	Disadvantages
FWS	- Combine aerobic and anaerobic conditions - More efficient nitrogen removal than SSF - More suitable in warmer climates - Well suited for small communities - Aesthetic appeal, recreational and environmental education activities	- Large extension of land required for construction - High susceptibility to climate conditions - Potential exposure of water to human contact
HSSF	- Greater treatment surface - High organic consumption rates - Decreased odor production - Decreased insect proliferation - Higher tolerance to climatic conditions	- Limited aeration - Poor potential for nitrification - Large extension of land required
VSSF	- Minimized treatment area than HSSF - Better oxygen transfer, higher nitrification than H-SSF - Suited to small communities where inexpensive land and low cost media are readily available	- Higher construction costs, restrict large spatial development - Less efficient in removal of suspended solids than H-SSF -Often need further polishing in H-SSF

Adapted from Kadlec and Knight, 1996.

3.7.3 General contaminant removal

Physical, chemical, and biological processes are combined in wetlands to remove contaminants from wastewater. Theoretically, treatment of wastewater within a constructed wetland occurs as wastewater passes through the wetland medium and the plant rhizosphere. A thin aerobic film around each root hair is aerobic due to the leakage of oxygen from the rhizomes, roots, and rootlets. Decomposition of organic matter is facilitated by aerobic and anaerobic micro-organisms present. Microbial nitrification and subsequent denitrification release nitrogen as gas to the atmosphere. Phosphorus is coprecipitated with iron, aluminum, and calcium compounds located in the root-bed medium. Suspended solids are filtered out as they settle in the water column in surface flow wetlands or are physically filtered out by the medium within subsurface flow wetland cells. Harmful bacteria and viruses are reduced by filtration and adsorption by biofilms on the rock media in subsurface flow and vertical flow systems. Principal contaminant removal and transformation mechanisms in FWS and SSF constructed wetland are summarized in Table 8.

Table 8 Description of principal contaminant removal and transformation mechanisms in free water surface (FWS) and sub-surface flow (SSF) constructed wetland.

Contaminant	FWS system	SSF system
Organic Material	Bioconversion by aerobic facultative, and anaerobic bacteria on plant and debris surfaces (soluble BOD) Adsorption, filtration, and sedimentation (particulate BOD)	Bioconversion by facultative, and anaerobic bacteria on plant and media surfaces Adsorption, filtration, and sedimentation (particulate BOD)
Trace organics	Volatilization, adsorption, photolysis, biodegradation	Adsorption, biodegradation
Suspended solids	Sedimentation, filtration	Filtration, sedimentation
Nitrogen	Nitrification/denitrification, microbial/plant uptake, volatilization	Nitrification/denitrification, microbial/plant uptake, volatilization
Phosphorous	Sedimentation, soil sorption, plant and microbial uptake	Filtration , sedimentation, media sorption, uptake
Heavy metal	Adsorption of plant and debris surfaces, sedimentation, plant uptake	Adsorption of media surfaces, sedimentation, plant uptake
Pathogens	Natural decay, predation, UV irradiation, sedimentation	Natural decay, sedimentation , predation

Source: Crites and Tchobanoglous, 1998.

3.7.4 Roles of vegetation in constructed wetlands

The presence of vegetation in constructed wetland has been associated with increased final effluent quality and higher nutrient removal rates (Bachand and Horne, 2000; Lin *et al.,* 2002). Plant communities are fundamental for ensuring that many processes involved removing pollutants are effectively carried out by both direct and indirect effects (Gersberg *et al.,* 1986). On one hand, vegetation largely affects the hydrology by controlling the path of water preferentially follows, slowing down the water flow velocity and promoting the sedimentation of particles. On the other hand, plant submerged structures act as physical filters and provide surfaces for the establishment of microbiota. In addition, rhizomes and roots of macrophytes also participate in soil stabilization, reduce the impact of the wind on the water column and minimize the resuspension of solid particles. The contribution of plants to water treatment also involves active metabolic actions that favour microbial activity. Plants contribute to the production of organic carbon through exudates for heterotrophic metabolism (Reed *et al.,* 1995), increase oxygen availability in the sediments, thereby enhancing mineralization and nitrification (Reddy *et al.,* 1989), and promote denitrifiaction by pulling nitrates from the water column into anaerobic zones in the sediment through the root (Martin *et al.,* 2003). The influence of submerged structures in the water column includes an active oxygenation process, which allows microbiota found in natural wetlands to develop.

Vegetation selected for the constructed wetland will be emergent hydrophytic plants suitable for local climatic conditions and tolerant of the concentrations of nutrients, pesticides, and other constituents in the stormwater. Principal plants to be used include cattail, maiden cane, bulrush, and reed. Although natural wetlands typically have a wide diversity of plant life, attempts to reproduce the natural diversity in a constructed wetland have proven unnecessary. Cattails alone or in combination with either reeds or bulrushes will often dominate in an established system. Free floating plants, such as water hyacinth and duckweed, have proven useful in municipal treatment systems; however, they are not to be used in constructed wetlands associated with these requirements due to the need for harvesting. For aesthetics and beautification, one should consider blueflag iris, canna lily, ginger lily, and wildflowers on dikes and other disturbed areas which are outside of maintenance activity areas. Nutrient uptake is not a major consideration in plant selection. The roots and stems in the water column serve as a medium for bacterial growth and serve as a media for filtration and adsorption of solids and enhanced settling. The stems and leaves at or above the water surface provide shade and thus reduce growth of algae. Wetland plants provide for the transfer

of oxygen to and from the submerged parts of the constructed wetland plants. Plants can be planted with a dibble bar, trencher, or a one-row tree planter and should be established on about 3.0 feet centers. The planting depth will vary, depending on species but all roots should be covered with 2-4 inches of soil mixed with available organic matter. Several experimental results showed that pollutant removal efficiencies of constructed wetland systems, which have different emergent plants, are various. The removal efficiencies of major emergent macrophytes can be summarized in Table 9.

Table 9 Comparison of pollutant removal efficiency from different emergent plants

Removal efficiency (%)	*Typha* spp.	*Scirpus* spp.	*Phragmites* spp.	*Canna* spp.	*Vetiveria* spp.
BOD		> 60 [3,4]	85.8 [5]	90.5±4.8 [7]	97-98[8]
COD	68 [1]		94.4 [5]	75.5±7.9 [7]	
T-N	89 [1]	85-97 [3,4]	64 – 86 [5,6]	44.3±5.3 [7]	81-82[8]
NH3-N	98 [2]			56.9±13.4 [7]	
T-P	67 [2]	93-99 [3,4]	17 [5]	56.7±8.2 [7]	
SS	86-92 [1]	60 [3,4]			93-94[8]

[1] Kerdsup, 2000; [2] Koanetsuwan, 2001; [3] Kantawanichkul, 2003; [4] Buddhawong ,1996; [5] Panapawuttikul, 1996; [6] Urbance-Bercic and Bulc, 1995; [7] Saranakomkun, 2005; [8] Wongpankamol, 2005

The results are not readily comparable due to different constructed wetland designs and operations were used. Since variation of removal efficiency of these emergent plants depending on various factors such as type of constructed wetland, flow rate, media, hydraulic retention time, wastewater strength, pollutant loading etc. However, it can be observed that BOD and COD removal efficiencies of *Phragmites* spp., *Canna* spp., *Vetiveria* spp. are relatively high.

Wetland environments may emit considerable amounts of both CH_4 and N_2O, gases formed under the anoxic conditions in the sediments of inundated areas. Many of the wetlands have populations of emergent plants that were either deliberately planted or naturally colonised the area (Kadlec and Knight, 1996). These plants are morphologically adapted to growing in anoxic sediments in various ways, including the development of aerenchymous tissues that supply their roots with oxygen. However, this aerenchyma can also act as conduits for CH_4 and N_2O, thereby increasing the flux strength of these gases from the wetland to the atmosphere. Several researches explored different emergent plants in various constructed

wetland systems emitted fluxes of CO_2 CH_4 and N_2O with temporal and spatial variations. Greenhouse gases fluxes from various emergent macrophytes can be summarized in Table 10.

Table 10 Comparison of greenhouse gas fluxes (mg $m^{-2}d^{-1}$) from FWS and SSF constructed wetland, rice paddy and natural wetland.

Wetland	Location	Plant	CO_2 flux	CH_4 flux	N_2O flux	Reference
CW FWS	Finland	*Menyanthes.trifoliate* *Carex lasio carpa* *Potentilla palustris*	7,270-13,600	140 - 400	0.34 -0.45	Liikanen *et al.*, 2006[1]
	Sweden	*Typha latifolia* *Spirogyra* sp. *Glyceria maxima*		–375 -1739		Johansson *et al.*,2004[2]
	Thailand	*Digitaria. Bicoarnis* *Typha.angustifoli Linn*		64.8-1,817		Kaewkamthong , 2002[3]
	Japan	*Phragmites australis* *Zizania latifolia*		0-1,560	0-3.36	Inamori *et al.*, 2007[4]
	Japan	*Typha latifolia* *Zizania latifolia* *Phragmites australis*		433-2,540 1,621-6,487 1,063-1,697		Wang *et al.*, 2007[4]
	Japan	*Phragmites communis*		0-4		Zhu *et al.*, 2007[3]
CW SSF	Czech Republic	*Phragmites australis*	96-7,416	0-2,232		Picek *et al.*, 2007[5]
	Estonia	*Phragmites australis* *Typha latifolia*	-146-25,200	-0.14-2,093	0.02-62.4	Teiter and Mander, 2005[6]
	Estonia	*Phragmites australis* *Scirpus sylvaticus*	600-2,000	1.4–4.1	1.3–1.4	Mander *et al.*, 2008[6]
Rice paddy	Japan		16,264		0.20-0.31	Raghareutai *et al.*, 2004
	Thailand			14.4-1,020		IPCC, 1995
Natural wetland	USA	*Typha sp.* (Marsh)		0–1700		Schipper and Reddy, 1994
	Australia	*Eleocharis sphacelata* (Floodplain wetland)		4–1056		Boon and Sorrell , 1995
	Germany	*Phragmites australis* (Prairie wetland)		40–650		Kim *et al.*, 1998

[1] constructed wetland purify draining waters from the adjacent peat mining area.
[2] constructed wetland treating municipal wastewater from sewage treatment plant
[3] pilot scale constructed wetland treating domestic wastewater
[4] experimental scale constructed wetland treating non-point sewage at the rural area
[5] horizontal subsurface flow treating combined sewage and stormwater runoff
[6] horizontal subsurface flow purify wastewater from a hospital and hybrid system treated municipal wastewater

The rates of greenhouse gases emission for among constructed wetland, rice paddy and natural wetland, at various climates are tabulated in Table 10. Most studies were conducted in temperate zone rather than tropical climate. The results show highly fluctuating

due to spatial and season variation. However, it can be observed that constructed wetland tend to emit greenhouse gases relatively higher than rice paddy and natural wetland.

Variations of greenhouse gas fluxes from constructed wetlands are influenced by plant species and CW systems. Besides these factors, gaseous fluxes vary by several factors such as seasonal change (Zhu *et al.,* 2007; Liikanen *et al.,* 2006; Gui *et al.,* 2007), operational design, hydraulic retention time (Zhu *et al.,* 2007 ; Kaewkamthong , 2002), plant growth rate and plant biomass (Gui *et al.,* 2007; Liikanen *et al.,* 2006).

4. Research objectives

The objectives of this research are as following;

1) To quantify CO_2, CH_4 and N_2O fluxes from wastewater treatment constructed wetlands,

2) To estimate diurnal and seasonal fluctuations of N_2O, CH_4 and CO_2 fluxes from wastewater treatment constructed wetland,

3) To investigate the effect of plant species on microbial distribution and CO_2, CH_4, N_2O fluxes.

5. Research hypotheses

The hypotheses of this research are:

1) wastewater treatment constructed wetlands are sources of CO_2, CH_4 and N_2O.

2) greenhouse gas fluxes from constructed wetland have diurnal and seasonal fluctuations influenced by several factors such as plant, wastewater characteristics, and some environmental factors; and

3) plant species affect amount and activity of rhizobacterium involved greenhouse gas production and consumption.

6. Scope and limitation of the study

This research will be performed on experimental scale of free water surface flow (FWS) and horizontal sub-surface flow (HSSF) constructed wetlands used to treating artificial domestic wastewater located in Suranaree University of Technology. Different emergent plants will be compared between *Phragmites* spp., *Vetiveria* spp., *Cyperus* spp., *Vetiveria* spp., or *Canna* spp.

7. Research procedure

7.1 Study site and constructed wetlands design

The experiment scale of constructed wetland will be located at a vacant area in Suranaree University of Technology. Three emergent plants will be evaluated. The treatment cells have identical dimensions approximately of 2.0 m × 0.5 m × 0.8 m (length × width × depth). Layer of sand, the medium layer (small gravels) and large gravels will be laid in the bottom of FWS-CW. The wastewater surface is approximate 20 cm higher than the sand layer. The treatment cells (monoculture) will be planted *Phragmites* spp., *Vetiveria* spp., *Cyperus* spp and *Canna* spp. with two replicate of cells. Control cells will carry out of non-vegetation control (CL). Technical parameters of beds show in table 11.

Table 11 Technical parameters of the constructed wetland

	FWS	SF
Length of each bed (m)	2 m	2 m
Width of each bed (m)	0.5 m	0.5 m
L/W ratio	4:1	4:1
Media depth (m)	0.45 m	0.65 m
Water Depth (m)	0.20 m	0.55 m
Average flow (m^3/d)	0.003	0.003

The experiment comprises of 2 stages. First stage is to compare greenhouse gas flux emitted from free-water surface flow (FWS) and sub-surface flow (SF) constructed wetlands. The experiment beds consist of four FWS beds, which are 2 replicates of plant and non-plant (control) and four SF beds, which are also 2 replicates of plant and non-plant (control). The emergent plant for this stage is *Cyperus* spp. The second stage is to investigate the effect of plant species on greenhouse gas emission from free-water surface flow constructed wetlands. Emergent plants for this stage will be *Phragmites* spp. , *Vetiveria* spp. or *Canna* spp.

Artificial wastewater will be used in the experiment. Wastewater with BOD concentration of 200 mg L^{-1} will be prepared and sent to the cells by gravimetric flow and the volume will be controlled by the water level. The flow rate Q for the free water surface (FWS) wetland units are calculated by the following equation

Free water surface flow system

From $HRT = \frac{LW(d_m n + dw)}{Q}$ (Lim and Polprasert, 1996)

where;
- HRT = hydraulic retention time, d
- L = basin length, m
- W = basin width, m
- d_m = media depth, m
- d_w = water depth from media surface, m
- n = void fraction in the media (as a decimal fraction)
- Q = average flow through the unit, m^3/d

Sub-surface flow system

From $HRT = \frac{LWnD}{Q}$ (Metcalf & Eddy,1991)

where;
- HRT = hydraulic retention time, d
- L = basin length, m
- W = basin width, m
- D = depth of basin, m
- n = porosity of the bed
- Q = average flow through the unit, m^3/d

At the beginning of the experiments, the average height of the cultivated are about 1.5 m above the water level. After achieving steady-state conditions, the first run with fourteen simultaneous experiments will be started.

7.2 Tracer study for finding actual retention time

Salt tracer experiment is a cost-effective tool widely used in studies of flow and transport in free surface flows. Chloride represents a useful tracer, since it is relatively inert and not used by biota in a great degree. Thus, actual retention time will be established using the salt tracer based on the difference in the electric conductivity (EC). Breakthrough curves will be measured by electric conductivity probes, and the electric conductivity values obtained are then converted to chloride concentrations by using calibration curves. The concentration versus conductivity relationships proved quite stable, with very little scatter (R^2

≈ 0.999) (Schmid *et al.*, 2004). A salt tracer is injected at an accurately measured constant rate. Monitoring efforts will begin just prior to release of the tracer. Conductivity probe is submerged at 4.5 m (near centerline) and the background conductivity will be monitored. The tracer response curve is displayed during passage and after return to the background conductivity. Then, the procedure is stopped and the response curve can be evaluated.

7.3 Gas fluxes measurement

The gas fluxes will be measured using a static chamber technique. The chambers consist of two parts. The upper part will be constructed from acrylic and made gas-tight by silicon glue. The bottom part is made from aluminum. This frame will be inserted into the gravel substrate 1 day before measurement started and be removed afterwards. During observations, the chamber is placed on top of frame. Two chambers will be installed in the vegetation bed at the entry and exit point of wastewater and another chamber will be installed in non vegetation at the middle of bed. Size of chamber modules is 0.25 m x 0.25 m x 2 m. will be used. Inside the chamber, a small electronic fan will be installed to ensure a thorough heat and gas mixing together with a thermometer and a sampling hole.

The measured CO_2 flux is the total CO_2 release from aerobic and anaerobic decomposition processes, respiration of soil. This flux measured does not include photosynthesis and is not a measure of net CO_2 exchange between the ecosystem and the atmosphere.

Gas flux measurements will be made eight times at 3 hours intervals during one day for diurnal variations study. Seasonal variations of gas fluxes will be determined once a month during 1 year. Samples of the gases will be taken from the chamber for GC analysis. For the analysis, gas from the chamber is pumped into sampling glass vials. Chamber and ambient air temperatures will be measured at every 30-minute, sediment (10 cm below the surface) temperatures, soil ORP, and plant height will be measured at each sampling occasion.

Gas samples will be taken at the top of each chamber with polypropylene syringes equipped with three-way stopcocks. The sample from each cell will be taken three times at 0, 30 and 60 min intervals and collected in evacuated glass vial. All the samples will be analyzed within 24 hours. The CH_4 concentration will be analyzed by means of gas chromatograph equipped with a flame ionization detector (FID). The N_2O concentration will be analyzed by means of gas chromatography equipped with an electron capture detector (ECD). The CO_2 will be analyzed immediately in the field using an infrared gas analyzer. The gas flux will be calculated from the increase/decrease in the chamber gas concentration

over time using the frame surface area as equation 1. If the increase/decrease in the gas concentration is non-linear, the measurement is rejected. The gas fluxes will be calculated from the temporal increase or decrease of the gas mixing ratios inside the box using the equation (Singh *et al.,* 1998)

$$\text{Gas Flux (F) (mg m}^{-2}\text{ h}^{-1}) = \frac{BV_{std} * dC * MW * 1000 * 60}{10^4 * 22400 * A * dt} \quad \text{eq. 1}$$

$$BV_{STd}\text{ (Box Air Volume in cm}^3\text{ at STP)} = \frac{BV * B.P. * 273}{(273+T) * 760}$$

Where BV = Box Air Volume in cm^3

$B.P.$ = barometric pressure (mm Hg)

T = box air temperature at the time of sampling in °C;

MW = molecular weight of gas

dC = change in gas concentration from 0 min sampling to the t min sampling;

dt = sampling period (min)

A = area covered by the box in m^2.

7.4 Water analysis

Daily wastewater samples will be collected from the influent and effluent points, and analyzed for BOD concentration until the steady-state conditions reached. After the steady-state conditions, the parameters to be analyzed in influent and effluent wastewater samples include BOD, TSS, NH_3-N, TP, pH, DO once a month. Details of analyses are given in Table 12.

Table 12 Method of analysis

Parameter	Method
BOD5	Dilution Method
COD	Open Reflex Method
NH_3	Phenate method
TSS	Total Suspended Solids Dried at 103°C
TP	Ascorbic Acid Method
pH	pH Meter
DO	DO meter

Source: Standard Method for Examination of Water and Wastewater (APHA, AWWA and WEF, 2005)

7.5 Sediment sampling and analyses

The litter layer will be removed and the soil sample of soil surface (0-5 cm) and at the depths of 10, 20 and 30 cm below the surface will be samples using a PVC tube. To ensure the representative of soil samples, three replicate tubes per experiment cell will be taken. These samples will be used for chemical analysis and DNA extraction. For chemical analysis, sampled soil in the same cell is mixed together and stored immediately in a cooler with ice. After sieving the soil are stored at -20 °C until processed further. Soil organic matter and Kjeldahl-N are analyzed in all soil samples using the standard methods.

7.6 DNA extraction from soil sampler

DNA will be extracted from soil using SDS-based DNA extraction method as described by Zhou *et al.,* (1996). Sediment samples of 5 g are mixed with 13.5 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% Hexadecylmethylammonium Bromide (CTAB)) and 100 ml of proteinase K (10 mg/ml) (in Oakridge tubes) by horizontal shaking at 225 rpm for 30 min at 37°C. After the shaking, 1.5 ml of 20% sodium dodecyl sulfate (SDS) is added, and the samples are incubated in a 65°C water bath for 2 h with gentle end-over-end inversions every 15 to 20 min. The supernatants are collected after centrifugation at 6,000 x *g* for 10 min at room temperature and transferred into 50-ml centrifuge tubes. The soil pellets are extracted two more times by adding 4.5 ml of the extraction buffer and 0.5 ml of 20% SDS, vortexing for 10 sec, incubating at 65°C for 10 min, and centrifuging as before. Supernatants from the three cycles of extractions are combined and mixed with an equal volume of chloroformisoamyl alcohol (24:1, vol/vol). The aqueous phase is recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 x g for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 500 µl.

7.7 Quantification of microbial population by real-time PCR

In recent years, real-time polymerase chain reaction (RT-PCR) has been emerged as a promising tool for studying soil microbial communities. RT- PCR is a highly sensitive method that can be used for the detection and quantification of anaerobic microbes from environmental samples without cultivating them (Yu *et al.,* 2005; Kandeler *et al.,* 2006). RT-PCR is based on the real-time detection of a reporter molecule whose fluorescence increases

as PCR product accumulates during each amplification cycle. RT-PCR relies on the use of fluorescently labeled selective markers, which allow the continuous quantification of gene copies belonging to a certain group or species during the amplification reaction. The amplification kinetics can be related to the original copy number in the sample by using the appropriate standards.

RT-PCR technique will be used to detect population of microbial (i.e. methanotroph, methanogen, nitrifier bacteria and denitrifier bacteria) in the active layers of wetlands soil. Primer and target gene use in this study are shown in Table 13.

Table 13 Primers used for real-time PCR

Primer	Target gene	Target group	Reference
NirS832F NirS3R	*nirS*	Denitrifiers	Braker *et al.*, 1998 Liu *et al.*, 2003
ME 1 MCR1R	*mcrA*	Methanogens	Springer *et al.*, 1995 Hales *et al.*, 1996
amo/pmof amo/pmor mmof mmor 16Sf 16Sr	*amoA* or *pmoA* *mmoX* *16S rDNA*	Methanotroph	Holmes *et al.*, 1995 McDonald *et al.*, 1995 Lane, 1991
amoA-1F amoA-2R	*amoA*	Ammonia-oxidizing bacteria	Rotthauwe *et al.*, 1997

All samples analyses including gases, soils and water are given in Table 14.

Table 14 Plan of individual experiments

Sample	Parameter	Frequency
Gas	CH_4, N_2O, CO_2	1/month
Water	BOD,COD, TSS, NH_3, TP, pH, DO	1/month
Soil	DNA of soil microbial OM, TKN	2/year (growth & senescence season) 2/year (6^{th} month & end of experiment)

7.8 Statistics

The SPSS statistical package will be used in the statistical analyses of the data. Pearson correlation coefficients (two-tailed significance) are determined for gas fluxes, air and surface soil temperatures. Differences in gas fluxes were tested with *t*-test, and analysis of variance (ANOVA) using Tukey's post hoc test.

8. Expected results

The benefits of this research are:

1) data on magnitude of CO_2, CH_4 and N_2O fluxes from constructed wetland,

2) reveal diurnal and seasonal fluctuations of N_2O, CH_4 and CO_2 fluxes from constructed wetland,

3) explore effect of plants species and seasonal variation on microbial distribution and CO_2, CH_4, N_2O fluxes,

4) obtain greenhouse gases mitigation option from constructed wetland.

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10. Experiments

Activity	2009												2010			
	Jan	Feb	Mar	April	May	June	July	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	April
1. Test equipment	←															
2. Construct wetland experiment cells		←	→													
3. Sampling				←	→	→	→	→	→	→	→	→	→	→		
4. Analyses				←	→	→	→	→	→	→	→	→	→	→		
5. Thesis writing															← →	
6. Defense																← →

Student ..... (Mrs. Sukanda Chuersuwan)

6 / 3 / 2009

Advisor ..... (Dr.Pongthep Suwanwaree)

6 / 3 / 2009

Ph.D. Thesis Proposal

MCM-41-SUPPORTED BIMETALLIC CATALYSTS CONTAINING PLATINUM AND BASE METAL OXIDES FOR ETHANOL OXIDATION

ตัวเร่งปฏิกิริยาโลหะคู่ที่มีแพลทินัมและโลหะออกไซด์ที่เป็นเบสบนตัวรองรับ MCM-41 สำหรับการออกซิเดชันของเอทานอล

By
Miss Kamolwan Rintramee

School of Chemistry
Institute of Science
Suranaree University of Technology
February 2009

Ph.D. Thesis Proposal

Miss Kamolwan Rintramee **ID. No.** M4910060
School of Chemistry Institute of Science
Thesis advisor: Associate Professor Dr. Jatuporn Wittayakun

1. Thesis title

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2. Introduction

This Ph.D. thesis proposal includes preparation of MCM-41 with rice husk silica and utilization as a support for bimetallic catalysts containing platinum (Pt) and base metal oxide notated as $PtMO_x$/MCM-41 where M is copper (Cu), cobalt (Co) or manganese (Mn). Precursors of the bimetallic catalysts will be loaded on MCM-41 by sequential impregnation method. Chemical and physical properties of the MCM-41 support and prepared catalysts will be investigated by several techniques such as X-ray diffraction (XRD), nitrogen adsorption-desorption, and transmission electron microscopy (TEM). In catalysis, the bimetallic catalysts will be tested for oxidation of ethanol (C_2H_5OH) under conditions similar to those of the exhaust gases from vehicles using ethanol as fuel or oxygenated agent for the control of emission of unburned ethanol and aldehyde. In addition, the activities of the $PtMO_x$/MCM-41 catalysts will be compared to those of commercial catalysts. Both reactants and products from the reaction will be analyzed by gas chromatograph (GC). The reaction temperatures that correspond to 50% of ethanol conversion is used as a measure of ethanol oxidation activity and is denoted as $T_{50\%}$.

2.1 Background of ethanol oxidation

The catalytic oxidation is important for elimination of pollutant gases such as CO, aldehyde, and unburned ethanol which are emitted from ethanol-fueled vehicles. These pollutants are considered to be harmful to human health and recognized as contributors to pollution and the photochemical formation of smog (Trawazynski *et al.*, 2005). On the basis of the products formed during the reaction experiments, the oxidation of ethanol over the various catalysts involves one or more reactions in equation 1-3 (Rajesh and Ozkan, 1993). In excess of oxygen, only a complete oxidation is expected but it also depends on reaction conditions such as temperature. Both partial and incomplete oxidation should be minimized because they produce acetaldehyde and CO, respectively,

Complete oxidation	$C_2H_5OH + 3O_2$	$\rightarrow$	$2CO_2 + 3H_2O$	(1)
Partial oxidation	$C_2H_5OH + 1/2O_2$	$\rightarrow$	$CH_3CHO + H_2O$	(2)
Incomplete oxidation	$C_2H_5OH + 2O_2$	$\rightarrow$	$2CO + 3H_2O$	(3)

In general, noble metal catalysts such as Pt, Pd or Rh exhibit high activity for ethanol conversion to CO_2 and water with low acetaldehyde formation. However, they are easily poisoned by undesired reaction intermediates such as surface ethyl from ethanol dehydration which leads to deactivation by coking. Thus, base metal oxides catalysts have been developed for ethanol oxidation because of their resistance to poisoning and lower price than noble metal. For example, $CuO\text{-}MnO_2$ catalyst was reported to be only slightly less active than Pt/Al_2O_3 catalyst for the same volume in the combustion of ethanol (McCabe and Mitchell, 1984). Many researchers have studied a catalyst mixture between a small amount of noble metal and metal oxide to improve catalytic activity and properties of both components because they compensate each other's drawback. The role of metal oxides is to diminish the CO inhibition which is typical for Pt catalysts at low temperatures (Mergler *et al.*, 1996). On the other hand, Pt helps metal oxides with multi-oxidation states to accelerate the oxygen transfer from gas phase to the catalyst (Menezo *et al.*, 1993). For instance, $Pd\text{-}Cu/CeO_2\text{-}Al_2O_3$ catalyst is 50°C lower than $Pd/CeO_2\text{-}Al_2O_3$ catalyst in reaching 50% conversion of CO (Garcia *et al.*, 2000).

Table 1 demonstrates conditions of ethanol oxidation for various catalysts and the testing results. The $T_{x\%}$ is the temperature that gives x % ethanol conversion and the T_{Yx} is the temperature at which x % CO_2 yield are obtained.

Table 1 Conditions for ethanol oxidation on various catalysts and testing results.

Catalysts	Conditions	Conversion	Comment	Reference
0.1 wt%Pt/Al_2O_3	0.1 V% C_2H_5OH : 1V% O_2 in N_2 (1000 ppm ethanol)	$T_{90\%}$~145°C T_{Y90} ~227°C	Pt/Al_2O_3 reduces unburned fuel during engine cold-starting and warmup.	McCabe and Mitchell, 1984
0.3 wt%Pt/Al_2O_3 (K/Al = 0.1)	500 ppm ethanol in air	$T_{100\%}$~ 210°C T_{Y100} ~ 196°C	Addition of K to Al_2O_3 to minimize undesired products.	Avgouropoulos *et al.*, 2006
Commercial hopcalite (CuO-MnO_2)	0.1 V% C_2H_5OH : 1V% O_2 in N_2	$T_{90\%}$~148°C T_{Y90} ~230°C	Hopcalite catalyst has similar activity to Pt/Al_2O_3.	McCabe and Mitchell, 1984
Mn_xCu_yO (x = 9, y = 1; atomic ratio)	C_2H_5OH : O_2 : H_2 = 1:20.8:78.2 (100 ml min^{-1})	$T_{100\%}$~ 192°C T_{Y100} ~ 208°C	Poor crystalline of Mn gives the best performance in ethanol combustion to CO_2.	Morales *et al.*, 2008
Cu/Al_2O_3	0.35 V% C_2H_5OH : 3.83 V% O_2 : 95.82 V% N_2	$T_{95\%}$~ 250°C T_{Y95} ~250°C	Cu favors complete ethanol oxidation.	Rajesh and Ozkan, 1993
Cr/Al_2O_3	0.35 V% C_2H_5OH : 3.83 V% O_2 : 95.82 V% N_2	$T_{95\%}$~ 250°C T_{Y50} ~250°C	Cr favors dehydrogenation or partial oxidation.	Rajesh and Ozkan, 1993
0.5wt% Pd-1wt% Cu/CeO_2-Al_2O_3	1 V% CO : 0.1 V% NO : 0.45 V% O_2 in N_2	$T_{98\%}$~ 174°C	Cu improves activity by enhancing the rate of CO oxidation.	Garcia *et al.*, 2000
0.5 wt% Pd/Co_3O_4-CeO_2	1 V% CO : 5 V% O_2 in N_2	T_{Y100} ~90°C	A synergy effect between Pd and Co_3O_4-CeO_2 is responsible for the activity enhancement.	Luo *et al.*, 2008

From table 1, a number of catalysts have been tested, and the activity of noble metal combined with metal oxide merited attention as catalysts for total oxidation. In this thesis proposal, combination between Pt and metal oxides such as Co, Cu or Mn

will be prepared by dispersing both components on MCM-41 support to achieve complete ethanol oxidation.

The modification of Pt catalyst with Co, Cu and Mn has been reported to improve catalytic activity for the oxidation reaction (Ferrandon, 2001). Manganese oxides were among the most efficient transition-metal compounds in catalytic combustion and they were considered to be environment-friendly materials (Morales *et al.*, 2006). Cobalt oxides could act either as catalysts for total oxidation reactions and as sorbents for sulphur (Pope *et al.*, 1976). Because of similarity to copper oxides, they have been considered as substitutes for noble metal catalysts in emission control (Peiyan *et al.*, 1987).

2.2 Background of MCM -41

MCM-41 is a mesoporous molecular sieve consisting of hexagonal arranged cylindrical uniform channels. It has high surface area, narrow pore size distribution and thermal stability (Kumar *et al.*, 2004). MCM-41 is potentially applicable as a catalyst support because the high surface area can improve a dispersion of metal catalyst resulting in better performance. For instance, when Cu-Mn was deposited on MCM-41 support, the catalytic activity of toluene oxidation was improved more than on β-zeolite and SiO_2 support because high dispersion of Cu-Mn was obtained on the support with high surface area (Li *et al.*, 2006). In general, alumina (Al_2O_3) is widely used as a support for ethanol oxidation, however, it causes undesired by-products as a result of its acidic properties. The MCM-41 may serve as a better choice of support which may be more suitable for ethanol oxidation because it has weaker acidity than Al_2O_3.

2.3 Proposed oxidation mechanism

Figure 1 shows proposed mechanism of ethanol chemisorption on Pt to produce various surface species. Those surface species could dissociate further and react with surface oxygen to produce CO_2 and H_2O. Although some surface species could react with each other to form undesired products, the modification with metal oxides is expected to minimize the by-products.

The role of base metal oxides is as oxygen storage. The oxygen can react with surface species to produce CO_2 and H_2O. The regeneration of the base metal oxide can be done by a reaction with oxygen in the reactant stream as shown in figure 2.

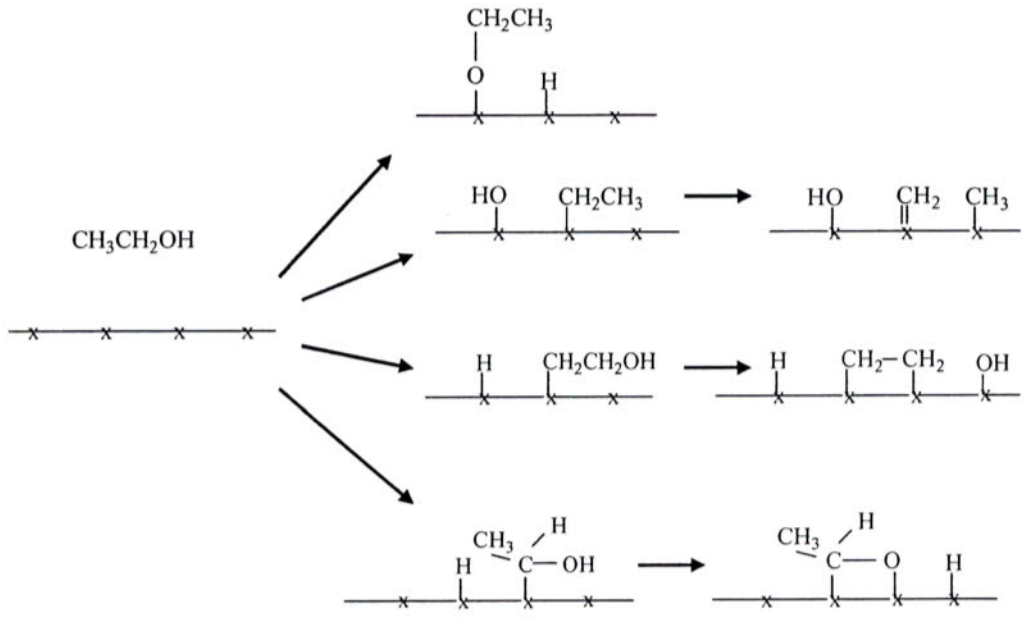

Figure 1 Proposed mechanism of ethanol chemisorption on Pt (Nagal and Gonzalez, 1985).

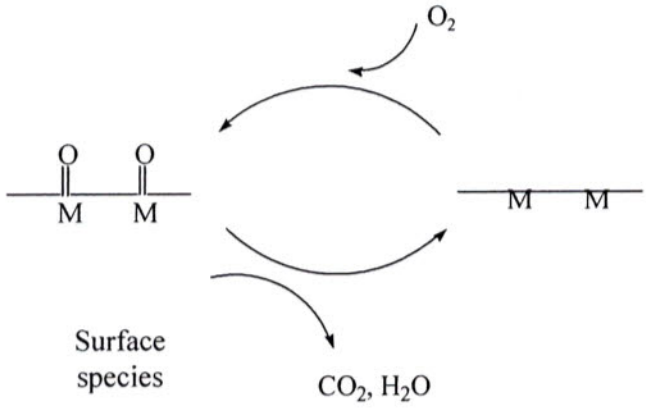

Figure 2 Proposed role of base metal oxide as oxygen storage and its regeneration by oxidation.

3. Research objectives

1. To synthesize and characterize MCM-41 using rice husk silica.
2. To prepare and characterize monometallic and bimetallic catalysts containing platinum and base metal oxides.
3. To test the monometallic and bimetallic catalysts for ethanol oxidation.
4. To study reaction parameters for optimum conditions.
5. To understand reaction pathway.

4. Scope and limitation of the study

1. MCM-41 will be synthesized with rice husk silica from a method in literature with modification (Shylesh and Singh, 2004).
2. Catalysts containing platinum and base metal oxides will be prepared by sequential impregnation, i.e., impregnation of metal oxide precursor follow by Pt precursor. The Pt loading on the catalysts catalyst will fix at 0.1 wt% and the MO_x loading will be varied from 2 to 10 wt%.
3. The catalytic testing on ethanol oxidation will be studied in a fixed bed reactor under conditions similar to those in ethanol-vehicle exhaust and compared to those of commercial silica and alumina-supported catalysts.
4. The reactants and products from reaction will be analyzed by an on-line gas chromatograph (GC).
5. The reaction temperatures that correspond to 50% of ethanol conversion will be used as a measure of ethanol oxidation activity.

5. Research procedures

5.1 Apparatus and Equipments

The apparatus and equipments for the extraction of rice husk silica will be glasswares, condenser, round bottom flasks, a heating mantle, a muffle furnace, and a hot-air oven.

The apparatus and equipments for MCM-41 synthesis will be glasswares, polypropylene bottles, magnetic stirrers and bars, a hot-air oven, pH paper, and teflon-lined autoclave a muffle furnace, and a centrifuger.

The apparatus and equipments for catalytic testing will be a tube furnace equipped with a thermocouple and a temperature controller, mass flow controllers, a

quartz tube reactor, a syringe pump (Samtronic ST670), thermocouple, and GC (SRI 310C).

5.2 Chemicals

The chemical for rice husk silica preparation will be hydrochloric acid (HCl) 37% supplied by Carlo Erba. The chemicals for MCM-41 synthesis will be cetyl trimethylammonium bromide (CTAB) supplied by Fluka; sodium hydroxide, anhydrous pellet (NaOH) and sulphuric acid (H_2SO_4) 96%, both supplied by Carlo Erba.

The chemicals for catalyst preparation will be dihydrogen hexachloroplatinate (IV) ($H_2PtCl_6 \cdot 6H_2O$) supplied by Alfa; cobalt(II) nitrate hexahydrate ($Co(NO_3)_2 \cdot 6H_2O$), copper(II) nitrate trihydrate ($Cu(NO_3)_2 \cdot 3H_2O$), and manganese(II) nitrate tetrahydrate ($Mn(NO_3)_2 \cdot 4H_2O$), all supplied by Merck.

The chemicals for catalytic testing will be ethanol (C_2H_5OH) 99.9% supplied by Merck; oxygen (O_2) 99.99%, hydrogen (H_2) 99.99%, and nitrogen (N_2) 99.99%, all gasses supplied by Thai Special Gas.

5.3 Extraction of rice husk silica

The preparation of silica from rice husk will be similar to a method in literature (Wittayakun *et al.*, 2008). Rice husk will be washed by H_2O, dried at 100 °C overnight, and refluxed at 70 °C by 3M HCl solution. The obtained solid material will be washed with water until pH~7, dried at 100 °C overnight, and calcined at 550 °C for 6 h. The sample will be referred to as rice husk silica.

5.4 MCM-41 synthesis

MCM-41 will be synthesized by hydrothermal method modified from literature (Shylesh and Singh, 2004). First, a clear solution of sodium silicate will be prepared by mixing 3 g of rice husk silica with 6 g of aqueous NaOH and stirred in a polypropylene bottle. Subsequently, this mixture will be added to an aqueous CTAB solution (4.5 g) and stirred at room temperature for 4 h. After stirring the mixture, 5N H_2SO_4 solution will be added to adjust the pH to 11. The resulting mixture will be transferred to a teflon-lined autoclave and then heated at 100 °C for 3 days. The solid product, as-synthesized MCM-41 will be separated by centrifugation, washed thoroughly with DI water and dried at 100 °C. Finally, the organic template will be

removed by calcination in air at 540 °C for 6 h. The resulting MCM-41 will be characterized by XRD, TEM and nitrogen adsorption-desorption.

5.5 Catalyst preparation

Monometallic catalysts including Pt (0.1 wt%) and MO_x (from 2 to 10 wt%) will be prepared by impregnation. Bimetallic catalysts will be prepared with a fix loading of Pt at 0.1 wt% and varied loading of MO_x from 2 to 10 wt% by sequential impregnation method. $H_2PtCl_6 \cdot 6H_2O$ and precursor of other metal oxides will be dissolved in DI water to produce desired concentrations and sufficient amount to ensure the complete wetting of the MCM-41 support.

The MO_x/MCM-41 will be first prepared by impregnating the MCM-41 support with an aqueous solution of metal oxides precursor, dried at 70 °C and then 100 °C overnight and calcined at 400 °C for 3 h. Subsequently, the $PtMO_x$/MCM-41 will be prepared by impregnating the MO_x/MCM-41 with an aqueous solution of $H_2PtCl_6 \cdot 6H_2O$ followed by drying and calcination at 400 °C for 3 h. A physical mixture of MO_x/MCM-41 and Pt/MCM-41 (1:1 w/w) will be prepared by grinding both catalysts in an agate mortar. All prepared catalysts will be characterized by various techniques including XRD, TEM, and nitrogen adsorption-desorption.

5.6 Characterization of MCM-41 and MCM-41-supported catalysts

Catalysts will be characterized by various techniques including XRD, TEM, and nitrogen adsorption-desorption as the following detail.

5.6.1 Characterization by XRD

Powder XRD patterns will be obtained from a Bruker axs D5005 diffractometer using Cu Kα radiation. The x-ray will be generated with a current of 35 mA and a potential of 35 kV. The samples will be scanned from 1 to 15 degrees (2θ) with an increment of 0.02 and a scan speed of 0.5 sec/step. The powder patterns of the samples will be recorded at the same time and with the same amount of material, so that the intensity of the peaks could be compared.

5.6.2 Characterization by nitrogen adsorption-desorption

Physical characteristics of the samples will be determined by nitrogen adsorption-desorption isotherm at -196 °C at relative pressure from 0.01 to 0.99 on an AUTOSORB-1 analyzer. Before measurement, each sample will be degassed with

heat at 250 °C under vacuum for 3 h. The surface area will be obtained with BET method from the adsorption data in the relative pressure range from 0.02 to 0.2. The pore size and pore volumes will be calculated from the desorption branches of the isotherm using Barrett-Joyner-Halenda (BJH) method.

5.6.3 Characterization by TEM

The morphology of MCM-41 and catalysts will be investigated with a TEM JSM 6400. Samples will be dispersed in ethanol by sonication, dropped on a copper-only-carbon grid and dried with UV light. The energy of primary electron will be 120 KeV to produce images with magnification up to 100000 fold.

5.7 Catalyst activity testing

The catalysts will be tested in a continuous-flow, fixed bed reactor made of a quartz tube. The connections of reactor parts are shown in Figure 3. Before the test, the catalyst powder will be ground, pressed into pellet, crushed and sieved to 250-450 mesh size. Approximately 100 mg of catalyst pellet will be packed on a quartz wool bed. Prior to reaction, the catalyst will be reduced under H_2 at 500 °C for 2 h. Ethanol will be introduced into the feed system by a syringe pump. The feed gas mixture containing synthetic air and ethanol vapor which gives O_2/ethanol ratio of 6 will be fed to the reactor. The reaction temperature will be increased from 50 °C to total conversion of ethanol. All of the reactor inlet and outlet lines will be heated to prevent condensation and to preheat the feed. The reagents and products of reaction will be analysed by an on line gas chromatograph. The reaction temperatures that correspond to 50% of ethanol conversion will be used as a measure of ethanol oxidation activity and is denoted as $T_{50\%}$.

First the ethanol oxidation experiments will be conducted over the MCM-41 support as a reference study. Then the role of MO_x loading will be studied with three different loadings (from 2 to 10 wt%) to determine optimum amount of MO_x. The oxidation will also be studied over 0.1 wt% Pt/MCM-41.

The ethanol oxidation over the bimetallic catalysts will be studied with catalysts containing 0.1 wt% Pt and different loading of MO_x (from 2 to 10 wt%) prepared by sequential impregnation method. The catalytic performance of bimetallic catalysts will be compared with a physical mixture between monometallic catalysts to confirm the benefit of bimetallic interaction between both metals. In addition, the

ethanol oxidation will be compared with commercial silica and alumina-supported catalysts.

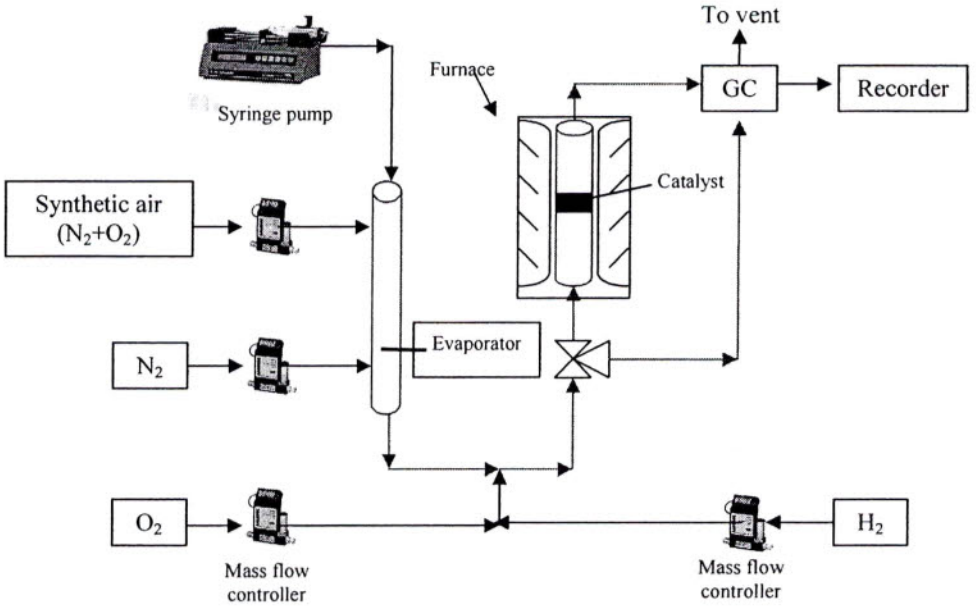

Figure 3 Reactor set up for ethanol oxidation.

6. Expected results

1. Both physical and chemical properties of MCM-41 support and MCM-41 supported catalysts will be understood.
2. The role of each catalyst component in catalysis of ethanol oxidation will be determined.
3. Parameters in catalytic testing that provide optimum performance will be obtained.
4. Reaction pathways will be understood.

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8. Research plan

Thesis procedures begin in 2008

Step	Activities	Period (months)					
		1-5	6-10	11-15	16-20	21-25	26-30
1.	Literature review	←	—	—	—	—	→
2.	Proposal preparation			←	—	→	
3.	Purchasing glasswares, chemicals, tools for experiment and silica preparation	←	—	→			
4.	MCM-41 synthesis and characterization		←	→			
5.	Reactor assembling and testing		←	→			
6.	Catalyst preparation and characterization			←	—	→	
7.	Catalytic testing for ethanol oxidation and characterization of spent catalysts				←	—	→
8.	Data analysis and thesis writing				←	—	→
9	Submission of thesis results for publication and presentation in international conference					←	→

Student's signature.....

(Miss Kamolwan Rintramee)

18 February 2009

Thesis advisor's signature.....

(Assoc. Prof. Dr. Jatuporn Wittayakun)

18 February 2009

Ph.D. Thesis Proposal

GEOSPATIAL MODELING FOR OPTIMAL LOCATION AND PLANNING OF SCHOOLS IN EDUCATIONAL SERVICE AREA OFFICE 2 NAKHON PATHOM PROVINCE THAILAND

การใช้แบบจำลอง GEOSPATIAL เพื่อหาที่ตั้งที่เหมาะสมของโรงเรียนในเขตพื้นที่การศึกษา 2 จังหวัดนครปฐม ประเทศไทย

By

MISS SIRIRAT PHONGPIPATTANAPAN

School of Remote Sensing

Institute of Science

Suranaree University of Technology

December 2008

Ph.D. Thesis Proposal

Miss Sirirat Phongpipattanapan ID.D4810148
School of Remote Sensing Institute of Science
Thesis Advisor: Asst.Prof.Dr.Songkot Dasananda

1. Thesis Title

GEOSPATIAL MODELING FOR OPTIMAL LOCATION AND PLANNING OF SCHOOLS IN EDUCATIONAL SERVICE AREA OFFICE 2 NAKHON PATHOM PROVINCE THAILAND

การใช้แบบจำลอง GEOSPATIAL เพื่อหาที่ตั้งที่เหมาะสมของโรงเรียนในเขตพื้นที่การศึกษา 2 จังหวัดนครปฐม ประเทศไทย

2. Introduction

Education is key in developing a country because it is the main root in the economic, social and political development or it might be said correctly educating people becomes foundation in developing all areas.

In Thailand, its currently educational conditions can not be counted facilitating the national development both quantitatively and qualitatively. Rationally, education handling at present is found with low quality unmet the needs of learners, societies, the country and slower than the globalization waves. The Ministry of Education sets policy on education reforms in 4 areas, i.e. management reforms, curriculum reforms, instruction reforms, and teaching profession and education personnel development. Strategies applied are decentralization, participation and quality assurance focusing learner-centered and strategy aiming at education excellence and quality learners (สมศักดิ์ คลประสิทธิ์, 2547). Planning and education organizing are therefore important become promising tool to recommend solution for waste and inefficiency in education. (Anderson, 2003)

Education wastes are tremendous when the school mapping is unset. The Commission of Founding for Education Reforms (2001) reveals that prudence must be taken in organizing education about method of organization and source allocations for education which are keys because they affect its quantity and quality and impact the national social and economic structure. Therefore, resource selections from

different sources must be geared to better effectiveness of educational organization in order to expand and avail education for all and with fairness. Further, in 1999, Thailand has launched education reforms instituting new Educational Service Areas (ESA) which probably require survey to analyze that whether children in each ESA gain opportunity to totally be fundamentally educated, how many children have attended classes and so on. It counts that the desired educational organization requires lifetime learning process spearheading to promote quality citizens to gain knowledge and ability in living and to bring benefits to societies which is the human resource development acquiring efficiency and meeting the needs of the nation.

Commission of Founding for Education Reforms (2001) recommends that locating school need not adhere to subdistrict zone or village but to the community density. Any zones with density of less than 100 households per 12.5 km^2 should not establish new schools and schools should be located at the center of the community. The small size schools should be surveyed and mapped. They should be dissolved to the larger schools because the small ones are difficult to gain quality and students living near the school are not registered to study with the schools near their homes.

With the above reasons, the researcher recommends to study locations of schools comprehensively to plan school locations applying the *p*-median model for studies and organizing education in the Educational Service Area Office 2 in Nakhon Pathom Province at the level of Basic Education. The main objective of the study is to find solutions to problems arisen and improving the educational management in the chosen area to help reducing further criticality of the problems. In this work, there are 4 main topics to be reviewed for further use as follows:

(1) GIS application in planning the school's location

(2) P-median Model

(3) Markov Model

(4) Gravity P-median Model

3. Literature Review

3.1 GIS application in planning the school's location

In recent years, geography information system (GIS) has become essential tool in planning should location as seen in several reports. For examples, Moller and

Jensen (2006) used GIS on the Allocation Program to found relationship between school locations and number of students in Copenhagen, Denmark. They find that the school locations fit their surroundings and students with residences nearest to any schools need to be admitted to those schools. Transport networks are another key to travel to schools. In addition, walking, bicycling, taking bus and taking private cars to school affect the travels and the expenses. Had schools not been considerably distant from the residences, it would save both time and money. Further, it is useful for educational planners to decide which schools should admit more students or which ones should reduce their admission. This betters the school quality.

This is corresponding with Krak (2005) who employed Network Analysis in his numeric planning on transport networks in Copenhagen. He found that the new map best models the network of the transportation routes fit traveling because least time is spent, convenient, safe, and economy. Also, the map could be used as a database to map proper school service area.

Gilo (1997) investigated master planning fit location for schools in Tel Aviv, Israel using geographic information. He found that GIS is the best instrument and best efficiency to master planning fit locations for schools. Conditions for consideration are distance of walking for students from home to schools, school sizes (admit ability), transportation networks and public spaces. Adopting these factors is by reasoning that many schools need change of location such as blind lane, few roads to access and adjacent to highway unable to cross it to school even residing on the opposite location. Such reasons affect the school locations and by the school location analysis, Gilo proposes that school location must be changed for properness. It is further found that some service areas of some schools are too wide turning students spend surplus time to reach their schools. Models he found are:

1. Two new schools should be established to admit not more than 540 students and the most distance is 1,500 meters.

2. Three new schools should be established in fit location after applying location allocation method.

Rattana Rujirakul studied GIS application to evaluate the locations and elementary education service areas in Muang District, Nakhon Ratchasima by studying models from research papers and developed models using population and

transportation variables by allocation analysis. Results found in the study were used to evaluate the locations and elementary education service areas in Muang District, Nakhon Ratchasima and to create the map locating proper locations and elementary education service areas, which can be applied to improve the improper ones. (รัตนา รุจิรกุล, 2548)

3.2 The P-Median Model

Location-Allocation Model (LA Model) is a mathematical model applied in resource management analysis in order to balance demand and supply and maximize the efficiency. P-Median is a kind of LA model applied for locating the service point in order to best shorten the demand point to the service point (a service-point allocation to each existing demand points to turn total distance least value). Examples of the model's application are as follows:

Yasenovskiy and Hodgson (2007) investigated the school location planning using the primary method of locating the demand points of the study (real condition which might be changed by space and time). Then, required service points were be set (1 point, 3 points, 5 points...) before using the above technique to locate each best fit service points (matching the selective points). Later, all results were compared with the locations of the real existing service points on to what extent they are matched as well as recommending the proper procurement or improvement of the location.

Harvey, Hung, and Brown (1974) employed the p-median model to fix number and the center location of a hospital in Sierra Leone by studying a one-fold level of the problem. They were also aware on interactions between the low-high levels of the hospital. Reilly, (1976) employed the p-median model to analyze the problem of the hospital location and its emergency ambulance for best services by conditioning on the shortest distance and least time to travel from the incident spot to the hospital. Calvo and Marks (1973) employ the p-median model with multi-purposes in applying with the medical service stations. The hierarchical model showed that the p-median model able to best access the service stations, minimize cost but maximize benefits.

Banerji and Fisher (1991) employed both the p-median model and the

hierarchical model to plan proper location for service station in India. He sets the Hierarchical Model as a network, i.e. high level affairs providing the lower level of services. He found that the demand points are enabled to best use of the service points with the shortest distance. Similarly, Pizzolato *et al.* (2008) applied the process of the Operation Research to establish public schools in Rio de Janeiro, Brazil, which is a poor country. Students therefore simply walk to schools from home. And in finding location where the totals sum of distance between residences and schools is least value. So, they use the p-median model for the investigation observing the existing number of schools and use it to estimate the results by investigating the spread of the schools. Results suggest best locations and recommend on short-term and long-term administration.

Peng *et al.* (2007) employed location-allocation modeling for primary school accessibility and catchments analysis-a case study: Wuhan, China was selected as a case study to employ the location-allocation model in order to achieve two main objectives. The first one is to examine availability and efficiency levels to school and to assess the demand-supply balance between students and schools capacity. The second one is to identify a new potential location and evaluate the feasibility of location-allocation model for planning additional schools in Wuhan. Four experiments with different hypotheses were implemented to test the above objectives. The results showed that the service area of each school can be delineated according to the school capacity and the accessibility of schools is highly correlated with the school capacity. This provides urban planners tools to make decisions concerning where to increase or decrease school capacity, to combine schools or plan new schools.

3.3 The Markov Model

Goodman (1992), define Markov analysis as a mathematic approach in changing data in the past and the present data in order to be use in estimating the changing data in future, which A. Markov a Russian mathematician has used it to estimate markets to study the changes in customers and to help planning human resource for business affairs. Chatman and Jung (2001) have applied Markov Analysis to estimate workforce and state that the Markov Analysis is more advantageous than

other approaches on seeing the migration and change of the estimated population and clarifies the workforce at the micro level and needs no interdependent variables like the Regression Analysis and the Economic Estimation. It helps collect data faster. In education, applied the Matrix of Markov Analysis to estimate workforce in education to find the model in changing number of school and to estimate the requirements of personnel at the micro level. Estimation of personnel number is by different variables, e.g. age, subjects, and campus.

Ketsanee Wasanathip (1996) used the Markov analysis in a creation of Markovian transition matrix for forecasting science and mathematics teachers in secondary school, educational region five. The purpose of this research was to create a Markovian transition matrix to forecast the demand of science and mathematics teachers in secondary schools under the jurisdiction of the General Education Department, educational region five, in the academic year 2540-2549. The research instrument was the teacher inventory form which was used by the researcher to collect the data from report on education statistics. The data were analyzed by descriptive statistics, frequency distribution, graphic presentation, chi-square test and Markov analysis technique of forecasting.

3.4 Gravity P-Median Model

Gravity model is a model based on a hypothesis that a customer chooses a facility by considering both the attractiveness of the choices and the distances to those facilities. Drezner (2006) used the gravity model in studying multiple facilities location in the plane using the Gravity median two problems are considered in this article .The first problem is the p median where the total distance traveled by customers is minimized. The second problem focuses on equalizing demand across facilities by minimizing the variance of total demand attracted to each facility.

Reilly (1976) proposed the gravity rule. Suppose that a customer resides in an intermediate town near two large cities. The probability he will patronize one of the cites is proportional to the city's size and inversely proportional to the squared distance from that city. This gravity rule had been used by Huff (1966) to model consumer behavior in selecting a store to patronize. He asserted that the probability that a customer patronizes a stone is proportional to the store's floor area and

inversely proportional to some power of the distance to the store. He applied the gravity model to consumer choice behavior and generalized the distance decay function from the square of the distance to some power of the distance depending on the retail category.

Pandered (1997) showed that the gravity model is a motion phenomenon based on distance – if farther distance, the distance decay value. By these concepts, other discipline scholars apply to explain the law of motion. For example, Ripley (1993) applied to explain the motion event such as the influence of retailing in the city. It is corresponding with Hartshorne (1990) who modified the gravity model for the use in investigating retailing. He found that the service point of the retailing shop in general is small and people will never walk far to buy goods. Customers attracted by high quality goods will walk farther. In addition, Wimonporn Paisopa (2003) used the gravity model to model travels of city dwellers, outskirt dwellers and rural dwellers in the city plan of Chiangmai. She found that variables affecting the Travel Model are number of students, parents' income, accessibility which influence in selecting service points.

Wallapha Pornpatcharaphong (2005) applied the gravity model to analyze scope of the service point of the secondary schools in Nakhon Ratchasima Province and found that the school fame affects decision in selecting a school.

Drezner and Drezner (2007) used the gravity p-median model to calculate gravity towards customers divide their patronage among the facilities with the probability that a customer patronizes a facility being proportional to the attractiveness of that facility and to a decreasing utility function of the distance to the facility.

4. Research Objectives

4.1 To establish GIS-database based on appropriate criterion for location and allocation planning of schools.

4.2 To identify optimal schools location and allocation in present and future using geospatial modeling

4.3 To compare present schools location with location derived from geospatial modeling.

5. Scope and Limitations of the Study

5.1. The study area is the Educational Service Area Office 2 in Nakhon Pathom Province engulfing 4 districts: Nakhon Chaisi, Sam Phran, Bang Len and Phuttamonthon with 1,174.09 km^2 areas covered. (Figure 1)

5.2. This is a selective investigation including 144 schools in the study area, which are (for Academic Year 2007)

5.2.1. 138 primary schools

5.2.2. 35 junior secondary schools (including the extended education)

5.2.3. 15 senior secondary schools

5.3. Data contributed by related offices are valid.

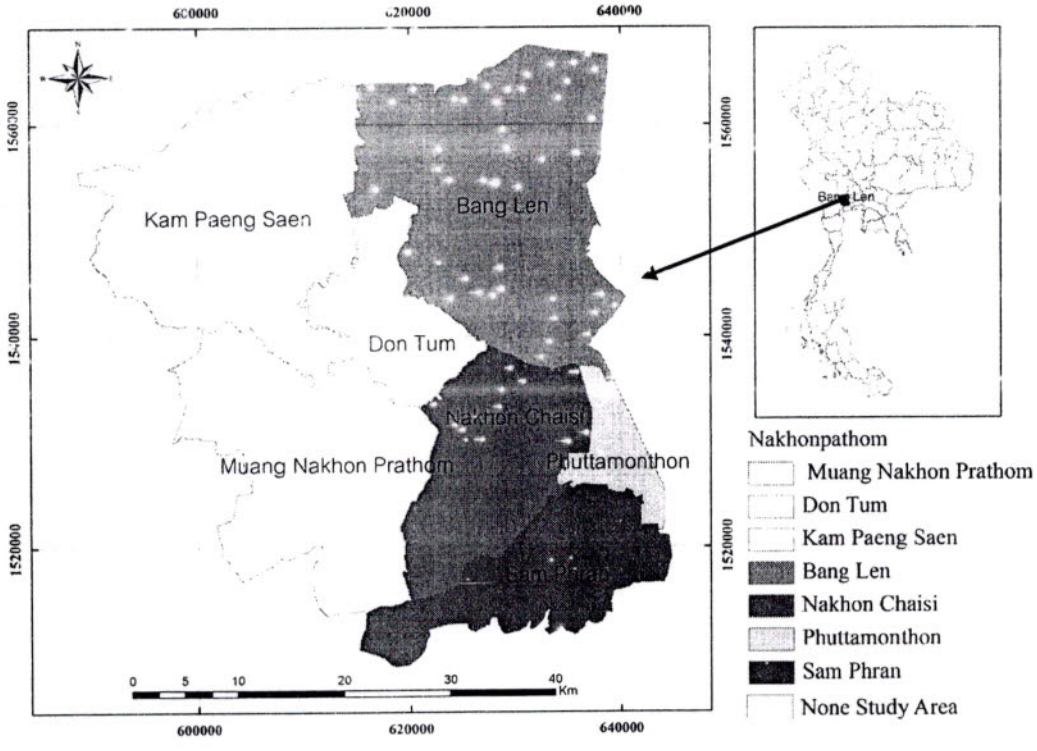


Figure 1 Location Map of the study area, Nakhon Pathom Education Service Area Office 2

6. Research Methodology

Analysis processes used to achieve the four main purposes of the study are: (As show in Flowchart 1)

6.1 GIS – based database formation

6.1.1 Collect interested information from related offices which include both primary and secondary data (see Table 1 for detail).

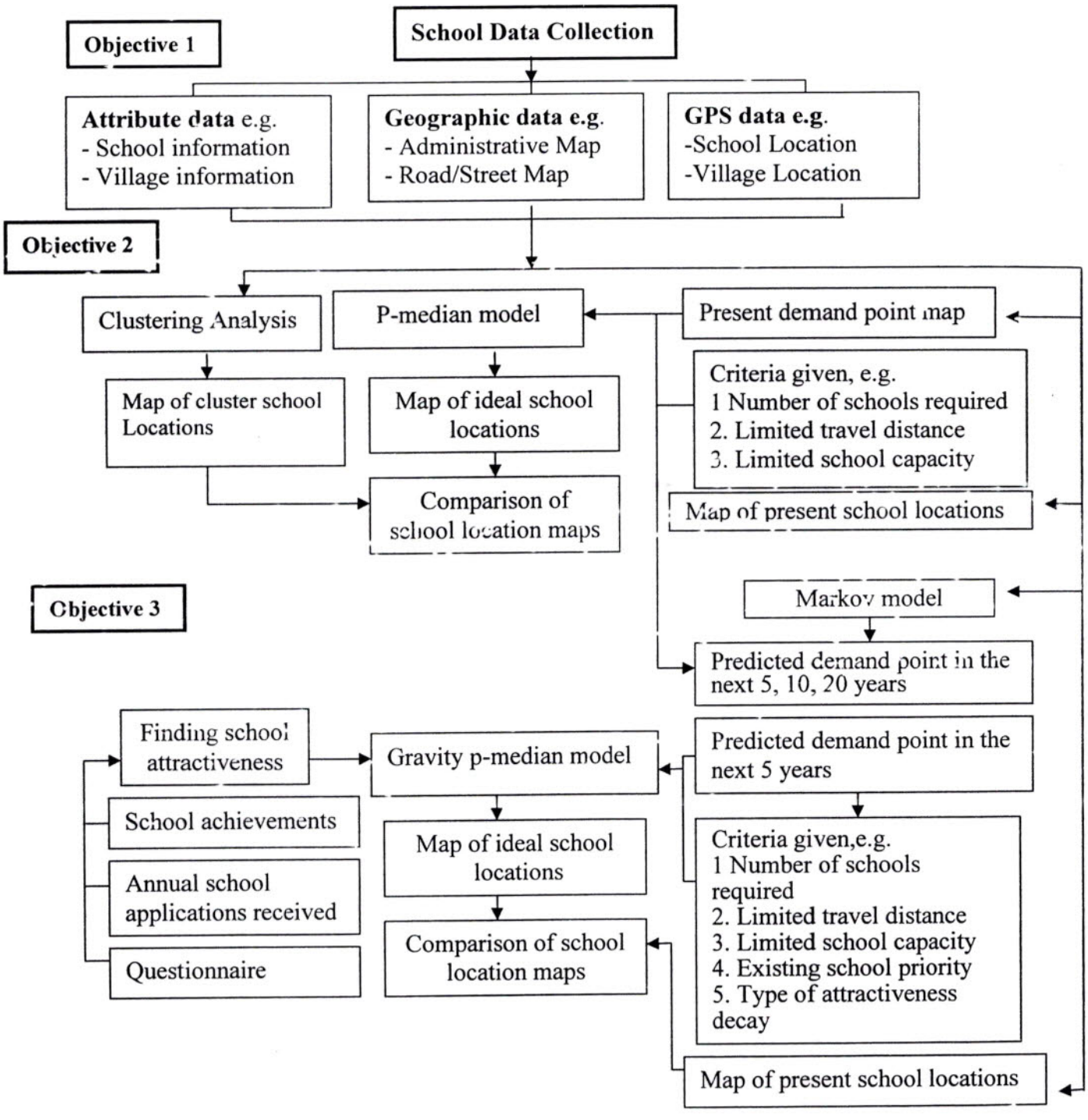
6.1.2 Categorize data obtained from 6.1.1 and store them in form of GIS–based database (including several GIS layers) for further use.

6.2 Evaluation in efficiency of present school location pattern.

6.2.1 Evaluation based on basic factors including 2 steps:

6.2.1.1 Consider the suitability of school locations by average distances.

6.2.1.2 Analyze correlation between communities and schools regarding demand and supply of schools; the more students going to school, the more effective the school is.

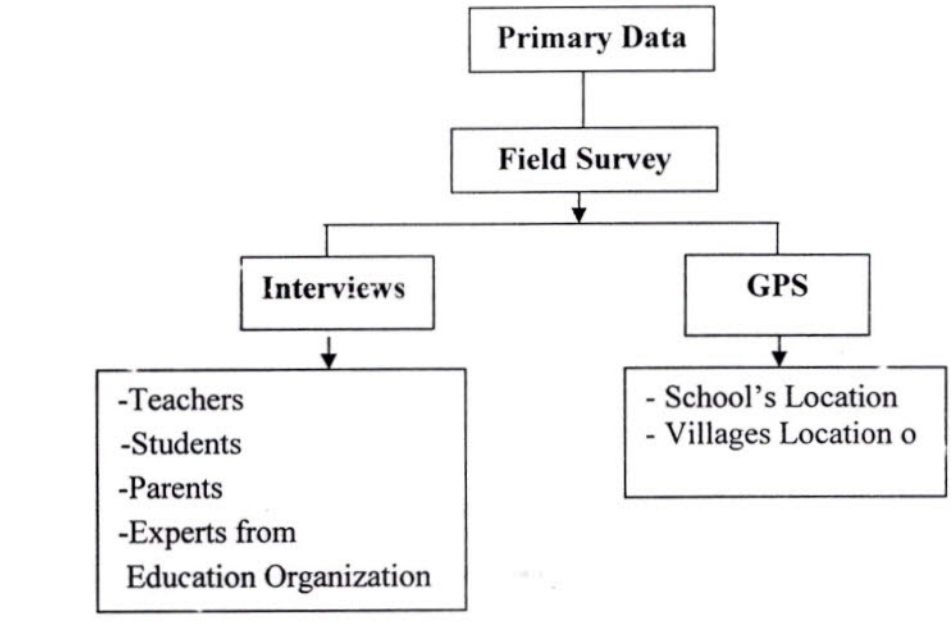


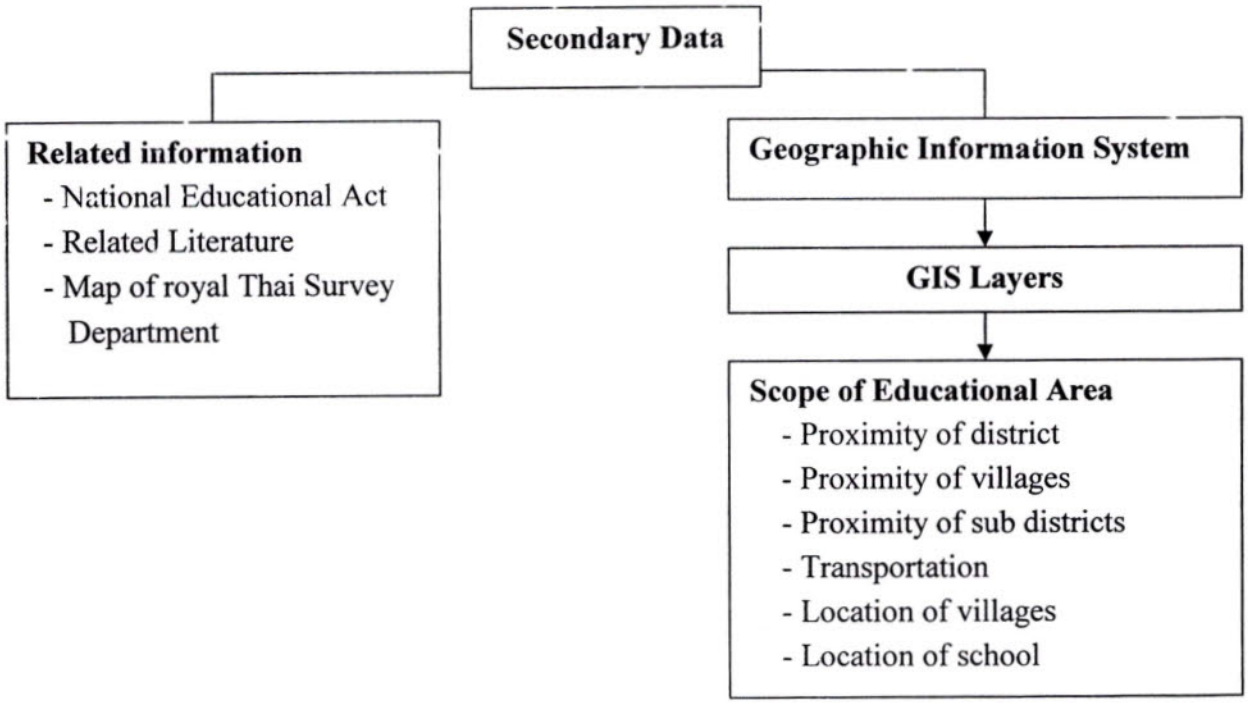
Flowchart 1: Conceptual Framework

Table 1 Detail of school data collected

Capacity	Data type	Source
1.Physical data		
- Geography	- mean Elevation	Land Development Department 1:50000 Year 2005
- Administrative area	- maps displaying proximity of the province, district, subdistricts and villages	
-Transportation routes	- proximity accessibility of the schools	
- Land uses	- different types of land uses, e.g. paddy fields, pomelo orchards, etc.	
2.Demgraphic data	- number of students in each school by admission	The Bureau of Registration Administration
	- population density in each village	
	- number of population in each village by year	
	- annual migration of population	
	- annual birth rate	
	- annual dead rate	
3. School data	- number of schools	Nakhonpathom Education Service Area Office 2
	- location and type of schools	
	- number of students per class	
	- rise and fall rate of population	
	- educational achievement	
	- number of teachers	
	- number of admission	
	- number of graduation	
	- number of fail and drop-out	
4.Student data	- students' residences	- Questionnaire
	- way to travel to school	- Interview
	- occupation and income of parents	

Flowchart 2 Data collection process





6.2.2 Evaluation based on clustering technique including 3 steps :(See Flowchart 3 for more detail)

6.2.2.1 Prepare school location map and define clustering criteria :(See Table 2)

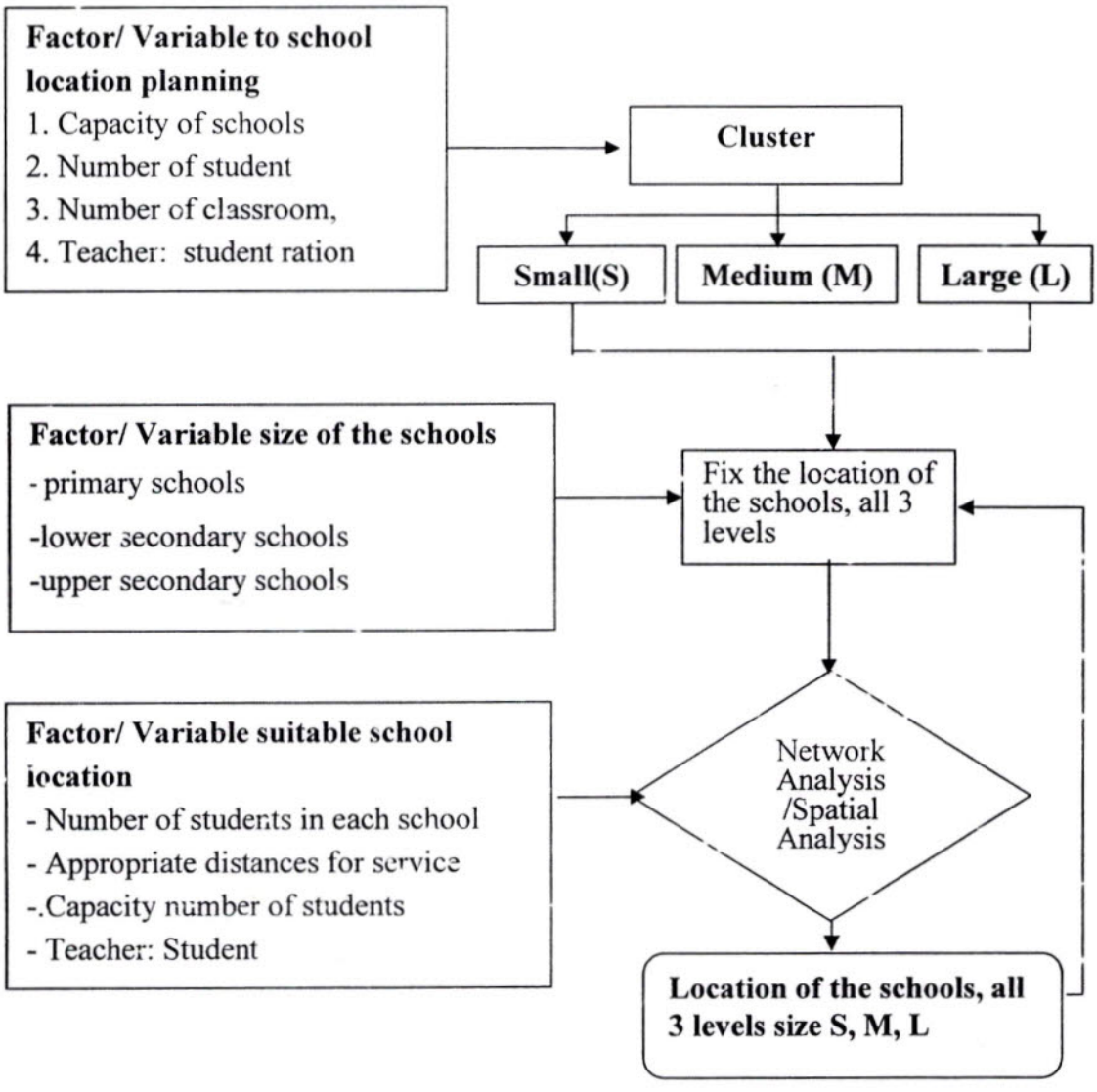
6.2.2.2 Determine predictions areas to handle students and plan annuals classes using 2 following factors:

1) current school locations - prioritize original locations to reduce site selection.

2) distance - potential location must be able to reach within suitable distances according to the act.

Table 2 Clustering Criteria

Criteria	Small	Medium	Large	Source
1.School capacity (person)	1-120	121-600	601-1,500	National Educational Act
2.Service distances (km.)				
-primary schools	3	3	3	National Educational Act
-lower/upper secondary schools	5	5	5	
3.Teacher:Student ratio(person)				
-primary schools	1:25	1:25	1:25	National Educational Act
-lower/upper secondary schools	1:40	1:40	1:40	



Flowchart 3 Methods of clustering to plan annual classes

6.2.3 Evaluation based on p-median model including 3 steps:

6.2.3.1 Find ideal school locations for each educational level using set of demand points generated and specific number of school given (e.g. 1,2,3....) where some criteria, for examples, limited traveling distance and school capacity will also be introduced

6.2.3.2 Compare ideal locations fond in step 1 with the existing (real) location in term of average traveling distance, school capacity and school service area.

6.2.3.3 Suggest plans for better school management based on results found in step 1 and 2, e.g., dissolving or regrouping some redundant schools or relocation of some improperly-placed schools.

6.3 Finding proper future school locations

This objective is achieved using 2 methods, which are Markov model, for prediction distribution pattern of school-age population in the future (e.g., in 5,10 years), and p-median model, for generating ideal school locations to assist such distribution found. Details of each procedure are as follows:

6.3.1 Predicting distribution pattern of school-age population using Markov model (See Flowchart 4 for more detail)

6.3.1.1 Prepare necessary data for running Markov model, e.g. present distribution pattern and expected trend in the school-age population growing.

6.3.1.2 Generate future distribution pattern (in 5, 10, 20 years) and make new demand-point map according to each scenario selected.

6.3.2 Finding proper school location using p-median model

6.3.2.1 Find ideal school location for each educational level using set of demand points found in 6.3.1.2 for each scenario selected. In this process, some criteria, for examples limited traveling distance, school capacity and existing-school priority will also be introduced.

6.3.2.2 Consider plans for proper school management for each scenario chosen based on results found in step 1, e.g., dissolving or regrouping some redundant school or relocation of some improperly-placed schools.

6.3.3 Application of gravity p- median model

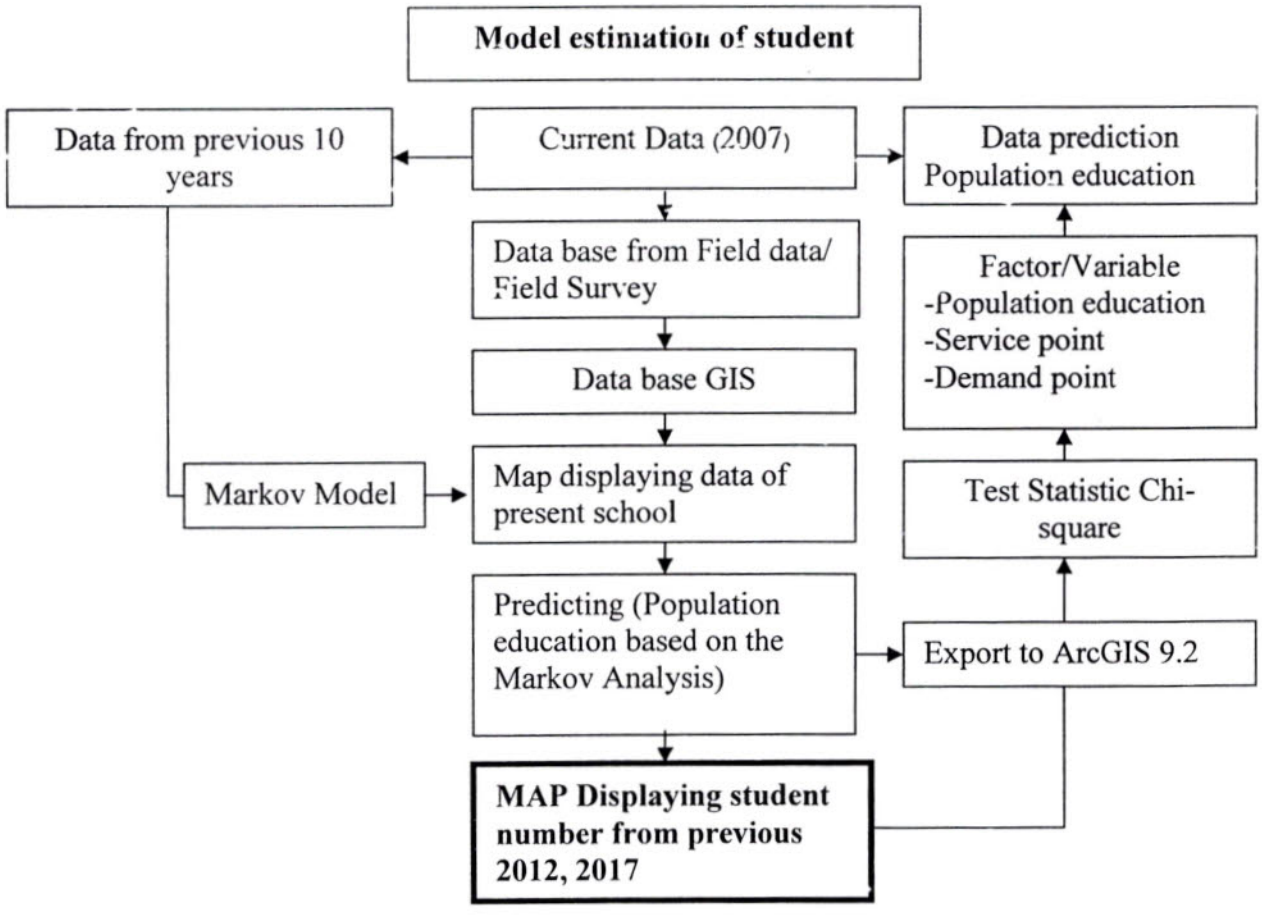
The purpose of this objective is to introduce gravity p- median model in the prediction of future school location to be established in the study area, where only upper-secondary schools are considered. To achieve this goal, 3 main steps are performed as follows:

6.3.3.1 Determine attractiveness weight of each school involved based on factors like school achievements, school's annual applications received, and results from questionnaire distributed

6.3.3.2 Find ideal school locations using set of demand points in the future obtained from 6.3.1.2 for the scenario chosen. In this process, some criteria, for examples, type of attractiveness decay function (e.g. inverse–square or exponential form) and existing school priority will also be introduced.

6.3.3.3 Compare results found in 6.4.2 with those from 6.3.2.1 to see effect of school attractiveness on pattern of proper school locations found.

Flowchart 4 Factors used population estimation on the educational age group by Markov Model



7. Expected Results

7.1. Having effective school data system based on GIS application

7.2. Finding efficiency of present school locations, principally in term of average traveling distance and school capacity for the use in planning and creating better school management system.

7.3. Finding proper school locations in the future to assist the growing number of school-age population at present.

7.4 Having more understanding in problems of present school management system and how to apply geoinformatics technology to resolve some specific problems found.

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9 Research Plan

Steps	Activities	Period																							
		1/2008				2/2008				3/2008				1/2009				2/2009				3/2009			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	Literature review / methodology																								
2	Design methodology tools																								
3	Thesis Proposal defense																								
4	Collecting data																								
5	Data analysis																								
6	Thesis writing																								
7	Thesis defense																								
8	Thesis correction and submitting																								

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(Miss Sirirat Phongpippattanapan)
Ph.D. Student
(17 / March / 2009)

.....

Asst.Prof.Da.Songkot Dasananda)
Ph.D. Thesis Advisor
(17 / March / 2009)

Appendix

1. **P-median model**
2. **Gravity P-Median Model**

1 Factors to assess optimal model in locating future schools applying the P-median model

Topic of Variable	Variable/Factors	Source
Dependent Variable :Y Evaluate appropriate model for scoping the location of prospective schools by using the p-median model	$MinZ = \sum_{i=1}^{n} \sum_{j=1}^{m} w_i \cdot d_{ij} \cdot x_{ij}$ Subject to 1) $\sum_{j=1}^{m} yj = P$ restricts the number of facilities to p 2) $\sum_{j=1}^{m} Xij = 1 \; \forall i$ ensures that every demand location i is served 3) $Yi \geq X_{ij}, \; \forall i, \forall j$ node I can be assigned to j only if there is an open facility at j (if X_{ij} = 1, than $y_i = 1$) 4) $Yj = 0, 1, \; \forall j$ facility site location decision variable 5) $X_{ij} = 0, 1, \; \forall i, \forall j$ allocation decision variable Where i = demand location j = candidate facility location n = number of demand locations m = number of candidate facility locations p = number of facilities located w_i = weight at demand node i d_{ij} = shortest distance between demand location i and candidate j j = 0, otherwise	
Independent Variable :X 1. Distance	- The shortest distance between school and total students studying in the school according to the national educational act - primary 3 km - Junior high school 5 km - Senior high school 5 km	Educational Act
2Demand point (student)	- Number of Student form each village admitting in each school	Field Survey
3 Service point(school)	- Location of School - Number of School for each level	Field Survey

2 : Gravity P-Median Model

Topic of Variable	Variable/Factors	Source
Dependent Variable :Y School attraction	$\min_{P}\left\{f(P)=\sum_{i\in N}\left[w_i\frac{\sum_{k\in P}u_{ik}d_{ik}}{\sum_{k\in P}u_{ik}}\right]\right\}.$ Given that W_i = The amount of students in the community i D_{ij} = The distance from the community i to the community j ($d_{ij}=d_{ji}$) U_{ij} = The scores of the schools in the community j give by the Community i (U_{ij} need not be equal to U_{ji}) The result of the study infers the amount of the students in each school and the reasons why the students choose the specific schools	
Independent Variable :X Amount Student	applicants in each school (all three levels)	Nakhonpathom Education Service Area Office 2

แก้ไข

Ph.D. Thesis Proposal

ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTICANCER ACTIVITIES OF HOAN-NGOC, *PSEUDERANTHEMUM PALATIFERUM* (NESS) RADLK.

ฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบ และต้านมะเร็งของฮว่านง็อก, *PSEUDERANTHEMUM PALATIFERUM* (NESS) RADLK.

By

Miss Patcharawan Sittisart

School of Biology

Institute of Science

Suranaree University of Technology

April 2009

Ph.D. Thesis Proposal

Miss Patcharawan Sittisart I.D.No. D4910435

School of Biology Institute of Science

Thesis Advisors

A) Asst. Prof. Dr. Benjamart Chitsomboon Thesis advisor

B) Prof. Dr. Norbert E. Kaminski Thesis co-advisor

1. Thesis title

ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTICANCER ACTIVITIES OF HOAN-NGOC, *PSEUDERANTHEMUM PALATIFERUM* (NESS) RADLK.

ฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบ และต้านมะเร็งของฮ่วานง็อก, *PSEUDERANTHEMUM PALATIFERUM* (NESS) RADLK.

2. Introduction

2.1 Significance of the study

Fruits, vegetables and grains have been reported to contain a wide variety of antioxidant components, including phytochemicals such as phenolic compounds and flavonoids. Phytochemicals have been considered to be beneficial for human health and help decrease the risk of chronic diseases such as cancer, cardiovascular and

various inflammatory diseases (Kubola and Siriamornpun, 2008; Atmani et al., 2009). Chronic inflammation is considered as one of the major contributors of carcinogenesis by causing cellular damage leading to cell proliferation (Jackson, Seed, Kircher, Wiloughby, and Winkler, 1997). The proinflammatory cytokines such as IL-1β, IL-6, TNF-α, GM-CSF and growth factors produced in inflammatory state can also stimulate vascular endothelial growth factor (VEGF) function. VEGF is an important angiogenesis-inducing protein functioning in inducing and promoting new blood vessel growth for tumor angiogenesis, and hence sustaining the progression of cancer (Sheeja, Guruvayoorappan, and Kuttan, 2007). Among various angiogenesis inducer, VEGF is the best characterized factor that can stimulate nitric oxide (NO) synthesis. NO is an important biological molecule with dual roles. At modest concentrations, NO could be a pro-malignant whereas at very high concentrations, however, NO could act as a potent anticancer agent, promoting apoptosis and inhibiting angiogenesis (Sheeja et al., 2007; Coulter et al., 2008). Therefore, there are relationships among oxidative stress, inflammation, angiogenesis processes and the development of cancer.

Many researches indicated that the extracts from medicinal plants, especially flavonoids, have antioxidant, anti-inflammatory and anticancer activities. For example, gallic acid, catechin and caffeic acid from fractions of bitter gourd (*Momordica charantia* L.) had free radical scavenging properties (Kubola and Siriamorpun, 2008). The phenolic compounds from medicinal plants in Algeria possessed high antioxidant activities (Atmani et al., 2009). The crude methanolic extract from the pericarp of *Garcinia mangostana* has powerful antiproliferation as evidenced by inducing apoptotic cell death in human breast cancer (SKBR3) and a

potent antioxidation by inhibiting intracellular reactive oxygen species (ROS) production (Moongkarndi et al., 2004). The extracts from Labrador tea leaves (*Ledum groenlandicum* Retzius) containing high concentrations of phenolic compounds also showed a strong antioxidant activity, inhibited the NO release in LPS-stimulated RAW264.7 macrophages, and were active against colon carcinoma cell line DLD-1 and Lung carcinoma cell line A-549 (Dufour et al., 2007). In addition, *Andrographis paniculata* crude extract, its major component andrographolide and minor component andrograpanin had antioxidant and anti-inflammatory activities. They also possessed anticancer activity by inducing apoptotic cell death in human cancer cells *in vitro* and inhibiting angiogenesis *in vivo* (Zhou, Zhang, Ong, and Shen, 2006; Sheeja et al., 2007; Liu, Wang, and Ge, 2008). The extract from *Andrographis paniculata* inhibited the B16F-10 melanoma cell which induced capillary formation in C57BL/6 mice. It down regulated various proangiogenic molecules (VEGF and NO), proinflammatory cytokines (IL-6, TNF-α and GM-CSF) and upregulated antiangiogenic molecules (IL-2 and TIMP-1). The antiangiogenic activity of the extract was also demonstrated *in vitro* using the rat aortic ring assay (Sheeja et al., 2007). Yoon et al. (1995) reported that the extract from Korean mistletoe (*Viscum album coloratum*) inhibited metastasis of B16-BL6 melanoma, colon 26-M3.1 carcinoma and L5178Y-ML25 lymphoma cells in mice by inhibition of tumor growth and the number of blood vessels that oriented towards the tumor mass.

Beta-sitosterol is one of phytosterols found almost in all plants such as pecans, *Serenoa repens* (saw palmetto), avocados, *Curcurbita pepo* (pumpkin seed), *Pygeum africanum*, cashew fruit, rice bran, wheat germ, corn oils, soybeans, seabuckthorn and wolfberries (http://en.wikipedia.org/wiki/Beta-sitosterol). Beta-sitosterol is mainly

known for health benefits. It has been used to reduce cholesterol, increase immunity, reduce inflammation, prevent oxidative damage and lower risk of cancer. Also, it has been known to reduce growth of human prostatic and colon cancer cells. (http://medicine.oxac.uk/bandolier/band924.html; http//www.drlam.com/opinion/beta_sitosterol.asp). In Europe, β-sitosterol plays a major role in the herbal therapy of benign prostatic hypertrophy (BPH). In the BPH treatment, β-sitosterol may improve prostate symptom by increasing peak flow and decreasing mean residual urinary volume. However, prostatic volumes in the patients treated with β-sitosterol were not significantly changed compared with placebo group (Berges, Windeler, Trampisch, Senge, and the β-sitosterol study group, 1995; Lowe and Ku, 1996; Wilt, Macdonald, and Ishani, 1999).

In Vietnam, Hoan-Ngoc is considered as a new medicinal plant. It is referred as a miraculous plant in folk medicine for curing various diseases such as wound, trauma, stomachache, colitis, blood pressure, nephritis, diarrhea, diabetes and cancer. The pathology of these diseases are mainly caused by oxidative stress and inflammation. Leaves of Hoan-Ngoc display antioxidant activity when tested in human blood peroxidase model (Dieu, Loc, Yanasaki, and Hirata, 2006; วงศ์สถิต ฉั่วสกุล และ อรัญญา ศรีบุศราคัม, 2551). Also, Hoan-Ngoc leave extract (HLE) contained β-sitosterol and apigenin-7-O-β-glucoside which have various pharmacological properties. Apigenin-7-O-β-glucoside may metabolize in body into apigenin which has antioxidant activity. A mixture of apigenin-β-glucoside, β-sitosterol and stigmasterol in leaves of *Buddleja globosa* was reported to have anti-inflammatory activity as suggested by reducing TPA-induced edema in mouse. (Backhouse et al.,

2008). Therefore, there is a high potential that HLE may have antioxidant, anti-inflammatory and anticancer activities. Though the Vietnamese have used Hoan-Ngoc leaves as a folk medicine for a long time, the pharmacologic studies of its claimed properties are still limited, and there are still no reports of toxicity study.

2.2 Literature review

Pseuderanthemum palatiferum (Nees) Radlk. (*Eranthemum palatiferun* Nees.) known as Hoan-Ngoc is a native plant of Vietnam belonging to the Acanthaceae family. During the latter half of the 1990's, the plant was found in Cuc Phuong forest in northern Vietnam. After the discovery, the plant has been cultivated throughout the country as both a medicinal and ornamental plant (Dieu, Loc, Yanasaki, and Hirata, 2005; Dieu et al., 2006). Hoan-Ngoc was taken into Thailand about 20 years ago by a Vietnam Era veteran and passed northeast of Thailand such as Surin, Buriram, and Sisaket provinces. Its Thai name is "Wan Ling" or "Payawanorn". Hoan-Ngoc is a shrub tree of 1-3 m high. Stem is quadrangular, glabrous, and green in color. Leaf arrangement is opposite, simple and green foliage color. Shape of leaf is lanceolate to elliptic, 3-5x5-15 cm, acuminate terminal, attenuate at base and entire margin. Flower is inflorescence and irregular. Corolla is white-violet in color (วงศ์สถิต ฉั่วสกุล และ อรัญญา ศรีบุศราคัม, 2551).

Vietnamese people believe that Hoan-Ngoc is a miracle-medicinal plant. Chewing its fresh leaves, or drinking its juice prepared fresh or boil is supposed to cure many diseases such as wound, trauma, stomachache, colitis, blood pressure, nephritis and diarrhea. In addition, Hoan-Ngoc has also been used for treatment and

prevention of various diseases in animals. (Dieu et al., 2005; Dieu et al., 2006). Hoan-Ngoc can widely grow in Thailand and Thai people have used Hoan-Ngoc leaves for prevention and treatment of many diseases such as chronic pain in older adults, trauma, nerve disease, influenza, high or low blood pressure, digestive disorder, diarrhea, bone fracture, stomachache, nephritis, hepatitis, diabetes and cancer (มูลนิธิเพื่อสุขภาพไทย, 2552). In Thailand, Hoan-Ngoc has gained its reputation for alleviating or curing cancer, the number one cause of death among Thai people (สำนักงานพัฒนาระบบข้อมูลข่าวสารสุขภาพ, 2548). Similar to various herbal medicine, anticancer effects of Hoan-Ngoc leaves have been referred among consumers without adequate scientific evidence. At present, there are very limited published studies of Hoan-Ngoc and most of them are written in Vietnamese. Dieu et al. (2006) and Dieu (2008) reported that the chemical composition of Hoan-Ngoc was a mixture of stigmasterol and β-sitosterol, β-sitosterol-3-O-β-glucoside, apigenin-7-O-β-glucoside, 1-triacontanol and salicylic acid. The leaves also contained pseuderantin, a proteinase with high thermal stability and proteolytic activity. Moreover, the leaves contained 30.8% of the dry matter as crude protein, minerals such as Ca, Mg, Fe and Cu, and amino acids such as lysine, methionine and threonine (Dieu et al., 2005). Dieu et al. (2006) reported that the dried and fresh-leaves have comparable effect in the treatment of diarrhea in piglets, and the efficacy was almost equivalent to Coli-norgent and Cotrimxazol which are the best prevalent drugs used in treating diarrhea. The ethyl acetate and n-butanol fractions of Hoan-Ngoc leaves contained high flavonoid contents and had antioxidant activity in human blood peroxidase model.

Singh et al., 2002). Angiogenesis is controlled by multistep processes an involving with imbalance of pro and angiogenic factors which include several growth factors and cytokines. VEGF is the most important protein among various inducers of angiogenesis. It induces new blood vessel growth and regulation of hemopoietic stem cell development, extracellular remodeling and inflammatory cytokine generation (Sheeja et al., 2007). Therefore, VEGF is an important target for antiangiogenic approach in cancer treatment. Recent studies showed that several polyphenols (e.g., isoflavones, flavonoids, flavones, flavonones and catechins) from fruits, vegetables and herbs (soybeans, grapes, peanuts, tea, cranberry and black current) had antiangiogenic and anticancer activities (Cao et al., 2002). Angiogenesis and inflammation are codependent processes. Chronic inflammation may promote angiogenesis leading to tumor. Proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF act as autocrine growth factors for tumor cells. Inflammatory cells in the stromal area of many tumors may produce proangiogenic cytokines and growth factor inducing new blood vessel growth for tumor which in accordance with the studies that IL-1β, IL-6 and TNF-α could stimulate VEGF (Jackson et al., 1997; Balkwill and Mantovani, 2001; sheeja et al., 2007). Epidemiological studies revealed a strong association between chronic inflammatory conditions and carcinogenesis in several human cancer (sheeja et al., 2007). Vasodilation of smooth muscle induced by NO is a prerequisite for endothelial cell to enter the angiogenic cascade. (Griffioen and Molema, 2000; sheeja et al., 2007). Furthermore, angiogenesis can also promote inflammation as VEGF stimulate nitric oxide synthase (NOS) activity leading to NO production. Various studies also demonstrated the positive correlation of increased NOS activity with increased vascular density and tumor growth. (sheeja et al., 2007).

Antioxidant and anti-inflammatory compounds in fruits, vegetables and grains had anticancer activity, especially polyphenol in phenolic compound and β-sitosterol in phytoestrol. The mechanisms of anticancer activity may be direct cytotoxicity, apoptotic induction and/or inhibition of angiogenesis (Cao et al., 2002). Examples of medicinal plants that had antioxidant, anti-inflammatory and anticancer activities are: *Andrographis paniculata*, *Ledum groenlandicum* Retzius, *Piper longum*, resveratrol in grapes, epigallocatechin-3-gallate from green tea leaves (Cao et al., 2002; Sunila and Kuttan, 2006; Dufour et al., 2007; sheeja et al., 2007). Hoan-Ngoc leaves contain many important compounds such as flavonoid, β-sitosterol and apigenin-7-O-β-glucoside which have high efficiency against free radicals, inflammation and cancer. Therefore, the cited reputation of Hoan-Ngoc leaves for cancer treatment may relate to the antioxidant and anti-inflammatory properties of these constituents. This study aims to determine total phenolic and flavonoid contents, and further explore the antioxidant activities of HLE. *In vitro* anti-inflammatory activities of HLE will be investigated by inhibiting NO production and proinflammatory cytokines. Anticancer activities of HLE will be examined by apoptotic induction and antiangiogenesis in tumor cells both *in vitro* and *in vivo*. Moreover, this study will also determine mutagenic effect of HLE to evaluate the toxicity of long-time consumption.

Keywords: Hoan Ngoc (*Pseuderanthemum palatiferum* (Nees) Radlk.), toxicity, mutagenicity, antioxidant activity, polyphenols, flavonoids, anti-inflammation, nitric oxide (NO), RAW264.7 cell, anticancer, apoptosis, antiangiogenesis, proinflammatory cytokines, B16F-10 melanoma

3. Research objectives

The objectives of the study are:

1 To determine the total phenolic and flavonoid contents from HLE.

2 To examine the antioxidant activity of HLE by using DPPH, FRAP and DCFH-DA assays.

3 To study *in vitro* anti-inflammatory activity of HLE by inhibiting NO production and proinflammatory cytokines in LPS and/or IFN-γ stimulated RAW264.7 cells.

4 To investigate anticarcinogenic activity of HLE *in vitro*: cytotoxicity and induction of apoptotic cell death in PC-3 and B16F-10 melanoma.

5 To investigate anticarcinogenic activity of HLE *in vivo*: inhibition of new blood vessel growth, proinflammatory cytokines and angiogenic factors using B16F10 melanoma inducing angiogenesis in C57BL/6 model.

6 To study mutagenic activity of HLE.

4. Research hypothesis

The extract contains high total antioxidant, anti-inflammatory and anticarcinogenic activities but has no mutagenicity.

5. Scope and limitations of the study

This thesis will only focus on the study of basic toxicity and biological activities of the extracts from Hoan-Ngoc leaves. Antioxidant, anti-inflammatory, and mutagenic activities will be limited to *in vitro* studies. Anti-inflammatory activity will

be limited to LPS and/or IFN-γ stimulated RAW264.7 cells. Anticancer activity will be studied using both *in vitro* and *in vivo* models. *In vitro* studies will be limited to cytotoxicity and induction of apoptotic cell death in PC-3 and B16F-10 melanoma. *In vivo* anticancer study will be focused on antiangiogenesis using B16F-10 melanoma inducing capillary formation in C57BL/6 murine model.

6. Research procedure

6.1 Preparation of crude Hoan-Ngoc leave extracts

Fresh leaves of Hoan Ngoc will be purchased from Yasothon province, Thailand. Specimen will be identified by Dr. Kongkanda Chayamarit of the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Powder air-dried leaves will be extracted by using 80% ethanolic maceration and water maceration. Fresh leaves will be extracted fresh by blending with 80% ethanol and water. The extracts will be centrifuged and the supernatant will be filtered through Whatman No. 1 filter paper and concentrated by using vacuum rotary evaporator. Then, the extracts will be dried by lyophylization and the residues will be stored in a refrigerator at -20 °C till use in subsequent experiments. The residues of water maceration extract and water fresh extract will be dissolved with water, and ethanol maceration extract and ethanol fresh extract will be dissolved with dimethyl sulfoxide (DMSO) when use in experiments.

6.2 Determination of phytochemicals

6.2.1 Total phenolic content

Total phenolic content of individual extract will be determined by the method of Folin Ciocalteau (Mariod, Matthaus, Eichner, and Hussein, 2006). Briefly, test solution of 100 µl will be added to 2.0 ml of 2% Na_2CO_3 and mixed thoroughly. After 2 min, 100 µl of 50% Folin-Ciocalteau reagent will be added, mixed, and allowed to stand at room temperature for 30 min. Absorbance will be measured at 750 nm by a spectrophotometer against a blank consisting of all reagents and solvents without the extract. A standard curve of the reference compound, gallic acid, will be prepared, and the concentrations of phenolic content in the extracts will be determined from the curve. Results will be expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract.

6.2.2 Total flavonoid content

Total flavonoid content will be determined using a colorimetric method (Liu et al., 2002). Briefly, 0.25 ml of the extract will be diluted with 1.25 ml of distilled water. Then 75 µl of a 5% $NaNO_2$ solution will be added to the mixture. After 6 min, 150 µl of a 10 % $AlCl_3 \cdot 6H_2O$ solution will be added and the mixture will be allowed to stand for another 5 min. Half of milliliter of 1 M NaOH will be added, and the total will be made up to 2.5 ml with distilled water. The solution will be well mixed, and the absorbance will be measured immediately against the prepared blank at 510 nm using spectrophotometer in comparison with the standard prepared similarly with known catechin concentrations. The results will be expressed as milligrams of catechin equivalent (CE) per gram of dry extract.

6.3 Antioxidant activity

6.3.1 DPPH scavenging assay

The DPPH method will be determined as described by Sanchez-moreno, Larraui, and Saura-Calixto (1999). The extract of 0.1 ml at different concentrations will be added to 3.9 ml of DPPH solution ($2.5x10^{-2}$g/L methanol). The mixture will be shaken vigorously and left to stand at room temperature for 45 min in the dark. The mixture will be measured spectrophotometrically at 515 nm. The free radical scavenging activity will be calculated as following:

DPPH radical scavenging activity (%) = $[(A_{control}-A_{sample})/A_{control}]x100$

Where $A_{control}$=the absorbance of control (methanol)

A_{sample}= the absorbance of different concentrations of sample extract

A standard curve of ascorbic acid and trolox will be prepared by plotting the percentage (%) of free radical scavenging activity of ascorbic acid and trolox versus its concentration. The final result will be expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) and trolox equivalent antioxidant capacity (TEAC) in 1 g of dry extract (Bakar, Mohamed, Rahmat, and Fry, 2009).

6.3.2 FRAP (Ferric reducing/antioxidant power) assay

The ferric reducing ability of HLE will be measured colorimetrically (Fang et al., 2009). The FRAP reagent will include 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution will be prepared by mixing acetate buffer, TPTZ solution, and $FeCl_3 \cdot H_2O$

solution (10:1:1, v/v/v). HLE of 0.1 ml will be added in 3 ml FRAP reagent and mixed. Readings will be recorded on the spectrophotometer at 593 nm, and the reaction will be monitored for 10 min. Ascorbic acid and trolox standard solution will be used to prepare the calibration curves. The ferric reducing ability of HLE will be expressed as mg AEAC and TEAC per gram of dry extract.

6.3.3 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) assay

Antioxidant activity will be evaluated using the DCFH-DA assay as described by Dufour et al. (2007). Briefly, L-929 cells will be plated in 96 microwell plates and incubated for 24 hrs at 37°C and 5% CO_2. Cells will be washed with Hank's balanced salt solution (HBSS) at pH 7.4 and incubated for 30 min with HBSS (pH 7.4) containing DCFH-DA. Cells will then be washed again with HBSS. To assess antioxidant activity, cells will be incubated either with various concentrations of HLE, or trolox in the absence or presence of *tert*-butylhydroperoxide (*t*BH). Fluorescence will be measured after 1 and 4 hrs on the automated 96-well plate reader using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

6.4 Animals

C57BL/6 mice will be used for *in vivo* angiogenic study. C57BL/6 mice (4-6 weeks old, 20-25 g body wt.) will receive mouse chow and water *ad libitum*. The animals will be housed in a temperature controlled room with a daily of 12 hrs light and dark cycle in Animal Care Facility at Suranaree University of Technology (Sheeja et al., 2007).

6.5 Cell lines

Murine fibrosarcoma L-929 cell lines will be used to investigate antioxidant activity using the DCFH-DA assay. L-929 cells will be grown in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Liu et al., 2008).

The mouse macrophage cell line RAW264.7 gamma NO (-) will be used to investigate the anti-inflammatory effect of HLE on inhibition of NO and proinflammatory cytokines production *in vitro*. Cells will be cultured and maintained in RPMI-1640 medium supplemented with 10% heat-inactivate FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Exponentially growing cells will be used for experiments when they reach about 80% confluence (Tsai, Lin-Shiau, and Lin, 1999).

Human prostate adenocarcinoma cell line, PC-3, will be used to investigate apoptotic effect of HLE. PC-3 will be maintained in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Ezekwudo et al., 2008; Shukla and Gupta, 2008).

B16 F-10 melanoma cell line will be used in apoptotic and angiogenic studies. B16F-10 will be maintained in Dulbeco's Modified Eagles Medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Sheeja et al., 2007).

All cell lines will be grown and maintained at 37°C in an atmosphere of 95% air and 5% CO_2.

6.6 Determination of anti-inflammatory activities

6.6.1 *In vitro* cytotoxicity

In order to find the optimal concentrations of HLE for subsequent experiments, RAW264.7 cells will be incubated in the absence or presence of various concentrations of HLE for 24, 48 or 72 hrs. Cell viability will be evaluated by the trypan blue exclusion (Wibuloutai, 2006), MTT methods (Peng, Fan, and Wu, 2006) or propidium iodide staining (Duchler and Stepnik, 2008).

6.6.1.1 Trypan blue exclusion method

The cell suspension will be mixed with trypan blue and cell viability will be evaluated in a hemocytometer. The viable cells (exclude trypan blue) and nonviable cells (blue) will be counted under a light microscope. The percent viable cells will be calculated according to the following formula:

$$\text{Percent viable cell} = \frac{\text{Total cells} - \text{dead cells}}{\text{Total cells}} \times 100$$

6.6.1.2 MTT colrimetric assay

Serial dilutions of HLE will be added into each of 96-well plates. Cells will be plated in a 96-well plate and incubated in the presence or absence of HLE for 24, 48 or 72 hrs. MTT dye solution will be added in each well and the plates will be further incubated at 37 °C, 5% CO_2 for 4 hrs. After incubation, the medium will be removed. DMSO will be added into each well to dissolve insoluble formazan crystal to give a uniform dark purple color before reading at 590 nm by a microplate reader (Peng et al., 2006). The percentage of cell viability was calculated according to the following equation (Komutarin et al., 2004).

$$\text{Percent cell viability} = \frac{\text{average OD for test group}}{\text{average OD for control group}} \times 100$$

6.6.1.3 Propidium iodide (PI) staining

Cells viability will be determined with PI staining as described by Duchler and Stepnik (2008) with some modifications. Briefly, cells will be plated in a 96 well plates at 10^6 cells per well and incubated in the absence or presence of serial dilutions of HLE at 37 ˚C, 5% CO_2 for 24 or 48 hrs. Cells will be centrifuged at 1500 rpm for 10 min and the pellets will be washed in PBS containing 1% BSA and 0.1% sodium azide (PBS-BSA-NaN_3). Then, PBS staining will be removed and cells will be incubated with 100 µl of PI solution (1 mg/ml stock solution diluted to 1:200) for 10 min in the dark. After incubation, cells will be washed in PBS staining and transferred to 5 ml flow-tube. Cell viability will be analyzed by flow cytometry.

6.6.2 Inhibition of nitric oxide production

RAW264.7 cells in a 24 well plate will be pretreated with or without various concentrations of HLE or other antioxidant controls at 37°C for 24 hrs. Twenty-four hrs later, cells will be stimulated with LPS and/or IFN-γ and further incubated for another 24 hrs. The NO production will be determined by measuring nitrite concentration using the Griess reagent (Wibuloutai, 2006). Briefly, 100 µl of cell-free supernatant from each well will be transferred to another 96-well plate. Then an equal volume of Greiss reagent (3.5 ml of phosphoric acid, 1 mg of sulfanilamide and 0.1 mg of N-(1-naphyl)-ethylenediamine (NED) in 100 ml MQ water) will be added. The absorbance of sample was measured at 545 nm with a spectrophotometric microplate

reader. The amount of nitrite will be calculated from the $NaNO_2$ standard curve (Komutarin et al., 2004).

6.6.3 Inhibition of proinflammatory cytokines

RAW264.7 cells in a 24 well plate will be pre-incubated with or without various concentrations of HLE for 24 hrs. Cells will then be treated with LPS and/or IFN-γ. The culture supernatants will be collected and proinflammatory cytokines (IL-1β, IL-6 and TNFα) will be measured by commercial ELISA kits (Park, Lee, Hong, Kim, 2002; Wang et al., 2008).

6.7 Anticancer activities

6.7.1 *In vitro* cytotoxicity

Both PC-3 and B16F-10 melanoma will be used to evaluate the *in vitro* cytotoxicity of HLE in order to find optimum concentrations to use for *in vitro* anti cancer activity. Cells will be plated in a 96 well plate in the absence or presence of serial dilutions of HLE for 24, 48 or 72 hrs. Cells viability will be evaluated by trypan blue exclusion (Wibuloutai, 2006), MTT methods (Peng et al., 2006) or PI staining (Duchler and Stepnik, 2008).

6.7.2 Analysis of DNA fragmentation by agarose gel electrophoresis

Both PC-3 and B16F-10 melanoma will be incubated for 48 hrs in the absence or presence of various concentrations of HLE. Cells will be washed, and pelleted by centrifugation. Induction of apoptosis will be detected by visualizing DNA fragmentation in agarose gel electrophoresis (Shukla and Gupta, 2008). Briefly, cells

pellets will be incubated with DNA lysis buffer and centrifuged to collect the supernatant. The obtained supernatant will be incubated overnight with RNase and proteinase K for 2 hrs. After that, DNA will be extracted with phenol:chloroform (1:1), precipitated with 95% ethanol and centrifuged. The pellet will be air dried and dissolved in Tris–EDTA buffer. The total amount of DNA will be resolved over 1.5% agarose gel, containing ethidium bromide in TBE buffer. The bands will be visualized under UV trans-illuminator followed by Polaroid photography.

6.7.3 Detection of apoptosis by flow cytometry

In addition to DNA fragmentation, detection of apoptosis will be confirmed with annexin-V-staining as described by Ezekwudo et al. (2008). Cells will be labeled with annexin V-FITC according to the manufacture's instructions. Briefly, both PC-3 and B16F-10 melanoma will be incubated for 48 hrs in absence or presence of various concentrations of HLE. Cells will be harvested and washed with cold PBS. Then, cells will be washed and resuspended in annexin-V binding buffer. After that, Cells will be incubated with annexin-V-FITC in the dark for 15 min. At the end of incubation, cells will be further stained with PI solution and then analyzed by flow cytometry to discriminate between live and apoptotic cells.

6.8 Antiangiogenesis activities

6.8.1 *In vivo* toxicity

C57BL/6 mice will be injected intraperitonealy with different concentrations of extract for 5 days to find the optimum doses for *in vivo* angiogenic study. The

animals will be observed for general signs of toxicity and mortality. The control group will receive only the vehicle.

6.8.2 *In vivo* antiangiogenesis study

Induction of angiogenesis will be performed by injecting B16F-10 melanoma cells intradermally on the shaven ventral surface of C57BL/6 mice. For vehicle control group, animals will be received water or DMSO. Other groups will be treated with HLE by intraperitoneal injection at difference doses for 5 days.

Blood will be collected from caudal vein of mice after 24 hrs and the 9th day of tumor induction. Serum will be separated and used for investigation of various cytokines (IL-1β, TNF-α and IL-6) and the angiogenic factor VEGF by using ELISA Kits (Sheeja et al., 2007).

On 9th day, mice will be sacrificed immediately after intravenous injection of 1% Evan's blue solution. Ventral skin will be removed and the number of tumor directed capillaries per cm^2 around the tumor will be counted using a dissection microscope. At the same time, the tumor size will be assessed by averaging the diameter of the short and long axes of the remainder of the injected cells. (Yoon et al., 1995; Sheeja et al., 2007)

6.9 Mutagenic activity

The mutagenicity assay with *Salmonella typhimurium* strains TA98 and TA100 will be performed as described by Bouhlel et al. (2008). The experiments will be performed with and without an exogenous metabolic system, the S9 fraction. Bacteria culture and sodium phosphate buffer (for assay without S9) or S9 mix (for

assay with S9) will be added to top agar (supplemented with L-histidine and D-biotin) containing different concentrations of HLE. The resulting complete mixture will be poured on minimal agar plates. The plates will be incubated at 37°C for 48 hrs and the revertant bacterial colonies of each plate will be counted. Negative control without HLE will be prepared in the same manner as the assay mixture to give the number of spontaneous revertant. Benzo[a]pyrene (B[a]P) will be served as the positive control. The extract will be considered as mutagenic if the number of revertants per plate was at least doubled in TA98 and TA100 strains over the spontaneous revertant frequency. Data will be collected with a mean ± SD of 3 plates (n = 3).

7. Location of research

This study will be conducted at the Center for Scientific and Technological Equipment (F9 building), Suranaree University of Technology and Center for Integrative Toxicology at Michigan State University.

8. Statistical analysis

Results will be reported as mean±SD. Differences will be considered statistically significant for $p<0.05$ by using analysis of variance (ANOVA) and *t*-test. All treatments *in vitro* and *in vivo* will be repeated at least two times.

9. Expected results

1. Basic datas for the toxicity and mutagenicity of HLE will be direct benefits to the consumers.

2. Information on the biological activities of HLE can be used as basic pharmacological data for future research.
3. Scientific data obtained from HLE will help to support and strengthen the knowledges and practices of using Hoan-Ngoc which has become popular medicinal plant in Thailand.
4. Scientific data obtained from HLE will be basic knowledges to apply for development of anti-cancer drugs in the future.

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11. Research plan

Thesis procedure begins on April 2009 to September 2011

Activities	2009									2010												2011								
	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9
1. Plant extraction																														
2. Analyzing of phytochemical contents and antioxidant activity																														
3. Testing anti-inflammatory activity *in vitro*: cytotoxicity, inhibition of NO production and proinflammatory cytokines																														
4. Anticarcinogenic studies *in vitro*: cytoxicity and induction of apoptotic cell death																														
5. Anticarcinogenic studies *in vivo*: general toxicity and antiangiogenesis																														
6. *In vitro* antimutagenicity study																														
7. Summary and analyze																														
8. Thesis preparation																														

Student signature.....Patcharawan sitisart.....

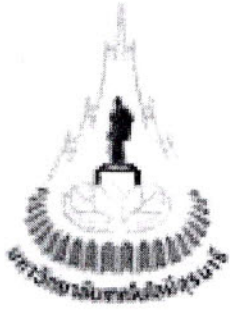
(Miss Patcharawan Sittisart)

.....20 / April / 2009.....

Thesis advisor signature.....Benjamart Chitsomboon.....

(Asst. Prof. Dr. Benjamart Chitsomboon)

.....20 / Apr. / 2009.....



แก้ไข.

M.Sc. Thesis Proposal

USE OF LICHEN AS INDICATOR FOR AIR POLLUTION MONITORING IN NAKHON RATCHASIMA PROVINCE

การใช้ไลเคนเพื่อเป็นดัชนีชี้วัดการตรวจมลพิษทางอากาศในจังหวัดนครราชสีมา

By

Miss Amornrat Pitakpong

School of Biology

Institute of Science

Suranaree University of Technology

3 April 2009

M.Sc. Thesis Proposal

Miss Amornrat Pitakpong **ID.No.M4930372**
School of Biology **Institute of Science**
Thesis advisor
Asst. Prof. Dr.Nathawut Thanee Advisor
Asst. Prof. Dr.Wanaruk Saipunkaew Co- Advisor

1. Thesis title

USE OF LICHEN AS INDICATOR FOR AIR POLLUTION MONITORING IN NAKHON RATCHASIMA PROVINCE

การใช้ไลเคนเพื่อเป็นดัชนีชี้วัดการตรวจมลพิษทางอากาศในจังหวัดนครราชสีมา

Key words : Lichen / Bioindicator / Air Pollution / Nakhon Ratchasima.

2. Introduction

Millions of year ago, a single-cell organism was born and subsequently developed into a many-celled organism as it evolved and reproduced along with the aromatic physical changes of the earth. There are around 5 million species on the earth, one of which is a living thing called a "lichen" which first appeared about 400 million year ago.

Lichens (pronounced as "Liken") are, by definition, symbiotic organisms composed of a fungal partner which is mycobiont, and one or more photosynthetic partner. The photobiont may be either a green alga or a cyanobacterium. (Brodo et al., 2001).The cyanobacteria symbiont component may specialize in fixing atmospheric nitrogen for metabolic use and occur as crusty patches or bushy growths on trees, rocks and bare ground. Over the years these two components grew up together in a harmonious association and are referred to as symbiosis, or, more simply, the "together living components", and are classified in the Fungal Kingdom (Mason, 1979). Lichens are organisms that are often mistaken for plants. Most people believe they are a type of moss. In fact, they do not even belong to the kingdom Plantae because they are not considered as a single entity but as fungi having symbiotic relationship with green algae or cyanobacteria photobionts (Brodo et al., 2001) that form the body or thallus. The fungal partners are mostly (over 95%)Ascomycetes. Most of the rest are Basidiomycetes. As far as science has been able to discover few, if any, of the fungi involved can survive and reproduce in the wild on their own. Each lichen species contains a different species of fungi and so it is according to the species of fungi that lichens are classified. This classification is generally based on characteristics of the thallus and reproductive organs. There are between 13,500 and 17,000 species of lichen depending on the classification adopted. About 20% of fungal species are involved in lichen partnerships.

There are approximately 100 species, 40 algal genera and cyanobacteria as photobionts (Nash, 1996) but about 85 percent of lichens are observed to associate only with green algae (Salix, 2004). The most common photobionts are the genera *Trebouxia, Trentepohlia,* and *Nostoc*. *Trebouxia* and *Trentepohlia* belong to the green algae and *Nostoc* to the cyanobacteria (Nash, 1996).Approximately 60 percent of known lichens have *Trebouxia* as their photobiont (Ahmadjian and Paracer, 1986) and about 10 percent contain cyanobacteria (Nash, 1996). Even the fastest growing ones are very slow compared to flowering plants. Many discussions on growth are focused on radial growth, particularly in the case of crustose lichens. The typical radial annual growth rates for lichens in unpolluted habitats are as follows: crustose: 0.5 mm to 2.0 mm, foliose: 0.5 mm. to 4.0 mm, fruticose: 1.5 mm to 10.0 mm (Paul, 2008)

Lichens grow by extending their thallus outwards, from either its tips or edges and rate of growth can vary from 0.5 mm per year to 500 mm per year (Richardson, 1975). Their slow growth rate equates with their long life.

A lichen absorbs most of its mineral nutrients from the air and rainfall. Pollution in the atmosphere can be especially dangerous to lichens because they retain, and can accumulate, deadly amounts of heavy metals, sulfur, radioactive elements, NO_2, and ozone. Sulfur dioxide (SO_2) is especially lethal to lichens because it lowers pH and deteriorates chlorophyll, which causes photosynthesis to cease. Anti-sulfur dioxide legislation in the last 25 years is allowing lichens to return to formerly polluted areas. Lichens have also been used to monitor the amount of pollutants in an environment by observing the condition of lichens as well as their chemical composition (Hawksworth, 1970).

Lichens are increasingly used as air quality biomonitors (Bartoli et al., 1997) because they have several advantages over electronic monitors which are expensive and their use and maintenance are not simple or cheap. In contrast, biomonitors are available without cost and there are millions of them already functioning throughout the world (Ockenden et al., 1998).Lichens are increasingly used worldwide as air quality biomonitors because they are efficient, easy and cheap, but validation studies of the methodology are scarce (Julián et al., 2002).

Lichens can be used as bioindicators of air quality because they are sensitive to atmospheric pollution, including heavy metals, radiation, and ozone. The component of air pollution responsible for the greatest damage to lichens is sulfur dioxide (SO_2) released by coal-burning power plants (Path, 2002).

Nowadays, the major pollutants are nitrogen compounds from road traffic and intensive farming. These compounds do not destroy all lichens because some species positively thrive on nitrogen. So different pollutants create different patterns of lichen growth and, by understanding them scientists are able to chart the health of the environment. Some lichens grow very slowly so that

they can indicate the history of the substrate and the environment where they are found. They have been used as indicators of ancient woodlands that have never been clear-felled.The reindeer and the caribou of the northern latitudes are well known for feeding on lichens, especially in winter when food is scarce. In fact, about 90 percent of their winter diet consists of lichens (Brodo et al., 2001). Lichens are also useful to their neighbours, recycling nutrients used by other plants and providing homes for spiders, mites, lice and other insects. Human beings extracts the most incredible range of wool dyes from lichens and also eat some of the edible species, while drug companies use lichens to make antibiotics or sunscreen cream.

The high sensitivity of lichens is related to their biology. Alteration of the symbiotic balance between the photobiont and mycobiont may readily lead to a breakdown of the association. Therefore, lichen decline may be a result not only from the occurrence of toxic concentrations, but also from altered nutrient supplies that favor one symbiont over the other. As long-lived and perennial organisms, lichens are exposed to air pollutions all year. Unlike many vascular plants, they have no deciduous part and hence cannot avoid pollutant exposure in this way. The lack of stomates and cuticle means that aerosols and gases may be absorbed over the entire thallus surface and may readily diffuse down to the photobiont layer. Furthermore, uptaking primarily involves physicochemical processes with limited biological control by the lichens.

Lichens are widely used as environmental indicators or bio-indicators. If air is very badly polluted, there may be no lichens present. Just green algae may be found. If the air is clean, shrubby, hairy and leafy, lichens become abundant. A few lichen species can tolerate quite high levels of pollution and are commonly found on pavements, walls and tree barks in urban areas (Brodo et al., 2001). The most sensitive lichens are shrubby and leafy while the most tolerant lichens are all crusty in appearance.

3. Literature review

3.1 Systematic character

Infrastructure that could be compared to a body of lichen is called the thallus. Thallus includes fungus and algae that allocated space as their habitat. Lichen classification, according to principle of taxonomy, is based on exterior character, interior structure and chemical composition of the thallus.

3.2 Exterior structure

Colour of Thallus: When in dry a condition, thallus is grey or brown. When it is in wet condition or with humidity, colour of algae clealy appears and

causes thallus to be green. The bright colour of lichen is caused by lichen products, e.g. orange or yellow substance is parietin pigment that could be seen a lichen in the areas under the bright light. The substance is helpful in screening of the UV to protect algae. In lichen with the blue-green algae, colour substance or natural substance is not found (Lichen Research Unit and Lichen Herbarium, 1993).

Size of Lobe: The thallus (foliose) includes lots of lobe joined together. However, lobes' ends may separate from each other. Therefore, sizes of lobe can be visibly viewed and that is one of the characters used for lichen classification. Size of lobe is measured from the separating points of lobes (Lichen Research Unit and Lichen Herbarium, 1993).

Isidia: It is similar to groups or pieces of short needle distributed on surface of thallus including of algae and hypha covered by cortex and serves for asexual reproduction and releases to the air caused by cracking of epidemis or cortex (Lichen Research Unit and Lichen Herbarium, 1993).

Phyllidia: It is in a form of scale, similar to a small lobe that emerges from thallus. It serves for asexual reproduction (Lichen Research Unit and Lichen Herbarium, 1993).

Fibril: It looks like a short branch shot from lateral side of fruticose thallus. It is mostly found in *Usnea* genus. It serves for asexual reproduction (Lichen Research Unit and Lichen Herbarium, 1993).

Cyphellae: It is as a small hole with rim and appears at low surface of thallus. It is the permanent character of the genus *Sticta*. It serves in releasing air or exchange gas (Lichen Research Unit and Lichen Herbarium, 1993).

Rhizine: It is a part of hypha at lower part joined up, looks similar to rootlet in different forms. It serves for attaching to substrates (Lichen Research Unit and Lichen Herbarium, 1993).

Tomentum: It looks like a cotton pad or Scotch-brite at the lower part of thallus. It can be in different colours, from pale-brown to black. It serves as a holder to habitat or helper to absorb humidity (Lichen Research Unit and Lichen Herbarium, 1993).

Cilia: It looks like eyelash emerged at rim of thallus (Lichen Research Unit and Lichen Herbarium, 1993).

Hypothallus: It is part of fast growing hypha. It can be brown or black without any algae living together. It can be found at rim of thallus (Lichen Research Unit and Lichen Herbarium, 1993).

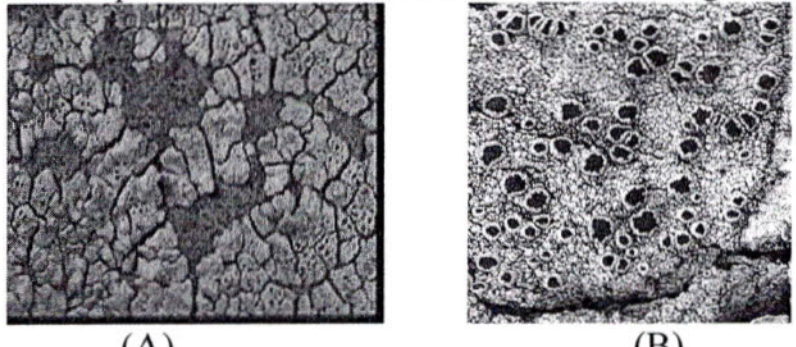
3.3 The structure and morphology of lichens

The form of a lichen is determined by the genetic material of the fungal partner, association with a photobiont is required for the development of that form. When grown in the laboratory in the absence of its photobiont, a lichen fungus develops as an undifferentiated mass of hyphae. If combined with its photobiont under appropriate conditions, its characteristic form emerges, in the process called morphogenesis (Brodo et al, 2001). In a few remarkable cases, a single lichen fungus can develop into two very different lichen forms when associating with either a green algal or a cyanobacterial symbiont. Quite naturally, these alternative forms were at first considered to be different species, until they were first found growing in a conjoined manner. Lichens are divided into three major growth forms: crustose, foliose, fruticose, more advanced books on lichens classify them into two further groups, squamulose and leprose (Brodo et al., 2001).

3.3.1 Crustose

Crustose lichens are "crust-like" forms.They tightly attach to or embed in substrates, and have no lower cortex. Crustose lichens consist of about 75 percent of all lichens on earth. Crustose lichens attach so tightly to rocks, trees, sidewalks, or soils where they grow up on. They, therefore, can not be removed without damaging the substrates.

Cracked crusts, like the species of *Acarospora* (or *Pleopsidium*), are separated into segments (areoles) and are called areolate. Crustose lichens that grow up immersing in rocks with only their fruiting bodies above the surface are called endolithic, and those that grow up immersing in plant tissues are called endophloidic or endophloidal. Loose, powdery lichen crusts without layered structure are called leprose. Crustose lichens are shown in Figure 1.

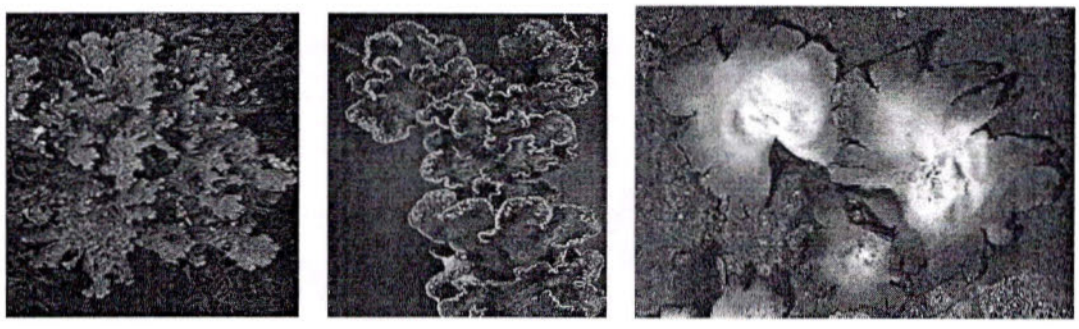


(A) (B)

Figure 1 (A) *Acarospora* or *Pleopsidium*, (B) *Haematomma*
Source : (A) Lichens of North America (2005)
(B) Lichen Research Unit and Lichen Herbarium (1993)

3.3.2 Foliose

Foliose lichens are "leaf-like" in both appearance and structure. They compose of lobes. They relatively loosely attach, usually by rhizines, to substrates. Their lobes have upper and lower sides and usually grow more-or-less parallel to the substrates. Umbilicate lichens attach to substrates only at central points. Foliose lichens are shown in Figure 2.



(A) (B) (C)

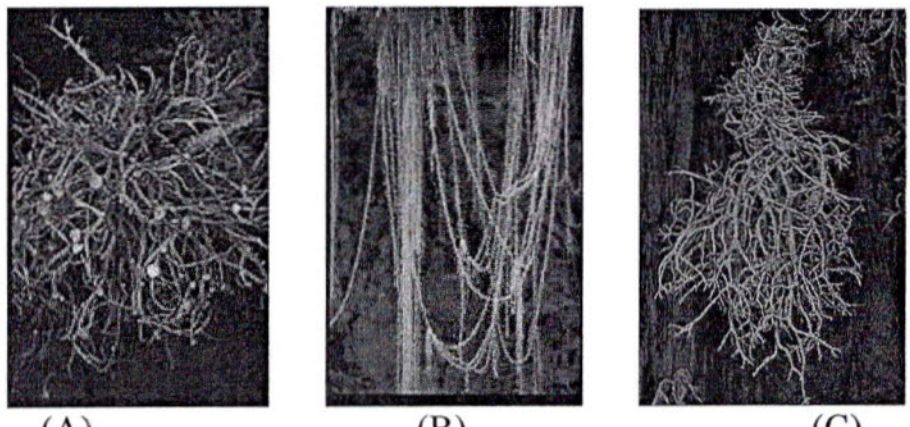
Figure 2 (A) *Lobaria linita*, (B) *Melanelia* , (C) *Umbilicalia*

Source : (A) Lichens of North America (2005)

(B) and (C) United State Forest Service (2008)

3.3.3 Fruticose

Fruticose lichens are "hair-like" and the most are three-dimensional. They are usually round in cross section (terete), and most are branched. Their thalli may be upright, shrubby, or of pendulous strands. Foliose lichens are shown in Figure 3.

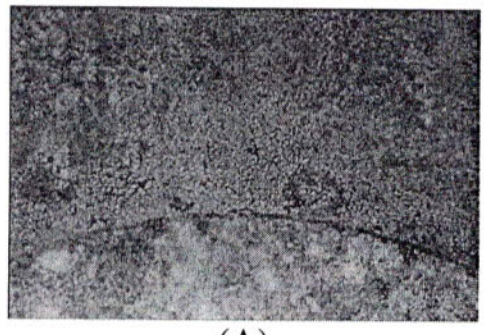


(A) (B) (C)

Figure 3 (A) *Ramalina stenospora*, (B) *Usnea longissima*, (C) *Letralia*
Source : (A)and (B) Lichens of North America (2005)
(C) United State Forest Service (2008)

3.3.4. Leprose

Loose, powdery lichen crusts without layered structure are called leprose. These lichens have no fruiting bodies, have a complex chemical content and are difficult to classify. Leprose lichens are shown in Figure 4.

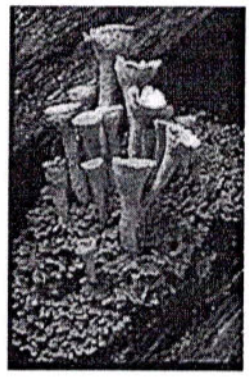


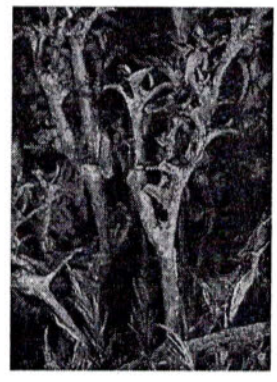
(A)

Figure 4 (A) Leprose
Source : The Council for Scottish Archaeology (2007)

3.3.5. Squamulose

Squamulose lichens have "scale-like" lobes called squamules that are usually small, overlapping and lacking a lower cortex. These are a sub-division of the Crustose lichens, peeling up at their outer edges to form "squamules" (e.g. *Cladonia* or *Toninia).*





(A) (C)

Figure 5 (A) *Cladonia carneola*, (B) *Cetraria*

Source : (A) Lichens of North America (2005);

(B) Vegetative Morphology I (2008)

Morphologically, lichens are made up of a few distinct characters. The most obvious is the thallus. The form of the thallus is a result of the fungal species involved. The thallus is the body of the lichen. Most of what you see, if

it isn't reproductive structures, is thallus. The fungal hyphae (filaments) branch and then fuse together (anastomose) when they meet to form a mesh of hair-like threads. The top surface is normally a layer of tightly packed hyphae called a 'cortex'. Below this is the algal layer where the photobiont lives. Below this is the medulla an area of loose hyphae in which nutrients are stored. Sometimes a lower cortex exists, in others the medulla rests on the surface. In crustose and squamulose lichens there is no lower cortex. In foliose lichens there is a lower cortex and in fruticose lichens the lower cortex is replaced by a central one. (Mason and Vernon, 1973)

Filamentous lichens are totally different. They consist of chains of algal cells wrapped around with fungal hyphae. In lichen species of the genus *Cladonia* are a successful group which have a primary and secondary thallus. The primary thallus is small and clings closely to the substrate while the secondary thallus is shrubby growth like fruticose lichens. Once the lichen is established the primary thallus often dies off.

Despite the wide diversity of the basic growth forms, all lichens have similar internal morphology. The bulk of the lichen's body is formed from filaments of the fungal partner, and the relative density of these filaments defines the layers within the lichen. (Wolseley et al., 1996)

At its outer surface, where it comes in contact with the environment, the filaments are packed tightly together to form the cortex. The dense cortex serves to keep out other organisms, and helps to reduce the intensity of light which may damage the alga cells.

The algal partner cells are distributed just below the cortex in a layer where the fungal filaments are not so dense. This is very similar to the arangement in a plant leaf, where the photosynthetic cells are loosely packed to allow air circulation.

3.4 Interior structure of thallus

There are two types of thallus depending on self-arrangement of fungus and algae:

3.4.1 Heteromerous thallus: Fungus and algae formed separately and can be divided into three levels, (Fig. 6), including:

Upper and lower cortex includes tightly pressed hypha to protect or dam up the algae inside.

Algal layer includes phycobionts that selves-arranged in line or in groups.

Meldulla is the level with loosely bound hypha. The level has secondary metalites that cause it different colours but mostly the white, yellow or orange is found.

3.4.2 Homoiomerous thallus

Thallus is not obviously divided into levels. It includes cyanobacteria algae or blue-green algae such as *Nostoc* as a component. These lichens are called gelatinous lichens e.g. *Collema* and *Leptogium*.

Below the algal layer is the medulla, a loosely woven layer of fungal filaments. In foliose lichens, there is a second cortex below the medulla, but in crustose and squamulose lichens, the medulla is in direct contact with the underlying substrate, to which the lichen is attached. The cross section of lichen is shown in Figure 6.

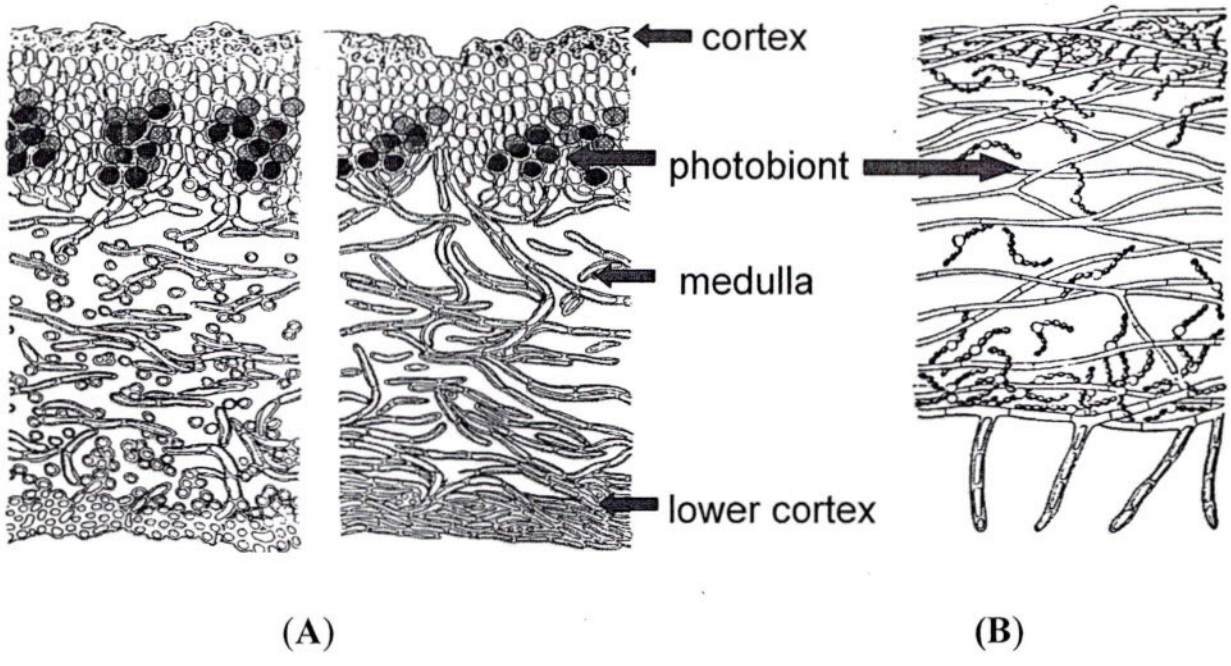


Figure 6. **(A)** Heteromerous thallus, **(B)** Homoiomerous thallus

Source : University of California museum of Poleontology (1905)

3.4.3 Crustose

Crustose lichens, as their name implies, form a crust on the surface of substrate where they are growing up. This crust can be quite thick and granular or actually can embed within the substrate. In the latter case, the fruiting bodies still rise on the surface. In many crustose lichens, surface of the thallus break up into cellulars, crazy-paving like pattern. Crustose lichens tend to grow out from

their edges and have fruiting bodies in the centres. Crustose lichens are very difficult to be removed from substrates.

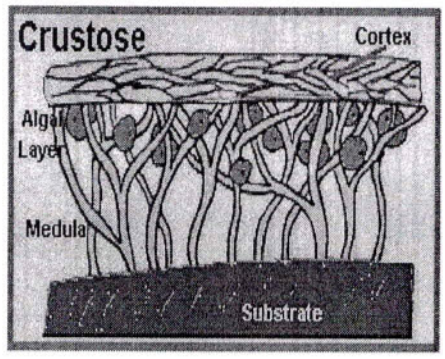


Figure 7 Cross Section of Crustose
Source : Earth-Life Web Productions (2008)

3.4.4 Foliose

These have an upper and lower cortex. They are generally raised to some extent above the substrate but connected to it by rhizines (specialised root-like hyphae). They are easier to remove from their substrate when collecting because of this.

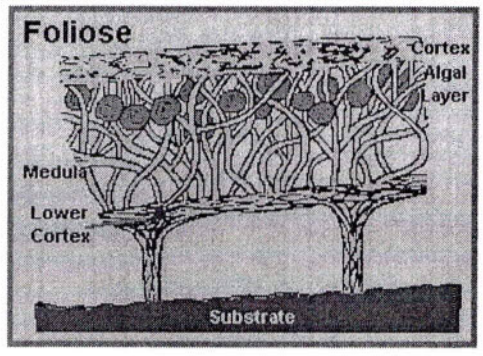


Figure 8 Cross Section of Foliose

Source : Earth-Life Web Productions (2008)

3.4.5 Fruticose

Fruticose lichens are shrubby lichens. They attach to substrates by a single point and rise, or more usually, dangle from these substrates. Some foliose lichens can be stubby like fruticose lichens. However, close examination will reveal that the algal part exists only on one side of the flattish thallus. Whereas in fruticose lichens, it exists as a ring around the thallus, even when it is flattened as in *Ramalina* sp.

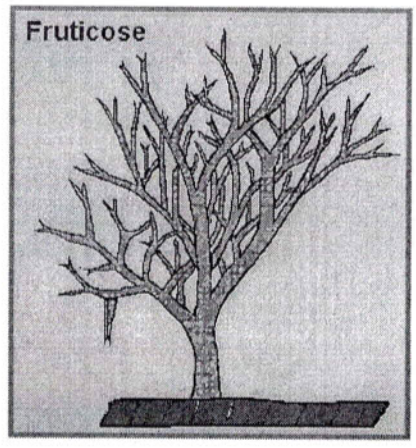


Figure 9 Cross Section of Fruticose.

Source : Earth-Life Web Productions (2008)

3.4.6 Leprose lichens

Leprose lichens are an odd group of lichens which have never been observed to produce fruiting bodies. Because knowledge of the form of the fruiting bodies is essential to the identification of fungi, these lichens have not yet been identified properly, or at least not yet given full scientific names. These fungi not only lack an inner cortex, but also lack an outer one, i.e. no cortex, only an algal cell layer and sometimes a weakly defined medulla.

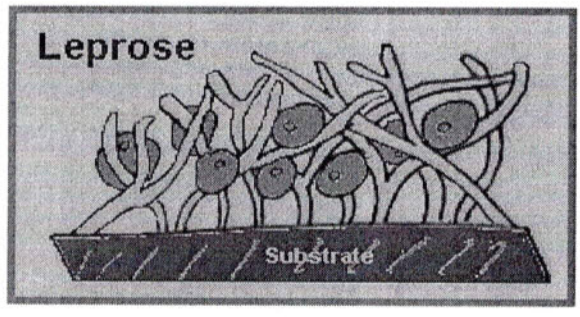


Figure 10 Cross Section of Leprose
Source : Earth-Life Web Productions (2008)

3.4.7 Squamulose

Some lichens have portions of their thallus lifted off the substrate to form 'squamules'. They are, otherwise, similar to crustose lichens in term of possessing upper cortexes but no lower cortex.

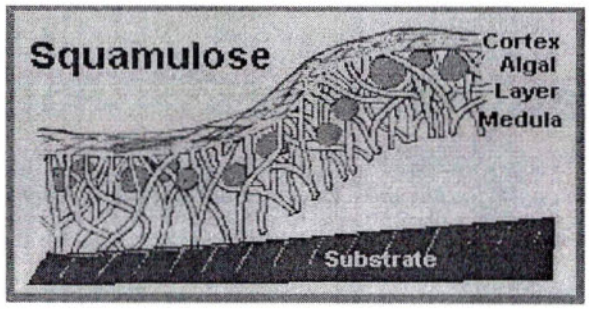


Figure 11 Cross Section of Squamulose
Source : Earth-Life Web Productions, 2008.

3.5 Reproduction

Nearly all lichen fungi (which are called mycobionts) are members of the order Ascomycotina and commonly known as Ascomycetes, though a few are members of Basidiomycotina and called Basidiomycetes. The two orders make different types of fruiting bodies, which are spore-producing structures. The most common kind of fruiting body in Ascomycete lichens is the apothecium (plural, apothecia) made by Ascomycetes, which is generally shaped like a disc, usually with a rim around the edge. Discs can become very contorted and appear as cracks in the surface of the thallus, called lyrellae or as nearly spherical blobs. Lichen reproduction may occure either by sexual (spore forming bodies) and asexual (vegetative reproductive bodies) reproduction. By asexual reproduction, fragments of thallus containing both photobiont and the mycobiont separate and form new lichens. This may happen when a piece of the thallus is accidentally broken off, but specialized structures that have evolved in lichens, namely isidia and soredia, usually play important role in this type of reproduction. (Eichorn et al., 2005)

In most lichens reproduced by sexual reproduction, tiny spores are produced in an ascus. The asci form inside structures was called ascomata. The most common type of ascoma, called an apothecium, is shaped like an open disc. For sexual reproduction, only the fungal partner is reproduced. Spores that germinate must find the appropriate photobiont in order to form a new lichen. Since this is an undependable type of reproduction, vegetative reproduction is very important.

Lichens have long been considered one of the most valuable air pollution biomonitors. As such, they have been widely used to assess trace element atmospheric contaminants. The advantages of using lichens over conventional air sampling techniques are that lichens are perennial and can be found in most terrestrial habitats. They also present easy sampling, low cost and the possibility of monitoring wide areas. Besides that lichens do not have root systems and thus they are able to uptake elements and accumulate them in their tissues. The

high degree of trace element accumulation enables the determination of several elements with high precision and accuracy. Consequently, several papers have been published on monitoring trace elements using lichens in different geographic areas (Loppi et al., 2000; Garty, 2001; Carreras and Pignata, 2002; Yenisoy-Karakas and Tuncel, 2004; Conti and Cecchetti, 2001; Bergamaschi et al., 2004).

Lichens vary in their sensitivity to SO_2 pollution; in general, crustose and squamulose lichens are least sensitive, foliose lichens are more sensitive, and fruticose lichens are most sensitive (The Georgia Conservancy, 2001).

Richardson (1992) stated that lichens can also indicate past pollution by faded or abnormal colouring and patchiness in the centre of the thallus.(Table 1)

Table 1 Some Lichens Indicative of Different Levels of Pollution

Highly Polluted	**Moderately Polluted**	**Slightly Polluted**	**Minimal or No Pollution**
Hypogymnia physodes	*Evernia prunastri*	*Parmelia caperata*	*Usnea subfloridan*
Xanthoria parietina	*Foraminella ambigua*	*Graphis scripta*	*Parmelia perlata*
Lecanora dispersa	*Lecanora chlarotera*	*Bryoria fucescens*	*Degelia plumbea*
Diploicia canescens	*Ramalina farinacea*	*Physconia distorta*	*Ramalina fraxinea*
Lepraria incana	*Lecidella elaeochroma*	*Opegrapha varia*	*Teleoschistes flavicans*

Lichens show remarkable differences with respect to their sensitivity to heavy metals. Some species are highly tolerant to high concentrations of transition metals including Cu (Purvis and Halls, 1996), Fe (Hauck et al., 2007), and Mn (Paul and Hauck, 2005). The Cu- and Fe-tolerant lichens include hyperaccumulors inhabiting metal-rich rock and slag (Lange and Ziegler, 1963). Other lichen species respond with reduced net photosynthesis or nitrogen fixation, chlorophyll degradation, and damage of thylakoids and plasmalemmas to relatively small amounts of heavy metals (Garty, 2001).

Lichens are among the most frequently used biomonitors of atmospheric pollution (e.g. Hawksworth and Rose, 1970; Ferry et al., 1973; Nimis et al., 2000; Van Herk et al., 2002; Jeran et al., 2007). They have even been proved useful as indicators of human health (Cislaghi and Nimis, 1997). Classic lichen-based monitoring has generated pollution maps showing areas largely devoid of epiphytic lichens, the so-called ''lichen deserts'', in and around cities, in Great Britain for example (Hawksworth and Rose, 1970), Germany (Kirschbaum et al., 1996), the Netherlands (Barkman, 1958) and in many other countries.

3.6 Ecology

Lichens play an important role colonising new surfaces. Among the metabolites excreted by some lichens are acids. Acids have the capacity to degrade the surfaces on which they are located, thus releasing minerals for uptake by the thallus. Acidic digestion has the effect of causing the slow disintegration of the surface, especially of limestone and other calcareous materials. "Rusting" of surfaces is probably unimportant in terms of the total uptake of minerals by lichens. Most minerals are extracted from solutes in rain or surface water flow. (Baron, 1999)

Lichens grow extremely slowly. Any one thallus may be many decades old. The outer edge is probably the only active component of the thallus, unless the lichen has started to overgrow itself. The inner part is commonly inactive. Lichens have the potential to withstand a wide range of environments. Thus they adapt rapidly to local and seasonal changes in temperature and water availability, they are found in bleak artic and desert environments. (Baron, 1999)

The thallus has the capacity to cope with the frequent aridity of the environment. Foliose thalli will curl as the thallus dries, and then flatten as it rehydrates. Photosynthesis follows the pattern of wetting and drying. While changes in form enable a return from dehydration, the presence of trehalose, and possibly a range of polyols, is also important. These metabolites enable the cytoplasm to desiccate, while protecting the functionality of the enzymes. Thus, primary production of lichens is highly dependent on the moisture levels of the environment, but they can survive desiccation. (Nash, 1996)

The slow rate of growth and the reliance on minerals in rain or high humidity has consequences for survival of lichens in polluted environments. Lichens absorb all minerals in rain, and the presence of pollutants, including sulphur, will result in the decline of the thallus. Because of their sensitivity to pollutants, most lichens are uncommon in areas affected by acid rain and aerial pollutants. (Ferry et al., 1993) However, some lichens grow on surfaces containing high concentrations of metals, and must be adapted to those metals: pollution of a single type is likely to select lichens that can tolerate the pollutant. Changing pollution will remove most. In cities, the pollution profile is variable and changing over time. Thus lichens are disappearing from cities. (Nash, 1996)

Remnants of lichen communities within cities are associated with protected habitats. Church yards for instance may house a wide diversity of lichens. Lichens are not welcome inhabitants of city surfaces, however. The capacity of lichens to "rust" the surface leads to loss of the structural integrity of stone and concrete. Attempts to remove lichens, and prevent the re-colonisation of grave stones and other surfaces, are rarely successsful. (Baron, 1999)

3.7 Factors limiting the distribution of lichens

Lichens have specific requirements for their habitats. Although they can occur on a variety of substrates, each substrate must have the individual components in the right amounts that growing lichen needs. These requirements are: water, air, nutrients, light, and substrates.

3.7.1 Water

Because lichens do not have a waxy cuticle like plants, they cannot conserve water during drought periods. On the other hand, lichens can absorb everything through their cortex, including water and water vapor. Many lichens are found in foggy areas like the coast, but not farther inland simply because there is not enough water in the air to support them.

When lichens are wet, they "turn on" and start photosynthesizing and growing. When lichens are dry, they "turn off", become brittle and go dormant. This process is known as "poikilohydry", and other organisms such as mosses and liverworts operate in the same way.

The simplest way to tell if lichen is dormant or growing is by looking at its color. The darker black or brighter green lichen is, chances are that it is photosynthesizing. Of course, if it is wet and pliable, that is a good indication too.

If lichen looks pale and is dry and brittle, then it is dormant and waiting for the next rain or fog event before it starts photosynthesizing. (United State Forest Service, 2008)

3.7.2 Air

Lichens need clean, fresh air to survive. They absorb everything through their cortex. From beneficial nutrients to harmful toxins, lichens absorb it all. They also absorb water in the air, which is why so many are found in fog belts along oceans and big lakes.

Look around the big cities of the world. Very few lichens can survive near factories, next to highways, and other sources of pollution. The ones that do survive have a higher tolerance to the pollutants in the air, like heavy metals and acid rain. (United State Forest Service, 2008)

3.7.3 Nutrients

Just like all living things, lichens need nutrients to survive and grow. The main nutrients include nitrogen, carbon, and oxygen.

Nitrogen is especially important since it is necessary for the production of proteins and organic acids, and not just for lichens, but for life on this planet. Lichens, like plants, have difficulty retrieving nitrogen for their use. That is why cyanobacteria are so useful. Like plants, lichens use cyanobacteria to "fix" nitrogen so it can be used. Fixing nitrogen is the process of changing unusable nitrogen into a usable form of nitrogen. Plants like legumes and rye grass use cyanobacteria to fix nitrogen from the soil. Lichens use cyanobacteria to fix nitrogen from the air. (United State Forest Service, 2008)

3.7.4 Light

Similar to plants, all lichens photosynthesize. They need light to provide energy to make their own food. More specifically, the algae in the lichen produce carbohydrates and the fungi take those carbohydrates to grow and reproduce.

Different lichens need different amounts of light. That is why you will find lichens on exposed rock and desert soils, as well as on a leafy tree or in its shadow on the mossy ground below. The color of lichen is also dependent on the amount of light it receives. For example, *Lobaria pulmonaria* is normally in a shaded environment, yet when it grows in an exposed environment, the color is different, usually darker, and browner. Different species that adapt to brighter, hotter environments are generally more pigmented. This could be a mechanism of the fungus to protect the algae from getting too much light and burning out. (United State Forest Service, 2008)

Most lichens achieved their maximum photosynthetic rate at light intensity 200 – 400 $\mu mol\ m^{-2}\ s^{-1}$ (Nash, 1996).

3.7.5 Substrates

Every lichen lives on top of something else. The surface of that "something else" is called a substrate. Just about anything that holds still long enough for a lichen to attach to and grow is a suitable substrate. Trees, rocks, soil, houses, tombstones, cars, old farm equipment and more can be substrates. The most common natural substrates are trees, rocks, and soil.

Having lichens growing on your rocks, trees and ground around your property is a good thing. That means the air you breathe in is healthy and clean. Although lichens can cause some damage to buildings and man-made structures, it is a very slow process and does not endanger those substrates.

Different species of lichens prefer, or only grow on different substrates. Thus some species will be found on smooth barked trees, some on rough barked and some on only one species of tree.

Also some lichens grow on basic rocks while others only grow on acidic rocks and some have particular mineral requirements, thus *Acarospora sinopica* only grows on rocks with a high iron content. However where ever they grow lichens grow slowly so what ever it is they are growing on, the 'substrate' needs to have been around for a few years. Lichens grow differently at different times in their lives. When young and very small they grow slowly, then once they are reasonably well established they grow much more quickly, obviously when they are dying, for what ever reason they grow more slowly again, or not at all.

Soil is another important substrate for lichens. It provides moisture, nutrients, space to grow, and depending on the location, shelter as well.

One unique habitat lichens can colonize is dune systems. If stable for a long enough time, shifting sands can be "held down" by soil crusts, allowing other communities to establish themselves over the top.

Soil crusts consist of cyanobacteria, mosses, and lichens. Be careful, though. Once these soil crusts are disturbed, they do not come back for many years and the process has to start over again. The shifting sands themselves pose a risk by blowing over the crust communities and covering them up, preventing light from getting to the organisms underneath and killing them. (United State Forest Service, 2008)

3.7.6 Temperature

Lichens survive in an extremely wide range of temperatures. They have been known to survive temperatures as low as -190 °C for several hours and as low as -78 °C for several days. Going to the other extreme they can also survive temperatures as high as 100 °C if they are dried out, and even when moist temperatures of 40 °C - 50 °C do not worry them. (United State Forest Service, 2008)

Desert lichens can tolerate temperatures as high as 90 °C (when dry, wet them and they cook!) and -195 °C (in liquid nitrogen). Lichens are a dominant part of Antarctica ecosystems (Guardian and John, 2006).

Natural disturbance regimes such as fire, insect outbreaks, fungal infections and wind continuously add dead wood to natural forests. These regimes are heavily controlled in managed forests, where instead dead wood is mainly created through final felling, clearing and thinning (Alexandro, 2007).

3.8 Benefits of lichens

Lichens have been used for various purposes since the ancient age. The benefits of lichens could be classified as follows:

3.8.1 Food

Lichens consist of no actual carbohydrate or even cellulose. However, they have lichenin at hyphae cell walls of fungus which could be used as food. For example, in the northern hemisphere, *Cetraria islandica* or Iceland moss has been taken as food and as a medicine to better food digestion in a body. Besides, it has been pound to be mixed with flour for making "Sea biscuit". (Thailand Graduate Institute of Science and Technology, 2002)

3.8.2 Medicine

The ancient Egyptians used lichens as ingredients in medicines and herbs. In the 15th Century, people used lichens for treatment of *Usnea barbata, Lobaria pulmonaria, Xanthoria parietina, Peltigera canina,* etc. In Thailand, people in local areas have used lichens as herbal medicine, i.e. *Usnea*. (Thailand Graduate Institute of Science and Technology, 2002)

A recent study on the *Umbilicaria esculenta* species shows that it produces substances that can inhibit the growth of HIV virus (Brodo et al., 2001).

3.8.3 Dyes

Lichens have been used, since the ancient Egypt age, as dyes. The one well-known is *Rocella tinctoria* and others. Lichens in this family give colors called orchill that are in purple tone. France and Holland have produced lichens in term of industry. With their property of being sensitive to the pH, they are therefore used as colors of the litmus. In medical study and research nowadays, lichens have been used for chromosome dying. (Lichen Research Unit and Lichen Herbarium, 1993).

3.8.4 Ingredients in perfume

In France, the *Evenia prunestri* lichen or usually called oak moss and *Lobaria pulmonaria* are ingredients in perfume. Apart from making good smell, they keep the smell stay longer. (Thailand Graduate Institute of Science and Technology, 2002 ; Sharnoff 1997; Brodo et al.,2001)

3.8.5 Indication of stone age and antique

When any material surface is open to the air, lichen will sponge on, grow up and increase its number, the longer, the more number. Lichen usually used in this case is *Rhizocarpon geographicum.* And this method is called Lichenometry. (Thailand Graduate Institute of Science and Technology, 2002)

3.8.6 Hair cleaning

In the 17th Century, lichen powder, *Ramalina calciaris*, was used to human hair to make it beautiful and clean, and get rid of dandruff. Besides, the *Evernia prunestri, Physcia ciliaris* or *Usnea* were used as well. With properties of lichens in smell absorbing and preserving, they have been produced in term of

industry in Montpellier in France. (Thailand Graduate Institute of Science and Technology, 2002)

3.8.7 Tanning and brewing

With property as being astringent of *Certraria islandica* and *Lobaria pulmonaria*, they could be used for leather tanning. Moreover, it is found that *Lobaria pulmonaria* is used instead of hop in beer brewing. In 19th Century, lichen was used in production of popular alcoholic beverage in Sweden. Lichen used in that case was *Cladonia rangiferina*, etc. (Thailand Graduate Institute of Science and Technology, 2002)

3.8.8 Poison

Although lichens produce various kinds of organic acids causing a bit irritation after taken, most lichens contain no poison. Two types of lichen found to have poison are *Letharia vulpine* and *Cetraria pinastri* that were used as poison to foxes by the European. (Thailand Graduate Institute of Science and Technology, 2002)

3.8.9 Dissemblance of some kinds of animal

In virgin forest in tropical area of New Guinea, lichen occurs on backs of a kind of insect. It seems to be a dissemblance. (Lichen Research Unit and Lichen Herbarium, 1993).

3.8.10 Index of air quality

Air pollution examination by lichens can be proceeded by 3 ways:

1) Survey lichen types in various areas as basic data on lichen types and regularly survey in the future to observe changes of lichen types
2) Examine quantity of accumulated substances in lichens
3) Transplant lichens from the places with good air to the places with pollution and observe changes in physiology

Lichens have some characters appropriate to be indicators of air quality such as no protecting cell-layer. Therefore, they can directly obtain pollutants, slowly grow up and have long life-time. In a year, crustose and foliose groups radially grow up for only 0.5-5.0 mm. As for fruticose group, they lengthwise grow up for 1-2 cm. (Hawksworth and Rose, 1976; Nash, 1996). So, various substances outside are accumulated within thallus of lichens. Moreover, it is realized that lichens are living things with most sensitivity to air pollution (Ferry et al., 1993). Factors influence lichen growth include humidity, light, temperature, receiving of nutrients from outside, season variance and annual season variety (Hale, 1973).

Properties as biological indicators of lichens (Verein Deutscher Ingenieure, 1995):

1. Lichens directly obtain minerals and nutrients from atmosphere.

2. Lichens do not have wax and cuticle to protect interior structure as multi-cellular plants do. Pollution from the atmosphere, thus, gets into cells and destroy their necessary process of living, for example photosynthesis and growth.

3. In condition with humidity, lichens will be very sensitive to air pollution since they increase working rates of different processes in cells.

4. Working rates of different processes in cells operate at low temperature. Lichens are, therefore, possibly disturbed by pollution in cold season.

3.9 Studies on lichens in Thailand and other countries

3.9.1 Studies on Lichens in other Countries

It has been more than 200 years that scientists have studied on relationship of lichen growth and pollutions and on capabilities of lichens used as indicating biological indices in different countries worldwide. The following evidences appeared in primary age. In 1921, E. Darwin noted about incapability of lichens in growing up near the areas around metal melting machines in Anglesey North Wales Island. Later, in 1970, D. Tuner and Borrer found that lichens were sensitive and related to air quality. As for the restudy by W. Borer in 1812, it was observed that lichens were more difficultly found in areas where air was unclean (Hawksworth and Rosse, 1976).

Hawkworth and Rose (1976) studied on basis of living patterns, structures and kinds of lichens on adhering substrates used as representatives of the studied areas in order to make air quality maps by using lichens. Later, Pilegssd (1978) used *Lecanora conizaeoides* lichen as an indicator of pollutant accumulation in industrial factory areas in Frederiksvaerk, Denmark. It was found that results of heavy metal concentrations in *L. Connizaeoides* were variant according to spaces from pollution resources i.e. more heavy metal concentrations in *L. connizaeoides* were found in the areas near industrial factories comparing to the areas far away.

According to Rossbach et al. (1999), lichens were used to follow up fine particles and found that lichens accumulated heavy metal in relation to dust quantity found in the air i.e. lichens in areas with much dust accumulated much heavy metal. Besides, little lichens were found in those areas.

Aptroot and Herk (2006) studied on effects of global warming on lichens by considering response of lichens to weather changes in Western Europe. It was found that some kinds of lichens increased and some kinds decreased in term of quantity. In the same year, Giordani (2006) made a study on variety of lichens as indicator of the air pollution in Genova, Italy, and found

that variety of lichens depended on various factors such as rainfall class and temperature. Different and various lichens could be found in rural and forest areas. In rural areas, the main effect was from SO_2. Interestingly, forest areas with deforestation and wildfire demonstrated strong effect to lichens. These areas should be mostly improved.

Larsen et al. (2006) made a study on lichens and bryophyte on oak trees in London, England, in terms of their distribution and frequency that also related to air pollution and bark's acidity. The study findings concluded that traffic pollution and the pH of barks influenced distribution of lichens and bryophyte.

Frati et al. (2006) studied lichens as indicators of ammonia and nitrogen around pig farms in Italy and found that appropriate lichen to be an index indicating ammonia pollution was *Physconia grisea.* As for the *Xanthoria parietina* and *Flavopararmelia caperata,* they accumulated more nitrogen when ammonia concentration was higher.

3.9.2 Studies on lichens in Thailand

Studies on lichens in Thailand should be divided into two phases: lichen samples collecting in Thailand by foreign botanists; and surveying, studying and researching by Thai botanists.

Lichen samples collecting in Thailand by foreign botanists

In 1899-1900, kinds of botany were surveyed in Thailand by a Danish botanist. After that, in 1901, Vainio from Finland made the first publishing and distributed the surveyed results of lichens collected from the Chang Island in Trad Province of Thailand. Vainio reported that 95 species of 29 genus of lichen were found. Among them, there are many new strains of the world. Moreover, in 1921, Vainio made another report on lichen survey in Doi Suthep areas. In 1930, Poaulson reported results of lichen survey in the Tao Island, Suratthani Provience (Lichen Research Unit and Lichen Herbarium, 1993).

In 1962, Sato reported lichen survey results in Doi Suthep and Doi Inthanon areas. Later, in 1964, Hale and Kurokawa made a survey and collected lichen samples in Thailand. The samples, later on, were kept in the Smithsonian Institute, Washington D.C. and the Tokyo National Museum. And, in 1978, Warncke collected lichens samples in the northern part of Thailand and assigned Yoshimura to examine species and strains of those samples. Those samples, later on, were kept at the Arthus University in Denmark and at Kochi Gakun College in Japan (Thailand Graduate Institute of Science and Technology, 2002).

In 1997, Wolseley and Aquirre-Hudson collected lichen strains in Thailand, according to the project of using lichens as indicators of environmental changes with funding of the National History Museum, England.

The studied areas mostly were in the northern part of Thailand and areas of Huai Kha Khaeng Wildlife Sanctuary. The lichen samples of this collection are presently kept at the National History Museum in England, the Royal Forest Department in Thailand and Chiang Mai University in Thailand (Lichen Research Unit and Lichen Herbarium, 1993).

Lichen samples collecting in Thailand by Thai botanists

Lichen sample collecting in Thailand by Thai botanists began in 1990 in order to use lichens as indicators of air pollution in Bangkok, under the support of Ramkhamhaeng University. At that time, researchers needed to send lichen samples abroad for examining of their scientific names. Therefore, it was realized that all studies and research could not be conducted without basic knowledge in taxonomy (Thailand Graduate Institute of Science and Technology, 2002).

Nimitre (2001) studied on vertical lichen distribution under the dense and clear canopies of *Cratoxylum* sp. and *Schima wallichii* in the second batch forest of the Khao Yai National Park. It was found that, areas under clear canopy, more foliose was found than crustose at every high level, except for areas under dense canopies. Both crustose and foliose were found at every high level. The lichens which were found at every light intensity were *Dirinaria* sp., *Graphis* sp., *Graphis* sp., *Parmptrema* sp. and *Pyxine* sp. and in areas under dense canopy was *Coccocarpia* sp.

Thanwarat (2002) worked on distribution and frequency of *Hyperphycia adglutinata* Flörke and *Lecanora* cf. *leprosa* Fe'e on mango trees in the city areas of Chiangmai Province. It was found that *H. adglutinata* generally distributed and had high frequency in heart of city areas with rather high pollution. As for *L.* cf. *leprosa,* it was less found and with low frequency in the heart of city areas but found with high frequency in some areas outside heart of the city. Thus, *L.cf. leprosa* tended to be used as good indicators of air quality and *H. adglutinata* tended to be used as minor indicators of air quality. Besides, Sudarat (2002) made a similar study as the one of Thanwarat but studied on the *Pyxine cocoes* Swartz and *Dirinaria picta* Swartz in areas of Chiangmai city. Findings showed that *P. cocoes* distributed around and had higher frequency than *D. picta. P. cocoes* tended to be used as good indicators of air pollution and *D. picta* could be used as indicators which were sensitive to air pollution.

Palee (2002) conducted a continuum study in Chiang Mai city and outside areas. In 2001, it was found that pollution in Chiang Mai decreased. By evaluation of air quality from quantity of chlorophyll and pheophytin, it was presented that lichens in outdoor areas had more chlorophyll than the ones in indoor areas. On the other hand, the lichens in indoor areas had higher pheophytin than the ones in outdoor areas.

Kantharee et al. (2003) suggested the use of lichens as indicators of air quality. Biological variety of lichens increased according to distances from Bangkok city to the Khao Yai National Park. The survey was done on trees with lichens on them, 20 trees per surveyed area in city, outside city, rural areas and remote areas from city (Khao Yai National Park). These areas were 10, 50, 100 and 200 km. away from the heart of city. The 7, 8, 20, and 55 species of lichen were found in each area respectively. There were 7 species able to grow up in city areas i.e. *Dirinaria picta, Buellia punctata, Cryptothecia* sp., *Laurera benguelensis, Lacanara pallida, Trypethelium tropicum* and *Graphis librata* . In areas of outside city, five same species found in city areas were found and the *Eschatogonia* sp., *Laurera* and *Graphis intricate* were additionally found. In rural areas, 20 species were found and 55 species were found in areas of Khao Yai National Park. All together, there were 520 species found in total areas of Khao Yai National Park. The lichens found in every area were *D. picta, L.benguelensis* and *T. topicum.* As for the *B. punctata,* it could only be found in city areas while the *Eschatogonia* sp. was not found in city areas but found in other three surveyed areas. It, therefore, could be used as an good indicator of air quality.

Wetchasart (2005) studied on comparison of lichens on *Dipterocarpus costatus* with rough shell and banyan trees with smooth shell at different levels from ground layer. The study was conducted at canopy of *Dipterocarpus costatus* at 30-40 metre from ground level. The main factors of lichen distribution were light, humidity, temperature and wind. It was found also that canopy had appropriate conditions for growth of most lichens and influenced the prior mentioned factors under the canopy.

Pomphueak (2005) studied on using lichens in evaluating of air quality in city areas and areas around Lampang city in Lampang Province by identifying lichen diversity values (LDVs). It was a study of lichens on 234 mango trees. Study area was divided into 39 units with size of 1 x1 square kilometre. Twenty one species were found. Then, air quality was classified into eight air levels. Passive collection of air was conducted to measure concentration of NO_2 and SO_2 . The findings concluded that NO_2 influenced lichen diversity in the studied areas.

Thanomsap (2006) studied on distribution and frequency of *Pyxine cocoes* Swartz and *Lecanora* cf. *leposa* Fee on mango trees in municipality areas of Lamphoon Province. The studied areas were 500 x 500 square metre, 30 squares , 6 trees in a square. All together were 180 trees. Findings presented that the *P.cocoes* distributes overall studied areas, both in and outside the city and high frequency was found in city areas. It was, therefore, concluded that the *P.cocoes* and *L.* cf. *leprosa* tended to be indicators of air quality by *L.* cf. *leprosa* indicated better quality of the air.

4. Research question

4.1 Is it possible to use lichens as bio-indicators in Nakhon Ratchasima Municipality to explain and measure air pollution?

4.2 What is the level of lichen density in dry evergreen forest in the Sakaerat Environmental Research Station of Nakhon Ratchasima Province ?

4.3 What is the lichen distribution and frequencies in Nakhon Ratchasima Municipality?

5. Research objectives

The objectives of this research are:

5.1 To examine and compare air quality in Nakhon Ratchasima Municipality by using lichens as bio-indicators.
5.2 To select lichen species which can be used as bioindicator for air quality monitory and produce distribution map of selected indicator species.
5.3 To study lichen diversity in dry evergreen forest in the Sakaerat Environmental Research Station of Nakhon Ratchasima Province.
5.4To provide basic information on lichen diversity of Nakhon Ratchasima Province for future studies.

6. Scope and limitation of the study

- This study is conducted to study air quality using lichens as bioindicators and lichen diversity in Nakhon Ratchasima municipality areas with different degree of human impact.

- In addition, the investigation of lichen diversity in dry evergreen forest in Sakaerat Environmental Research Station of Nakhon Ratchasima Province will be conducted in order to observe the difference between lichen community in disturbed are in Nakhon Ratchasima municipality and undisturbed area in the Sakaerat Environmental Research Station.

The experiment scale are 12 months or January to December 2009.

7. Materials and methods

7.1. Materials

7.1.1 Map of Nakhon Ratchasima Municipality
7.1.2 Map of the Sakaerat Environmental Research Station, Nakhon Ratchasima Province

7.1.3 Grid frames size of 20 x 50 cm^2
7.1.4 Hand lens
7.1.5 Compass
7.1.6 Tapelines
7.1.7 Knives (for sample collecting)
7.1.8 Camera
7.1.9 pH meter
7.1.10 Stereo microscope
7.1.11 Compound microscope
7.1.12 Notebooks and pens or pencils
7.1.13 Envelopes (paper bags)

7.2. Research methodology

7.2.1 Research Sites

In this study, research sites are divided into 2 parts i.e. in town areas with high pollution of Nakhon Ratchasima province and in dry evergreen forest area with basically no pollution of Sakaerat Environmental Research Station, Nakhon Ratchasima Province.

7.2.1.1 In town area of Nakhon Ratchasima

A. Conditions and Geography

According to the National Economic and Social Development Plan, Nakhon Ratchasima municipality is a central city of business progress in the Northeast Region of Thailand. It situates between latitude 14-16 °N and longtitude 101-103 °E at around 150-300 metres above sea level. It is about 259 km. far from Bangkok by car and about 264 km by train. It takes about 30 minutes by plane from Bangkok. Town area mainly slopes to the east. The northern part of town is low land and the southwest part of town is high land.

B. Size of Area

Nakhon Ratchasima municipality covers around 37.50 km^2 or comparable to 2,343 rais and 2 ngarn in Thai measure scale or 4.96% of total area of muang district (which covers around 755.596 km^2) or around 0.18% of total area of Nakhon Ratchasima province (which covers around 20,493.9 km^2)

C. Border of Nakhon Ratchasima municipality

in the north: borders on Nongjabok, Muenwai, Bankhor sub-districts, Muang Nakhon Ratchasima district

in the south: borders on Nongpailom sub-district, Muang Nakhon Ratchasima district

in the east: borders on Huathalae sub-district, Muang Nakhon Ratchasima district

in the west: borders on Banmai sub-district, Muang Nakhon Ratchasima district

D. Population

There are 170,095 people who inhabit the Nakhon Ratchasima municipality, 89,570 are females and 80,525 are male. In the area, there are 58,810 family households, according to the house registrations, and there are 33,773 families (as of September 2007).

E. Transportation

Since Nakhon Ratchasima municipality is a community situated in a province which is a gate to the northeast region, it is convenient and fast for traveling and transportation to many provinces in the north, central and east regions (the area of the Eastern Sea Board Project- ESB) including other provinces in the northeast region. Roads in Nakhon Ratchasima municipality are classified into main roads and local roads. The main roads are also classified into arterial roads and spreading roads. Format of road network is divided into 2 parts:

Part 1 is road network, neatly designed in table format, covers the ancient town area surrounded by moats. Most of the roads are in good conditions.

Part 2 is the road network the covers the ancient town area. Roads were constructed for city enlargement to the west of the city which lacked good planning. These roads, therefore, were not well-arranged. This road network connects to the Friendship road, Suranarai road, Friendship-Chokchai road, including other roads, alleys from town e.g. Mukkamontri, Atsadang, Ratchanikul, Kamhaengsongkram, Sappasit, Changpheuk, Ratchadamnern, Benjarong, Prajak, Kudan roads, etc.

F. Environment

- average rain fall per year: 894.5 mm

- waste water per day: 38,049 m^3 (2006)

- waste water pumping stations: 9 stations

- treated water per day: 15,781 m^3 (2006)

- solid waste per day: 220 tons (area for refuse disposal has been allowed by the Treasury Department, under the controlling of the 2nd Army Area, Royal Thai Army. At present, the area of 100 rais have been used from total area of 189 rais)

- Due to lachy of air monitory station in Nakhon Ratchasima municipality, air pollution data is not available.

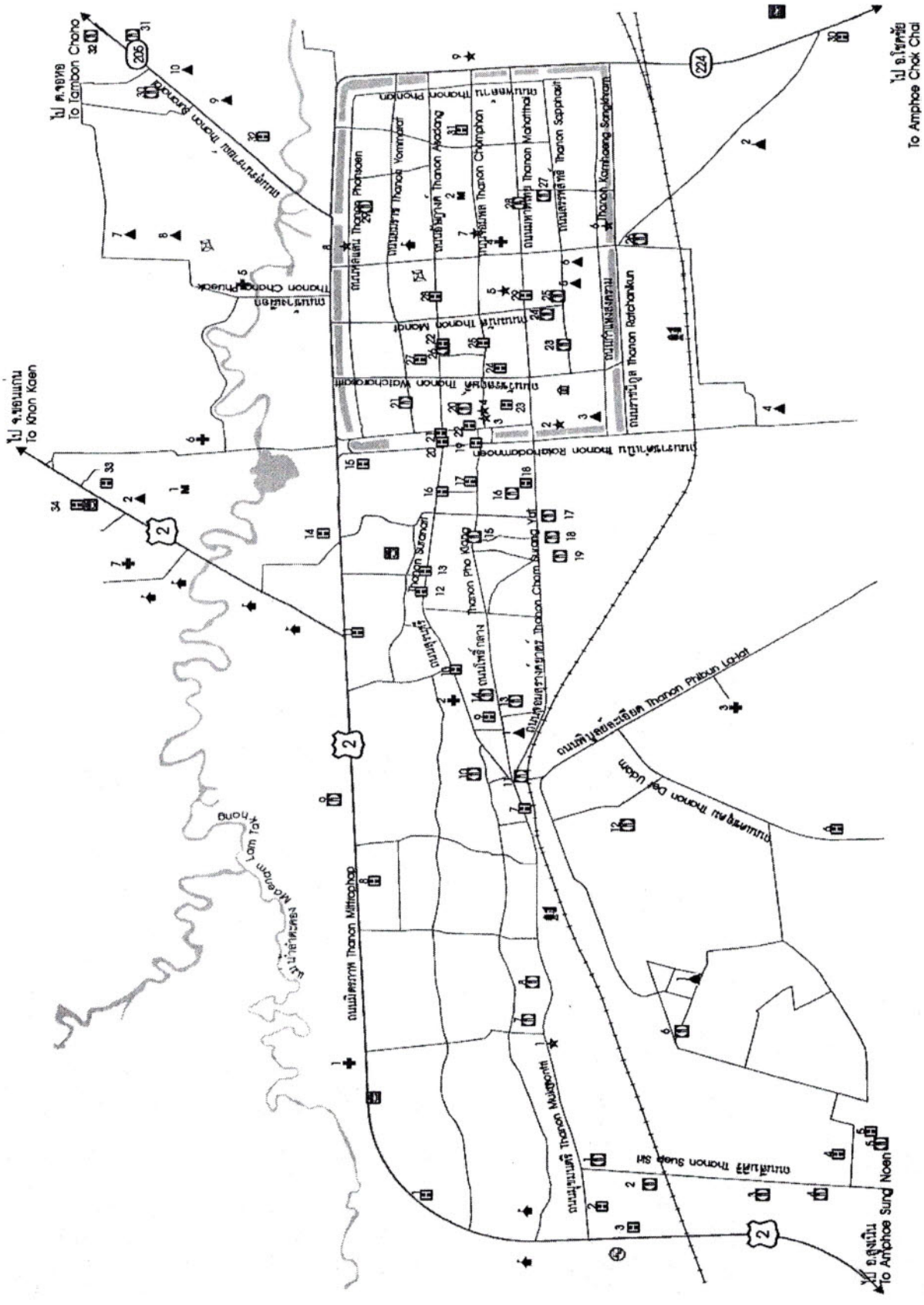


Figure 12 Location of the Nakhon Ratchasima municipality

Source : Koratcity (2008)

7.2.1.2 Sakaerat Environmental Research Station

A. Location and Border

Sakaerat Environmental Research Station located in Phooluang Sub-district, Pakthongchai District, and in Wangnamkhiew and Udomsap Sub-districts, Wangnamkhiew District, Nakhon Ratchasima Province. It is about 60 km from Nakhon Ratchasima downtown in the southwest on the highway no.304 (Chacherngsao-Nakhon Ratchasima). And, it is about 300 km. from Bangkok.

Sakaerat Environmental Research Station covers the area of 78.06 km^2 or about 48,800 rais. The boundary line in the east border along the highway no. 304 is 10 km long (Figure 3.2).

In 1976, the UNESCO, under the project of Man and Biosphere—MAB guaranteed Sakaerat Environmental Research Station as one of the world biosphere reserves which covers 48,800 rais. However, in 2000, the UNESCO/MAB announced a policy of increased of biosphere reserves and enlargement of the existing biosphere reserves areas. Thus, the area of the Sakerat Biosphere Reserve was enlarged from 48,800 rais to 481,969 rais or comparable to 771 km^2.

Table2 The enlarged area has covered areas of 11 sub-districts of Wangnamkhiew and Pakthongchai Districts of Nakhon Ratchasima Province (Figure 14) as follows:

Wangnamkhiew District	**Pakthongchai District**
1. Udomsap sub-district	1. Phooluang sub-district
2. Wangnamkhiew sub-district	2. Takhob sub-district
3. Wangmee sub-district	3. Toom sub-district
4. Thaisamakhee sub-district	4. Sukkasem sub-district
5. Rarerng sub-district	5. Lamnangkaew sub-district
	6. Ngiew sub-district

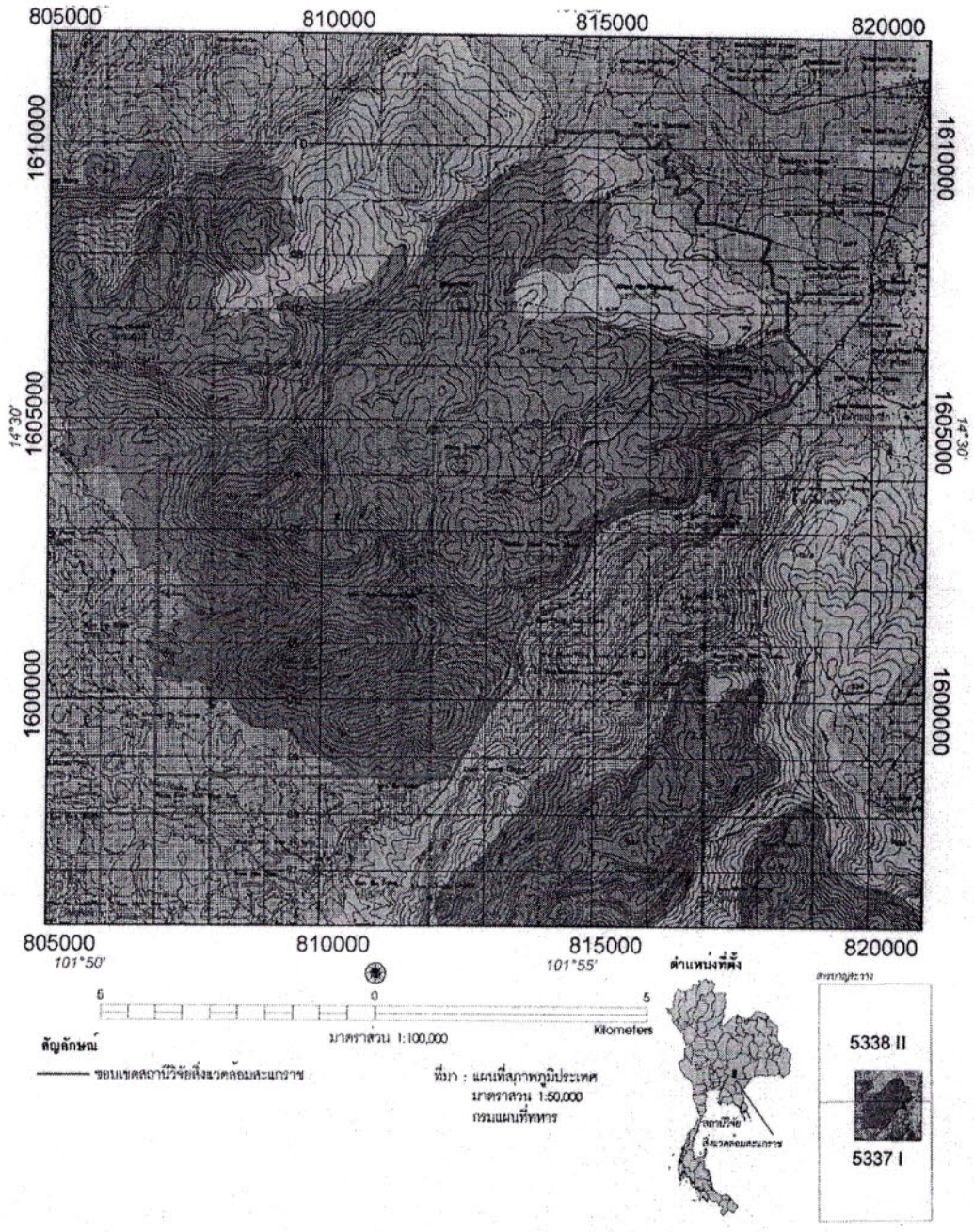


Figure 14 Area of the Sakaerat Environmental Research Station
Source : Sakaerat Environmental Research (2008)

B. Geography

The Sakaerat Environmental Research Station covers the area about 48,800 rais or 87.06 km^2 which is the south border of the Korat plateau. It is at about 280-762 metres from the mean sea level. There are high mountains in the south of the Station area i.e. Kliad (762 metres) Khiew (729 metres), and Soong (725 metres) mountains. The gradients are between 10-30% and 30-45% respectively as shown in Figure 2.2.

In the newly specified areas of the Sakaerat Biosphere Reserve that covers 771 km^2, there are mountains in the north, including the area of the Sakaerat Environmental Research Station. The mountains lie along from the northwest to the southeast. The highest mountain is the So Mountain, about 807 metres from the mean sea level, which is in the west of the Lampraplerng Dam. As for in the southwest of the Sakaerat Biosphere Reserve, it is a plain area between mountains or the Wangnamkhiew Shallow Lake and is 300 metres, in average, high from mean sea level.

C. Land Use

According to the data in 2000, land use in the Sakaerat Environmental Research Station is divided into 5 types (Figure 2.5):

1. dry evergreen forest	46.82 km^2 or	29,260	rais
2. dry dipterocarp forest	14.51 km^2 or	9,066	rais
3. grown forest	14.46 km^2 or	9,038	rais
4. grass land	0.93 km^2 or	582	rais
5. bamboo forest	1.12 km^2 or	697	rais
6. buildings	0.25 km^2 or	157	rais
Total	78.06 km^2 or	48,800	rais

As for the area of the Sakaerat Biosphere Reserve which covers 771 km^2 outside the Sakaerat Environmental Research Station, land use is different. The area includes forest, both natural and grown forests. Most forests are on mountains in the northwest and the southeast of the Sakaerat Environmental Research Station. However in the southern part in the Wangnamkhiew Shallow Lake, it is agricultural area used for corn and cassava,etc. Moreover, economic plants such as grape, longan and lychee, etc. are also grown in the area.

7.3 Research methodology and research/data collection areas

The research will be divided into 4 main parts:

1. Air quality mapping in area of Nakhon Ratchasima Municipality.
2. Studying frequency and distribution of some lichen types in the area.
3. Studying lichens in the area of the Sakaerat Environmental Research Station, Nakhon Ratchasima Province.
4. Measuring air quality with research tools in every studied area.

7.3.1 Air quality mapping in area of Nakhon Ratchasima municipality

The area in Nakhon Ratchasima municipality was identified as the traffic congestion area. It covers the area of 37.50 km^2 or 2,343 rais and 2 ngarns comparable to around 4.96% of the total area of Muang district (Muang district and covers the area of 755,596 km^2) or around 0.18% of total area of Nakhon Ratchasima province (Nakhon Ratchasima province covers the total area of 20,493.9 km^2). (Nakhon Ratchasima municipality, 2007)

Air quality mapping is a study on pollution conditions in the area of Nakhon Ratchasima Municipality by observing lichen found by using research method applied from the VDI method (Verein Deutscher Ingenieure, 1995). The method was developed and adjusted in order to be suitable for environmental studies in Thailand.

7.3.1.1 Divide the research area in the Nakhon Ratchasima Municipality into 30 grid squares (size: 500 × 500 m) (Figure 15)

7.3.1.2 Select mango trees *(Mangifera indica Linn.)* to survey for lichens. Barks of mango trees has appropriate pH for lichen growth and could be easily found in town areas (Saipunkaew, 1994). four mango trees will be selected for a grid square. Altogether, there will be 120 mango trees in this research.

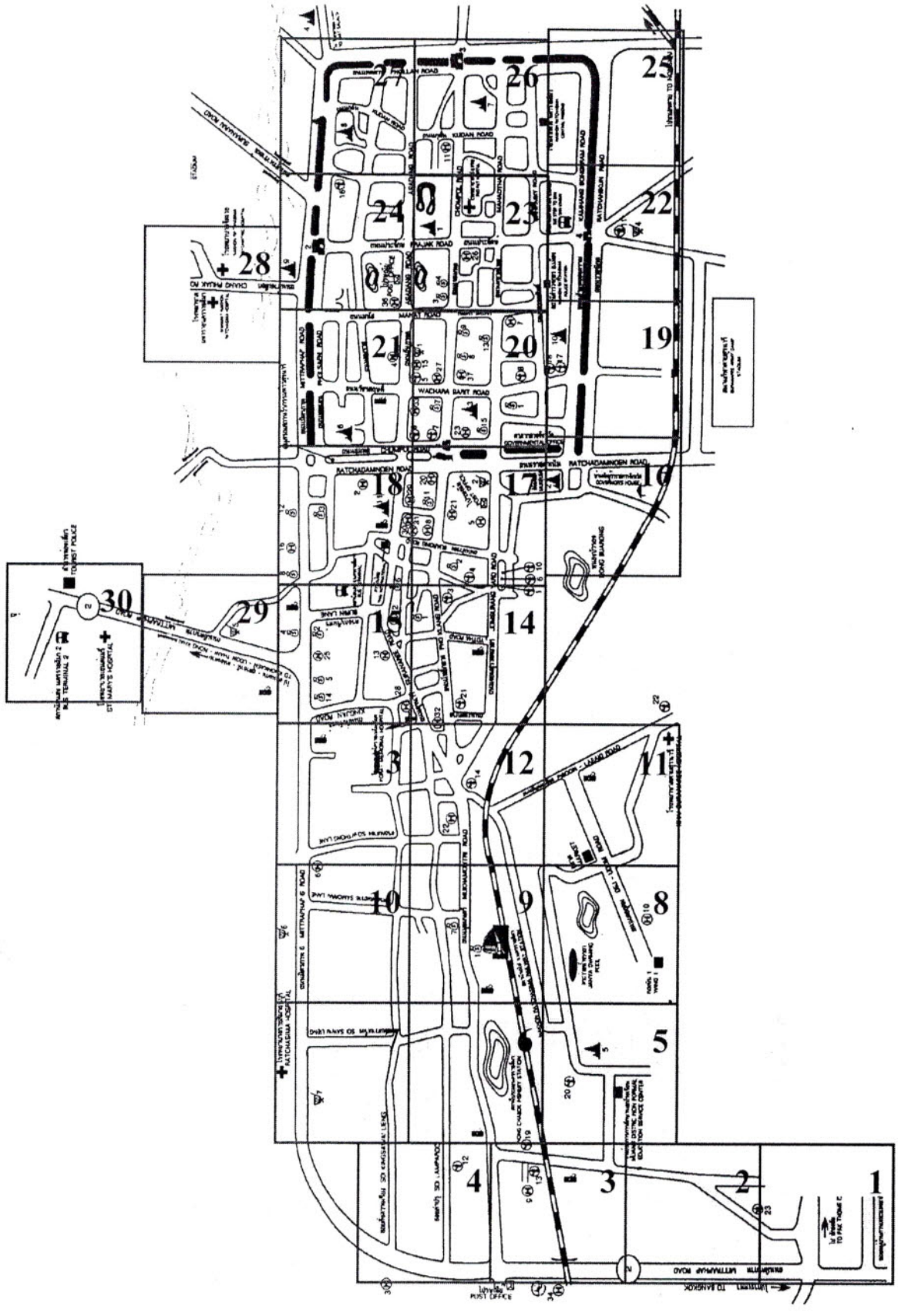


Figure 15 The area in Nakhon Ratchasima municipality

Source : Koratcity (2008)

7.3.1.3 Survey for lichens by randomly selecting mango trees with trunk circumference of 50 cm or more when measured at 150 cm above ground-level (Saipunkaen, 1994). Trunk of each tree must be straight or in the case of crooked trunk not more than 5 degrees-crooked. Straight and crooked trunks cause different nutrient storage, and moistness. And different light quantities cause different lichen growths. Moreover, trunks must not be damaged since this can affect lichen growth (Verein Deutscher Ingenieure, 1995).

7.3.1.4 Collect lichen samples by using a grid frame size of 20×50 cm^2 with 10 small grids size of 10×10 cm^2. Place the grid frame on a truck of mango tree where most of lichens are found. The lower part of the grid frame is about 1 metre above the ground (Figure 16). Record environment around that mango tree and grid frame direction.

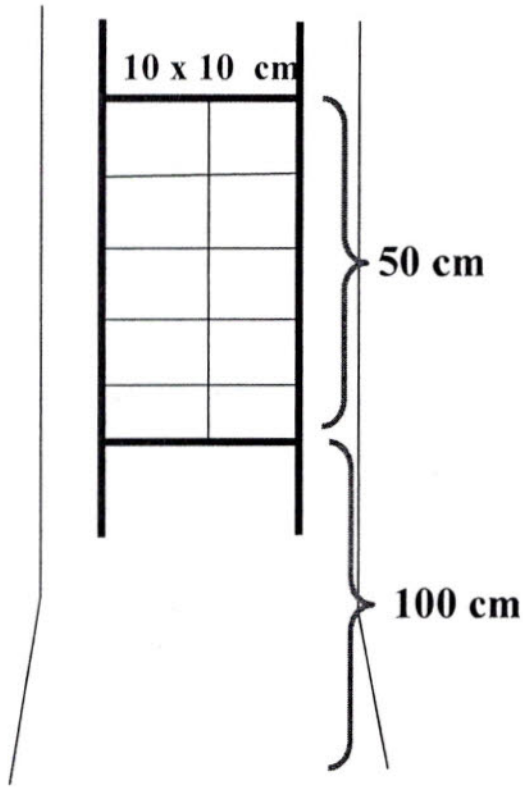


Figure 16 Location of grid frame
Source : Verein Deutscher Ingenieure (1995)

7.3.1.5 Categorize and record numbers and types of lichen found in the grid frame in term of frequency. In order to prevent errors in type classification, lichen sizes to be recorded need to have diameters not less than 3 mm (Verein Deutscher Ingenieure,1995).

7.3.1.6 Calculate for Air Quality Index (AQI) from lichen data of frequency found in each grid square by calculating the sum total of

lichen frequencies appearing in grid frame on mango trees. Calculation will be done using to the following equations:

Air Quality Index (AQI) in Table (j)

$$AQI = \frac{F_{ij}}{n_j} \quad (1)$$

Standard deviation of squares (S)

$$S_j = \sqrt{\frac{\sum \left(F_{ij} - AQI_j\right)^2}{n_j - 1}} \quad (2)$$

Lower class boundary (L_{1j}) and upper class boundary (L_{2j})

$$L_{1j}, L_{2j} = AQI_j \pm t_j \frac{S_j}{\sqrt{n_j}} \quad (3)$$

When

i	refers to each mango tree of the survey in Table j.
j	refers to number of square of the survey.
F_{ij}	refers to sum total of lichen frequencies on mango trees of the survey.
n_j	refers to number of mango trees of the survey in each square.
S_j	refers to standard deviation of squares of the survey.
L_{1j}, L_{2j}	refers to lower class boundary and upper class boundary of Air Quality Index, considering from values between L_2-L_1.
t_j	refers to values from Table t by studying distribution of independent variables from n_i -1.

7.3.1.7 Classify levels of air quality by Air Quality Indices. Width of air quality classes could be calculated from means of standard deviations of all grid squares of research area. The equations are as follows:

Standard deviations of all grid squares in studied area (Sp)

$$S_p = \sqrt{\frac{\sum_j \sum_i \left(F_{ij} - AQI_j\right)^2}{m\left(n_p - 1\right)}} \quad (4)$$

Classified levels of air quality by Air Quality Indices (AQC)

$$AQI = t_p \times \frac{S_p}{\sqrt{n_p}} \qquad (5)$$

When

S_p refers to mean of standard deviations of all grid squares in studied area.

n_p refers to mean of number of trees in each grid square of all squares in studied area.

m refers to number of total surveyed squares in studied area.

t_p refers to value(s) from Table t by studying distribution of independent variables from $n_p - 1$

Classification of air quality of each surveyed grid square in studied areas, it could be calculated from Table 3, starting from the first class with bad air quality.

Table 3 Air quality classification

0	<AQI≤	Width of the 1st Class
Width of the 1st Class	<AQI≤	Width of the 2nd Class
	Continuous	

7.3.1.8 Presentation of air quality will be managed by air quality classification. Air quality values are based on impact scale that have ranges at 0-12.5, 12.5-25.0, 25.0-37.5, and 37.5-50.0 (Verein Deutscher Ingenieure, 1995). Those values have been studied in European countries for more than 25 years, in order to get the most appropriate ranges of value . However, the calculated values were wide ranging and studied from countries in humid climates not been studied in countries in tropical zone. Thus, Thailand could use the impact scale as the basic values only. Therefore, the scale should be applied for calculation of the appropriate values in the future.

The received widths represented different levels of air pollution, replaced with different places in the studied map. In classification of air quality, values of quality classes possibly caused collaborative quality result for two classes. For example if the air quality class is 0-14.5, that is between standard value of the high pollution red class (12.5) and the rather high pollution orange class (25.0), it could be interpreted that the studied air quality has high to rather high pollution, etc. Assigning colors to each square is to represent characteristics of air quality class in each square or enable are to build isoline.

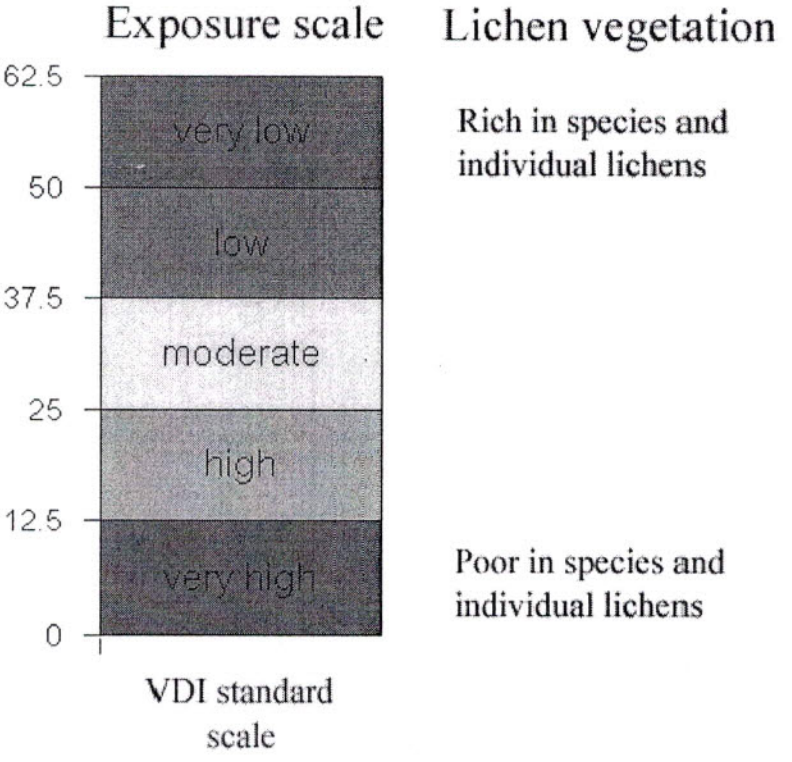


Figure 17 Impact scale.
Source : Verein Deutscher Ingenieure (1995)

7.3.2 The study on frequency of number and distribution of some types of lichen in Nakhon Ratchasima municipality.

After that, have air quality mapping and select lichen species which can be used as bioindicator for air quality monitory in Nakhon Ratchasima Municipality. In this study, the Geographic Information System (GIS) program will be used in mapping distribution of lichen.

7.3.3 The study on lichens in area of Sakaerat Environmental Research Station, Nakhon Ratchasima Province

The dry evergreen forest in the area of Sakaerat Environmental Research Station, Nakhon Ratchasima Province was specified as an area with rather different environments from the city and less human activities ocume. Therefore, it is very interesting to study if lichen community are different or similar to those of the city sities.With the basic study by Species area curve method, the following quadrate will be specified:

3 plots of dry evergreen forest, area size 20 × 20 m per plot

In each quadrate, the following procedures will be done:

1) Survey every perennial with 50 cm and more circumference. Categorize types of perennial by local names, common names and scientific names and attach a number to each perennial for later records.
2) At 150 cm above ground level, study lichens by using grid frame size 20 ×50 cm.
3) Record types of lichen along the procedures .

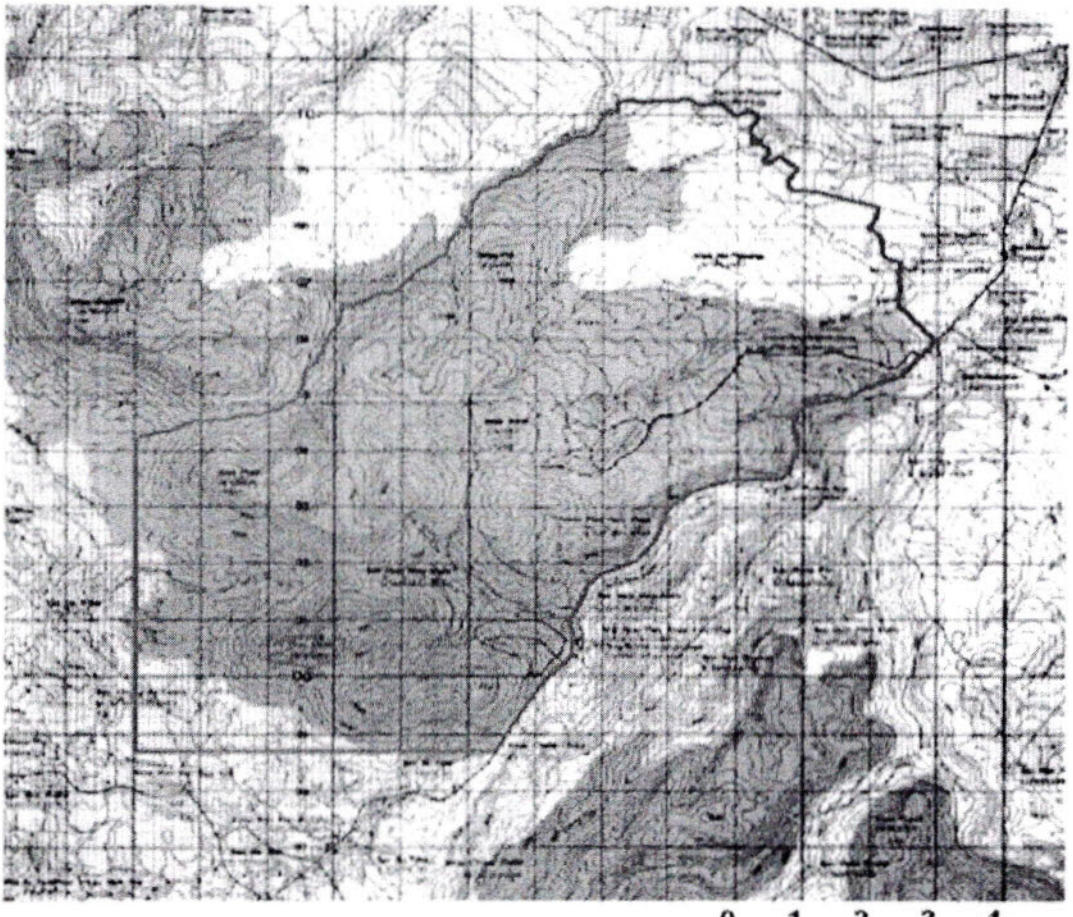


Figure 19. The area of Sakaerat Environmental Research Station, Nakhon Ratchasima Province
Source : Sakaerat Environmental Research (2008)

7.3.4 Measurement of other environment factor around the investigated trees in Nakhon Ratchasima municipality.

This study will record the environment around the trees on which lichens grow or expected to result in lichen growth. The data record will consist of area characteristics, areas around the studied trees, conditions effected by traffic, characteristics of barks, directions of tree trunks where lichens are found, space from each studied tree to road, circumferences, and pH of barks. The measurement of barks' pH will be modified by the Staxäng's method (1969). The method includes collecting 2-3 mm -thick barks, baking them at

temperature 80°C for one day, powdering 2 gram of dry bark and putting the powder and 10 cm^3 of distilled water into a test tube and left it for 24 hours. Measuring for pH of the solution and making a record. After that, analyzing relation between data of frequencies of 6 interested lichen types and the environment. The data will be statistical analyzed.

7.3.5 Air quality measurement by tool in all studied areas

Air qualities in the areas near all types of studied trees in the Sakaerat Environmental Research Station and Nakhon Ratchasima Municipality, will be measured by the thermoelectrically cooled/meated 3 gas sampler.

Air quality in measurements will be SO_2 and NO_2

The values received from measurement will be compared to the National Ambient Air Quality Standards specified by the Pollution Control Department, Ministry of Natural Resources and Environment.

8. Expected research success and worthiness

1. Biological diversity data of lichens in different areas of Nakhon Ratchasima Province will be obtained.
2. Types and groups of lichen that could be used as indicator for environmental condition monitory in city and natural areas will be identified.
3. Basic data will be learnt for further research at higher levels.
4. The data resulting from the research will be published both at national and international levels for interested people.

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10. Research Plan

Since January to December 2009, 12 month.

Step of study plan	Period (month)											
	1	2	3	4	5	6	7	8	9	10	11	12
1.Literature review	———	———										
2.Study site survey		———	———									
3.Detailed field investigation and sampling			———	———	———	———	———	———	———			
4.Laboratory work				———	———	———	———	———	———			
5.Data analysis and synthesis							———	———	———	———		
6.Thesis writing and result report.										———	———	———

Advisor's signature.....
(Asst.Prof.Dr. Nathawut Thanee)
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Student's signature.....
(Miss Amornrat Pitakpong)
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M. Sc. Thesis Proposal

EFFECTS OF THE CRUDE EXTRACT FROM THE FRUIT RIND OF RAMBUTAN (*Nephelium lappaceum* L.) ON OBESITY IN MALE WISTAR RATS

ผลของสารสกัดจากเปลือกเงาะที่มีต่อโรคอ้วนในหนูขาวเพศผู้พันธุ์วิสตาร์

By

Miss Aree Thinkratok

School of Biology

Institute of Science

Suranaree University of Technology

December 2008

M. Sc. Thesis Proposal

Miss Aree Thinkratok **ID. No. M5010226**

School of Biology **Institute of Science**

Thesis Advisor: Asst. Prof. Dr. Rungrudee Srisawat

1. Thesis Title

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ผลของสารสกัดจากเปลือกเงาะที่มีต่อโรคอ้วนในหนูขาวเพศผู้พันธุ์วิสตาร์

2. Introduction

2.1 Background/problem

Obesity is a complex, multifactorial, chronic disease involving environmental (social and cultural), genetic, physiologic, metabolic, behavioral and psychological components. It has been increasing at an alarming rate throughout the world over the past two decades to the extent that it is now a pandemic, affecting millions of people globally. It seems that approximately 300 million people are obese and 1 billion adults are overweight in the world (Bagchi and Preuss, 2007). Obesity becomes a problem of enormous economic, social and health consequences in our society.

Several literatures have presented well-documented links between obesity and increased mortality, accelerated aging and morbidity due to hypertension, dyslipidemia, type 2 diabetes associated with insulin resistance, coronary heart disease, congestive heart failure, stroke, osteoarthritis, pulmonary dysfunction (obstructive sleep apnea and hypoventilation syndrome), certain types of cancer (breast, endometrium, colon, prostate), gastrointestinal diseases (fatty liver, cirrhosis, gastroesophageal reflux, gallstones), menstrual abnormalities, increase in surgical risk, impaired fertility, increased pregnancy risks, brain disease (Alzheimer's disease), physical discomfort, depression and suicide (Devlin *et al.*, 2000; Kopelman, 2000; Roth *et al.*, 2004; Wickelgren, 1998).

Successful obesity treatment plans incorporate diet, exercise, behavior modification with or without pharmacologic therapy and/or surgery. Many attempts have been made to correct the metabolic disparity of the obesity condition, producing a number of reagents including Sibutramine, Fluoxetine and Atomoxetine (appetite suppressor), Orlistat (gastrointestinal lipid uptake inhibitor), Rimonabant (cannabinoid (CB1) receptor antagonists), Topiramate and Zonisamide (antiepileptic drug targeting multiple proteins), Fibrates (peroxisome proliferators-activated receptor α (PPAR α) agonists) and Bupropion (dopamine-noradrenaline-reuptake inhibitor) (Adan *et al.*, 2008; Bray *et al.*, 2003; Chapman, 2003; Davidson *et al.*, 1999; Gadde *et al.*, 2001; Gadde *et al.*, 2006; James *et al.*, 2000; McElroy *et al.*, 2006; Padwal and Majumdar, 2007; Van Gaal *et al.*, 2005). However, administration of these drugs is known to often cause undesirable side effects such as dry mouth, anorexia, constipation, insomnia, dizziness, nausea, diarrhea, tachycardia, hypertension, depressive symptoms, anxiety, palpitations and memory impairment (Adan *et al.*, 2008; Bray *et al.*, 2001; Bray *et al.*, 2003; Davidson *et al.*, 1999; Gadde *et al.*, 2001; Gadde *et al.*, 2006; James *et al.*, 2000; McElroy *et al.*, 2006; Van Gaal *et al.*, 2005). Therefore, the use of plant extracts is probably a better way to replace these anti-obesity drugs.

Fruits and vegetables are particularly rich sources of antioxidant components, including polyphenols (Bagchi and Preuss, 2007; Kubola and Siriamornpun, 2008).

Polyphenols found in fruits (e.g. apple, cherry, orange, pear) and vegetables (e.g. roselle, pumpkin, Nomame Herba, tea, peanut, *Nelumbo nucifera*) can inhibits obesity (Alarcon-Aguilar *et al.*, 2007; Calapai *et al.*, 1999; Choi *et al.*, 2007; De Oliveiera *et al.*, 2003; Han *et al.*, 2001; Jayaprakasam *et al.*, 2006; Moreno *et al.*, 2006; Ohta *et al.*, 2006; Ono *et al.*, 2006; Yamamoto *et al.*, 2000). Thus, consumption of fruits and vegetables containing high amount of polyphenol may contribute to the prevention of obesity.

The fruit rind of rambutan was reported to contain a large variety of substances possessing antioxidant activity, such as ascorbic acid (vitamin C), tannin and phenolics (Palanisamy *et al.*, 2008; Thitilertdecha *et al.*, 2008; Wall, 2006). It is hoped that natural antioxidants found in the fruit rind of rambutan could be candidate for treatment of obesity. Therefore, the effect of the crude extract from the fruit rind of rambutan on obesity will be investigated.

2.2 Literature Review

2.2.1 Rambutan (*Nephelium lappaceum* Linn.)

The rambutan (*Nephelium lappaceum* Linn.) belongs to the same family (Sapindaceae) as the sub-tropical fruits lychee and longan. It is native to Southeast Asia. This fruit is an important commercial crop in Asia, where it is consumed fresh, canned, or processed, and appreciated for its refreshing flavour and exotic appearance (Palanisamy *et al.*, 2008).

The rambutan fruit is ovoid, or ellipsoid, pinkish-red, bright-or deep-red, orange-red, maroon or dark-purple, yellowish-red, or all yellow or orange-yellow; 3.4-8 cm long. Its thin, leathery rind is covered with tubercles from each of which extends a soft, fleshy, red, pinkish, or yellow spine 0.5-2 cm long, the tips deciduous in some types. The somewhat hairlike covering is responsible for the common name of the fruit, which is based on the Malay word *"rambut"*, meaning "hair". Within is

the white or rose-tinted, translucent, juicy, acid, subacid or sweet flesh, 0.4-0.8 cm thick, adhering more or less to the ovoid or oblong, somewhat flattened seed, which is 2.5-3.4 cm long and 1-1.5 cm wide (Morton, 1987).

The rambutan fruit (perhaps unripe) is astringent, stomachic, acts as a vermifuge, febrifuge, and is taken to relieve diarrhea and dysentery. The leaves are poulticed on the temples to alleviate headache. The astringent bark decoction is a remedy for thrush. A decoction of the roots is taken as a febrifuge. In Malaysia, the dried fruit rind is sold in drugstores and employed in local medicine (Morton, 1987; Palanisamy *et al.*, 2008). The fruit rind of rambutan can be considered as an easily accessible source of natural antioxidants and antibacterial agents (Thitilertdecha *et al.*, 2008). The fruit rind of rambutan was reported to contain a large variety of substances possessing antioxidant activity, such as ascorbic acid (vitamin C), tannin and phenolics (flavonoids and anthocyanins) (Palanisamy *et al.*, 2008; Thitilertdecha *et al.*, 2008; Wall, 2006).

Phenolic compounds, one of the most widely occurring groups of phytochemicals, are secondary metabolites that are derivatives of the pentose phosphate and phenylpropanoid pathways in plants. These compounds are of considerable physiological and morphological importance in plants (Balasundram *et al.*, 2006). Plant phenolics comprise a great diversity of compounds, such as flavonoids (anthocyanins, flavanols, flavonols, flavones, among other) and several classes of non-flavonoids (phenolic acids, lignins, stilbenes) (Chirinos *et al.*, 2008). Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, anti-obesity, cardioprotective and vasodilatory effects (Aberoumand and Deokule, 2008; Balasundram *et al.*, 2006). These beneficial effects have been attributed to the antioxidant activity of phenolic compounds.

Flavonoids are a group of phenolic compounds with antioxidant activity that have been identified in fruits and vegetables (Mojzis *et al.*, 2008). Naturally occurring flavonoids are generally classified into six classes according to their chemical structures i.e. flavanones, flavones, isoflavonoids, flavanols (flavans), flavonols and anthocyanins (Li and Jiang, 2007). Numerous studies have indicated that flavonoids have antioxidant, anti-carcinogenic, anti-viral, anti-inflammatory, anti-estrogenic or estrogenic activities and anti-angiogenic (Havsteen, 2002; Middleton *et al.*, 2000; Mojzis *et al.*, 2008). Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity in both *in vivo* and *in vitro* systems. The functionality of flavonoids in human health is supported by the ability of the flavonoids to induce human protective enzyme systems, and by a number of epidemiological studies suggesting protective effects against cardiovascular diseases, cancer, obesity, osteoporosis and other age-related disease (Aoki *et al.*, 2007; Morris and Zhang, 2006; Yao *et al.*, 2004).

Anti-obesity effect of phenolic compounds has been demonstrated by many studies. Polyphenols found in apple and tea reduced the weight of visceral adipose tissues and the triglyceride content of blood and liver in rats fed a high-fat diet. Polyphenols from apple and tea improved lipid metabolism through different manners of action. Apple polyphenols can inhibit the expressions of genes involved in fatty acid synthesis (Ohta *et al.*, 2006). The anti-obesity effects of the hot water-soluble extract from the roots of *Salacia reticulate* (SRHW) were demonstrated using obese rat models and an *in vitro*. Polyphenolic compounds in SRHW may be involved in the anti-obesity effects through inhibition of fat metabolizing enzymes (pancreatic lipase, lipoprotein lipase and glycerophosphate dehydrogenase) and enhance lipolysis (Yoshikawa *et al.*, 2002). Peanut (*Arachis hypogaea* L.) shell extracts (PSE), containing luteolin and flavonoid, also have anti-obesity effects as PSE can inhibit a number of lipases, including pancreatic lipase, lipoprotein lipase and, possibly, hormone sensitive lipase. PSE treated rats showed an increase in fecal lipid excretion respect to the control group. PSE significantly lowered body weight,

body weight gain, liver size, triacylglycerol content in the liver, as well as the serum glucose and insulin in high-fat diet fed in rats. The PSE actions may, at least in part, be attributed to the inhibition of fat absorption in the digestive tract and the reduction of the adipocyte lipolysis (Moreno *et al.*, 2006). The anti-obesity effect of *Nelumbo nucifera* leaves extract (NNE), containing several flavonoids and alkaloids, has been demonstrated. NNE inhibited the activities of α-amylase and lipase, and up-regulated lipid metabolism and expression of uncoupling protein 3 (UCP3) mRNA in mouse C2C12 myoblasts. NNE prevented the increase in body weight, parametrial adipose tissue weight and liver triacylglycerol levels in high-fat diet mice. UCP3 mRNA expression in skeletal muscle tended to be higher, when mice were administrated by NNE and were exercised. Therefore, NNE impaired digestion, inhibited absorption of lipids and carbohydrates, accelerated lipid metabolism and up-regulated energy expenditure (Ono *et al.*, 2006).

Anthocyanins, which belong to the flavonoid phenolic group of compounds, are natural pigments which are widely distributed in plants that are consumed in the human diet such as crops, beans, vegetables and fruits (Lee *et al.*, 2008). Chemically, they are polyhydroxylated or polymethoxylated glycosides or acylglycosides of anthocyanidins which are oxygenated derivatives of 2-phenylbenzopyrylium or flavylium salts. Anthocyanins are responsible for most of the red, blue, and purple colors of fruits, vegetables, flowers and other plant tissues or products. They are particularly abundant in berries and other fruits with red, blue or purple color and in red wines (Mazza and Miniati, 1993). The six anthocyanidins (pelargonidin, cyanidin, delpinidin, peonidin, petunidin and malvidin) commonly found in plants are classified according to the number and position of hydroxyl and methoxyl groups on the flavan nucleus. The most commonly occurring anthocyanidin in nature is cyanidin (Mazza, 2007). In particular, these anthocyanins are associated with a wide range of biological activities including antioxidant, anti-inflammatory, anti-carcinogenic activities, α-glucosidase inhibition, antimicrobial, improvement of vision, induction of apoptosis and neuroprotective effects (Lee *et al.*, 2008; Mazza, 2007; Talavéra *et al.*, 2004). In addition, these pigments may reduce the risk of coronary heart disease through modulation of arterial protection, inhibition of platelet

aggregation or endothelial protection, protect again obesity, memory enhancement, prevention of generation of free radicals, decreased lipid peroxidation, reduced pancreatic swelling and decreased blood sugar concentrations in urine and blood serum (Alarcon-Aguilar *et al.*, 2007; Jayaprakasam *et al.*, 2006; Lee *et al.*, 2008; Lila, 2004; Mazza, 2007; Talavéra *et al.*, 2004). *Hibiscus sabdariffa* calyces aqueous extract containing anthocyanins has an inhibitory effect on obesity in monosodium glutamate (MSG) induced obese animal model. *Hibiscus sabdariffa* extract significantly reduced body weight gain in MSG-induced obese mice and increased liquid intake in healthy and MSG-induced obese mice. Alanine aminotransferase (ALT) levels were significantly increased on the 15^{th} and 45^{th} days in obese mice (Alarcon-Aguilar *et al.*, 2007).

Ascorbic acid (vitamin C) is an antioxidant and a cofactor for various biochemical reactions, and acts as an electron donor for different enzymes (Kaidar-Person *et al.*, 2008). These vitamin is the most widely taken nutritional supplement and is available in a variety of forms such as tablets and fruit juice. Its absorption is by the intestines using a sodium ion dependent channel, and it is partially regulated by glucose. The presence of large quantities of glucose/sugar either in the intestines or in the blood can delay its absorption (Wilson, 2005). Other possible functions of ascorbic acid in the body include a role in the regulation of endogenous cholesterol synthesis, cardiovascular disease and protective role against common cold (Kaidar-Person *et al.*, 2008). Many health benefits have been attributed to ascorbic acid namely antioxidant, anti-atherogenic and anti-carcinogenic activity (Naidu, 2003). It was suggested that low plasma ascorbic acid is associated with high diastolic pressure. Multivariate control for age, BMI, other plasma antioxidants, and dietary energy, calcium, fiber, sodium, and potassium did not reduce the plasma ascorbic acid effects (Block, 2002).

Tannins are polyphenolic secondary metabolites of higher plants, and are classically divided into two groups which are hydrolysable tannins and proanthocyanidins. Hydrolysable tannins are esters of phenolic acids and a polyol, usually glucose. The phenolic acids are either gallic acid in gallotannins or other

phenolic acids derived from the oxidation of galloyl residues in ellagitannins. Proanthocyanidins (PAs) are far more common in our diet. They are polymers made of elementary flavan-3- ol units. (Khanbaee and van Ree, 2001; Santos-Buelga and Scalbert, 2000). Tannins are the active principles of plant-based medicines, especially in Asian (Japanese and Chinese), the tannin-containing plant extracts are used as astringents, against diarrhoea, as diuretics, against stomach and duodenal tumours. As tannins can precipitate heavy metals and alkaloids (except morphine), they can be used in poisonings with these substances (Khanbabaee and Ree, 2001). Anti-obesity effects of dietary teasaponin containing a mixture of teasaponin epicatechin gallate and epigallocatechin have been demonstrated. Teasaponin inhibited pancreatic lipase activity and plasma triacylglycerol levels. Teasaponin suppressed the increases in body weight, parametrial adipose tissue weights and diameter in adipose cell size induced by a high-fat diet. The anti-obesity effects of teasaponin in high-fat diet treated mice may be partly mediated through delaying the intestinal absorption of dietary fat by inhibiting pancreatic lipase activity (Han *et al.*, 2001). An anti-obesity effects of Nomame Herba containing primarily proanthocyanidin (condensed tannin) has been demonstrated. Nomame Herba can inhibit lipase activity, prevent and ameliorat obesity, inhibit fatty liver and hypertriglyceridemia in rats fed a high-fat diet (Yamamoto *et al.*, 2001).

2.2.2 Definition of Obesity

Obesity is defind as a conditions characterized by excess body fat that is quantified by the elevation in body weight of patients (Seidell and Flegal, 1997). In general, it is accepted that obesity results from positive energy balance in which energy intake exceeds expenditure (Wood *et al.*, 1998). It is characterized by enlarged fat mass and elevated lipid concentration in blood (Devlin *et al.*, 2000; Fujioka *et al.*, 2002). The amount of fat mass is increased when the number and/or size of adipocytes are multiplied by proliferation and differentiation. Differentiated adipocyte stores fatty acids (FAs) in the form of triglycerides (TGs) in their cytoplasm, with an involvement of various enzymes such as strearoyl-CoA desaturase-1 (SCD-1) and

fatty acid synthase (FAS). This overall lipid synthetic process is called lipogenesis (Weissman, 1999). Adiposity has been shown to have a strong linear correlation with elevated plasma levels of fatty acids. If blood fatty acids levels are elevated for prolonged periods by excessive energy intake, triglyceride can be accumulate in non-adipose tissues including liver and muscle, which can lead to pathological consequences such as the development of fatty liver or ketosis (Herdt, 2000).

The widely accepted means of assessing obesity is the body mass index (BMI). A very good correlation has been found between BMI and the percentage of body fat in a population. Table 1 provides the World Health Organization (WHO) classification of adults according to the BMI (Bagchi and Preuss, 2007):

$$\text{BMI} = \frac{\text{Kg}}{\text{m}^2} = \frac{\text{Body weight in Kg}}{\text{Height in m}^2}$$

Table 1. Classification of adults according to BMI

Classification	**BMI**	**Risk of co-morbidities**
Underweight	< 18.50	Low (but risk of other clinical problems increased)
Normal range	18.50 - 24.99	Average
Overweight	≥ 25	
- Pre-obese	25.00 - 29.99	Increased
- Obese class I	30.00 - 34.99	Moderate
- Obese class II	35.00 - 39.99	Severe
- Obese class III	≥ 40.00	Very severe

From table 1, the current value settings are as follows: a BMI lower than 18.5 suggests the person is underweight and may indicate malnutrition, an eating disorder, or other health problems, a BMI of 18.5 to 25 may indicate optimal weight

while a number above 25 may indicate the person is overweight; a number above 30 suggests the person is obese (over 40, morbidly obese).

2.2.3 The physiology of obesity

The amount of fat in the body (adiposity) is not, as was once thought, a passive result of bad habits or over-indulgence. Rather, it is precisely regulated as part of the process of energy homeostasis, a process whereby energy intake (food intake) is matched to energy expenditure (metabolism and exercise) and the size of the body's energy stores (the fat mass). The major organ regulating this system is the brain, although multiple organ systems participate in the process (Woods and Seeley, 2002). Figure 1 shows how circulating signals related to the size of the fat mass (adiposity signals) are integrated with signals from the gastrointestinal system (satiety signals) to control energy homeostasis (Schwartz *et al.,* 2000). Main brain pathways involved in eating behavior regulation are shown in figure 2 (Valassi *et al.*, 2008).

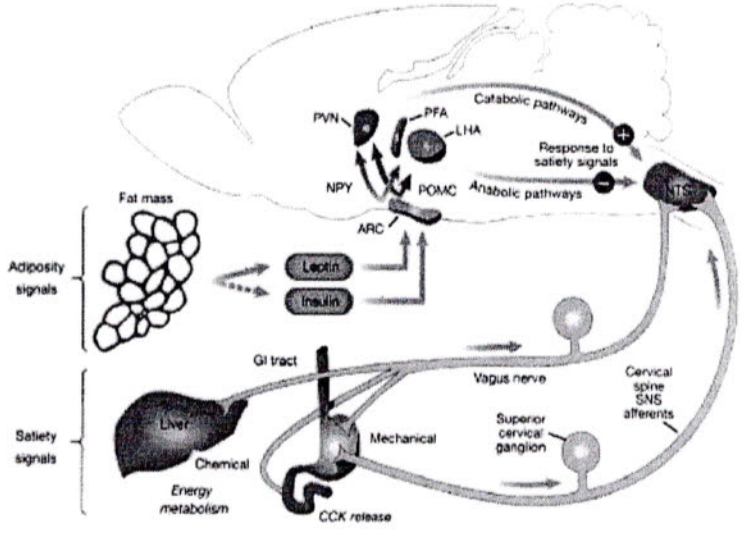

Figure 1. Pathways by which signals related to the fat mass are integrated with signals from the gastrointestinal system to control all aspects of energy homeostasis. Adiposity signals are connected through central autonomic pathways to centres that process satiety signals. Reduced input from adiposity signals (e.g. after weight loss)

increases meal size by reducing brain response to satiety signals (Schwartz *et al.*, 2000).

Adiposity and satiety signals enter the brain at different levels. Adiposity signals enter the brain at the level of the hypothalamus. Neural signals from the gastrointestinal system and the liver provide information about the food is being eaten, for example, the taste of the food, how much the stomach is distended, and the chemical content of the food. These satiety signals are sent to the hindbrain. The brain responds to the hormone signals *via* integrated neuropeptide pathways, leading to a number of outputs that are directly related to energy homeostasis. These outputs include neuroendocrine activation from the pituitary gland, motor behaviour (eating, exercise, etc.) and autonomic activity. In recent years it has become apparent that the autonomic nervous system has a much greater impact than was once thought upon many fundamental processes of metabolism, including lipolysis, the secretion of insulin and glucagons from the pancreas, and glucose synthesis and secretion from the liver. It is important to note that, while energy expenditure tends to decrease with ageing, mainly because of the absence of occupational activity and extreme physical exertion, energy intake does not tend to decrease to the same extent, for a number of reasons, including lifetime habits. Thus, there is a tendency over time for the body weight to increase (Woods and Seeley, 2002).

Satiety signals are generated in the gastrointestinal (GI) tract during a meal and regulate food intake on meal-to-meal basis, inducing a sense of fullness. After entering the GI lumen, nutrients trigger the secretion of several peptides which, in addition to other actions, activate vagal and sympathetic pathways afferent to the nucleus of the solitary tract (NTS) in the caudal brainstem where they provide information on the chemical and mechanical properties of the nutrients (Wood, 2004). NTS expresses both pro-opiomelanocortin (POMC) and leptin receptors, which suggests that this brain area, like arcuate nucleus (ARC), is able to integrate peripheral satiety and adiposity signals with hypothalamic and suprahypothalamic information (Schwartz *et al.*, 2000).

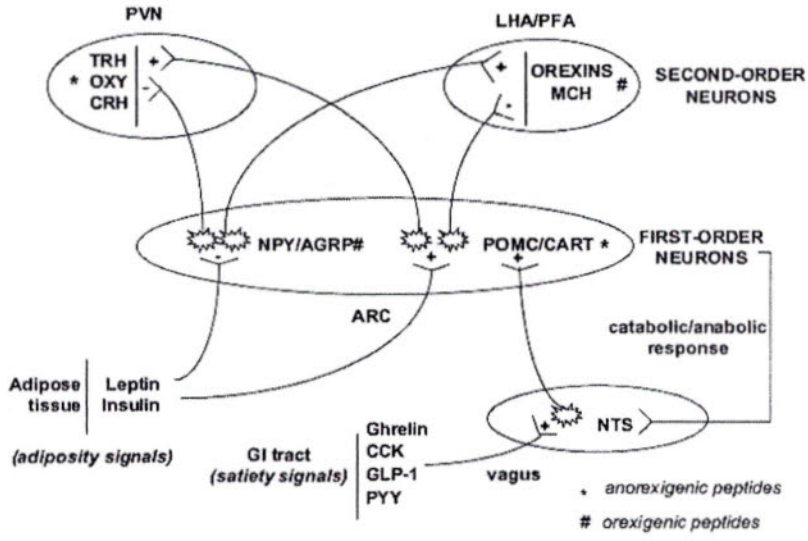

Figure 2. A schematic representation of the chief brain pathways involved in the regulation of eating behavior. (ARC: arcuate nucleus, NTS: nucleus of the solitary tract, CCK: cholecystokinin, GLP-1: glucagon-like peptide 1, PYY: peptide YY, PVN: paraventricular nucleus, LHA: lateral hypothalamic area, PFA: perifornical area, NPY: neuropeptide Y, AGRP: Agouti-related peptide, POMC: pro-opiomelanocortin, CART: cocaine- and amphetamine-regulated transcript, CRH: corticotropin-releasing hormone, TRH: thyrotropin-releasing hormone, OX: oxytocin, MCH: melanin concentrating hormone) (Valassi *et al.*, 2008).

Cholecystokinin (CCK) is mainly produced by neuroendocrine secretory cells lining the intestinal lumen. CCK peptides exert their action on 2 distinct receptor subtypes: CCK-A (alimentary), now called the CCK-1R, which is mostly expressed peripherally; and CCK-B (brain), renamed the CCK-2R, which is primarily present in the brain (Kissileff *et al.*, 2003). When nutrients enter the lumen and binds to specific receptors (CCK-1R) located on vagal sensory terminals delivering to NTS a sense of fullness (Valassi *et al.*, 2008). CCK reduces food intake *via* the parasympathetic nervous system during food ingestion (Kissileff *et al.*, 2003) and increases absorption by retarding stomach emptiness (Helm *et al.*, 2003).

Adiposity signals compose of leptin and insulin as shown in figure 1 and figure 2. Leptin, the *ob* gene product, is produced mainly in the adipose tissue and

enters the brain in proportion to its plasma levels. Leptin maintains long term control on adiposity and regulates adaptive metabolic changes in response to modifications of nutritional (Ahima and Osei, 2004). Leptin is also able to regulate short-term energy intake, modulating meal size according to changes in energy balance: with negative energy balance, low leptin signaling activates anabolic and inhibits catabolic circuits, enhancing neuropeptide Y and agouti-related peptide (NPY/AGRP) release and blocking the activity of pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript (POMC/CART) neurons with increase in meal size and decrease in energy expenditure (Schwartz *et al.,* 2000). Insulin, when body weight augments, insulin resistance occurs with attendant increase in insulin secretion. The hormone enters the brain in proportion to its circulating levels, contributing to reduce energy intake through the activation of catabolic pathways (Schwartz et al., 2000). Central administration of insulin significantly reduces feeding and body weight in animal models (Vettor *et al.,* 2002). Insulin and leptin both activate POMC neurons, but they seem to differentially regulate AGRP, with leptin inhibiting and insulin stimulating its synthesis (Valassi *et al.*, 2008 quoted in Wanting *et al.*, 2005).

Arcuate nucleus (ARC), adjacent to the third ventricle, is the chief hypothalamic area involved in the control of food intake and contains two interconnected groups of "first-order" neurons releasing NPY and AGRP, which enhance food intake, and the anorexigenic substances POMC and CART (Valassi *et al.,* 2008). The axons of these neurons project to "second-order" neurons, located in part in the paraventricular nucleus (PVN), where the anorexigenic substances thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and oxytocin are secreted, and in part in lateral hypothalamic area (LHA) and perifornical area (PFA), where the orexin molecules melanin-concentrating hormone (MCH) and orexins are produced. When adiposity signals reach ARC, anorexigenic peptides are released which activate a catabolic circuit. In contrast, the activation of anabolic pathway leads to the release of orexigenic peptides and occurs when adiposity signal concentrations in the brain are low (Valassi *et al.,* 2008).

NPY is a 36–amino acid hypothalamic orexigenic neuropeptide secreted in the arcuate nucleus (Angelopoulos *et al.*, 2005 quoted in Demont *et al.*, 1992), from which NPY neurons project to second-order neurons located in PVN, LHA, PFA, ventromedial (VMN) and dorsomedial (DMN) nuclei, and to other brain regions, setting in motion the anabolic pathway (Ramos *et al.*, 2004). Furthermore, 90% of NPY neurons co-express AGRP (Schwartz *et al.*, 2000). Central administration of NPY inhibits thermogenesis, enhances food intake and promotes adipogenesis in rats (Williams *et al.*, 2004). AGRP is another potent orexigenic peptide. Its release by ARC is inhibited by leptin infusion. AGRP influences food intake mainly through the competitive antagonism of central melanocortin receptors (Ollmann *et al.*, 1997).

POMC is the precursor of several molecules including alpha-melanocyte-stimulating hormone (α-MSH), which represents the main regulator of food intake and body weight (Valassi *et al.*, 2008; Lawrence *et al.*, 1999). In the brain, POMC is located primarily in the ARC. Two of the five melanocortin receptors (MC-Rs), MC3-R and MC4-R are expressed in the hypothalamic regions associated with feeding behaviour (e.g. PVN and VMN) (Lawrence *et al.*, 1999). CART are neropeptides involved in feeding, which are widely distributed in the brain, gut, pituitary, adrenals, and pancreas (Gautvik *et al.*, 1996). Ninety percent of CART neurons are co-localized with POMC neurons in ARC and project to second-order neurons likely mediating the anorexigenic effect of leptin (Aja *et al.*, 2001).

2.2.4 Adipose Tissue

Adipose tissue is specialized connective tissue (Albrignt and Stern, 1998). Adipose tissue functions can be classified into three aspects. First, it is related to lipid metabolism including triglycerides (TGs) storage and fatty acids (FAs) release. Second, it catabolizes TGs in order to release glycerol and FAs that participate in glucose metabolism in liver and other tissues. Finally, adipocytes secrete adipokines, which include hormones, cytokines and other proteins with specific biological functions (Morrison and Farmer, 2000). Adipose tissue has an important influence on

physiological processes such as development and growth of the adipocyte and energy homeostasis (Bays *et al.*, 2008). There are two types of adipose tissue depending on its cell structure, location, color, vascularization and function: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the primary site of energy storage in a lipid droplet of the adipocytes in the form of TGs, whereas BAT contains multilocular adipocytes or cells with various lipid droplets. It has a large number of mitochondria and is specialized in heat production and, therefore, energy expenditure. Nevertheless, in humans, BAT is present only in newborns for regulating thermogenic process (Gesta *et al.*, 2007).

- Lipogenesis

Lipogenesis is the synthesis of esterified FAs, which from TGs from carbohydrates or other energy sources acquired in the diet (Figure 3). In rats, lipogenesis occurs in liver and WAT, whereas in humans, lipogenesis contributes mildly to the fat balance (McDevitt *et al.*, 2001). It occurs predominantly in liver and to a lesser extent in adipose tissue, even with high-carbohydrate diets. In rodents, nutritional status and small changes in insulin levels are factors that influence lipogenesis rate (Vázquez-Vela *et al.*, 2008 quoted in Huber *et al.*, 1965). Lipid synthesis is augmented during postprandial state and after carbohydrate consumption and is inhibited under fasting conditions (Sebokova *et al.*, 1997). Lipid accumulation in adipose tissue depends on circulating FA uptake (Zechner *et al.*, 2000). FAs are provided by the enzymatic hydrolysis of TG contained in the chylomicrons by the lipoprotein lipase. After FAs enter the adipocyte, reesterification is necessary for lipid storage in TG form (Hirata *et al.*, 1999). Several enzymes involved in adipose tissue lipogenesis are induced by insulin. These are fatty acid synthase (FAS), acetyl CoA carboxylase (ACC) and malic enzyme (ME). Newly synthesized FAs are used as substrates in TG synthesis (Sul and Wang, 1998).

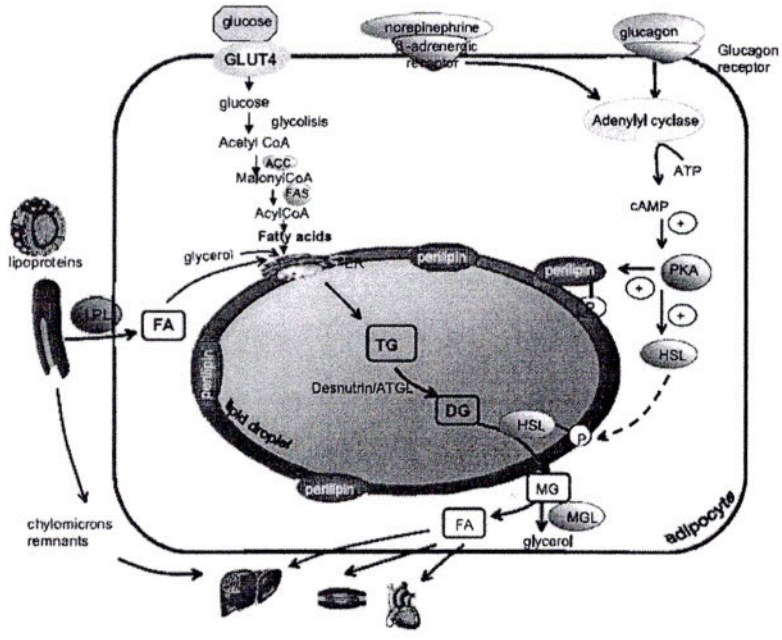

Figure 3: Lipogenesis and lipolysis (Vázquez-Vela *et al.*, 2008).

- Lipolysis

TG stored in the lipid droplet are first hydrolyzed by the enzyme adipose triglyceride lipase (ATGL), also known as desnutrin, releasing a diacylglycerol moiety and FA (Villena *et al.*, 2004). After hydrolysis by ATGL, diacylglycerols are then hydrolyzed sequentially by the hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL), producing FFAs and glycerol (Fredrikson *et al.*, 1986; Holm, 2003). Different lipases gain access to the lipid droplet when proteins that coat the vesicle (perilipins) are phosphorylated. Perilipin a normally prevents lipolysis of TG by surrounding the lipid droplet, preventing the access of lipases (Brasaemle *et al.*, 2000). β-adrenergic stimulation of adipocytes and the subsequent protein kinase A-dependent phosphorylation of HSL and perilipin trigger the translocation of HSL from the cytoplasm to the lipid droplet and induce neutral lipid hydrolysis (Egan *et al.*, 1992). During fasting, glucagon and catecholamines stimulate lipolysis in the adipocytes by activating via PKA several lipases, resulting in a mobilization of FFA from the adipocyte to the circulation, which are then bound to albumin and transported to muscle, liver, heart and other tissues for its oxidation or reesterification (Lafontan *et al.*, 2000).

• Pancreatic Lipase (PL)

Lipases are enzymes that digest fats, including triacylglycerol and phospholipids. The human lipases include the pre-duodenal (lingual and gastric) and the extra-duodenal (pancreatic, hepatic, lipoprotein and the endothelial) lipases (Mukherjee, 2003). PL (triacylgycerol acyl hydrolase), the principal lipolytic enzyme synthesized and secreted by the pancreas, plays a key role in the efficient digestion of triglycerides. It removes fatty acids from the α and α′ position of dietary triglycerides, yielding β-monoglycerides and long chain saturated and polyunsaturated fatty acids as the lipolytic products (Mukherjee, 2003; Shi and Burn, 2004; Thomson *et al.*, 1997). PL is responsible for the hydrolysis of 50-70% of total dietary fats. Figure 4 depicts the physiological role of PL. Thus, PL inhibition is one of the most widely studied mechanisms form the determination of the potential efficacy of natural products as anti-obesity agents (Birari and Bhutani, 2007).

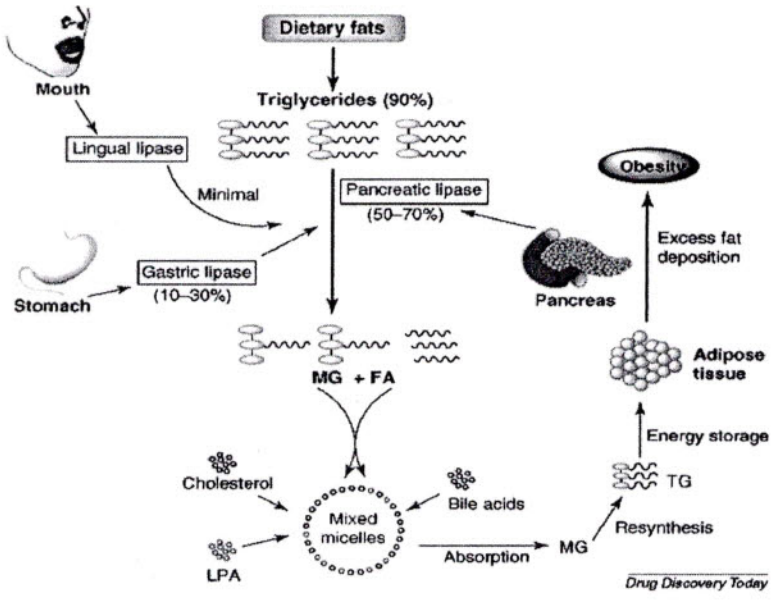

Figure 4. Physiological role of pancreatic lipase in lipid absorption (Birari and Bhutani, 2007).

2.2.5 Fos protein

Fos protein, the translational product of immediate early gene *c-fos*, exerts influence on cellular functions by regulating the induction of its downstream target genes as a transcription factor and its is induced rapidly and transiently in neurons after applying a variety of stimuli (Herrera and Robertson, 1996; Hughes and Dragunow, 1995). After translation, Fos protein couples with Jun protein to form a heterodimer nucleoprotein complex that binds with high affinity to a DNA-specific sequence identified as activating protein-1 (AP-1) site (Hughes and Dragunow, 1995; Sassone-Corsi *et al.*, 1988). AP-1 is a collective term referring to dimeric transcription factors composed of Jun, Fos or activating transcription factor (ATF) subunits that bind to a common DNA site (Karin *et al.*, 1997). Several *cis* elements mediate *c-fos* induction. Proximal to the *c-fos* TATA box (TGACGTCA) is a cAMP-responsive element (CRE) or ATF proteins, which all mediate *c-fos* induction *via* cAMP- and Ca^{2+}-dependent signaling pathways in response to neurotransmitters and polypeptide hormones (Sheng *et al.*, 1991). Expression of the immediate early gene *c-fos* and its protein product c-Fos has been extensively used to map stimulus-evoked functional activity in the brain (Nikolaev *et al.*, 2002). Fos can be identified by immunohistochemical to be in the nuclei of neurons (Bullitt, 2004).

The studies of Kim et al. (2005) reported that crude saponin of Korean red ginseng reduced the NPY-immunoreactive neurons of PVN, LHA and VMN in HFD-induced obesity rats. Administration of crude saponin of Korean red ginseng for 3 weeks reduced body weight, food intake, parametrical adipose tissues and serum leptin level in HFD-induced obesity rats. The effect of long term HFD has been shown in rats. HFD rats have decreased in the number of neurons carrying α-MSH and CART peptide in ARC of the hypothalamus (Tian *et al.*, 2004). The effect of dietary fats on c-Fos immunoreactivity has been shown in mouse. C-Fos immunoreactivity neurons in the dorsal part of lateral hypothalamic (dLH) area were rapidly increased by saturated fat feeding at 1 week whereas VMH activity was decreased. C-Fos immunoreactivity neurons have increased in PVN by high saturated

fat at 7 and 11 weeks. Substitution of saturated fat diet with the *n*-3 polyunsaturated fatty acids diet partially revered the increase in c-Fos immunoreactivity neurons in PVN of saturated fat fed mice, while it significantly increase c-Fos immunoreactivity neurons in ARC (Wang *et al.*, 1999).

2.2.6 High-fat diet (HFD)-induced obesity model

Obesity models compose of monosodium glutamate (MSG)- and high-fat diet (HFD)-induced obesity. MSG is a sodium salt of the amino acid L-glutamate (GLU), elicits a unique taste termed "umami" (Kondon and Torii, 2008). It has been demonstrated that neonatal administration of MSG to rodents destroys 80-90% of the ARC and damages other central structures (Dolnikoff *et al.*, 2001). Therefore, this way then doesn't like to use in the experiment. Now, HFD-induced obesity model can be used to generate obese rodent models (Buettner *et al.*, 2007). In rats fed HFD have shown obese, hyperphagic, hyperleptinemic, hyperinsulinemic, hyperglycemic and hypertriglyceridemic (Farley *et al.*, 2003). A multitude of different HFD have been used with relative fat fractions between 20% and 60% energy as fat, and the basic fat component varies between animal-derived fats (e.g. lard or beef tallow) and plant oils (e.g. corn or safflower oil) (Buettner *et al.*, 2007). Prolonged feeding with fat-enriched diets induces an increase in body weight in susceptible rats in the range of 10% to 20% over standard chow-fed controls. Obesity induction is most effective when the diet is started at a young age and continues for several weeks (Peckham *et al.*, 1962).

Anti-obesity effects of plant extracts have been demonstrated in many studies using HFD fed rodents models. Pine needle extract (PNE) treatment reduced adipose tissue mass, hyperlipidemia, and hepatic steatosis in obese rats fed HFD. PNE treatment suppressed differentiation of 3T-L1 adipocytes, in part by down-regulating expression of peroxisome proliferator-activated receptor γ (PPAPγ) mRNA (Jeon and Kim, 2006). Chitosan and oolong tea exerted anti-obesity and/or anti-hyperlipidemic actions that were mediated through delaying the intestinal absorption of dietary fat by

inhibiting pancreatic lipase activity (Han *et al.*, 1999a, b). Anti-obesity effects of *Dioscorea nipponica* Makino have been shown in rats fed HFD. *Dioscorea nipponica* Makino inhibited pancreatic lipase activity. *Dioscorea nipponica* Makino treated rats showed an increase in fecal fat excretion and plasma high-density lipoprotein cholesterol (HDL) levels and significantly lowered body weight, adipose tissue, plasma triacylglycerol levels, plasma triglyceride (TG), total cholesterol, very low-density lipoprotein cholesterol (VLDL) and low-density lipoprotein cholesterol (LDL) levels (Kwon *et al.*, 2003). In mice fed HFD, chikusetsusaponins inhibited pancreatic lipase activity. Chikusetsusaponins prevented the increase in body weight, parametrial adipose tissue weight and plasma triacylglycerol levels and significantly increased the fecal content (Han *et al.*, 2005).

3. Research Objectives

The experiments are designed to clarify the following:

1. To study the effect of the crude extract from the fruit rind of rambutan on lipase activity *in vitro*.
2. To study toxicity of the crude extract from the fruit rind of rambutan in rats.
3. To study acute effects of the crude extract from the fruit rind of rambutan on plasma triacylglycerol levels in healthy and HFD-induced obese rats.
4. To study subchronic effects of the crude extract from the fruit rind of rambutan on food intake, body weight, visceral adipose tissue and visceral organ weights, liver triacylglycerol and plasma parameters in healthy and HFD-induced obese rats.
5. To study acute and subchronic effects of the crude extract from the fruit rind of rambutan on Fos protein expression in the hypothalamus and the nucleus of the solitary tract of healthy and HFD-induced obese rats.

4. Research Hypothesis

The crude extract from the fruit rind of rambutan may have anti-obesity effects in high-fat diet (HFD)-induced obese rats.

5. Research Methodology

5.1 Plant Materials

5.1.1 Rambutan (*Nephelium lappaceum* L.) fruits were obtained from local market in Nakhon Ratchasima province during June-August 2008.

The fruit rind was washed with copious amounts of water and allowed to air dry at room temperature for 2-3 h. The fruit rind was then cut into small thin pieces and dried at room temperature for 2-4 days. The dried rind was powdered using an electric mill with a 1 mm mesh (Alarcon-Aguilar *et al.*, 2007; Palanisamy *et al.*, 2008; Tachakittirungrod *et al.*, 2007). The dried powder was extracted by maceration method with 85% aqueous ethanol (100 g dried powder/ 500 ml of 85% aqueous ethanol) for 7 days in the dark at room temperature. The obtained suspension was filtered through No.1 Whatman filter paper and the filtrate was collected. The filtrate was concentrated using a rotary evaporator and then converted to crude extract by freeze-dried. The obtained crude extract was stored at -20 °C until further used (Kähkönen *et al.*, 1999; Palanisamy *et al.*, 2008; Tachakittirungrod *et al.*, 2007).

5.1.2 Determination of total phenolic compounds

The total phenolic compounds of sample extract will be measured according to the Folin-Ciocalteu reagent method (Singleton and Rossi, 1965). Extract solutions will be prepared by dissolving 0.1057 g/ml of ethanol (analytical reagent). Extract solutions (1 ml three replicates) will be introduced into test tubes in the dark; 0.5 ml of 10% (v/v) Folin-Ciocalteu reagent will be added. Thirty minutes later, 3 ml

of 40% (w/v) sodium carbonate will be added. After mixing for 15 min; 10 ml of double distilled water will be added. Subsequently, the contents will be mixed and allowed to stand for 30 min at room temperature. The absorbance of extract solutions and a prepared blank will be measured at 725 nm using a spectrophotometer (UV-visible Spectrophotometer Model 1000, CECIL, England). The total phenolic compounds will be expressed as gallic acid equivalents (GAE) in milligrams per gram of sample extract, using a standard curve generated with 0.00, 6.25, 12.50, 25.00, 50.00 and 100.00 mg/L of gallic acid (Aberoumand and Deokule, 2008; Maisuthisakul *et al.*, 2008; Pastrana-Bonilla *et al.*, 2003; Thitilertdecha *et al.*, 2008).

5.1.3 Determination of antioxidant activity

The free radical scavenging activity of sample extract will be determined by DPPH (2,2-diphenyl-1-picrylhydrazyl radical) method (Brand-Williams *et al.*, 1995). The absorbance of sample extract and a prepared blank will be measured at 517 nm using a spectrophotometer (UV-visible Spectrophotometer Model 1000, CECIL, England). All determinations will be performed in triplicate. The ability to scavenge the DPPH radical will be calculated as percent DPPH scavenging using the following equation:

$$\%\text{DPPH scavenging} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the mixture containing extracts (Thitilerdecha *et al.*, 2008).

5.1.4 Determination of ferric reducing/antioxidant power

The total antioxidant potential of sample extract will be determined by using a ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996). FRAP assay measures the change in absorbance at 595 nm owing to the formation of a blue colored Fe^{2+}- tripyridyltriazine compound from colorless oxidized Fe^{3+} form by

the action of electron donating antioxidants. All determination will be performed in triplicate (Katalinic *et al.*, 2006; Tachakittirungrod *et al.*, 2007).

5.1.5 Determination of anthocyanins

The anthocyanins presenting in the sample extract will be measured at 538 nm using a spectrophotometer (UV-visible Spectrophotometer Model 4001/4, USA) (Lohachoompol *et al.*, 2004).

5.2 Animals

Experiments will be carried out on age-matched male Wistar rats (6 weeks old). They will be housed under standard laboratory conditions (12:12 h dark-light cycle, ambient temperature 20±1°C) with free access to food and water. The experiments will be carried out following the Animal Care and Use Committee Guidelines of Suranaree University of Technology.

5.3 Experimental Design

Experiment 1: Effect of the crude extract from the fruit rind of rambutan on lipase activity *in vitro*.

The assay for lipase activity using triolein as the substrate will be performed according to the method of Huerta *et al.* (2007). Assays will be initiated by adding triolein emulsion to porcine pancreatin and various concentrations of extract from the fruit rind of rambutan dissolved in Polysorbate 80. The contents will be vortexed and absorbance measured immediately at 450 nm and designated as T_0. The test tubes will be incubated at 37 °C for 30 min and at the end of the incubation absorbance at 450 nm will be again recorded and designated as T_{30}. $\Delta A_{450} = [\, A_{450}\,(T_0) - A_{450}\,(T_{30})]$ will be calculated for both control and the treatment. The inhibition of lipase activity (%) will be calculated by:

$$\% \text{ inhibition} = \left(\left[\frac{\Delta A_{450}{}^{\text{Control}} - \Delta A_{450}{}^{\text{Extract}}}{\left[\Delta A_{450}{}^{\text{Control}} \right]} \right] \right) \text{X } 100$$

Experiment 2: Toxicity studies of the crude extract from the fruit rind of rambutan.

Six weeks old rats (n=42) will be selected by stratified randomization and then divided into seven groups. Each group contained six rats. The first group will serve as control (Polysorbate 80) while the remaining six groups will be given 50, 100, 250, 500, 1000 and 2000 mg/kg of the crude extract from the fruit rind of rambutan dissolved in Polysorbate 80 by oral gavage, respectively. Clinical observations (increase activity and irritability, salivation, itching the nose and mouth on the cage floor, and diarrhea) will be conducted at 1, 2.5, 5, 10 and 24 h after administration and daily thereafter for 7 days. Mortality checks will be conducted twice a day (morning and afternoon) for 7 days, and again on the morning of day 8. The toxicity (LD_{50}) will be calculated as the geometric mean of the dose that result in 100% mortality and that causes no lethality at all. If any inconsistencies will be observed in the mortality patterns, then an estimation of the LD_{50} will be carried out using the probit-log analysis (Aniagu *et al.*, 2005; Lorke, 1983; Schauss *et al.*, 2007).

Experiment 3: Acute effect of the crude extract from the fruit rind rambutan on plasma triacylglycerol levels in healthy and HFD-induced obese rats.

Three weeks old rats will be divided into control (healthy rats) and high-fat diets (HFD) groups. The rats in control group will receive normal diets. The rats in HFD group will receive high-fat diets. In both groups will be given free access to water. The body weight will be measured weekly. Six weeks later, healthy and

HFD-induced obese rats will be selected by stratified randomization to be use in the experiment (Choi *et al.*, 2007).

Nine weeks old healthy rats (n=32) will be divided into four groups. Each group will contain eight rats. After fasting overnight, the first group served as control will be orally administered with Polysorbate 80, while the remaining three groups will be orally administered with low, middle, high concentrations of the crude extract from the fruit rind of rambutan dissolved in Polysorbate 80, respectively.

Nine weeks old HFD-induced obese rats (n=32) will be divided into four groups. Each group will contain eight rats. After fasting overnight, the first group served as control will be orally administered Polysorbate 80, while the remaining three groups will be orally administered with low, middle, high concentrations of the crude extract from the fruit rind of rambutan dissolved in Polysorbate 80, respectively.

Blood samples will be taken from the tail vein at 0, 0.5, 1, 2, 3, 4 and 5 h after administration of the Polysorbate 80 with or without the crude extract from the fruit rind of rambutan using a heparinized capillary tube, and centrifuged at 5,500 g for 5 min. The plasma triacylglycerol will be determined using a Triglyceride assay kit (Han *et al.*, 2001).

Experiment 4: Acute effects of the crude extract from the fruit rind of rambutan on Fos protein expression in the hypothalamus and the nucleus of the solitary tract in healthy and HFD-induced obese rats.

Nine weeks old healthy rats (n=32) and HFD-induced obese rats (n=32) will be treated similar to experiment 3.

After 90 min of each treatment, the rats will be sacrificed by decapitation. Trunk blood will be taken and centrifuged. Plasma will be separated and frozen at -20 °C until further assay for the plasma parameters.

The plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), very low-density lipoprotein cholesterol (VLDL), glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities will be measured by automatic blood analyzer (Jeon and Kim, 2006; Yamamoto *et al.*, 2000).

The brains will be removed and freezed on dry ice. The brains will be coronal sectioned (16 μm) using cryostat and stored in desiccated boxes at -20 °C until further use. Fos protein expression in the hypothalamus and the nucleus of the solitary tract will be used for determination of neuronal activity by Fos immunohistochemistry (Xiong and Hatton, 1996). Four sections from each areas (ARC, PVN, LHA, PFA and NTS) will be counted for Fos-immunoreactive neuron.

Experiment 5: Subchronic effects of the crude extract from the fruit rind of rambutan on food intake, body weight, visceral adipose tissue and visceral organ weights, liver triacylglycerol, plasma parameters and Fos protein expression in the hypothalamus and the nucleus of the solitary tract in healthy and HFD induced obese rats.

Nine weeks old healthy rats (n=32) will be divided into four groups. Each group will contain eight rats. The first group served as control will be orally administered with Polysorbate 80, while the remaining three groups will be orally administered with low, middle, high concentrations of the crude extract from the fruit rind of rambutan dissolved in Polysorbate 80 for 12 weeks. All animals will be given free access to normal diet and water throughout the experimental period.

Nine weeks old HFD induced obese rats (n=32) will be divided into four groups. Each group will contain eight rats. The first group served as control will be orally administered Polysorbate 80, while the remaining three groups will be orally administered with low, middle, high concentrations of the crude extract from the fruit

rind of rambutan dissolved in Polysorbate 80 for 12 weeks. All animals will be given free access to HFD and water throughout the experimental period.

Daily food intake and weekly body weight for each rat will be determined throughout the study. After 12 weeks of treatment, the rats will be fasted 10 h and sacrificed by decapitation. Trunk blood will be taken and centrifuged. Plasma will be separated and frozen at -20 °C until further assay for the plasma parameters.

The plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), very low-density lipoprotein cholesterol (VLDL), glucose, aspartate aminotransferase (AST)) and alanine aminotransferase (ALT) activities will be measured by automatic blood analyzer (Jeon and Kim, 2006; Yamamoto *et al.*, 2000).

The visceral adipose tissues (subcutaneous, perirenal, inguinal, epididymal and mesenteric adipose tissue) and visceral organs (liver, heart, spleen, lung and kidney) will be quickly removed, measured and recorded as a percentage of final body weight together with the absolute values (Kwon *et al.*, 2003; Yamamoto *et al.*, 2000).

The liver tissue will be stored at -20 °C until needed for the triacylglyceride (TAG) levels analysis. Liver samples will be cut using cryostat (20 μm). The cutting sections will be put on glass slides and fixed in 10% formalin for 20 min. Sections will be then stained with 0.2% Oil Red O solution for 30 min. The sections will be rinsed with 60% 2-propanol and water and then counterstained with hemotoxylin. Images will be obtained using a fluorescent microscope equipped with a digital camera and analyzed for the triacylglyceride (TAG) levels in liver (Jayaprakasam *et al.*, 2006).

The brains will be removed and freezed on dry ice. The brains will be coronal sectioned (16 μm) using cryostat and stored in desiccated boxes at -20 °C until further use. Fos protein expression in the hypothalamus and the nucleus of the solitary tract will be used for determination of neuronal activity by Fos immunohistochemistry

(Xiong and Hatton, 1996). Four sections from each areas (ARC, PVN, LHA, PFA and NTS) will be counted for Fos-immunoreactive neuron.

5.4 Statistical Analysis

Differences between groups will be assessed by ANOVA using the SigmaStat software package (Systat software, Point Richmoud, CA). Post hoc testing will be performed for inter group comparisons. A p-value less than 0.05 ($p < 0.05$) will be considered to be statistically significant.

6. Expected Results

1. The findings will provide the new evidence of the beneficial effects of the crude extract from the fruit rind of rambutan on anti-obesity as it may inhibit pancreatic lipase activity, decrease food intake, decrease body weight and visceral adipose tissues weights, decrease liver triacylglycerol, stimulate neuronal activity in the paraventricular nucleus of the hypothalamus and stimulate neuronal activity in the nucleus of the solitary tract, inhibit neuronal activity in the lateral hypothalamic area and the perifornical area of the hypothalamus.
2. The findings will provide the first evidence of acute and chronic effects of the crude extract from the fruit rind of rambutan on anti-obesity.
3. The findings will provide supporting data for long-term supplementary for nutritional antioxidants could be candidates for anti-obesity agents.

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8. Research Plan

Thesis procedure begins in July, 2008

Step	Activities	Period							
		2008		2009-2010					
		July-Sep.	Oct.-Dec.	Jan.-Mar.	Apr.-June	July-Sep.	Oct.-Dec.	Jan.- Mar.	Apr.-June
1	Literature review	←	→						
2	Plant & Animal preparation	←					→		
3	Experiment 1, 2			←		→			
4	Experiment 3, 4					←	→		
5	Experiment 5						←	→	
6	Data Analysis						←	→	
7	Writing up the thesis							←	→
8	Thesis Submission								↔

Advisor's Signature.....

(Asst. Prof. Dr. Rungrudee Srisawat)

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Student Signature.....

(Miss Aree Thinkratok)

...2../..มี.ค../..2552

Ph.D. Thesis Proposal

THE DISTRIBUTION, BEHAVIOR AND THREAT OF RED-SHANKED DOUC LANGUR *Pygathrix nemaeus nemaeus* IN HIN NAMNO NATIONAL PROTECTED AREA, KHAMMOUANE PROVINCE, LAO PDR

การแพร่กระจาย พฤติกรรม และสถานภาพการคุกคามของค่างห้าสี (*Pygathrix nemaeus nemaeus*) ในพื้นที่อนุรักษ์หินน้ำโน จังหวัดคำม่วน สาธารณรัฐประชาธิปไตยประชาชนลาว

By

Mr. Phaivanh Phiapalath

School of Biology

Institute of Science

Suranaree University of Technology

October, 2007

Thesis Proposal

Mr. Phaivanh Phiapalath — ID No. D4910046

School of Biology — Institute of Science

Thesis committee

A) Dr. Pongthep Suwanwaree — Chairperson

B) Dr. Arlyne Johnson — Committee

C) Assist. Prof. Dr. Carola Borries — Committee

1. Thesis Title

THE DISTRIBUTION, BEHAVIOR AND THREAT OF RED-SHANKED DOUC LANGUR *Pygathrix nemaeus nemaeus* IN HIN NAMNO NATIONAL PROTECTED AREA, KHAMMOUANE PROVINCE, LAO PDR

การแพร่กระจาย พฤติกรรม และสถานภาพการคุกคามของค่างห้าสี (*Pygathrix nemaeus nemaeus*) ในพื้นที่อนุรักษ์หินน้ำโน จังหวัดคำม่วน สาธารณรัฐประชาธิปไตยประชาชนลาว

2. Introduction

Red-shanked Douc Langur *Pygathrix nemaeus nemaeus* is extremely endangered by human while lacking of scientific studies. It is an endemic to Southeast Asia, cannot be found elsewhere in the world besides Laos and Vietnam.

Red-shanked Douc Langur belongs to the group of Old World Monkeys, in family Cercopithecidae, sub-family Colobinae (leaf-eating monkeys), genus *Pygathrix* and species *Pygathix nemaeus*. Red-shanked Douc Langur is classified on the Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) which is prohibited for any purposes of trade (IUCN, 2004). It is also considered as endangered species of IUCN Red list 2004 (IUCN, 2004). The main population of the species

(Timmins and Duckworth, 1999). Primates, especially endangered species are commonly used as flagship species for wildlife conservation (Mittermeier, 1988). Therefore, the loss of the species may indicate the severe decline of forest and ecosystem status in its home range.

Historically, Red-shanked Douc Langur was reported in Laos, Vietnam, Cambodia, and Southern of China; however, it is no longer in China (Fooden and Feiler, 1988) and no confirmation in Cambodia today. In Vietnam the species was reported in central highland areas (Lippold, 1995) while in Laos it distributes from the Central, counted from Nam Chat catchement in Bolikhamxay province, to the southernmost of Attapeu province at Cambodia border (Timmins and Duckworth, 1999). At present, the largest population of Red-shanked Douc Langur in the world is in Hin Namno National Protected Area and Nakai Nam Theun National Protected Area in Khammouane province including Nam Chat provincial protected area of Bolikhamxay province (Duckworth *et al.*, 1999; Timmins and Duckworth, 1999) located in the northern Annamite Mountain Range (*Sai Phou Luang in Laos*) in an area of over 3,000 km^2.

Two other sub-species of douc langurs reported in Vietnam and Cambodia are Black-shanked Douc Langur *Pygathrix nemaeus nigripes* and Grey-shanked Douc Langur *Pygathrix nemaeus cinereus* (Lippold, 1995). The Grey-shanked Douc Langur becomes rare in Cambodia.

In Lao PDR, a lack of information on distribution and ecology of Red-shanked Douc Langur in the wild is of concern for the species conservation. Very little is known about the species in the country (Timmins and Duckworth, 1999) as no study on the species has been undertaken so far. Whereas, some studies of Red-shanked Douc Langur had been undertaken in Vietnam (Lippold, 1997, 1998; Pham *et al*, 2000) especially the diets and general distribution (Lippold and Vu, 1995; Pham, 1993a). The field surveys of Red-shanked Douc Langur were carried out in Vietnam between 1995-1998. Behavior the species in captivity were quite well documented (Kavanagh, 1978). Both Black and Grey-shanked Douc

Langurs have barely been studied in the wild as well. In 2007, a scientific study on Grey-Shanked Douc Langur is being carried out in Vietnam. With this regard, a need for having sufficient information of distribution, ecology and threats of Red-shanked Douc Langur is very important for its conservation in the future.

3. Literature review

3.1 Classification.

According to Grove (2001), the order of primate is reclassified from Traditional system to new system "Cladistic system":

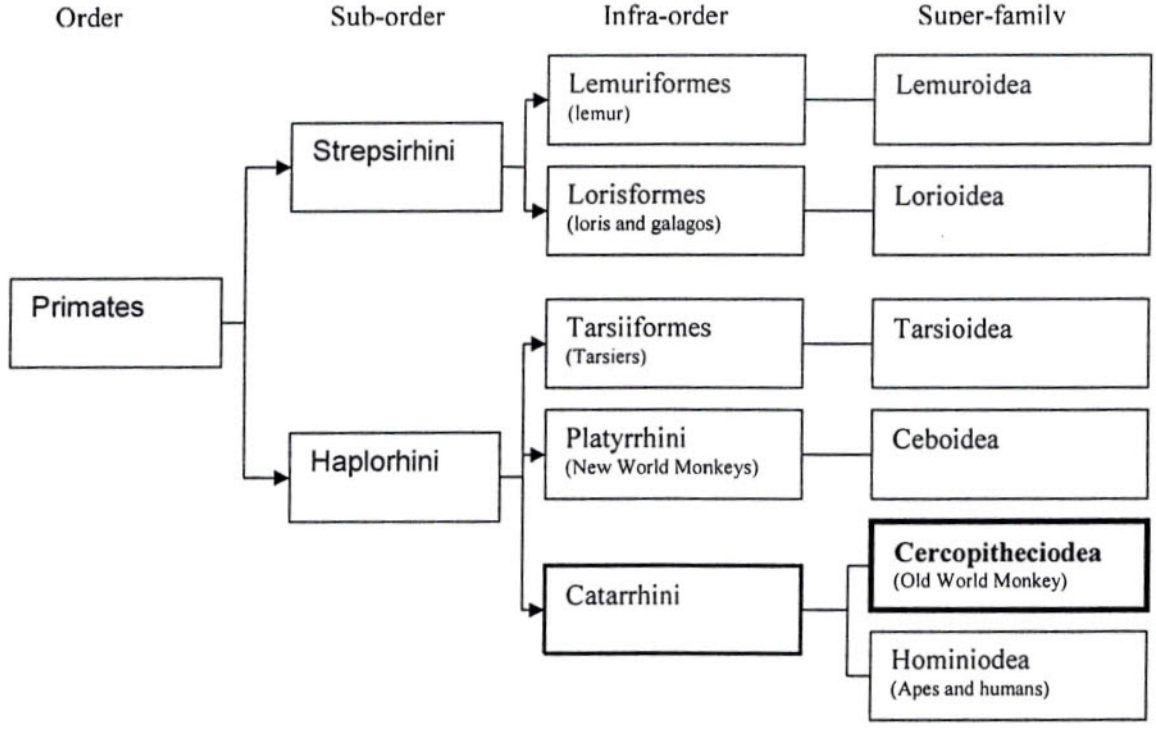

Figure 1. Cladistic system

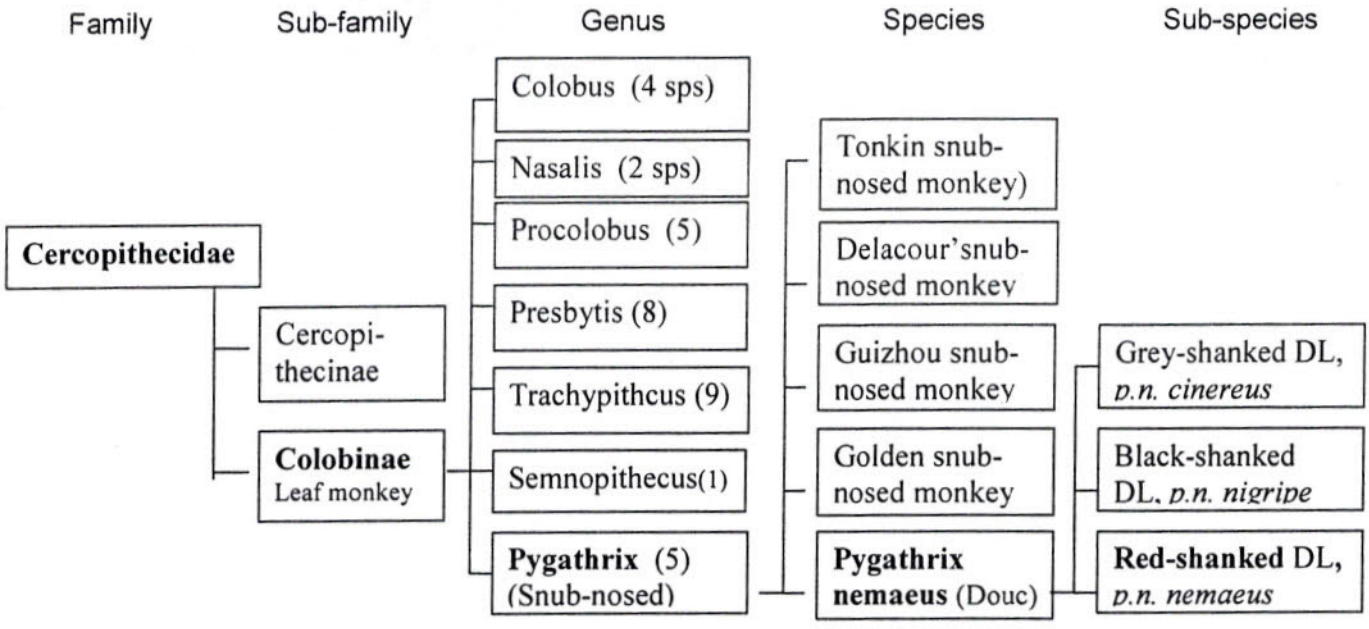

Figure 2. Classification of Cercopithecidae

Description of Red-shanked Douc Langur

Ankel-Simons (2000) and Lippold, (1998) described Red-shanked Douc Langur looks like a man with long white whisker, reddish yellow face, wearing a grey shirt, and black pants. Over the grey shirt or body part has black shoulder and white color on front upper neck down to chest. It wears deep maroon leg warmers from knees to ankles, and on its arms are elbows length white groves. Both feet and hands are black while face with white long tail.

Red-shanked Douc Langur adult weight ranges from 7-10 kg, as male slightly heavier than female (Lippold, 1977). Based on captivity observation, Red-shanked Douc Langurs are classified into three stages of life as infant (less than 1.5 year), juvenile (2 and 3 years), sub-adult and adults. They can live 25-30 years in the wild.

3.2 Distribution

Red-shanked Douc Langur in Laos and Vietnam has similar habitats. In Vietnam, the langurs were found in Bach Ma National Park (Pham, 1993b), Phong Nha Ke Bang (Pham *et al.*, 2000), Phu Mat (Lippold, 1998), Kong Cha Rang Nature Reserve and Kon Khi Kinh Nature Reserve (Lippold, 1995). These places are mainly in Vietnam's Central Highlands with altitude 500 -1000 m above sea level. In Laos, their habitats were between 14°25' and 18°25' N (Timmins and Duckworth, 1999). They can be found along the Vietnam border in the east, Cambodian border in the south, Nam Chat catchment in the north, and Mekong River in the west (Timmins and Duckworth, 1999). Recently, the species has been recorded and confirmed in 11 sites ranging from 200 m to1,600 m above sea level (Timmins and Duckworth, 1999). Nakai-Nam Theun National Protected Area including its adjacent area, Hin Namno National Protected Area and Nam Chat Provincial Protected Area, supports the largest population of the species in the world (Duckworth *et al.,* 1999; Timmins and Duckworth, 1999). The small population has also been reported in other 8 sites in Laos including Nam Kading in Bolikhamxay, Phou Xang He and Dong Phouvieng in Savannakhet, Xe Bang Nuan in Saravanh, Dong Hao Sao in Champasak, Dong Amphan and Nam Kong in Attapeu, and Phou Ahyon in Khammouane Province (Duckworth *et al.,* 1999).

Red-shanked Douc Langur is a diurnal and arboreal, leaf-eating monkey, living in a group up to 30 individuals in the past. For instance, the group sizes were found as large as 50 individuals in Kong Cha Rang Nature Reserve and Kon Khi Kinh Nature Reserve respectively (Lippold, 1995). In contrast, with high pressure of human activity, the number per group remains as low as 4-5 individuals (Lippold, 1997, 1998).

The Red-shanked Douc Langur habitats are mainly in primary forests but including secondary forest, semi evergreen hill forests, sub-montane evergreen forests, mixed deciduous forests, mixed evergreen forests, mosaics of primary evergreen forests, and

closed broad-leaved tropical forests (Lippold, 1998). These habitat types were mostly based on the surveys and identification made in Vietnam.

3.3 Behavior

Behavioral ecology of Red-shanked Douc Langur is somewhat similar to other primates. They like to stay and play within a family or group; engaged in hopping, running, jumping and climbing during first few months of infants (Kavanagh, 1978; Lippold, 1998). They are diurnal and arboreal as normally in the group of colobids spending at least 50% of the day feeding in the wild (Lippold, 1995). The species is active during the day living in the middle canopy of the trees. Usually, colobine aggression increases with group size; however, this kind of habit is rarely found among douc langurs. They do share a clump of trees for food (Lippold, 1997, 1998).

Different groups of douc langurs have considerably overlaps in habitat ranges, also the habitat overlaps with Black-shanked Douc Langur in Vietnam (Lippold, 1998). Groups move through the forest canopy along established routes (Lippold, 1998). Adult male is a leader as all group members fallowed when the adult male moves. Females and infants are often found in the center and juvenile males bringing up the rear during their locomotion (Lippold, 1998).

Group size is variable depending on habitat and human disturbance and that can range up to 50 members. Groups are mostly multi-male and multi-female with a sex ratio of 2.5 females for each male (Lippold, 1998). Females reach sexual maturity at about 4 years, while the males reach it at 4-5 years. Mating characteristics and sexual invitation start when the jaw is thrust forward and the head is shaken sideways with eyebrows are raised and lowered several times (Kavanagh, 1978). In captivity, the mean interbirth interval was 22 months and the gestation period was between 180 and 190 days (Lippold, 1989; Brockman and Lippold, 1975). Most births in captivity occurred between January and August, just before

fruiting season of some favorite foods (Lippold, 1989, 1977). During giving new birth, the female grasps the infant as it emerges and pulls it out (Brockman and Lippold, 1975), the newborns with eyes wide open, grasp hold of the female's fur, aiding in their own delivery (Brockman and Lippold, 1975), in the meanwhile females lick their newborns immediately after birth.

Similar to all primates, social grooming in the wild occurs most frequently in the afternoon before napping. During sleeping time, females sleep with their offspring, while males spend time in rough play; whereas, some females spend time grooming (Lippold, 1998). Some observation showed that females had a higher tendency to groom males in multi-male groups than in uni-male groups (Lippold, 1995).

3.4 Feeding

Pham (1993a) revealed the diet of Red-shanked Douc Langur from the stomach content analysis of dead animals as many as 50 species of food types. The main food types were leaves that still young, unripe fruits which they did not eat seeds, also buds and flowers. Eighty two per cent of intake were leaves (at least 75% are young leaves), 14% was fruits and seeds (mainly unripe but some over ripe) and 4% flowers. Red-shanked Douc Langur has a large stomachs partitioned with a number of sacks, allowing the toxic breakdown of leaf compounds (Pham, 1993a; Sterling *et al.*, 2006).

3.5 Threats

The population of douc langurs has declined remarkably since Indo-China war. They were killed by bombs and also being target for shooting practice of the army, hunting for sale and food, and also catching infants for pets (Lippold and Vu, 1995). Remaining forests are

subject to commercial logging, hunting and clearance. Hunting for bush meat and medicinal purposes is a major activity (Lippold and Vu, 1995).

In Vietnam, douc langurs are on the menu for the Viets and ethnic minority peoples. They are sold to local Viet populations for traditional medicine use. Central highlands in Vietnam support most important habitats of douc langurs; whereas, the government policy moving people from the northern is likely to increase deforestation. Moreover, forest loss in Vietnam, due to expansion of fruit tree plantations, illegal logging, and firewood collection, seriously affects the population (Lippold and Vu, 1995; Mittermeier *et al.*, 2005).

Beginning in the late 1960s, douc langurs were widely imported to the United States and European zoos; the exhibition was up from 8 to 15 zoos from 1968 to 1973 (Kavanag 1987). Currently successful colonies exist only in San Diego zoo in the US.

In Laos, Red-shanked Douc Langur is threatened due to hunting, habitat degradation, and human disturbance. Since the species is on the list of prohibited species and guns have been handed over from the last ten years– the pressures on the species have been slightly declined. In recent years, Red-shanked Douc Langur gets high pressure from Vietnamese intruders by hunting with army guns and snares, also collecting forest products along Vietnam borders. Douc langurs are often hunted in Laos for traditional purpose of tribal ethnic group "*Salang*" when giving birth.

3.6 Conservation

The recent report of IUCN 2003 states that nearly a third of all primate taxa are currently considered endangered. *Pygathrix nemaeus* is one of 8 species in the world has been reported to be critical threatened (Wolfheim, 1983; Jones, 1997). Many primates are listed as prohibited species of many nations while conservation projects have been implemented, including long-term studies of species, like the Primate Rescue Center in Vietnam. Also, some projects focus on raising awareness through developing educational

materials for different target groups, working with school children/villagers around conservation areas, and developing ecotourism project to provide income for local people.

3.7 Primate census methods.

There are many techniques designed for census sampling primates. Four most popular methods, for non-vocalized primates study, are Effective Distance or **Whitesides method** (Whitesides *et al*, 1988), Max Reliable Observer to animal Distance or **Max ROD** (Struhsaker, 1981), Max Reliable Observer to Transect animal Distance or **Max RTD** (Struhsaker, 1981), and **Transan** Computer software program (Johnson and Routledge, 1985).

Whitesides method	Group Density	$= \frac{N_t}{2(S/2+D)L_t}$ *while* $D = (FD)(N_f / N_f)$	(Whitesides *et al.*, 1988)
		$D =$ Effective distance equal to FD $S =$ Mean group spread in km $L_t =$ Sum length of all census combined $FD =$ Fall off distance or the largest number in the first 10 m interval $N_t =$ Total number of group seen $N_f =$ Number of sightings of group at distance less than the fall off distant	
Max. ROD method	Group Density	$= \frac{\text{Sum of group sightings}}{2\ (\text{length x width of 1 side of transect in })}$	(Struhsaker, 1981)
Max RTD method	Group Density	$= \frac{\text{Sum of group sightings}}{2\ (\text{length x width of 1 side of transect in })}$	(Struhsaker, 1981)
TRANSAN	Group Density is computed by a PC program using a non-parametric, shape restricted estimator		(Johnson and Routledge, 1985)

Within these, Max ROD and Max RTD are very similar, perhaps recognized as only **Max** ROD. The difference is only the Max ROD based on distance from an observer or transect to an animals while Max RTD based distance from line to an animals.

Fashing and Cords (2000) tested these methods of identifying diurnal primate density in the Kakamega Forest in Kenya. They concluded that Whitesides method had more accuracy of estimating the density of primates comparing to known densities calculated with long-term data on home range size and overlap. Nijman and Menken (2005) also had the same conclusion. However, this method may not be appropriate for studying the census of species with low density or rare species and inaccessible location.

Occupancy and encounter rates are also used for census study of primates where a straight line is unable to make; however, these two methods can not estimate species density. For limestone karst, there is no suitable method to study a density of species accurately since it is unable to make transects in a straight line, only rough estimate or occupancy of species can be made in limestone area using either general estimate per km walk or an Occupancy method of MacKenzie *et al* (2002).

4. Research objectives

4.1 To investigate the distribution of Red-shanked Douc Langur in Hin Namno National Protected Area, Khammouane province, Lao PDR.

4.2 To learn the behavioral activities of Red-shanked Douc Langur in the wild.

4.3 To identify the main threats to the population of Red-shanked Douc Langur and their magnitude in the study area.

5. Research hypotheses

5.1 The distribution of Red-shanked Douc Langur is specific to limestone mountain.

5.2 The behavioral activities of Red-shanked Douc Langur is different depending on seasons.

5.3 More frequent encounter of Red-shanked Douc Langur is in wet season.

5.4 The threat of Red-shanked Douc Langur is hunting but the level is low.

6. Scope and limitation of the study

6.1 **Distribution:** the study area is in Hin Namno National Protected Area with an area of 865 km^2. Six main camps are located in Nam Khoum, Nam Masay, Kuane Nong, Nong Boun and Kuanethoun. In order to cover wider areas, seven camps are added in for the total of 13 study camps. At least two line transect per camp site are walked twice a year in wet and dry seasons. Only rough estimate of species density will be made.

6.2 **Behavior study:** two groups of 15 individuals are selected. Group one (G1) is observed monthly through the year, while G2 is observed at least 6 months per year. The observation schedule is 5 to 10 days per month. Behavior activities in different seasons, in terms of opportunity for detection, food types, and attitude comparison and response to predators will be recorded.

6.3 **Identifying threats:** through the same transect walks, evidences of hunting such as man with a gun or cross bow, gunshot, human disturbance (camps or camp fire), habitat loss (log/felling trees) will be recorded.

7. Research methodology

7.1 Study area

The study area is located in Hin Namno National Protected Area, which joins the international borders with Phong Nha Ke Bang Natural World Heritage site in Vietnam. It is situated at latitude 17°15'-17°40' N and longitude 105°43' - 106°09' E, about 174 km from Khammouane province to the east or 300 km from Vientiane. Hin Namno has altitude ranged from 200 m to 1,000 m above sea level. A total area of 869 km^2, it borders between Lao and Vietnam and being parts of northern Annamite Mountain Range lied entirely within the Boualapha district of Khammouane province National Protected Area. The area is just next to Nakai Nam Theun NPA.

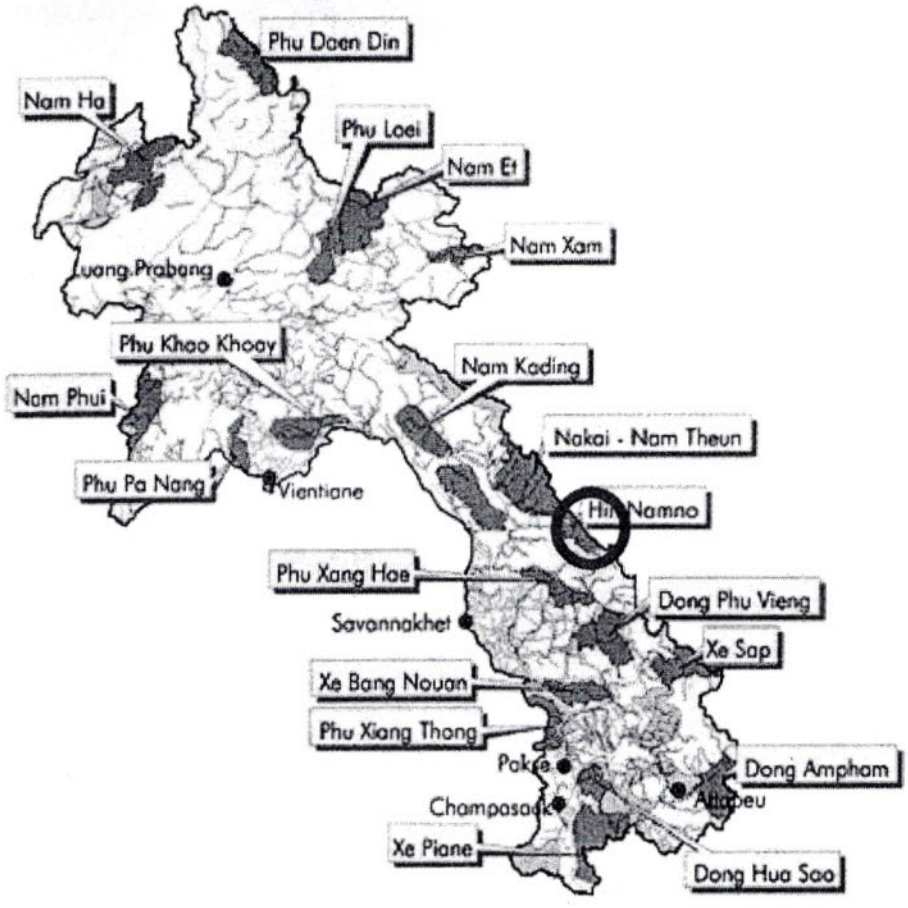

Figure 3. Hin Namno National Protected Area

7.1.1 Geography

The Hin Namno NPA is mainly dominated with steep slope mountains and limestone karst with outstanding scenic values. The area has one main river, Xe Bang Fai, lying outside the area, which form the boundary along a 18 km stretch, of which the river runs underground for 6 km. There is one permanent stream, Houy Talee, located in the northwest and some few seasonal streams lie in the area. The seasonal streams are Nam Huck, Houy Pakha and Houysan as these streams mainly drain into Xe Bangfai River. Some small lowland and riparian areas are found in core areas, called Nam Khoum and Nam Masai zone. The altitude range of 500 -1000 m above sea level covers 45% of the area and 200 m– 500 m is 50% (Berkmuller *et al.*, 1995).

7.1.2. Climate

There are two main distinct seasons (rainy and dry seasons). The rainy season lasts from May to November and the dry season from December to April with some rain and cool at night (Robichaud, 2005). There is influence of monsoon sweeps up from the Tonkin Gulf of Vietnam and cross the Annamite Range between September and November. It sometimes bring cold dry air (Walston and Vinton, 1999). In Annamite Mountain Range, an average of rainfall is 2,500 - 3,000 mm/year, particularly in Nakai Nam Thuen (NTPC, 2005 in Robichaud, 2005) but it is probably lesser in Hin Namno. High wind speeds across Vietnamese border at the northern part of Hin Namno NPA from December to March. The temperature in the area ranges from 15 to 43 ^{O}C.

7.1.3 Fauna and flora

Hin Namno NPA lies in two biogeographic zones, which are pockets of localized species endemism: the central Indochinese limestone and the Annamite Mountain region (Timmins and Khounboline, 1996).

Habitat: majority of the area is limestone karst with a range of vegetation cover from bare rock to shrubby forest, flat lowlands broken by sporadic karst outcrops. Among the limestone mountains, especially the central and some northern parts of the area, are covered with tall closed canopy evergreen forest, semi-evergreen forest, mixed deciduous forests; nonetheless, patches of secondary and degraded forest are found in the area (Timmins and Khounboline, 1996). Because some tribal inhabitants (*Salang*) used to live and cultivated inside the area for last 25 years, some scatter areas are covered with old fallow of 20-30 years old. In addition, the flat lowland areas are found in core area such as Nam Khoum, Nam Masay, and riparian habitats found along the Xe Bangfai River.

Fauna: Hin Namno NPA provides suitable home for various animals especially birds. Besides primates, there are Large Antlered Munjact, Crested Argus, Large Cats, Black Giant

Squirrels, and four species of hornbills (Timmins and Khounboline, 1996). There are 18 primate species known in Laos (Ruggeri and Timmins, 1996), about 9 of them were found in Hin Namno NPA such as "White/Yellow-checked Gibbons" *Hylobates leucogenys*, Red-shanked Douc Langur *Pygathrix n. nemaeus*, two forms of Francios' Langurs *Trachypithecus francios and Trachypithecus laotumhatinhensis*, Assamese Macaque *Macaca assamensis*, Stump-tailed Macaques *Macaca arctoides*, Rhesus Macaque *Macaca mulatta*, two species of Loris: Pygmy Loris *Nyticebus pygmaeus* and Slow Loris *Nyticebus Coucang*, and Phayre's Langurs *Trachypithecus phayri* (Timmins and Khounboline, 1996).

Flora: Little is known by scientists about flora in the area. So far, Walston and Vinton (1999) revealed 536 species of plants in the area including ground vegetation; however, the quadrats were mainly undertaken in adjacent areas. Walston and Vinton also classified habitat types of Hin Namno NPA into seven different forest types such as evergreen, mixed deciduous, deciduous, secondary, cultivated, bamboo, and limestone forest. The dominant tree species were in Agavaceae, Arecaceae and Poaceae families.

7.1.4 Threats to the biodiversity in the area

The major threat to wildlife population in the study area are hunting and some disturbances of Vietnamese intruders. Habitat clearance for cultivation were reported in the past; while recent problems are non-timber forest products (NTFPs) extraction, select certain value trees for timber chopping, and langurs hunting with bamboo traps and snares by local villagers especially *Salang* people. These threats may lead to severe reduction in population of many key species in the long run (Timmins and Khounboline, 1996).

7.1.5 Conservation status

Wildlife inventories were conducted in Hin Namno NPA in 1995 and 1996 by the cooperation of Wildlife Conservation Society and the Department of Forestry/Ministry of

Agriculture and Forestry (Timmins and Khounboline, 1996). As well, World Wildlife Fund for Nature (WWF) did complete survey in 1999. WWF Lao Program had introduced some management intervention, including land use planning activities to all villages in the area. So far, series of dialogue for cooperation, the Agreement and Management Framework with Vietnam for Transboundary conservation has been made; however, the implementation is still in a slow track. Right now, the dialogue on establishing International Transboundary Natural World Heritage Site in order of joining Hin Namno and Phong Nha Ke Bang is going on. Lastly, IUCN with funding from UNESCO are working with the government to designate Hin Namno NPA as a Natural World Heritage Site in 2007.

7.1.6 Local community

Approximately 7,240 inhabitants of 22 villages are living adjacent to Hin Namno NPA. They comprise of four main ethno-linguistic groupings such as Vietic, Brou, Tai-Kadai, and Hmong (Timmins and Khounboline, 1996). The livelihoods of them rely on the products from forests and shifting cultivation.

7.2 Study site selection

Six main study camps are selected for the Red-shanked Douc Langur distribution during the preliminary study. The names of the first six study camps are as below:

- Camp 1. Kuane Nong (2 lines, 4.5 km) 4.5 hours walk from Ban Dou.
- Camp 2. Nongboun (3 lines, 6.3 km) 3 hours walk from Ban Dou.
- Camp 3. Kuane Talee (2 line, 3.5 km) 2 hours walk from Ban Dou.
- Camp 4. Nam Khoum (3 lines, 7.2 km) 10 hours walk from Ban Dou.
- Camp 5. Nam Masay (3 lines, 5.7 Km) 14 hours walk from Ban Dou.
- Camp 6. Kuane Thoun (2 lines, 3.5 km) 2 hours walk from Ban Dou.

Seven other sites will be added to wider area in Hin Namno NPA. The proposed names of these study camps are as below, which may be subject to change:

- Camp 7. Kuaneke
- Camp 8. Nongluang
- Camp 9. Nongping
- Camp 10. Phathoung
- Camp 11. Chala
- Camp 12 Khoun-anh
- Camp 13. Nongkapoud

Description of the main study sites

Kuane Nong, Camp 1, is located close to the Vietnamese border on the east with the altitude 450 m on average. The areas are covered with limestone mountains, limestone karst, and land mountains, where evergreen, semi-evergreen, and hill evergreen forest occur in scatter providing suitable habitats for douc langurs especially for feeding. Some bamboo forests and secondary forests are found sporadically and shrubby forests or limestone forests are found widely on upper limestone mountains.

Nongboun, Camp 2, is on the way to Kuange Nong and this area is dominated with similar habitats as Camp 1 but here with some mixed deciduous forests.

Kuane Talee, Camp 3, is located in the northern part of the area and onwards to Vietnamese border. The areas are covered with evergreen or hill evergreen forest, with majority of secondary and bamboo forests. Mixed deciduous forest is also found including shrubby forests or limestone forests on upper limestone mountains.. The altitude range is between 325-550 m above sea level.

Nam Khoum, Camp 4, and **Nam Masai** Camp 5 are located in the core area, belonging to Ban Dou. The areas are covered with evergreen forest, surrounded by precipitous karst and some semi-evergreen forests. However, secondary forest and old

fallows with age of 40 years olds are also found in this site, because entirely lowland areas of this site used to be an old settlement of Ban Na.

Nam Masai, Camp 5, is on the way to Houy Kalor from Camp 4. It has quite flat lowland areas covered with evergreen forests with the altitude 400-550 m above sea level especially in Houy Kalor (Timmins and Khounboline, 1996). Secondary forest covers entirely lowland areas because some parts of this site used to be an old settlement of Ban Na.

Nong Boun, Camp 6, is located in western part, covered with evergreen, hill evergreen, mixed deciduous, and bamboo forests.

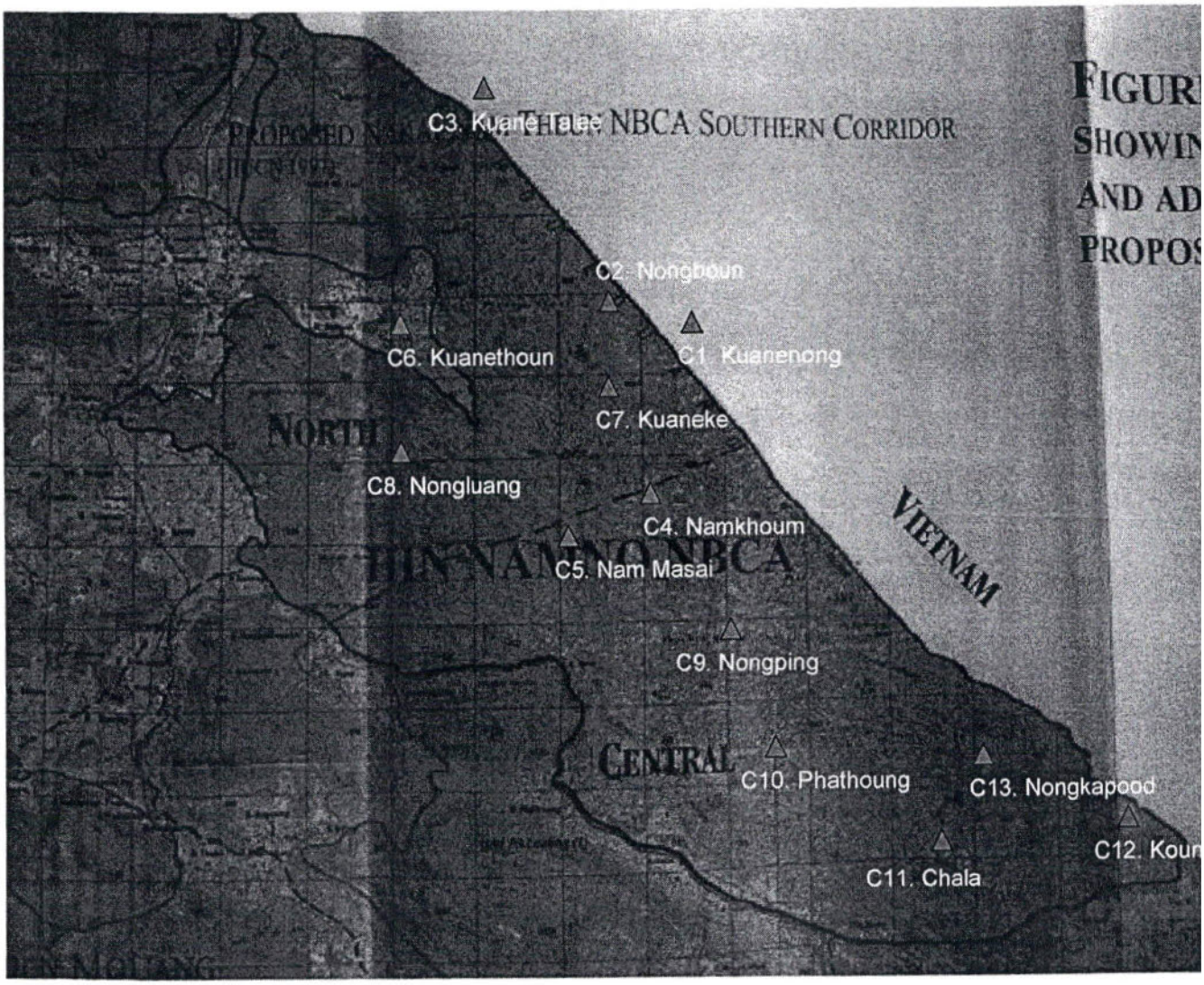

Figure 4. Location of the study plots

7.3 Project design and data collection

Sampling for distribution, behavior, and threats are designed in details as followed.

7.3.1 Distribution study

Thirteen study sites will be sellected randomly in the study area. Six of them were visited during the preliminary research but seven other sites will be added. Each study site has at least 2 or 3 line transects of 100 m x 2 km or 3 km. At least 28 transects will be used from 13 study sites (camps). The line will be walked twice a year during wet and dry seasons. Since the study sites are difficult to access and hard to get all a straight line due to limestone dominance - neither Whitesides method nor Struhsaker are appropriate for the study. Therefore, rough density estimate will be made using general calculation for per km walked per visiting block or Occupancy method (MacKenzie *et al*, 2002) will be applied.

$$E(C) = pN \text{ while } N = C/p$$

N = Abundance,
C = Count statistic
P = Detection probability; P(member of N appear in C)

When detection is 100% (p=1), the count statistic provides an accurate estimate of N. However, if p<1, the count statistic provides a biased estimate of N. N and C is in function of relationship, when N increase, C increase and *vice versa*.

All line transects will be marked every 50 m on a tree. Each line will be designed to different direction and to cross in different habitats, started from a camp with a distance of at least a half-km. Both a straight line and curve line are accepted. The transect walk will be conducting only in the morning from 7.30 AM to 11.30 AM, and in good weather with walking speed at 0.7-1 km/hour. During the walk, all necessary data will be recorded, including time of animal encounters, species, number of the species, and location (GPS and habitat types). Also, if possible, there is a need to know the activity of langurs, what kind of leaves is being

eaten. Rangefinder, compass, and GPS will be used to locate the animal and the data recording will be completed within 10 minutes.

7.3.2 Behavior study

G1 of camp 5 will be observed every month through the year, while G2 of camp 4 will be observed at least 6 months consecutively per year. Six different age classes of the animals such as adult male, adult female, sub-adult, juvenile, and infants (1&2) will be classified. The infant 1 is the animal with an age of less than 6 months and Infant 2 is less than 1.5 year. The observation takes 3 to 4 hours per day, at least 5 days a month. Daily observation will be started from dawn to dusk, if animals are still in contact. The scan sampling technique will be last within 10 minutes, with 30 minute interval. The focus of the observation is to record a group's daily activity: time use for feeding, moving, and socializing (see Form B).

7.3.3 Identifying threats

During the transect walks, evidences of hunting such as man with a gun or cross bow as well as camps or camp fire will be recorded as hunters. For human disturbance (including people, camp fire, and snare lines), and habitat loss will be recorded when finding logs/logging and felling trees.

7.3.4 Forest structure and climate

Six plant plots will be studied (Form C) in order to gain habitat structure and tree dominance in the area. Climatic information will be collected by using a format form (Form D). A barometer for min/max temperature recording and a rain gauge 90 mm for rainfall will be

installed at Ban Vangmaner. One villager will be hired for recording the data throughout the year. Temperature will be checked every day in the morning at 7 to record min and max, then to reset it. The rain gauge will be located in open area nearby and the data will be recorded every day; however, if heavy rain, checking more often is needed.

7.4 Materials and equipment

Topography map (1:50,000), Global Positioning System (GPS), binocular 3 units (10x50 1 unit and 7x18 2 units), rangefinder, compass, regular camera, digital camera, films, and batteries, tent, sleeping bags. Torch lights, candles, lighters, alarm clock, plastic bags, tape (50m and 5m), ruler (30 cm), line transect markers, strings, barometer (Min/Max), newspaper for plant specimen collection, plastic bags, rubber bands. Data record forms, plastic sheet, notebooks, waterproof pens, medicine etc.

7.5 Data analysis

Firstly, all data will be entered in an excel spreadsheet. Then, the data for census study will be analyzed and estimated roughly per km walked or using Occupancy method (MacKenzie *et al*, 2002) with software available. The behavior observation data will be analyzed using appropriate data analysis tool. T-test will be used, by SPSS program for Window, for comparing behavior differences of Red-shanked douc langur in two different seasons, based on time using for each activity of interest.

8. Expected results

8.1 Will gain knowledge of Red-shanked Douc Langur distribution.

8.2 Understanding behavior of Red-shanked Douc Langur in the wild.

8.3 Have information of their behavior in different seasons.

8.4 Perceiving threats to the population of Red-shanked Douc Langur.

8.5 Provide suggestions to conserve viable population of Red-shanked Douc Langur in the area.

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10. Research Plan

	Activities	2006			2007			2008			2009		
		1	2	3	1	2	3	1	2	3	1	2	3
1	Thesis proposal preparation	←	—	→									
2	Prelim				←	—	→						
3	Line transect					←	—	—	→				
4	Group observation					←	—	—	→				
5	Data entry/analysis								↔				
6	Writing thesis								←	—	—	→	
7	Thesis submission											←	→

Advisor's signature..... P. Suwan..... Student's signature Phaivanh Phiapalath

Dr. Pongthep Suwanwaree

2/7/07

Phaivanh Phiapalath

2/7/07

Appendices

1. Form A. Line transect
2. Form B. Group observation (focus on female relationship)
3. Form C. Climate data collection (temperature and rainfall)
4. Form D. Climate Data collection
5. Abbreviation and Category of activity budget

Form A: **Line Transect**

Investigator: Date: Name of the site Site no. GPS Weather

Total time contact Total time out Total time walk Walking speed

Time	Species/ threats	No seen	AM	AF/ sub-adult	J	I 2/ I 1	No. esti	Total esti	Total time contac	Distance to the animal/to the group	Compa Bearing	Activities (what do they do) **I**nactive, **F**eeding, **G**lomming, **R**esting, **P**laying, **S**leeping...	Feeding type, part, height	Habitat	Location GPS/ alt

Form B: **Group Observation**

Investigator: Date: Name of the site Site no. GPS Weather

Group description: Group Identity:
Member and sex:
No of infant (1&2) and sex:

Time	**Activities** (what do they do at that time and what details), **I**nactive, **F**oraging, **F**eeding, **S**ocial, **T**raveling, **O**ther (to specify)	Habitats/ Height/canopy	GPS/ Altitude Other	Weather S, C, R, T
30'	1. Adult female 2. Adult male			
	3. Sub-adults 4. Juveniles			
	5. Infant 2 6. Infant 1			
30'	1. Adult female 2. Adult male			
	3. Sub-adults 4. Juveniles			
	5. Infant 2 6. Infant 1			
30'	1. Adult female 2. Adult male			
	3. Sub-adults 4. Juveniles			
	5. Infant 2 6. Infant 1			
30'	1. Adult female 2. Adult male			
	3. Sub-adults 4. Juveniles			
	5. Infant 2 6. Infant 1			

Note: use this record form alongside the category of activity budget sheet to record the target animals every 30' interval for all activities (AF, AM, Sub-adult, Juveniles, Infant 2 and Infant 1) and each time to be complete within 10 minutes. Be careful not to record the same animals every time. Need to record a position (height) of which the animals are being described. Besides the recording time, there is also a need to describe some interesting activities of especially the adult female.

Form C: **Tree Specimen Collection**

Plot no. Area: Habitat/Slope GPS Date:

Plot description Plot structure/canopy

Specimen number	Local name) (the sp GBH>10 cm)	Scientific name	GBH	Height	Month: flower/fruit/fallen leaves etc	Douc use	(what part of trees is used by), others

Form D. Climate data collection (temperature and rainfall)

Month:______ Year:_________

Date	Temperature		Rainfall
	Min	Max	
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			

Month:_____ Year:_______

Date	Temperature		Rainfall
	Min	Max	
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			

Month:____ Year:______

Date	Temperature		Rainfall
	Min	Max	
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
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27			
28			
29			
30			
31			

Note: temperature of min and max to be recorded every day at the same time in the morning at 7, then reset it. Rainfall to be recorded every days and more often during the rainy season to ensure it will not overflow.

5. Abbreviation and Categories of activity budget

ia	**inactive**		**fo**	**foraging (searching and handling)**	
	no	normal	fl, fr, le, pi, ba, sh, dr, so, fi, am	flower, fruit, leaf, pith, bark, shoot, drink, soil, figs, animal matter	
	mo	monitoring with eyes open	**part**	**apply to fruit, flower, leaf**	
	mo?	eye open but unclear monitoring	wh, pu se. ps, ex, p, lb, bl	whole, pulp, seed, pulp and seed, exocarp, petiole, leaf base, blade	
	?	attention unknown	**age part**	**apply to fruit, flower, leaf**	
			bu, yo, ma, ol	bud, young, mature, old	
fe	**feeding (ingesting, chewing)**		____	underscore for unknown	
	fl, fr, le, pi, ba, sh, dr, so, fi, am	flower, fruit, leaf, pith, bark, shoot, drink, soil, figs, animal matter			
	Part	**apply to fruit, flower, leaf**	**ot**	**other (specify)**	
	wh, pp, se, pp+se, ex, p, lb, bl	whole, pulp, seed, pulp and seed, exocarp, petiole, leaf base, blade		sp	solitary play
	Age part	**apply to fruit, flower, leaf**		ag	autogroomming/ scratching
	bu, yo, ma, ol	bud, young, mature, old		ago	agonistic behavior (aggression)
	____	underscore, unknown		sex	sexual behavior
so	**Social (specify behavior and recipient**				
	gr	grooming			
	e	embracing		other	
	rp	rough play		A	Adult
	cp	Chase play		AM/AF	Adult Male/Adult Female
				Sub	Sub-Adult
In	**Inant**, body contact, height			SAM/SAF	Sub-Adult Male/Female
ca	Carry a baby			JM/JF	Juvenile Male/Female
nc	Nipple contact			I2	Infant 2 (6 months to 2 years)
bc	body contact (excluding trail)			I1	Infant 1 in orange color up to 6 month
bg	note if a monkey grooms the one you just take the activity for				
h	height from the ground to all the target animals (m)				
Od	**Oddity**				
	tr,br,c,h	travel, brachiation, call, height			

(Modified from Koenig, A. and Borries, C. 2007. Primate Ecology and Conservation, General teaching manual for workshop at Pu Kieo Wildlife Sanctuary)