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PROMOTION OF ADVENTITIOUS ROOT GROWTH BY COFFEE EXTRACTS

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ผลการทดสอบฤทธิ์ควบคุมการเจริญเติบโตของสิ่งสกัดเอทานอลจากเมล็ดกาแฟคั่วเบื้องต้นพบว่า ที่ความเข้มข้นสูงมีฤทธิ์ในการยับยั้งการเจริญเติบโตของเมล็ดพืช ขณะที่ความเข้มข้นต่ำมีฤทธิ์ในการส่งเสริมการเจริญเติบโตของเมล็ดพืช นอกจากนั้นมีฤทธิ์ในการส่งเสริมการเจริญเติบโตของฤาษีผสม *C. atropurpureus* สูงที่สุด (55%) ที่ความเข้มข้น 100 ppm ของสิ่งสกัดจากเมล็ดกาแฟ ส่วนของชบา *H. rosa-sinensis* พบที่ความเข้มข้น 1000 ppm (84%) ได้ศึกษาองค์ประกอบทางเคมีและใช้ฤทธิ์ทางชีวภาพติดตามการแยกสิ่งสกัดเอทานอล สามารถแยกสารได้ 2 ตัว องค์ประกอบส่วนใหญ่คือ คาเฟอีน ส่วนองค์ประกอบรองเป็นของผสมไม่สามารถจำแนกโครงสร้างได้ คาเฟอีนแสดงการส่งเสริมการเจริญเติบโตของฤาษีผสมสูงที่สุดที่ 10 ppm (61%) และของชบาสูงสุดที่ 100 ppm (82%) ส่วนฤทธิในการควบคุมการเจริญเติบโตของเมล็ดพืชของคาเฟอีนเหมือนกับสิ่งสกัดเอทานอล คาเฟอีน (61%) ส่งเสริมการส่งเสริมการเจริญเติบโตของรากและกระตุ้นการเกิดรากของกิ่งปักชำมากกว่าสารสกัดต่างๆที่ได้จากเมล็ดกาแฟคั่ว เช่น chlorogenic acid (5%), theobromine (39%), theophylline (37%) และ trigonelline (27%) จากการทดสอบสรุปได้ว่า สารที่มีฤทธิ์ในควบคุมการเจริญเติบโตที่สกัดได้จากกาแฟคือ คาเฟอีน

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

ปีการศึกษา.....2548.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

4572290023 : MAJOR BIOTECHNOLOGY
KEY WORD: CAFFEINE / PLANT GROWTH REGULATION / STEM CUTTINGS
NATTAKORN PUENGYEN: PROMOTION OF ADVENTITIOUS ROOT GROWTH BY COFFEE EXTRACTS. THESIS ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D. 59 pp. ISBN 974-14-3366-2.

The preliminary screening results on plant growth regulation of the ethanolic extract of roasted coffee indicated that at high concentration the growth inhibition of plant seeds were observed, while at low concentration the growth promotion clearly visualized. In addition, the highest growth promotion of *C. atropurpureus* (55%) stimulated by the extract from roasted coffee at 100 ppm was observed while that of *H. rosa-sinensis* was achieved at 1000 ppm (84%). The bioassay–guided fractionation of the ethanolic extract led to the isolation of 2 substances. Their structures were characterized by means of physical properties and spectroscopic data. The major compound was caffeine while the minor one was a mixture whose structure could not be identified. Caffeine showed the highest plant growth activity on *C. atropurpureus* at 100 ppm (61%) and *H. rosa-sinensis* at 10 ppm (81%). Plant growth regulation of plant seeds of caffeine exhibited the same the ethanolic extract. Caffeine (61%) enhanced the root growth promotions and stimulated the rooting of stem cuttings more than other compounds in roasted coffee such as chlorogenic acid (5%), theobromine (39%), theophylline (37%) and trigonelline (27%). It may be concluded that the main plant growth regulator of roasted coffee was caffeine.

Field of study.....Biotechnology..... Student's signature.....
Academic year.....2005..... .....Advisor's signature…….....

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LIST OF ABBREVIATIONS

^{o}C	degree Celsius
CH_2Cl_2	dichloromethane
$CDCl_3$	deuterate chloroform
cm	centimeter
^{13}C-NMR	carbon-13 nuclear magnetic resonance
DMSO-d_6	deuterate dimethylsulfoxide
EtOAc	ethyl acetate
g	gram
^{1}H-NMR	proton nuclear magnetic resonance
HPLC	high performance liquid chromatography
MeOH	methanol
mg	milligram
mL	milliliter
ppm	part per million
TLC	thin-layer chromatography
UV	ultraviolet
PGR	plant growth regulation
PGRs	plant growth regulators
EtOH	ethanol
H_2SO_4	sulfuric acid

CHAPTER I

INTRODUCTION

Thailand is an agricultural country with approximately 513,000 square kilometers of land of which 40.9 percent is cultivable. Since 1997 Thailand has been dealing with critical economic problems; nevertheless, the serious crisis could be allevitated by using self-dependent strategy such as improvements of crop production as rice, corn, mung beans, sugar cane, tapioca, maize and rubber. The utilization of chemicals to increase the yield of agricultural products is of utmost importance to support the economy of Thailand. As a result, between 1994 -1998, 38.6% of the imported chemicals (pesticides) were for agricultural sectors [1]. These agrochemicals included herbicides, insecticides, fungicides, nematocides and chemicals that could modify or control one or more specific physiological processes within a plant, plant growth regulator (PGR). Table 1.1 shows a total amount and value of imported PGR for Thailand [2]. It was found that a high tendency has been observed since 1999-2001.

Table 1.1 The total amount and value of imported PGRs for Thailand.

Year	Plant growth regulators PGRs	
	Quantity (kg.)	Value (baht)
1999	876,524	132.,206,283
2000	1,162,165	114,338,079
2001	1,460,108	170,354,338

PGRs are classified as pesticides, although their functions are very different. Pesticides essentially protect crops and prevent them from blight caused by diseases, insects and other factors. PGRs are applied on crops to increase yield and improve quality, thereby meeting commercial demand and quality standards. They also regulate the dormancy state of seed and buds, control the ratio of female flowers,

increase the ability of plant to withstand cold, dry condition, and promote blossoming. Other functions of plant growth regulators are to prolong the time of fruit stays on plants, make the plant shorter and stronger, prevent the plant from falling to the ground, prolong storage duration, and ease harvesting. In short, plant growth regulators are used to control and enhanced specific chemical processes in plant to meet the demands of human consumption.

1.1 PGRs and plant hormones

1.1.1 Plant hormones:

Plant hormones are organic chemicals produced by plants regulating the growth processes. Plant hormones are translocated within plant and are active at very low concentrations [3]. Plant hormones control physiological processes within the plant associated with growth and development. Plant hormones are synthesized in one part of the plant and translocated to another where activity occurs. Plant hormones have no nutritional role within the plant itself.

1.1.2 PGRs [4]

PGRs possess similar characteristic as plant hormones (controlling plant growth, development and growth promotion). PGRs are chemicals applied by a horticulturist to regulate plant growth. However, the difference between PGRs and plant hormones is PGRs are no made within the plant and do not meet all of the criteria of plant hormones listed above. Many PGRs are synthetic chemicals and include herbicides, defoliants, rooting compounds, and compounds used in tissue culture.

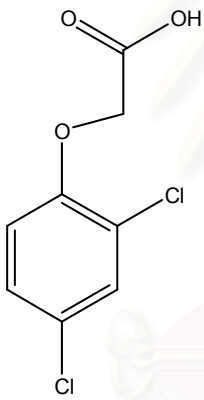
Plant hormones and PGRs used in plant propagation. In plant propagation, PGRs are most important because they are effective in parts per million or part per billion.

Plant hormones and PGRs are separate classifications of compounds involved in regulation of plant growth and development. Hormones and PGRs are broadly grouped as auxins, cytokinins, gibberellins, ethylene, and abscisic acid. PGRs either mimic or interfere with the function of plant hormones. Definitions and classifications are discussed below.

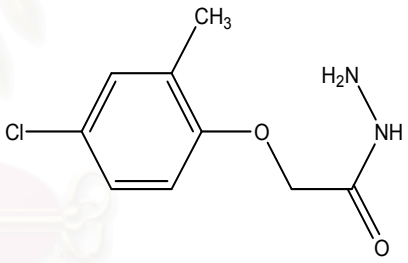
1.1.3 Categories of plant hormones and PGRs [5, 6]

a) Auxins

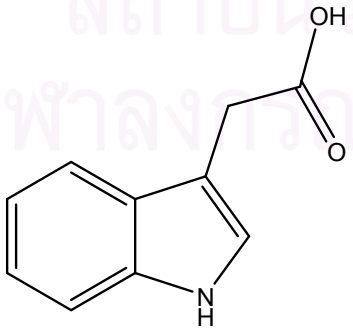
As a result of the role they exhibit in the process of phototropism, auxins were the first plant hormones discovered in the late 1800's by Charles Darwin and Ciesielski [7]. There are several different categories of auxins, and each category is composed of different structures. The primary, naturally occurring form of auxin (endogenous) is indole-3-acetic acid (IAA). However, several synthetic compounds exhibit auxin-like properties: indole-3-butyric acid (IBA), ά-naphthaleneacetic acid (NAA), 2- methyl-4-chlorophenoxyacetic acid (MCPA) and 2, 4-dichlorophenoxyacetic acid (2,4-D). 2,4-D is used as a herbicide in high concentrations, but in low concentrations may be utilized in plant propagation.



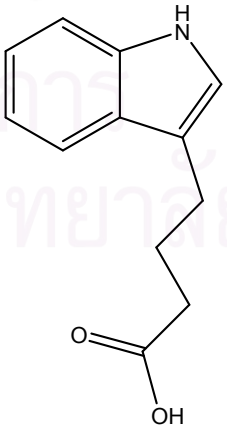
2, 4-dichlorophenoxyacetic acid



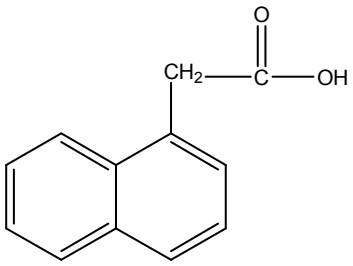
2- methyl-4-chlorophenoxyacetic acid



Indole-3-acetic acid



Indole-3-butyric acid



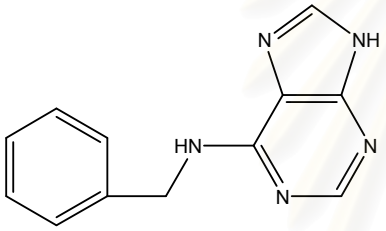
α-naphthaleneacetic acid

Function of auxins: auxin promotes elongation and cell enlargement. Auxins are involved in tropic responses. Auxins migrate away from light, which accounts for the uneven elongation of cells on the shaded side of a plant unevenly exposed to light. Auxin stimulates the production of secondary growth by simulating cambium cells to divide and secondary xylem to differentiate. Auxin produced in apical buds tends to inhibit the activation of buds lower on the stems. This is known as apical dominance. This effect lessens with distance from the shoot tip. Cytokinins counter the apical dominance effect of auxins and promote lateral bud development. Auxin promotes lateral, adventitious root development and flower initiation. Auxin promotes other hormone production, especially ethylene when auxin concentration increases. When loss of auxin make initiates leaf abscission. Fruit development requires auxin produced by the developing seed. Auxin pastes applied to developing ovaries can promote parthenocarpy (fruit development in the absence of viable seeds). Parthenocarpy is also induced by abscisic acid. Auxins are toxic in large concentrations.

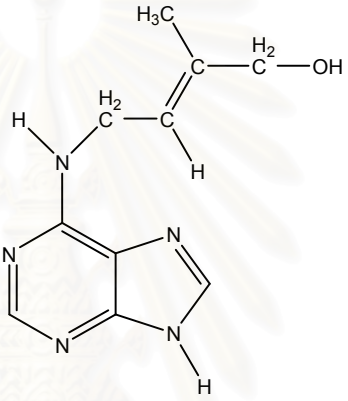
b) Cytokinins

Cytokinins are a group of phenyl urea derivatives of adenine, one of the molecules in DNA. The first cytokinin was chemically isolated in 1913 and cytokinins were studied using coconut endosperm for a number of years starting in the 1940's by Folke Skoog [8]. This isolate was shown to be a potent growth promoter and was used in tissue culture and embryo development studies. Skoog spent several years attempting to isolate this growth substance. They finally succeeded in isolating a breakdown product of DNA that promoted cell division. They named the substance kinetin and the related group of growth regulators, cytokinins, because they were involved in cell division. Kinetin was thus the first cytokinin discovered as a plant

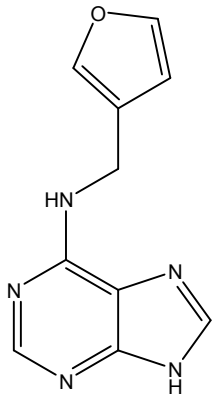
growth regulator. Later examination of plants produced the discovery of the naturally occurring cytokinin, zeatin (discovered in corn). Isopentenyladenine (2 IP) is another naturally occurring cytokinin which has been isolated in plants. Synthetic forms of cytokinins (PGRs) include benzyl adenine (BP or BAP) along with kinetin. Cytokinins are found in actively dividing tissues of seeds, fruits, leaves and root tips, and wound tissue sites. However, localized cytokinins are needed to release buds from dormancy.



Benzyl adenine



Zeatin



Kinetin

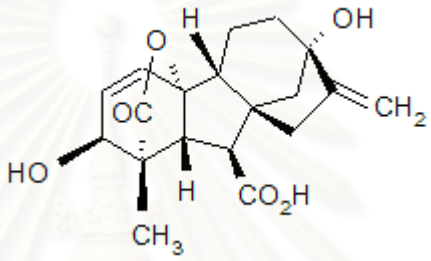
Function of cytokinins: cytokinins are most known for their use in tissue culture (promoting cell division) where a very strong relationship exists between auxins and cytokinins. High concentrations of auxins used in conjunction with low concentrations of cytokinins promote rooting in tissue culture. Conversely, low auxin concentrations combined with high cytokinin concentrations promote shoot development. In equal parts, auxin and cytokinin generally promote the production of callus tissue (undifferentiated cell growth).

In terms of plant propagation, auxins and cytokinins are the two most important hormones and PGRs. Auxins are used in traditional propagation and cytokinins and auxins in combination are used in tissue culture.

c) Gibberellins

Gibberellins were discovered prior to the 1940's by Kurosawa, a researcher studying on rice. He addressed that a fungus was responsible for abnormal rice seedling growth, called the "foolish seedling" disease. The fungus secreted a chemical

that caused the rice plants to grow abnormally long, and then collapse from weakness. The fungus was *Gibberella fujikuroi*, hence the hormone name. Naturally occurring Gibberellins in plants were later discovered and are classified as gibberellic acids (GA_3). Gibberellic acids are a diverse group of compounds with over 100 forms known to exist in plants. Gibberellins are produced in roots and younger leaves, but have the highest concentration in seeds. Most effects of gibberellins are shown only in concerted with auxins.



Gibberellic acid

Function of gibberellins: gibberellins, like auxin, promote cell elongation and division of stem tissue. GA_3 is the naturally occurring plant hormone in many plants. If applied as a PGR, GA_3 will cause certain dwarf species of plants to grow to the normal plant size. Other PGRs inhibit natural GA_3 synthesis, and therefore reduce growth if applied to seedling plants. In propagation, gibberellins are important to breaking dormancy after imbibitions of water by the seed coat. Gibberellins signal germination activities. In particular, application of gibberellins to seeds will counter the normal environmental cues, such as exposure to low temperatures.

d) Ethylene

Ethylene possesses the simplest structure out of the plant hormones and growth regulators being composed of 2 carbon atoms and 4 hydrogen atoms linked by a double bond. Ethylene is the only plant hormone that exists as a gas in most physiological conditions. Ethylene was discovered around 1900 by observations of plants defoliating around the proximity of natural gas leaks. After further investigation of compounds found in natural gas, ethylene was isolated, existing in natural gas as a contaminant. Ethylene is synthesized from the amino acid methionine.

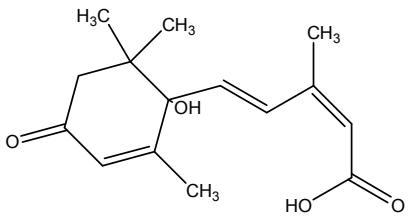
$H_2C=CH_2$

Ethylene

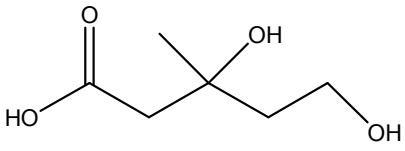
Function of ethylene: ethylene activates ripening in fruits by signaling chemical reactions that degrade the pectins of the middle lamella, softening fruit, and promoting the conversion of stored starches and/or oils into sugars that attract seed dispersers. Ethylene promotes female flower production in some members of the Cucurbitaceae, whereas high gibberellins may promote formation of male flowers. Ethylene functions to help germinating seeds overcome mechanical stress. Ethylene is responsible for initiating the programmed death (apoptosis) of sclerenchyma and xylem vessels and tracheids. Death involves intense cellular activity to degrade and salvage materials of the cytoplasm. Ethylene is the direct cause of leaf and fruit abscission. Ethylene promotes the degradation of the cell walls in the abscission zone cells. The declining levels of auxin in leaves signal the production of ethylene in the abscission zone cells as summer ends.

e) Abscisic acid (ABA)

Abscisic acid (ABA) is the most recently identified hormone. It was discovered in 1965 by researchers studying the processes of dormancy in certain plant species. Structurally, ABA is chemically complex and physiologically acts as an inhibitor on activity of other hormones within plants. ABA is a hormone that functions by inhibiting growth activities in times of environmental stress rather than by promoting growth. It often serves as an antagonist to the other growth promoting hormones in plants. ABA, which is also synthesized from mevalonic acid, got its name from the erroneous belief that it promoted the formation of abscission layers in leaves and fruits. It does not, although leaf abscission accompanies dormancy in many plants.



Abscisic acid



Mevalonic acid

Function of abscisic acid: ABA promotes seed dormancy activities. ABA levels are high when seeds mature, promoting lowered metabolism and synthesis of proteins needed to withstand the dehydration associated with dormancy. In other cases breaking dormancy is relative to the ratio of ABA (which keeps seeds in dormancy) and gibberellins (which promote germination). Low levels of ABA in maturing seeds promote premature germination.

1.2 Plant propagation [9]

Plants can be propagation, or multiplies in several different ways. Plant propagation is both an art and a science. There are two basic ways in which plant can be reproduced: sexual propagation and asexual propagation.

1.2.1 Sexual propagation of plant [10]

In past, most people are familiar with growing new plants from seed. Sexual propagation involves the exchange of genetic material between two parent plants to produce a new generation (seed). The seed is made up of the following three basic parts: the outer seed coat, which protects the seed; the endosperm, which is a food reserve; and the embryo, which is the young plant itself. When a seed matures and is put in a favorable environment, it will germinate and begin active growth. This radicle is the first part of the seedling to emerge from the seed. It will develop into the primary root from which root hairs and lateral roots will grow. The portion of the seedling between the radicle and the first leaflike structure is called the hyopcotyl. The seed leaves (cotyledons) encase the embryo and are usually different in shape from leaves produced by the mature plant. Plants producing one cotyledon fall into the group of monocotyledons. Plants producing two seed leaves are called dicotyledons.

1.2.2 Asexual propagation of plant [11]

Plant propagation refers to the reproduction of new plants from other means other than seed. Asexual propagation involves the vegetative parts of a plant including the roots, stems or leaves. A part of a single parent plant is made to regenerate itself into a new plant, which is genetically identical to the parent plant. There are several advantages of producing a large plant in a short period of time and may be the easiest

way to propagate some species of plants. The major methods of asexual propagation are cuttings, division, layering, grafting, budding and tissue culture.

1.2.2.1 Plant cuttings [12]

Cuttings can be made from any part of the plant. Most frequently, however, either a stem or leaf is used. A stem cutting includes a piece of stem plus any attached leaves or buds. Thus, the stem cutting only needs to form new roots to be a complete, independent plant. A leaf cutting uses just the leaf, so both new roots and new stems must be formed to create a new plant.

Propagation by stem cuttings is the most commonly used method to propagate many woody ornamental plants. Stem cuttings of many favorite shrubs are quite easy to root. Typically, stem cuttings of tree species are more difficult to root. However, cuttings from trees such as crape myrtles, some elms, and birches can be rooted. Type of stem cuttings

a) Herbaceous cuttings.

b) Softwood cuttings

c) Semi-hardwood cuttings

d) Hardwood cuttings

The three types of hardwood cuttings are straight, mallet, and heel. A straight cutting (A) is the most commonly used stem cutting. mallet (B) and heel cuttings are used for plants that might otherwise be more difficult to root. For the heel (C) cutting, a small section of older wood is included at the base of the cutting. For the mallet cutting, an entire section of older stem wood is included [13].

A **B** **C**

1.3 Physical environment [14]

The physical environment surrounding the plants that are being rooted has a large impact on how successful the cuttings are. There are four major physical conditions that affect plants: water, oxygen, light, and temperature. The cuttings need water that is obtainable at the base of the cutting and in the atmosphere. A high concentration of water vapor in the air around the plant reduces the rate at which the plant transpires. Oxygen, which is needed for respiration, is also needed at the base of the cuttings to aid in the formation of the roots. Light is necessary for photosynthesis; without photosynthesis the plant has no energy and energy is what drives the process of root formation. Root formation is most successful when there are longer intervals of light energy or continuous illumination. Temperature is important because, in part, it is what drives the rooting process. The optimum temperature for rooting is between twelve and twenty-two degrees Celsius. A temperature rising higher than that may inhibit root growth and temperatures lower than that may extend the amount of time it takes for the roots to form. Higher temperatures have a tendency to cause bud growth instead of root growth and more water loss in the leaves.

1.4 General characteristic of coffee [15, 16]

Coffee belongs to the botanical family *Rubiaceae*, genus coffea. Coffee is a glossy leafed shrub or small perennial tree and hardy plant. All species of coffea are woody, but they range from small shrub to large over to meters tall; the leaves can be yellowish, dark green, bronze or tinged with purple. First flowers 9-18 months after planting, and first crop matures during second or third year in the field. Coffee produces small flower, white and highly fragrant blossoms. The sweet fragrance resembles the sweet small of jasmine blossoms. Fruits of coffee called “cherry” or “berry”. Because at first to yellow and then light red and finally darkening to a glossy deep red. Each berry holds two locules containing the beans. The beans are actually two seeds within the fruits. There is sometimes a third seed or one seed.

The two most important species of coffee economically are *Coffea arabica* (Arabica coffee) which accounts for over 70% of the world production and *Coffea canephora* (Robusta coffee) most of the remaining 20%. Two other species which are

grown on a much smaller scale are *Coffea liberica* (Liberica coffee) and *Coffea dewevrei* (Excelsa coffee).

1.4.1 ***Coffea arabica*** (Arabica coffee, chromosomes (2n) = 44) and a large bluish dark green with oval shaped leaves that can reach a height of 4 to 6 meters, but with a deep root system. Fruits ripen 7-9 months after flowering in Arabica. Its fruits are oval and usually contain two flat seeds. After planting Arabica trees mature in 3 to 4 years when they produce their first crop. The plant can then produce fruits for 20 to 30 years. There trees prefer equatorial conditions with temperatures between 15 and 20 degrees Celsius, flourish in the tropics from sea level to 600-2000 m and annual rainfall of 150-250 cm.

1.4.2 ***Coffea canephora*** (Robusta coffee, chromosomes (2n) = 22) provides robusta beans. Robusta, which can grow up to 10 meters in height as a shrub or a tree, has a shallow root system. Fruits ripen 9-11 months after flowering in robusta. The fruits are round and take nearly a year to mature. The seeds are oval and smaller than the arabica beans. Robusta trees produce their first crop 3 to 4 years after planting and they remain fruitful for 20 to 30 years. There trees prefer equatorial conditions with temperatures between 23 and 30 degrees Celsius. Robusta, liberica and excelsa are more tolerant to heat, and flourish in the tropics from sea level to 1-100 m.

1.5 Characteristics and information of selected plants

1.5.1 ***Hibiscus rosa-sinensis***

Hibiscus rosa-sinensis belongs to the family “Malvaceae” common name “Hibiscus”, “Rose of China”, “China Rose”. Hibiscus is tropical trees or shrubs with lovely five-petaled flowers in white, yellow, red or magenta. The Chinese hibiscus is an extremely popular house plant. It is the state flower of Hawaii where some 5,000 varieties are known. Individual blossoms remain open for only a day, but under ideal conditions the plants are ever blooming. Here, given adequate light, plants can reasonably be expected to bloom from spring until late fall [17, 18].

Hibiscus rosa-sinensis

1.5.2 *Coleus atropurpureus* Benth.

Coleus atropurpureus Benth. belongs to mint family “Labiatae” common name “Flame Nettle”, “Painted Leaves”. Coleuses are tropical plants native to Java. Many people grow them as bedding plants in a shady place outdoors, but they make wonderful house plants as well. There are many varieties available varying in leaf shape, size and colour. Some are more pendulous than others and look terrific grown in a hanging basket [19, 20].

Coleus atropurpureus Benth

1.6 Literature Review

The study on PGR began with Went's discovery of auxin in 1928 [21] and Kogl's structural elucidation in 1934 [22] of the first natural phytohormone, indole-3-acetic acid (IAA).

In 1986, Tanimoto and Watanabe [23] studied the effect of plant hormones on elongation growth of root, compared with those of the shoot. They reported that auxin and gibberellin often have no remarkable effect on root elongation. Auxin revealed the inhibition effect on root elongation when used at high concentration.

In 1993, Preece [24] found that the temperature could also have an effect on the rooting of the cuttings. Improperly controlled temperature can affect rooting by slowing down the rooting process, making low rooting percentages, causing the root system to develop poorly, damage, or kill the cutting.

In 1994, Aminah [25] investigated the effect of IBA on stem cuttings of *Shorea leprosula.* At 20 µg per cutting was found to be the best with 70% of cuttings rooted and higher doses resulted in less rooting success. IBA enhanced the number of roots developed on each cutting.

In 1995, Laura [26] examined the effect of different concentrations of Dip'N grow (1% IBA and 0.5% NAA) on four types of Ivy. At the highest concentration of Dip'N grow significantly increased the length of the roots in all species. Muňoz found the positive effect of GA_3 on leaf growth of the seagrass *Cymodocea nodosa* (Ucria) Ascherson at concentration between 0.01 and 10 mgl^{-1}.

In 1997, Das [27] studied the effect of rooting in stem cuttings and air-layer of auxin (IBA, IAA, NAA) at various concentrations and in combinations of auxin with *Heritiera fome* and *H. littoralis* . IBA and NAA were found to exhibit a better result in the rooting than IAA and NAA combinations.

Boucher [28] examined the effect of 50% concentration of caffeine solution on the growth of soybean plants and observed that caffeine had a detrimental effect on the growth plant. Kukich [29] found the inhibition effect of caffeine on the growth of Rye grass plants.

According to the literature review, there is no report concerning PGRs from roasted coffee. This research was thus focused on the effect of the roasted coffee on growth promotion plants and to search for active principle responsible to this activity.

1.7 The Goal of Research

The goal of this research could be summarized as follows:

1. To study the plant growth regulators of roasted coffee from crude extract
2. To elucidate the structural formula of the isolated substances from the active ethanolic extract.
3. To search for substances which exhibit the plant growth regulator activity against *Coleus atropurpureus* Benth. and *Hibiscus rosa-sinensis.*

CHAPTER II

MATERIALS AND METHODS

2.1 Plant material

Coleus atropurpureus Benth. and *Hibiscus rosa-sinensis*. are selected as plant models. Stock plants were maintained under outdoor conditions so that succulent new shoots were available for cutting material throughout the testing period.

Bundles of cutting for treatment were prepared by removing the cutting from the plant with a sharp knife, making one cut on a slant and cutting 2 to 4 inch long. Then, the lower leaves were removed while 3 to 4 of the uppermost leaves were remained.

Germination tests were performed using various seedlings of *Tagetes erecta* Linn (Marigold). *Lycopersicon esculentum* Mill (Tomato), *Helianthus annuus* (Sunflower), *Luffa acutangula (L.)* Roxb (Ridged gourd), *Vigna unguiculata* var. *sesquipedalis* (String bean), *Raphanus sativus L.* var. *Longipinnatus* (Napa cabbage), *Cuourtita moschao* Decen (Pumpkim), *Cucumis sativus L* (Cucumber) and *Zinnia elegans* (บานชื่น).

2.2 Chemicals

Most solvents (dichloromethane, ethyl acetate and methanol) in this research were a commercial grade and distilled before use. Solvents for NMR performing were $CDCl_3$ and DMSO-d_6. Adsorbents such as silica gel Merck Kieselgel 60, No. 7734, No.7729 and No. 7731 were used for column chromatography and quick column chromatography, respectively. Thin-layer chromatography (TLC) was carried out on aluminium sheets precoated with silica gel 60 F_{254} (20 x 20 cm, layer thickness 0.2 mm) and spots on the plate were observed under UV light or visualized by dipping regent, 10% of sulfuric acid (H_2SO_4) in ethanol followed by heating.

2.3 Equipments

HPLC (Waters) was conducted on semipreparative using cosmosil 5C18-AR-II column (250 mm length, 10 mm i.d.; AllTech Associates,Waters) and UV detector. The proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C-NMR) including 2D-NMR experiments were carried out with a Bruker AC-F 200 FT-NMR spectrometer.

2.4 Bioassay on plant growth regulation

Plant growth regulator test was used as a main bioassay to verify the presence of bioactive compounds.

2.4.1 Primary screening on germination of selected plant seeds

Tested solutions of ethanolic extract were prepared at the concentration of 1000, 100, 10 and 1 ppm and 0 ppm was the control. 50 mL of tested solutions were added into a glass tube which diameter 30 mm and length 120 mm. A controlled tube contained an equal amount of the solvent used to prepare the tested solutions. Nine seeds of selected plants were soaked into a glass tube containing tested and controlled solutions for 3 hours, allowed to dry in air for 5 min. Then the seeds were placed into a petri dish (25 mm diameter) containing a filter paper disc wetted with 10 mL of distilled water. The petri dishes were covered by the lid and sealed with parafilm to prevent the evaporation of water. These petri-dishes were kept under daylight for 7-10 days. Growth was quantified by measuring dry weight of roots and shoots both treated and controlled plants. All tested and controlled experiments were run in 4 replications (10 seeds of each plant per test per replication) and averaged. Percent of growth promotion could be calculated from the following formula (see 2.4.2)

2.4.2 Root growth promotion of *Coleus atropurpureus* Benth test

Subfractions of ethanolic extract were dissolved in a proper solvent and diluted with water to make the concentration of 1000, 100, 10 and 1 ppm and 0 ppm was the control. 50 mL of prepared solutions was added into a glass bottle with 4 cm diameter. A controlled tube contained an equal amount of the solvent used to prepare the tested solutions. Stem-cuttings of *Coleus atropurpureus* Benth were dipped in tested and controlled solutions to a depth of 2 cm for 15 min, allowed to dry in air for

5 min. The treated plants were individually dipped in the rooting medium, containing distilled water in a glass bottle with 4 cm diameter. Suitable supporters were used to hold cuttings upright in solutions and then the plants were maintained under outdoor conditions. After 7-10 days, the treated plants were removed from the medium. Growth was quantified by measuring dry weight of roots both treated and controlled plants. All tested and controlled experiments were run in 4 replications (16 samples per test per replication) and averaged. Present of growth promotion could be calculated from the following formula [30].

$$\% \text{ Growth promotion} = [(T/C) \times 100] - 100\%$$

Where "T" was the mean roots dry weight of treated plant.

"C" was the mean roots dry weight of controlled plant

Growth promotion of 0% represents promotion growing

2.5 Extraction

Roasted Arabica coffee (200 g) was extracted by soxhlet for 3 days with 95% ethanol. The extract of roasted coffee was filtered off and evaporated by rotary evaporator to dryness to yield the ethanolic extract.

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CHAPTER III

RESULTS AND DISCUSSION

The main aim of this research was to find PGRs from roasted coffee. The preliminary screening of the ethanolic extracts of roasted coffee for plant growth promotion activity was examined against various plant seedlings. The ethanolic extract was in addition tested for plant growth promotion of *Coleus atropurpureus* in order to select the appropriate concentration displaying highest activity. The fractionation of the ethanolic extract was performed to follow the activity using plant growth promotion bioassay guided of *Coleus atropurpureus* The fractions displaying the highest growth promotion were selected for further examination to search for active compounds.

Roasted coffee (200 g) was extracted by soxhlet extractor for 3 days with 95% EtOH. The ethanolic extract as a dark brown viscous liquid 42.50 g was obtained.

3.1 Primary screening on promotion activity of selected plant seedlings

The effect of the ethanolic extract of roasted coffee at concentrations of 1000, 100, 10 and 1 ppm on plant growth activity of nine selected plant seeds was investigated. A solution without roasted coffee extract was used as control. Percent of plant growth promotion was determined based on the determination of dry weight of roots and shoots from both treated and controlled samples.

Table 3.1 Effect of the ethanolic extract on plant growth promotion of selected plant seeds.

Plant	% Growth promotion at level of concentration (ppm)				Control** (g)
	1000	100	10	1	
1. *Tagetes erecta* Linn.	-6.10	75.00*	2.13	-4.88	0.033
2. *Lycopersicon esculentum* Mill	-25.96	20.48*	6.40	4.02	0.055
3. *Helianthus annuus*	-50.06	20.96*	-13.25	-14.41	0.258
4. *Luffa acutangula (L.)* Roxb	-59.68	-40.01	16.54*	4.02	0.316
5. *Vigna unguiculata var. sesquipedalis*	46.88*	10.37	-8.56	-4.56	1.085
6. *Raphanus sativus L. var. Longipinnatus*	37.85*	2.58	7.37	2.08	0.159
7. *Cucurbita moschao* Decen	-29.66	7.99	59.73*	9.82	0.876
8. *Cucumis sativus L.*	-51.92	4.14	54.65*	45.59	0.169
9. *Zinnia elegans*	5.51*	-6.40	-13.69	-15.77	0.067

*Highest root growth promotion

** Dry weight of control (water)

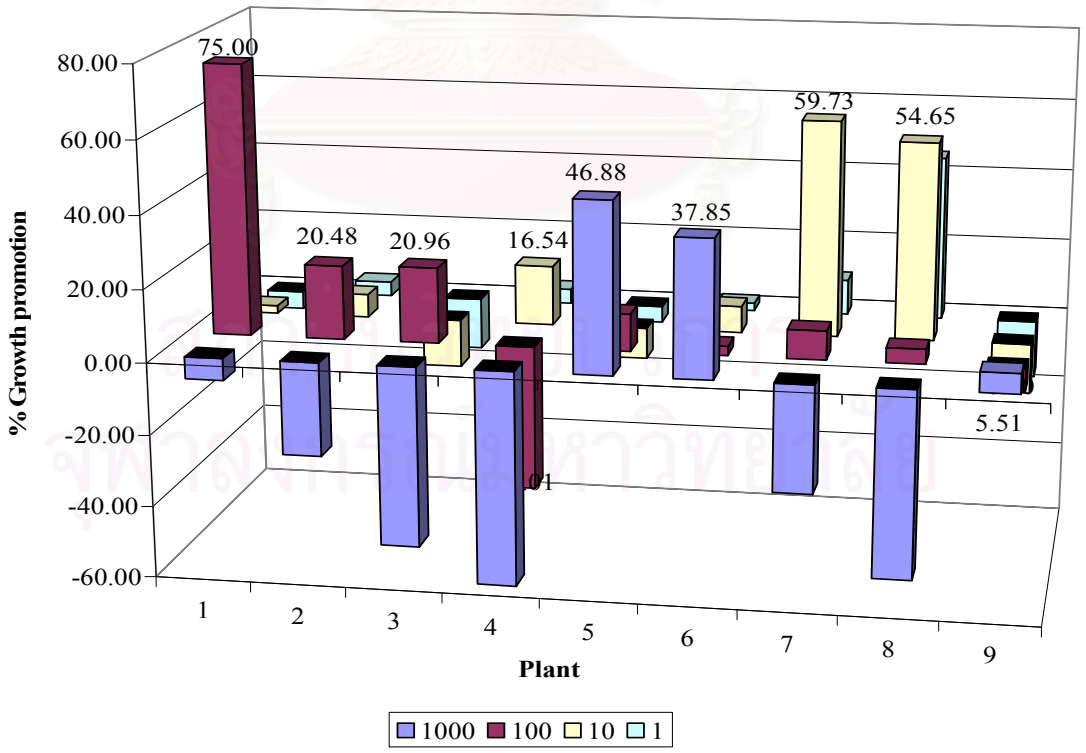


Figure 3.1 Effect of plant growth promotion of the ethanolic extract on plant seeds

Based on the results of the effect of the ethanolic extract of roasted coffee on the growth promotion of plant seeds was observed, the high growth promotion of *H. annuus*, *L. esculentum* and *T. erecta* were exhibited at concentration of 100 ppm. The high growth promotions of *R. Sativus* and *V. unguiculata* were at concentration of 1000 ppm. The concentration of 10 ppm of ethanolic extract was indicated the high growth promotion of *L. acutangul*, *C. sativus* and *C. moschao*. The low concentration of the ethanolic extract was exhibited the best promotion of seedling plants.

To sum up, the ethanolic extract should contain plant growth regulators. Further studies on the root growth promotion of the ethanolic extract for *C. atropurpureus* would be investigated.

3.2 PGR activity results of the ethanolic extract of roasted coffee on *Hibicus rosa-sinensis* Linn.

H. rosa-sinensis, a dicotyledonous plant was selected as a rerepresentative of plant shrubs. Generally, *H. rosa-sinensis* was propagated by stem cuttings.

The ethanolic extract of roasted coffee at concentration of 1000, 100, 10 and 1 ppm was prepared. Stem-cuttings of *H. rosa-sinensis* were dipped in test solutions and a control, to a depth of 2 cm for 15 min, allowed to dry in air for 5 min. The treated plants were individually dipped in the rooting medium containing soil in plastic trays (30×50 cm). Suitable supporters were used to hold cuttings upright in the rooting medium and then the plants were maintained under outdoor conditions. After 10-15 days, the treated plants were removed from the medium. All tested and controlled experiments were run in 4 replications (16 samples per test per replication). Growth was quantified by measuring dry weight of roots form both treated and controlled plants. Results are presented in Table 3.2 and Figure 3.2.

Table 3.2 The effect of the ethanolic extract on the root growth promotion of *H. rosa-sinensis*.

	Level of concentration (ppm)				Control*
	1000	100	10	1	(g)
% Growth promotion	83.77	39.09	13.72	9.04	0.299

* Dry weight of control (water)

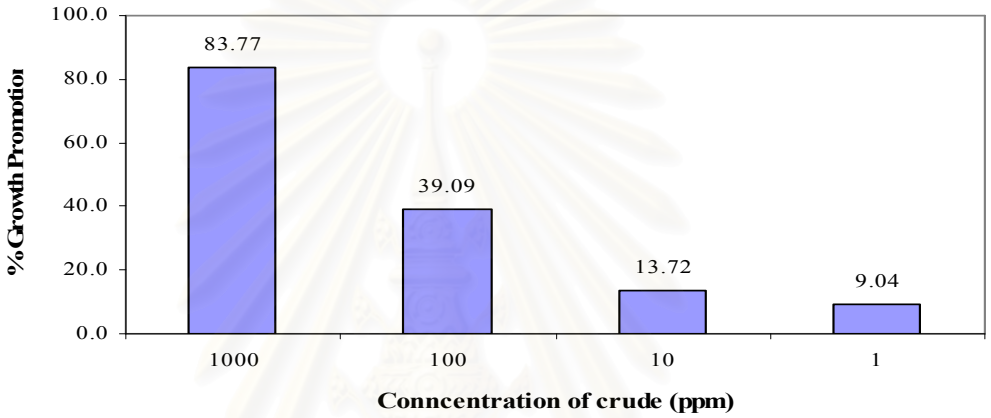


Figure 3.2 The effect of the ethanolic extract on the root growth promotion of *H. rosa-sinensis*

The highest activity for the root growth promotion of *H. rosa-sinensis* using the ethanolic extract was approximately 84% at the concentration of 1000 ppm. The average root and shoot number of *H. rosa-sinensis* were well coincided with the results presented above. The average root and shoot number of *H. rosa-sinensis* after treating with the ethanolic extract at four different concentrations is presented in Table 3.3 and Figure 3.3

Table 3.3 The average fresh and dry weight of the root of *H. rosa-sinensis* after treatment with the ethanolic extract

Average	Level of concentration (ppm)				Control*
weight (g)	1000	100	10	1	(g)
fresh weight	1.99	1.79	1.57	1.41	1.20
dry weight	0.55	0.42	0.34	0.33	0.30

* Dry weight of control (water)

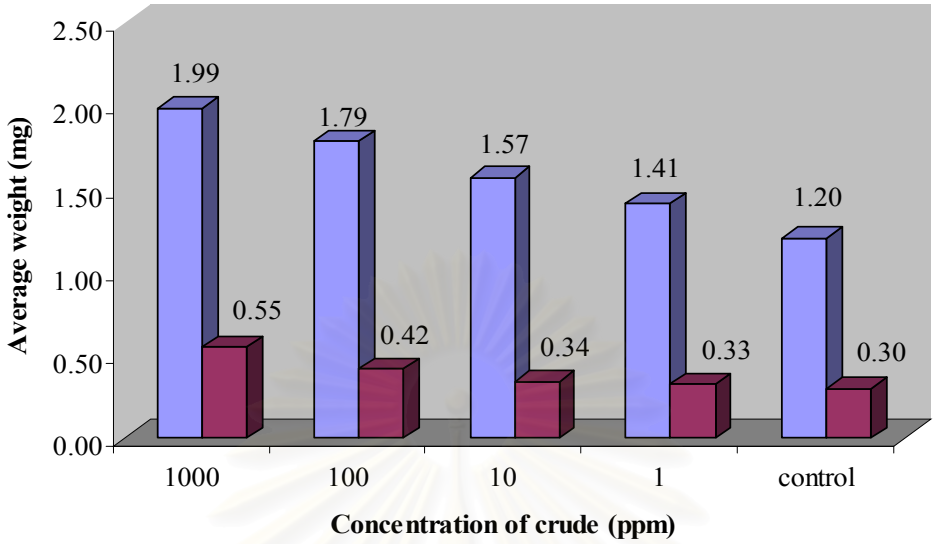


Figure 3.3 The average fresh and dry weight of the root of *H. rosa-sinensis* after treatment with the ethanolic extract

As the result, the ethanolic extract of roasted coffee clearly showed root growth promotion. Particularly at 1000 ppm, the highest activity approximately 84 % was observed. The maximum of average root and shoot number was also found at concentration of 1000 ppm (3.63 and 1.88, respectively). Therefore, it could be concluded that the ethanolic extract revealed a positive effect on the growth promotion of *H. rosa-sinensis* (see Appendix B).

H. rosa-sinensis was a shrubs and a dicotyledonous plant, while *C. atropurpureus* was the herbaceous stem and a monocotyledon plant. Further study on the effect of growth promotion of *C. atropurpureus* was conducted using the same concentrations of the extract used for *H. rosa-sinensis*.

3.3 PGR activity results of the ethanolic extract of roasted coffee on *C. atropurpureus*

The same levels of four concentration of the ethanolic extract: 1000, 100, 10 and 1 ppm were employed. The most appropriate concentration was considered from the average fresh weight, the average dry weight of *C. atropurpureus* and root growth

promotion. Water (0 ppm) was used as control. The results are shown in Tables 3.4, 3.5 and Figures 3.4, 3.5.

Table 3.4 The average fresh and dry weight of the root of *C. atropurpureus* after treatment with the ethanolic extract

Average weight (g)	Control* (g)	Level of concentration (ppm)			
		1000	100	10	1
Fresh weight	18.56	21.24	28.34	25.63	22.40
Dry weight	1.88	2.08	2.90	2.35	2.16

* Dry weight of control (water)

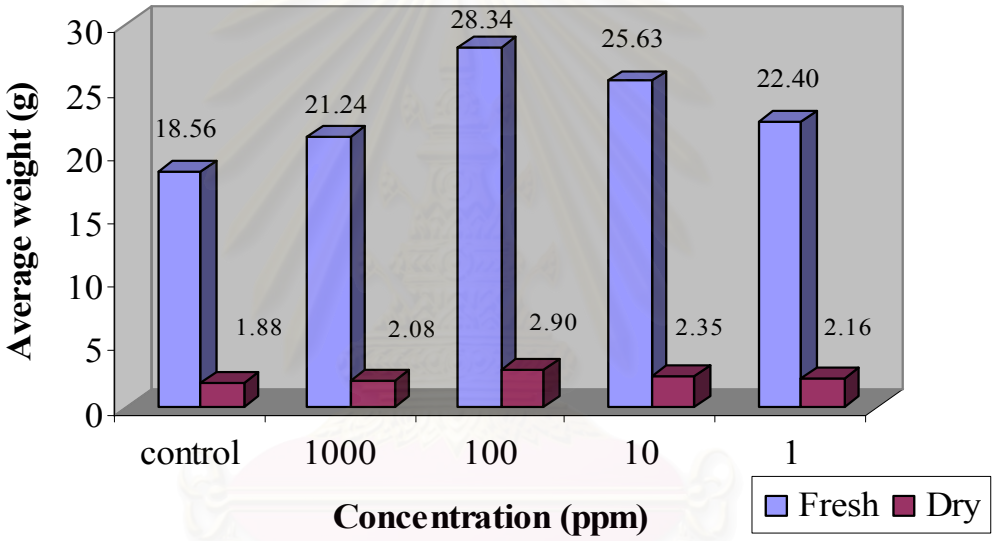


Figure 3.4 The average fresh and dry weight of the root of *C. atropurpureus* after treatment with the ethanolic extract

Based upon the results of varying concentration of the ethanolic extract on *C. atropurpureus*, the maximum dry weight (3.26 g) at concentration of 100 ppm was observed compared with control (1.88 g). The maximum fresh weight obtained was well related to that of dry weight. Every concentration of the ethanolic extract revealed a positive effect on growth promotion (see Appendix B).

Table 3.5 The effect of the ethanolic extract on the root growth promotion of *C. atropurpureus*

	Level of concentration (ppm)				Control*
	1000	100	10	1	(g)
% Growth promotion	10.84	54.52	25.36	15.19	1.88

* Dry weight of control (water)

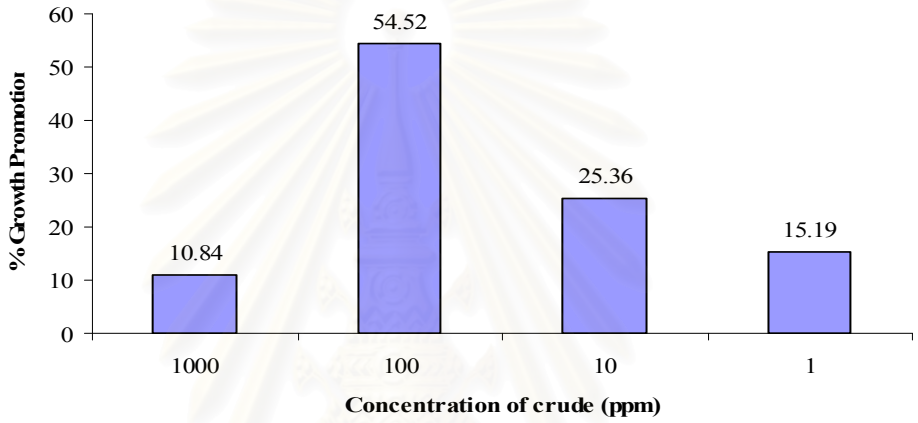


Figure 3.5 The effect of the ethanolic extract on the root growth promotion of *C. atropurpureus*

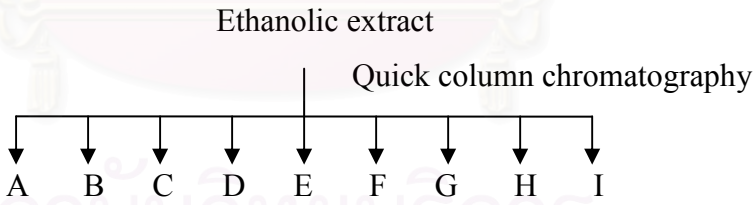
The root growth promotion of the ethanolic extract at different levels of concentration was examined. At 100 ppm, the highest root growth promotion was observed, approximately 55%. Thus, for further study, the concentration of 100 ppm would be used.

3.4 The separation of the ethanolic extract by quick column chromatography

The ethanolic extract (10 g) was first separated by quick column chromatography technique over silica gel 60 No 7729 and 7731 eluting with step gradient of CH_2Cl_2 – EtOAc to EtOAc - MeOH as solvent systems (approximately 1000 mL per system). Each subfraction was monitored by TLC and those which revealed similar components were combined to yield nine fractions as presented in Table 3.6 and Scheme 3.1.

Table 3.6 The separation of the ethanolic extract by quick column chromatography

Fraction code	Solvent system	Remarks	Weight (g)
A	Hexane - 2:8 Hexane/CH_2Cl_2	yellow oil	2.16
B	1:9 Hexane/CH_2Cl_2 - 8:2 CH_2Cl_2/EtOAc	brown viscous liquid	2.11
C	7:3 – 4:6 CH_2Cl_2/EtOAc	brown viscous liquid + precipitate	0.72
D	3:7 CH_2Cl_2/EtOAc	dark brown solid	0.19
E	2:8 CH_2Cl_2/EtOAc – EtOAc	brown solid	0.51
F	9:1 EtOAc/MeOH	dark brown solid	0.86
G	8:2 EtOAc/MeOH	dark brown viscous liquid + solid	0.43
H	7:3 EtOAc/MeOH	dark brown solid	0.72
I	MeOH	dark brown viscous liquid + solid	2.02



Scheme 3.1 The separation of the ethanolic extract

3.5 PGR activity of subfractions of the ethanolic extract

Nine subfractions derived from the separation of the ethanolic extract were subsequently assayed for plant growth promotion activity and the average root number against *C. atropurpureus* at concentration of 100 ppm. The results are presented in Table 3.7, Figures 3.6 and 3.7

Table 3.7 Root growth promotions of *C. atropurpureus* of subfractions A-G at concentration of 100 ppm.

Fraction	Concentration of 100 ppm						
	A	B	C	D	E	F	G
% Growth promotion	14.82	-11.65	-20.71	-5.36	14.81	73.38	-4.94

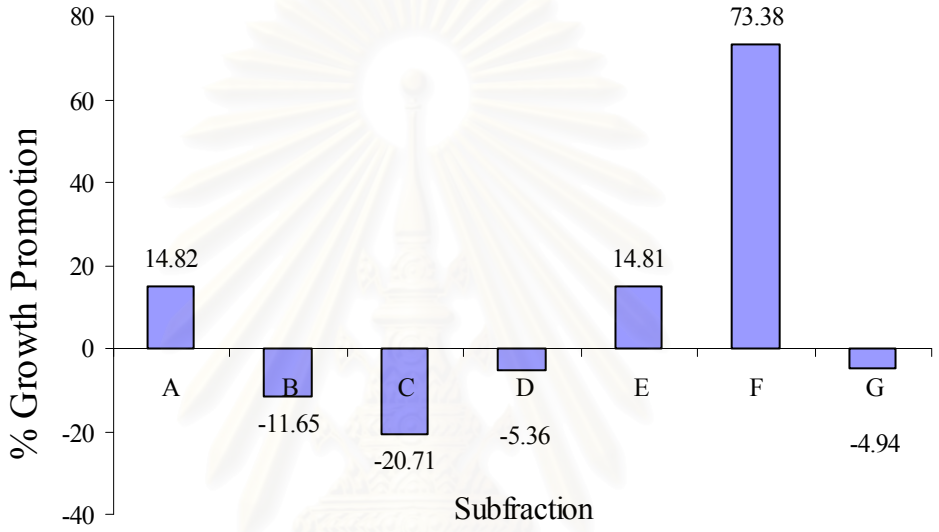


Figure 3.6 Root growth promotions of *C. atropurpureus* of subfractions A-G at concentration of 100 ppm

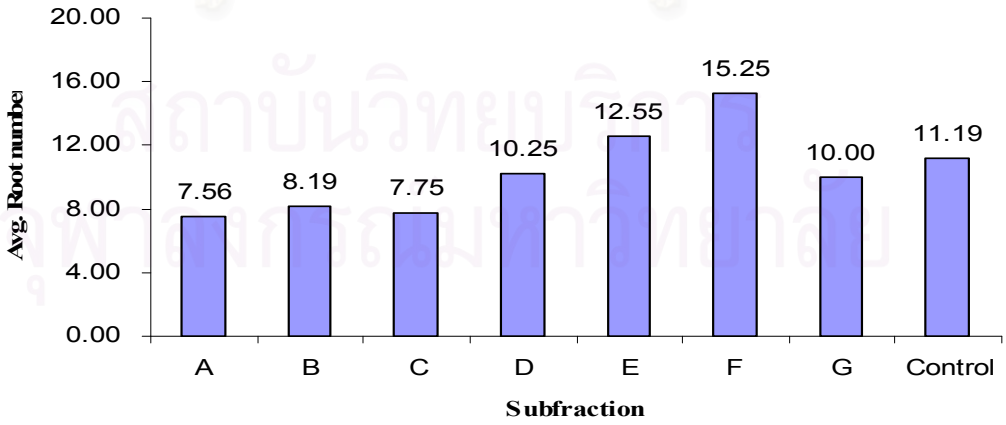


Figure 3.7 The average root number of *C. atropurpureus* of subfraction A-G at concentration of 100 ppm.

Table 3.8 The average fresh and dry weight of the root of *C. atropurpureus* after treatment with subfraction A-G at concentration of 100 ppm.

Average weight (g)	At 100 ppm of the ethanolic extract							Control* (g)
	A	B	C	D	E	F	G	
Fresh weight	15.99	16.88	15.59	18.59	22.60	33.06	18.27	19.23
Dry weight	1.58	1.64	1.47	1.96	2.13	3.22	1.77	1.86

* Dry weight of control (water)

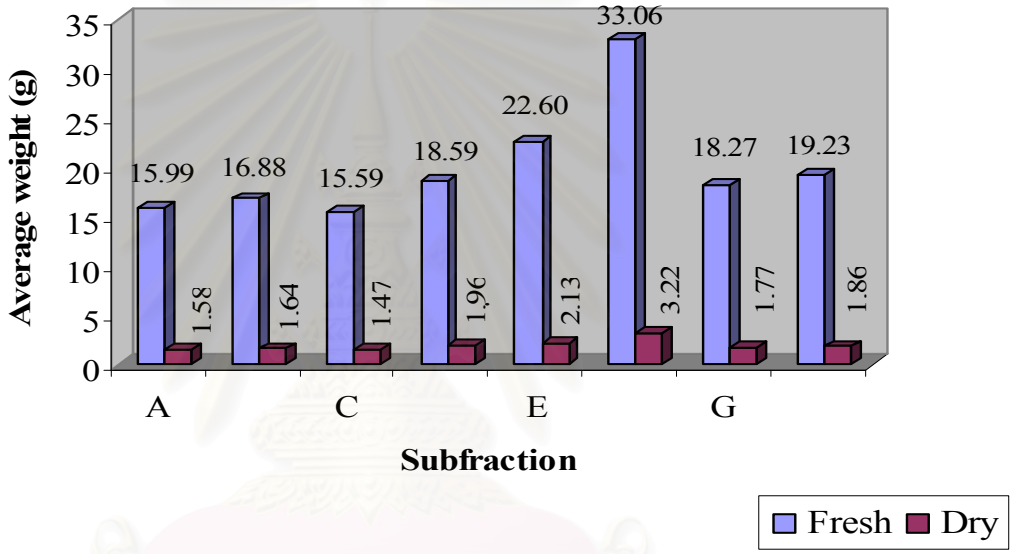


Figure 3.8 The average fresh and dry weight of the root of *C. atropurpureus* after treatment with subfraction A-G at concentration of 100 ppm

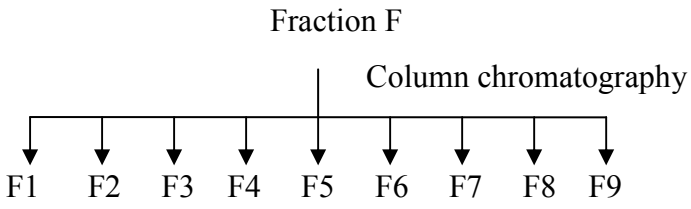
Form the results of root growth promoting activity against *C. atropurpureus*, fraction F achieved from the ethanolic extract exposed very good activity as observed from the maximum average root number (15.25, Figure 3.7) more than control. The growth promotion of fraction F was highly active of approximately 73% (see Appendix B). Fractions A, B, C, D and G decreased the average root number per cutting, but fraction A increased the root growth promotion more than control. Fractions H and I (not shown) inhibited or kill plants. Thus, fraction F was selected for further study.

3.6 The separation of fraction F by column chromatography

According to plant growth promotion activity of subfraction F, it was indicated that this fraction should contain active compound to assist the growth of *C. atropurpureus*. The separation by silica gel column chromatography was carried out using the eluent with step gradient of CH_2Cl_2 – EtOAc to EtOAc - MeOH as solvent systems (approximately 200 ml per system). Each fraction was monitored by TLC. The similar chromatographic patterns were combined to yield nine fractions as presented in Table 3.8 and Scheme 3.2.

Table 3.9 The separation of fraction F by column chromatography

Fraction code	Solvent system	Remarks	Weight(g)
F1	2:8 CH_2Cl_2 / EtOAc	pale brown viscous liquid	0.051
F2	2:8 CH_2Cl_2 / EtOAc	pale brown viscous liquid	0.051
F3	2:8 CH_2Cl_2 / EtOAc	pale brown viscous liquid + precipitate	0.066
F4	1:9 CH_2Cl_2 / EtOAc	pale brown solid + white crystal	0.281
F5	1:9 CH_2Cl_2 / EtOAc - EtOAc	dark brown solid	0.068
F6	EtOAc / MeOH	dark brown viscous liquid	0.055
F7	9:1 EtOAc / MeOH	brown viscous liquid + solid	0.095
F8	9:1 – 6:4 EtOAc / MeOH	brown viscous liquid	0.084
F9	MeOH	dark brown liquid + precipitate	0.095



Scheme 3.2 The separation of fraction F

As the result, fraction F was further fractionated into nine fractions. Fraction F4 gave the highest yield (0.281 g) (see Appendix B). Further study was performed by testing the root growth promotion of each fraction obtained against *C. atropurpureus* at concentration of 100 ppm.

3.7 Root growth promotion test of *C. atropurpureus* of fraction F

The results of testing the root growth promoting of *C. atropurpureus* at 100 ppm are shown in Table 3.9 and Figures 3.8 and 3.9.

Table 3.10 Root growth promotion of *C. atropurpureus* using of fractions F1-F9 at concentration of 100 ppm

Fraction	Concentration (100 ppm)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
% Growth promotion	0.09	-3.28	2.40	61.13	27.27	7.32	12.60	20.84	14.79

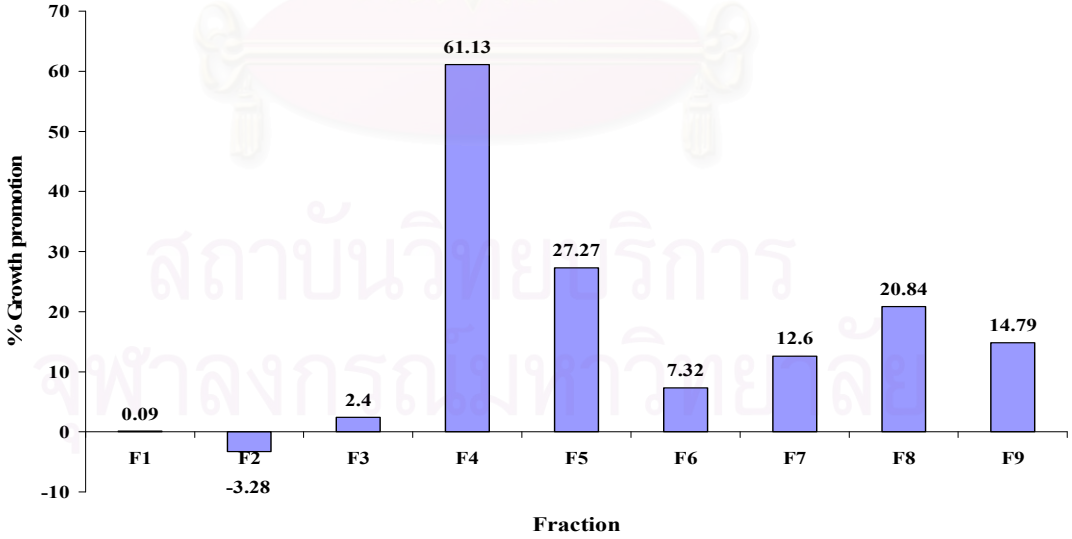


Figure 3.9 Root growth promotions of *C. atropurpureus* of fractions F1-F9 at concentration of 100 ppm

Table 3.11 The average fresh and dry weight of the root of *C. atropurpureus* using of fractions F1-F9 at concentration of 100 ppm

Average weight (g)	Concentration of 100 ppm									Control* (g)
	F1	F2	F3	F4	F5	F6	F7	F8	F9	
Fresh	19.39	19.57	19.59	30.96	23.56	19.69	22.70	23.78	21.67	19.12
Dry	1.79	1.73	1.83	2.88	2.28	1.92	2.02	2.16	2.05	1.79

* Dry weight of control (water)

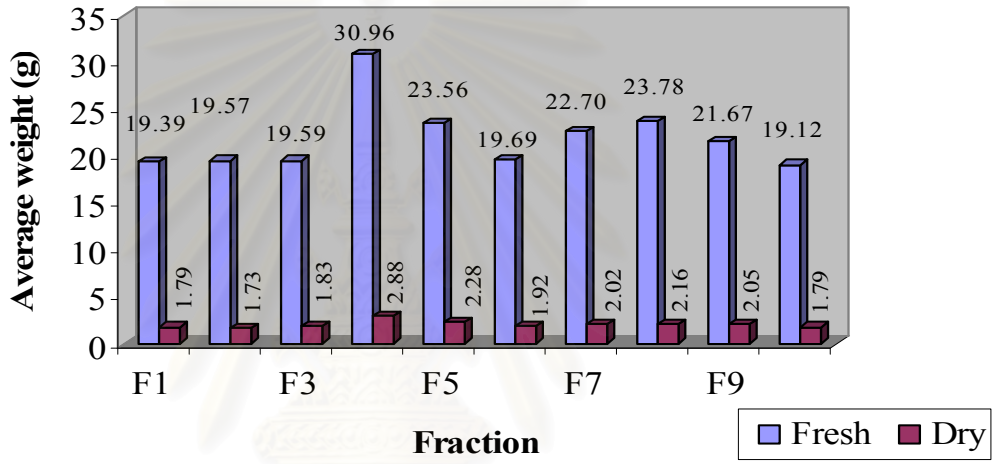


Figure 10 The average fresh and dry weight of the root of *C. atropurpureus* using of fractions F1-F9 at concentration of 100 ppm.

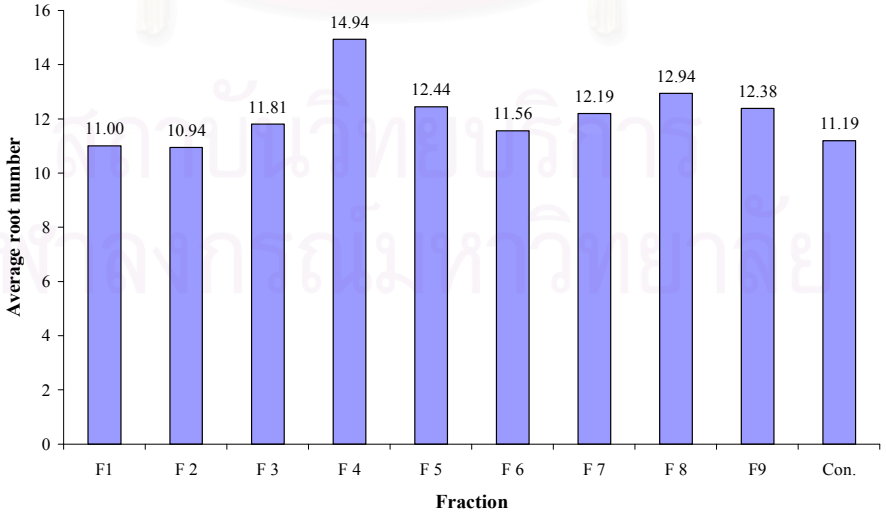


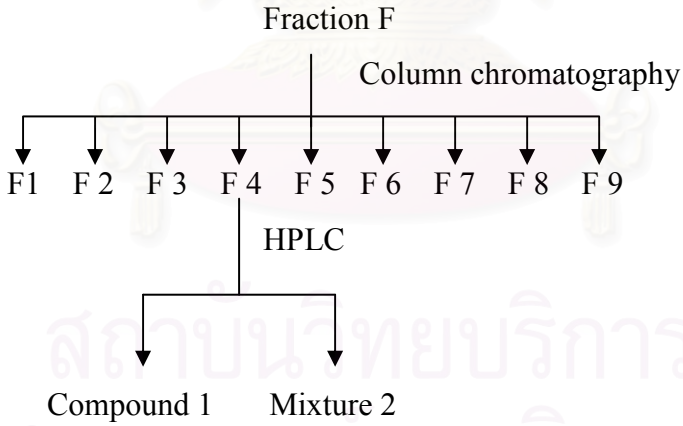
Figure 3.11 The average root number of *C. atropurpureus* after treating with fraction F1-F9 at concentration 100 ppm

As the results attained, fractions F1 and F2 did not reveal the difference in the average root number per cutting from the control. Fraction F4 showed markedly the highest of root growth promotion activity of approximately 61% and the maximum of average root number of 14.94.

To sum up, according to the bioassay-guided results, F4 should contain plant growth regulators. Thus, this fraction was subjected to the isolation and purification of bioactive compounds which affected the root growth promotion of *C. atropurpureus*.

3.8 Separation of fraction F4

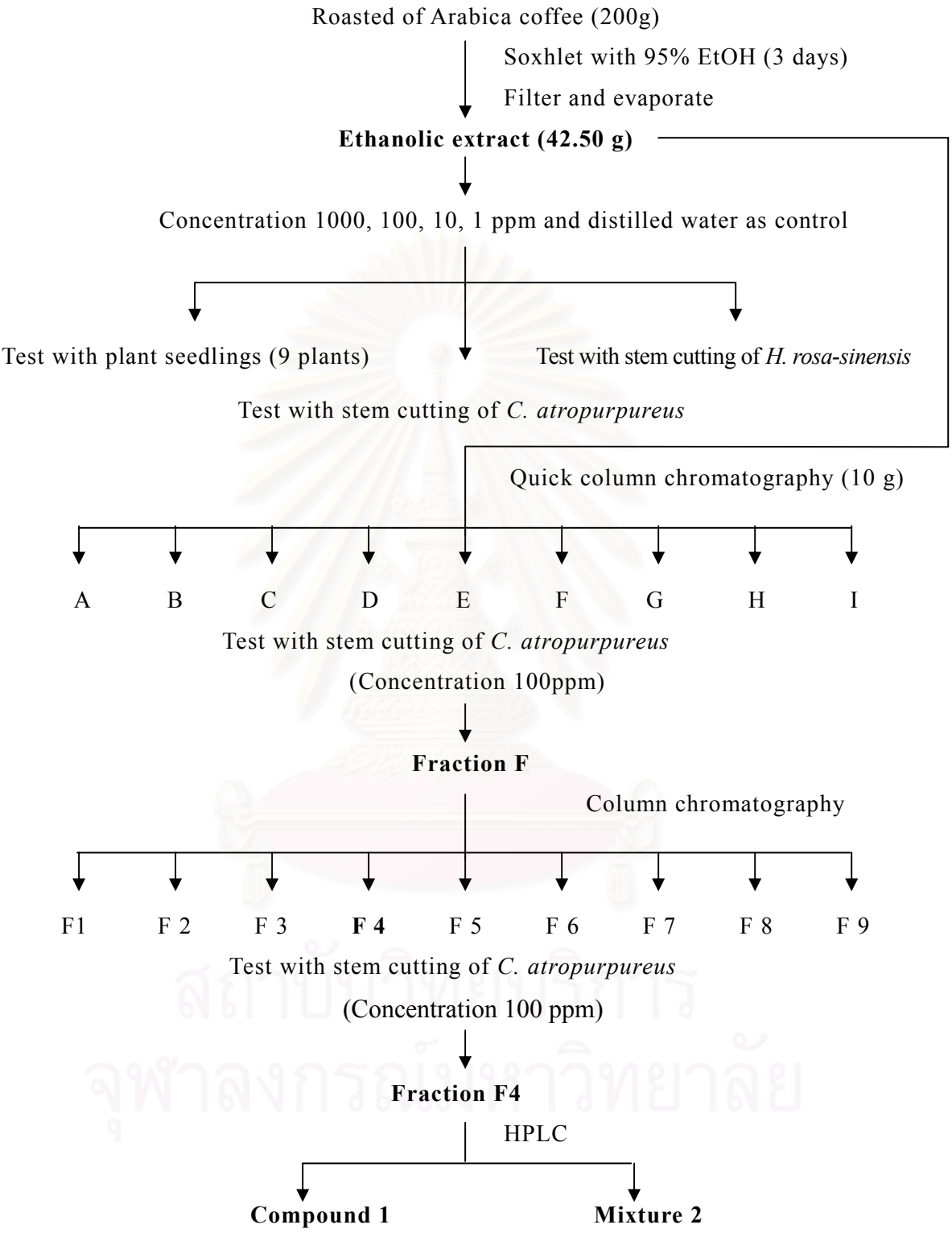
Fraction F4 was examined on TLC using 0.5% MeOH in EtOAc as a solvent system. TLC exhibited 3 spots: one spot could be detected under UV light whereas the others revealed were two spots detected with 10% H_2SO_4 in EtOH followed by heating to visualize the spot. Thus, fraction F4 was separated by HPLC on semipreparative cosmosil 5C18-AR-II column using a solvent system of 20% MeOH in H_2O trying to separate for the active compound. The results are presented in Scheme 3.3.



Scheme 3.3 The separation of fraction F4

Compound **1** was isolated as a white crystal and showed a single spot with R_f 0.57 (10% MeOH in EtOAc). Mixture **2** was also isolated as a trace brown solid with R_f 0.62 and 0.79, respectively (10% MeOH in EtOAc).

The summary of the separation of the ethanolic extract and tested procedures for plant growth regulation are depicted as shown in Scheme 3.4.

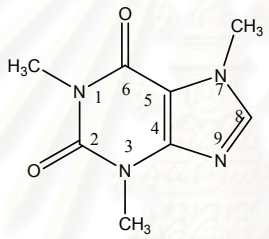


Scheme 3.4 Isolation procedure of the ethanolic extract

3.9 Structural elucidation of isolated compounds

3.9.1 Structural elucidation of Compound 1

Compound **1** was separated from fraction F4 as a white crystal. After recrystalligation with MeOH several times, 0.23 g of compound **1** was obtained (82.1% yield based on fraction F4). TLC of this compound was performed and compared with that of caffeine. It was observed that these two compounds gave the same R_f values in several solvent systems tried. In addition, ^{1}H and ^{13}C-NMR of the isolated compound manifestly indicated that this compound should be caffeine. The comparison of ^{1}H, ^{13}C-NMR spectral data of compound **1** and caffeine was tabulated in Table 3.10.



Compound **1**

Table 3.12 ^{1}H and ^{13}C-NMR spectral data of compound **1** and caffeine [31, 32]

Position	Chemical shift (ppm)			
	Compound 1		Caffeine	
	^{1}H	^{13}C	^{1}H	^{13}C
N-1	3.41	27.94	3.40	27.92
2	-	151.72	-	151.72
N-3	3.59	29.77	3.56	29.74
4	-	148.69	-	148.72
5	-	107.61	-	107.5
6	-	155.44	-	155.42
N-7	3.99	33.61	3.99	33.58
8	7.51	141.36	7.51	141.40
N-9	-	-	-	-

3.9.2 Structural elucidation of mixtures 2

Mixture **2** was obtained in limited amounts which was not enough for structural elucidation.

Therefore, compound 1 was selected plant growth regulation activity test against *C. atropurpureus*.

3.10 PGR activity of caffeine

3.10.1 Plant growth promotion activity of caffeine on plant seeds

The plant growth promotion of plant seeds at the concentration of 1000, 100, 10 and 1 ppm of caffeine are tabulated in Table 3.11 and Figure 3.10. At 0 ppm was the control Percent of plant growth promotion was determined based on the measuring dry weight of roots and shoots from both treated and controlled samples**.**

Table 3.13 Effect of plant growth promotion of caffeine on seeds of various plants

Plant	% Growth promotion at level of concentration (ppm)				Control** (g)
	1000	100	10	1	
1. *Tagetes erecta* Linn.	-15.24	25.61*	-12.80	-33.23	0.033
2. *Lycopersicon esculentum* Mill	56.31	63.62*	9.32	-13.71	0.055
3. *Helianthus annuus*	-47.62	-16.35	-3.68	23.79*	0.258
4. *Luffa acutangula (L.)* Roxb	-6.42	4.52	6.96	73.02*	0.316
5. *Vigna unguiculata var. sesquipedalis*	34.22	70.67*	6.40	2.29	1.085
6. *Raphanus sativus L. var. Longipinnatus*	5.86	45.78*	-12.78	-25.76	0.159
7. *Cucurbita moschao* Decen	-13.42	86.14*	46.07	26.46	0.876
8. *Cucumis sativus L.*	-51.15	-5.51	25.70*	3.97	0.169
9. *Zinnia elegans*	-42.72	-36.61	20.83*	-13.08	0.067

* Highest root growth promotion

** Dry weight of control (water)

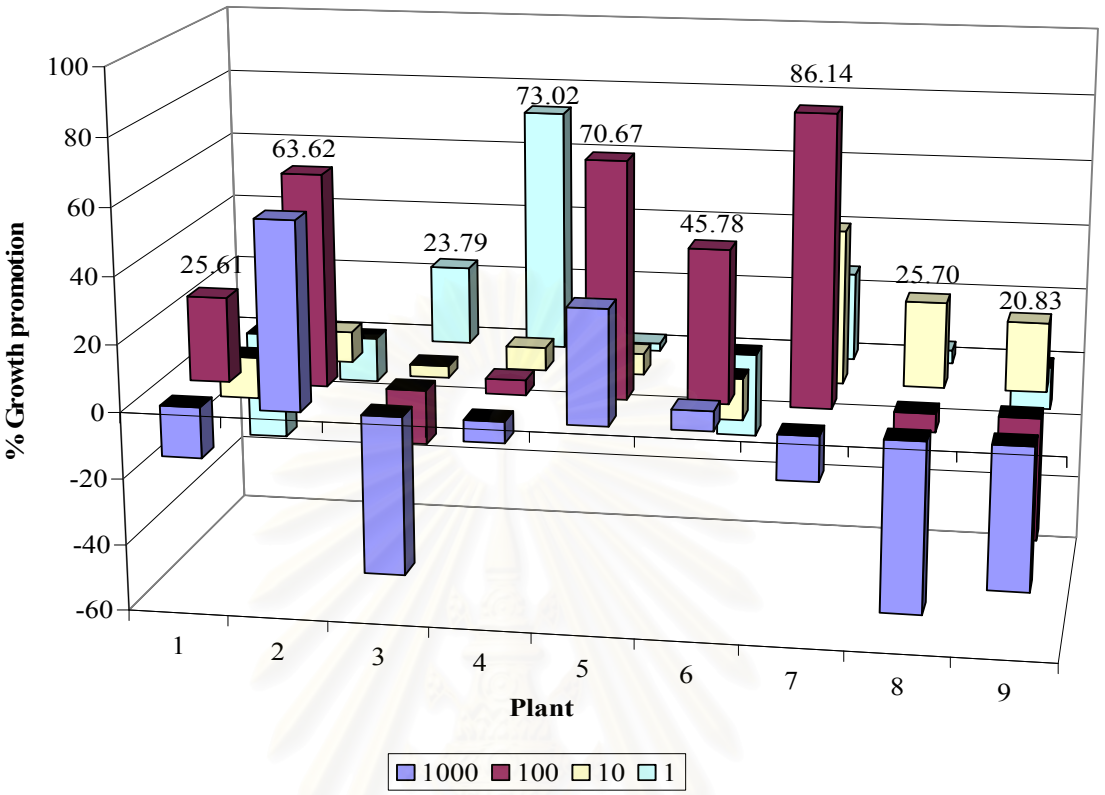


Figure 3.12 Effect of plant growth promotion caffeine on seeds of various plants

The growth promotion of the plant seeds of caffeine at different levels of concentration was examined. At 100 ppm of caffeine, the high level of plant growth promotion of *T. erecta, L. esculentum, V. unguiculata, R. sativus* and *C. moschao* could be observed whereas for *C. sativus* and *Z. elegans* the concentration of caffeine needed was 10 ppm. Interesting results could be attained when only 1 ppm of caffeine was needed to accelerate the growth promotions of *H. annuus* and *L. acutangula.* However, the root growth promotions of caffeine were indeed higher than that using the ethanolic extract.

3.10.2 Root growth promotion test of *C. atropurpureus*

. The results tested of the average root number and the percent growth promotion against *C. atropurpureus* of caffeine at concentration of 100, 10 and 1 ppm, are show in Table 3.12 and Figures 3.11, 3.12.

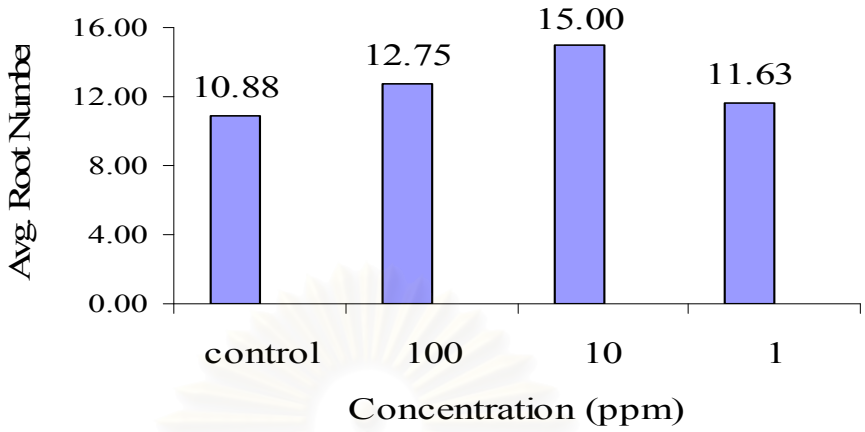


Figure 3.13 The average root number of *C. atropurpureus* using caffeine in a concentration of range 1 – 100 ppm

Table 3.14 Root growth promotion of *C. atropurpureus* using caffeine in a concentration of range 1 – 100 ppm

	Level of concentration (ppm)		
	100	10	1
% Growth Promotion.	14.56	51.16	8.70

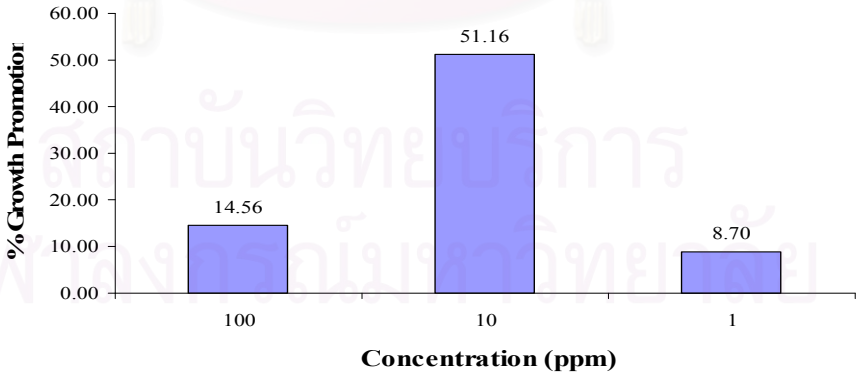


Figure 3.14 Root growth promotions of *C. atropurpureus* using caffeine in a concentration of range 1 – 100 ppm

Table 3.15 The average fresh and dry weight of the root of *C. atropurpureus* after treatment with caffeine

Average weight (g)	Control* (g)	Level of concentration (ppm)		
		100	10	1
Fresh weight	17.96	20.30	28.42	19.89
Dry weight	1.88	2.15	2.84	2.04

** Dry weight of control (water)

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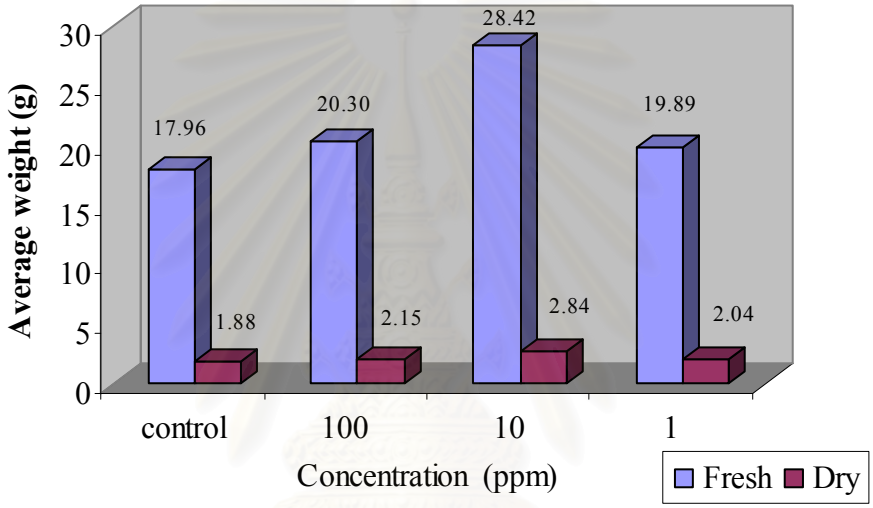


Figure 3.15 The average fresh and dry weight of the root of *C. atropurpureus* after treatment with caffeine

Based upon the aforementioned results, it was observed that there was a correlation between average root number of *C. atropurpureus* and the concentration of caffeine. The concentration of 10 ppm of caffeine exposed very good activity to the maximum of average root number (15) and root growth promotion was highly activity of approximately 51% (see Appendix B).

Caffeine revealed more effect on the average roots number of *C. atropurpureus* than that observed by fraction F4, but the root growth promotion of caffeine was on the contrary less than fraction F4, fraction F and the ethanolic extract. This implied that other substances present in roasted coffee may enhance the capability of caffeine on plant growth promoters

3.10.3 Root growth promotion test of *Hibicus rosa-sinensis* Linn

The test of root growth promoting and the percent growth promotion of caffeine at concentrations of 1000, 100, 10 and 1 ppm were performed on the stem-cuttings of *H. rosa-sinensis* and 0 ppm was the control. All tested plants were dipped in a caffeine solution to a depth of 2 cm for 15 min, allowed to dry in air for 5 min. The treated plants were individually dipped in the rooting medium containing soil in plastic trays (30×50 cm). Suitable supporters were used to hold cuttings upright in the rooting medium and then the plants were kept maintained under outdoor conditions. After 10-15 days, the treated plants were removed from the medium. All tested and controlled experiments were run in 4 replications (16 samples per test per replication). Growth was quantified by measuring dry weight of roots both treated and controlled plants. Results are show in Tables 3.13, 3.14 and Figures 3.13, 3.14.

Table 3.16 The average root number and shoot number of *H. rosa-sinensis* Linn using caffeine.

Average number of	Level of concentration (ppm)				Control* (g)
	1000	100	10	1	
Shoot	1.81	2.88	2.25	1.88	1.75
Root	1.19	1.75	1.44	1.13	1.13

* Dry weight of control (water)

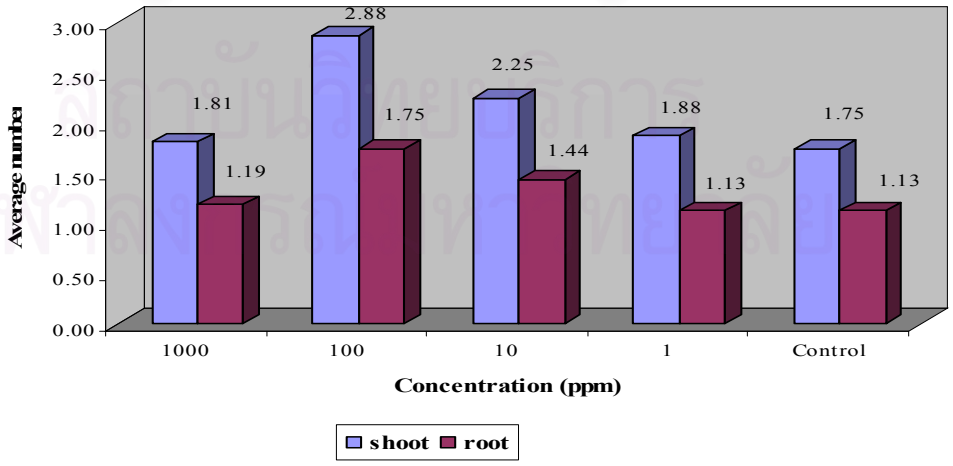


Figure 3.16 The average root number and shoot number of *H. rosa-sinensis* Linn after treating with the caffeine solution.

Table 3.17 The average fresh and dry weight of the root of *H. rosa-sinensis* after treatment with caffeine

Average weight (g)	Level of concentration (ppm)				Control* (g)
	1000	100	10	1	
fresh weight	1.16	1.99	1.80	1.30	1.12
dry weight	0.26	0.45	0.28	0.27	0.25

* Dry weight of control (water)

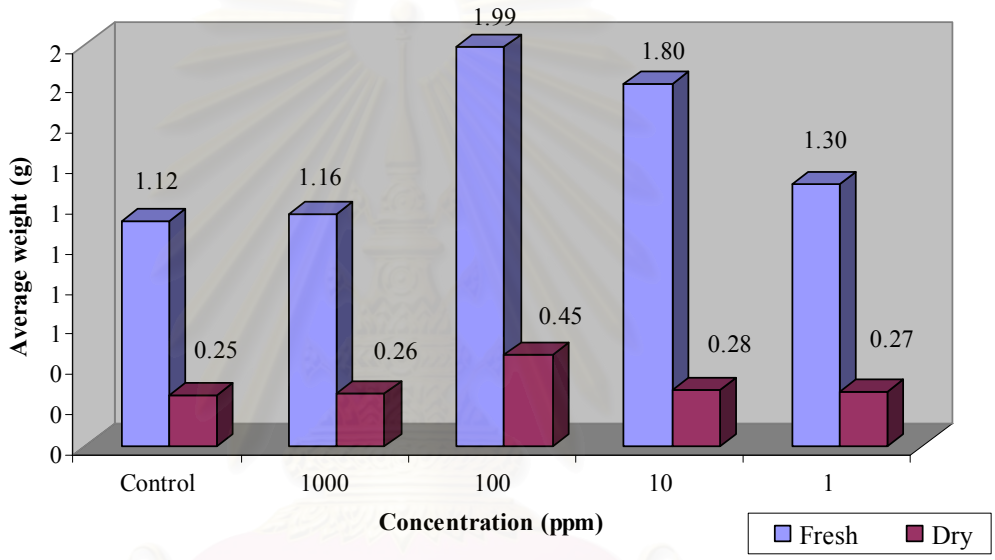


Figure 3.17 The average fresh and dry weight of the root of *H. rosa-sinensis* after treatment with caffeine

Table 3.18 The effect of the root growth promotion of *H. rosa-sinensis* after treating with the caffeine solution

	Level of concentration (ppm)			
	1000	100	10	1
% Growth promotion	2.38	81.93	11.98	7.13

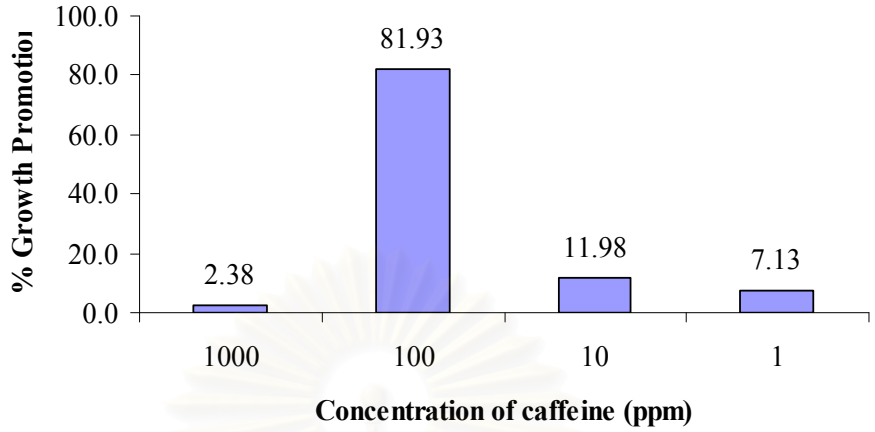


Figure 3.18 Root growth promotion of *H. rosa-sinensis* using caffeine in a concentration of range 1 – 1000 ppm

The caffeine 100 ppm exhibited the maximum of average root number of *H. rosa-sinensis* (2.88), shoot number (1.75) and root growth promotion (82%) (see Appendix B). Various concentrations of caffeine displayed that the root growth promotions were higher than control.

The effect of caffeine and the ethanolic extract on plant growth inhibition of various plant seedlings displayed that the inhibition was related to the concentration of caffeine and the ethanolic extracts. The higher the concentration was, the more inhibition was revealed.

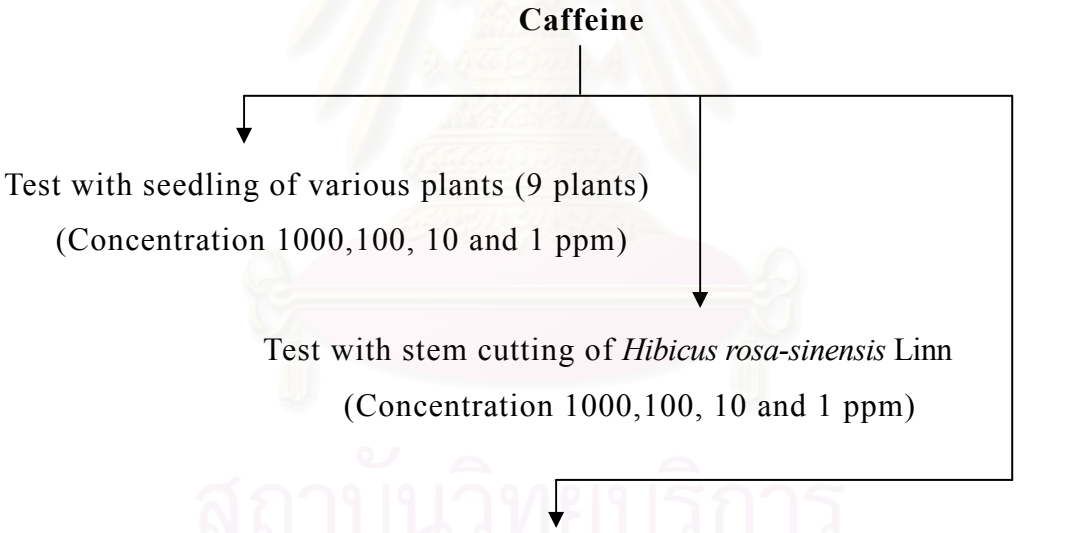
The average root number (2.88) of *H. rosa-sinensis* was observed when treating with caffeine which was less than those observed when treated with the ethanolic extract (3.63). The root growth promotions (82%) were however found to be comparable to that attained when the ethanolic extract was used (84%). The use of the ethanolic extract (1000 ppm) displayed higher root growth promotion than that of caffeine (100 ppm). Thus, the ethanolic extract may be utilized either as plant growth promotion or plant growth inhibition.

In addition, the ethanolic extract enhanced the root growth promotions and stimulation the rooting of stem cuttings more than caffeine did. It may thus conclude that the main plant growth regulator of roasted coffee was caffeine. Nevertheless,

mixture 2 or other minor constituents may also was enhanced the plant growth promotion activity of caffeine perhaps as synergist effect.

The function of caffeine to stimulate rooting and elongation of roots was found to be like auxin and cytokinin. Each plant species has a different threshold for auxin; if this threshold is exceeded, then the plant growth is inhibited by the application of additional auxin [33]. According to the literature review, caffeine exhibited the plant growth inhibitor while the results of the ethanolic extract in this study revealed the effect of caffeine on stem cuttings as plant growth promoter. The effect of caffeine on plant growth promotion depended on the amounts of caffeine used and the species of plant tested.

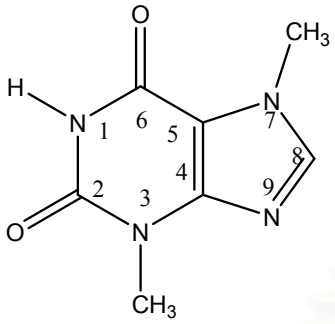
The summary of plant growth regulator of caffeine is depicted as shown in Scheme 3.5.



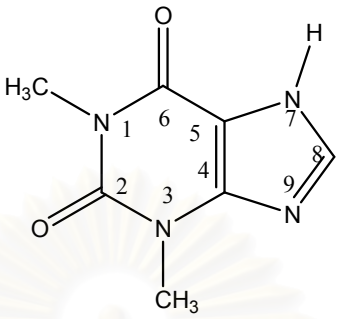
Scheme 3.5 Tested of plant growth regulator of caffeine

According to the literature review, certain compounds could be isolated from roasted coffee [34] such as caffeine (Ca), chlorogenic acid (Ch), theobromine (Tb), theophylline (Tp) and trigonelline (Tr). Further studies on the effect of plant growth promotion of the mentioned compounds on *C. atropurpureus* were performed

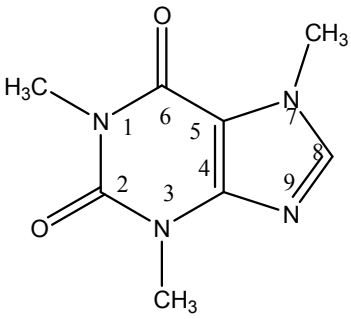
using the concentrations of 1000, 100, 10 and 1 ppm, 0 ppm was used as control. The results are shown in Table 3.15 and Figure 3.15.



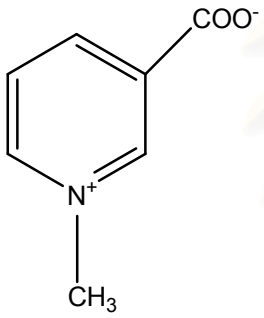
Theobromine



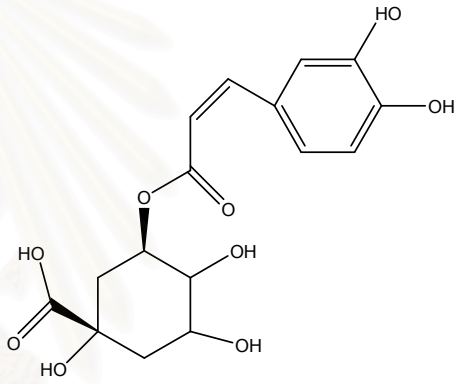
Theophylline



Caffeine



Trigonelline



Chlorogenic acid

Table 3.19 The effect of the growth promotion of substance of roasted coffee

Substance	% Growth promotion of level of concentration (ppm) compared with the control**			
	1000	100	10	1
Chlorogenic acid (Ch)	-15.33	-4.68	5.22*	0.11
Theophylline (Tp)	-15.33	33.66*	22.48	0.64
Theobromine (Tb)	-10.54	13.42	38.45*	3.31
Trigonelline (Tr)	-20.66	27.27*	15.55	18.22
Caffeine (Ca)	-5.21	16.62	54.96*	5.44

* High growth promotion

** Dry weight of control (water, 1.69 g)

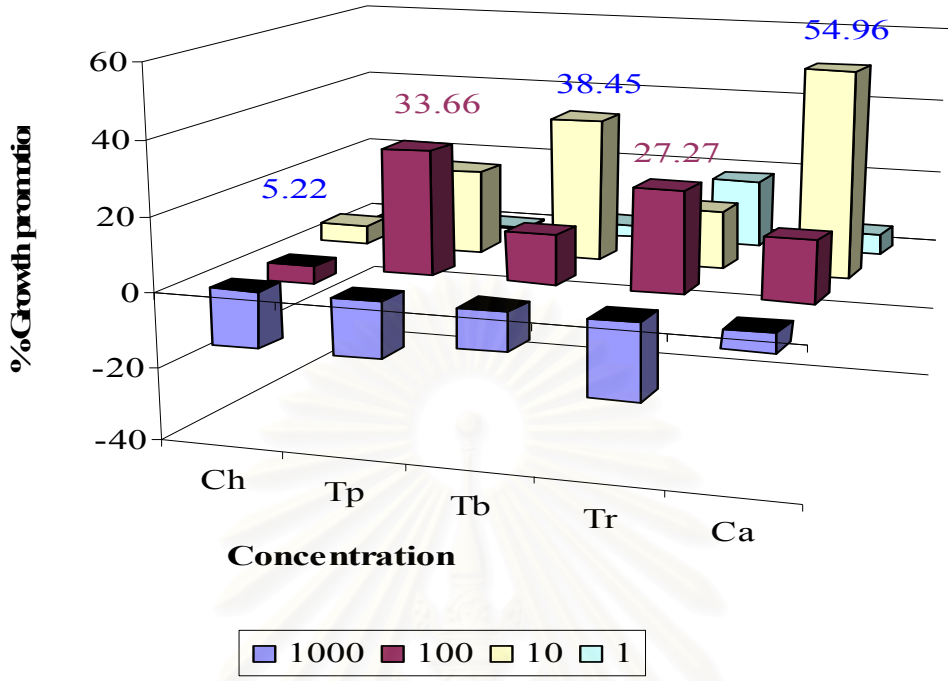


Figure 3.19 The effect of the growth promotion of substance of roasted coffee

At the concentration of 10 ppm of caffeine (Ca), the highest growth promotion of *C. atropurpureus* approximately 55% was manifestly observed. Other substances displayed lower potent activity. Interestingly, theobromine (Tb) also exhibit interesting growth promotion activity (39%) compared with the other substances.

CHAPTER IV

CONCLUSION

Effect of plant growth promotion was different for concentration of the ethanolic extract and type of plants. *Vigna unguiculata var. sesquipedalis, Raphanus sativus L. var. longipinnatus* showed high activity at 1000 ppm, *Tagetes erecta* Linn*, Lycopersicon esculentum* Mill. at 100ppm, , *Luffa acutangula (L.)* Roxb, *Cucurbita moschao* Decen*, Cucumis sativus L.* at 10 ppm. The concentration of ethanolic extract

Plant growth promotion of ethanolic extract on against *Coleus atropurpureus* Benth was highest activity to approximately 55% at the concentration 100 ppm. *Hibicus rosa-sinensis* Linn was highest activity to approximately 84% at the concentration 1000 ppm.

After fractionation and purification of active compounds from the ethanol extract, Compound 1 was identified to be caffeine.

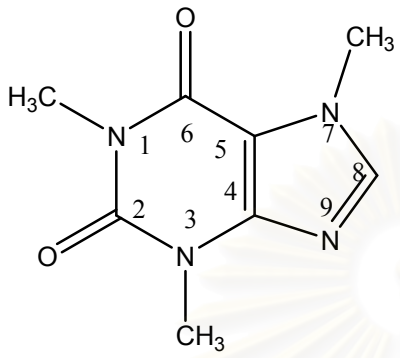
At the concentration 10 ppm of caffeine was highest activity of the plant growth promotion of *Coleus atropurpureus* Benth to approximately 51%, the maximum of the average root number (15) and at the concentration 100 ppm of caffeine showed highest activity of plant growth promotion of *Hibicus rosa-sinensis* Linn to approximately 82%.

The result of study is that Compound 1 or caffeine was plant growth regulators, but the effect of caffeine alone increased the average root number and root growth promotion less than the ethanolic extract. Nevertheless, mixture 2 was may be enhanced plant growth promotion activity of caffeine.

Mixture 2 cannot to identified and purification. The amounts of mixture 2 were found to be in fraction 4 no enough for used, when compared with the yield of Compound 1. The amount of mixture 2 was may be in other fraction.

Plant growth regulators of the ethanolic extract have each compounds [33], but the effect of compound of fraction 4 (caffeine) showed the highly activity when it

compared with the all fraction and other substances. Therefore, caffeine was the best plant growth regulator of roasted coffee.



Caffeine

Proposal for future work

From the result of plant growth regulators, Compound 1 or caffeine showed significant promotion effect against *Coleus atropurpureus* Benth and *Hibicus rosa-sinensis* and seedling of various plants. The possibly future work related to this research would be to further test of other plant that caused problem in Thai agriculture. Moreover, many compound of roasted coffee showed high activity of plant growth regulators, which plant growth promotion was sill very interesting. Future study on isolated and combination substances of varied fractions of roasted coffee were used to tested of plant growth promotion, which it found to be good combination of plant growth regulators.

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APPENDICES

APPENDIX A

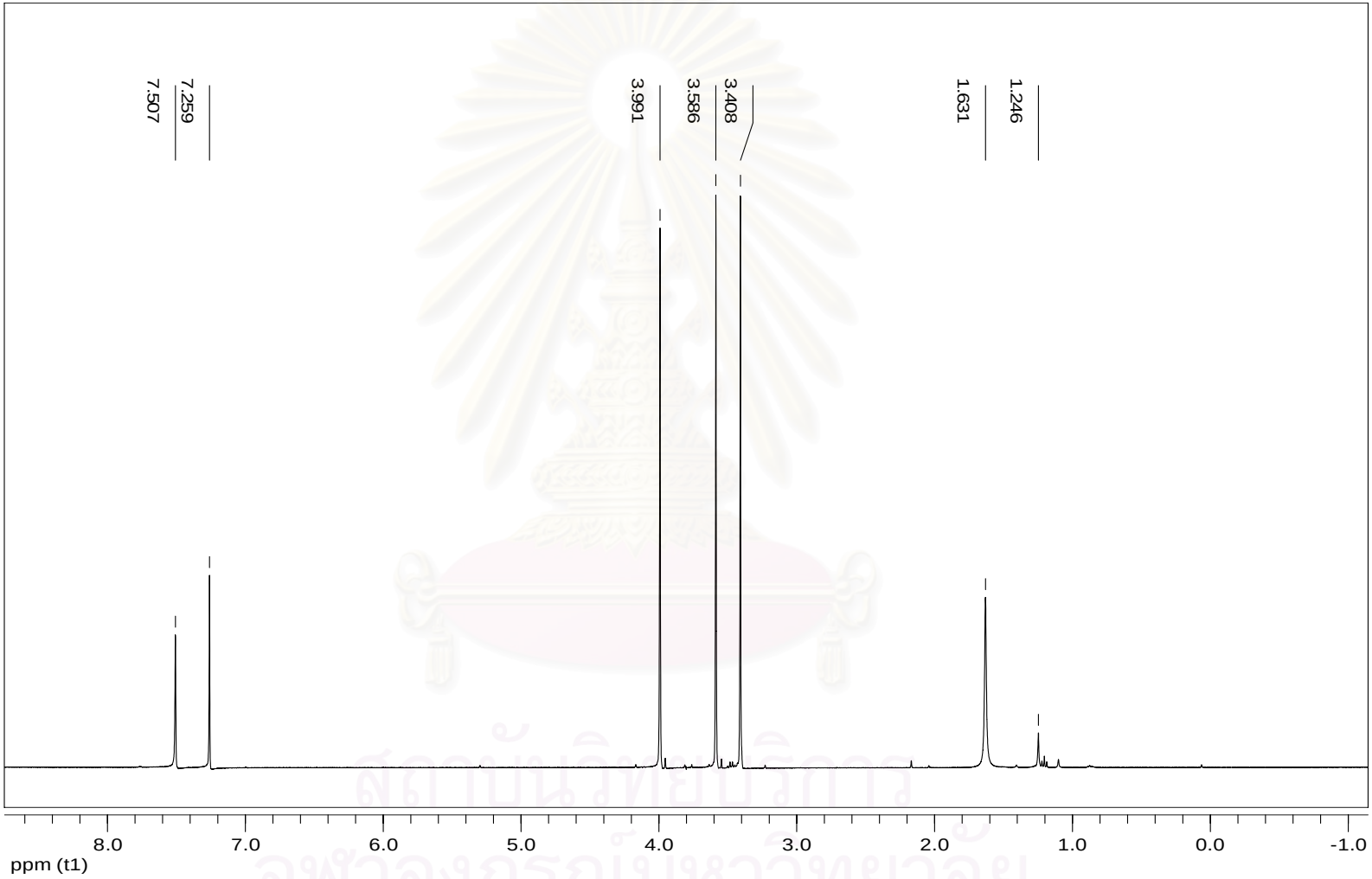

Fig. 3.20 ^{1}H-NMR spectrum of compound 1

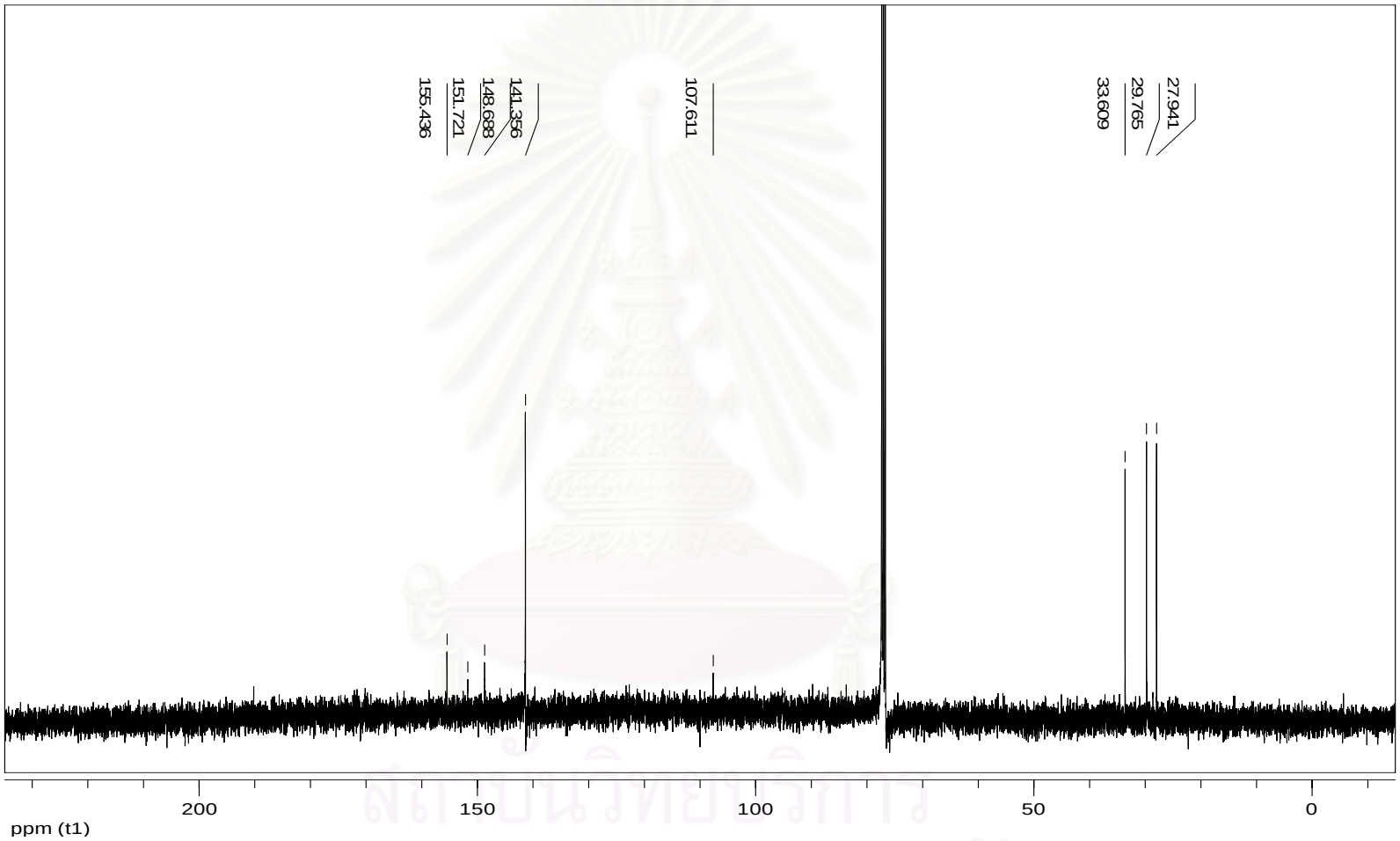

Fig. 3.21 ^{13}C-NMR spectrum of compound **1**

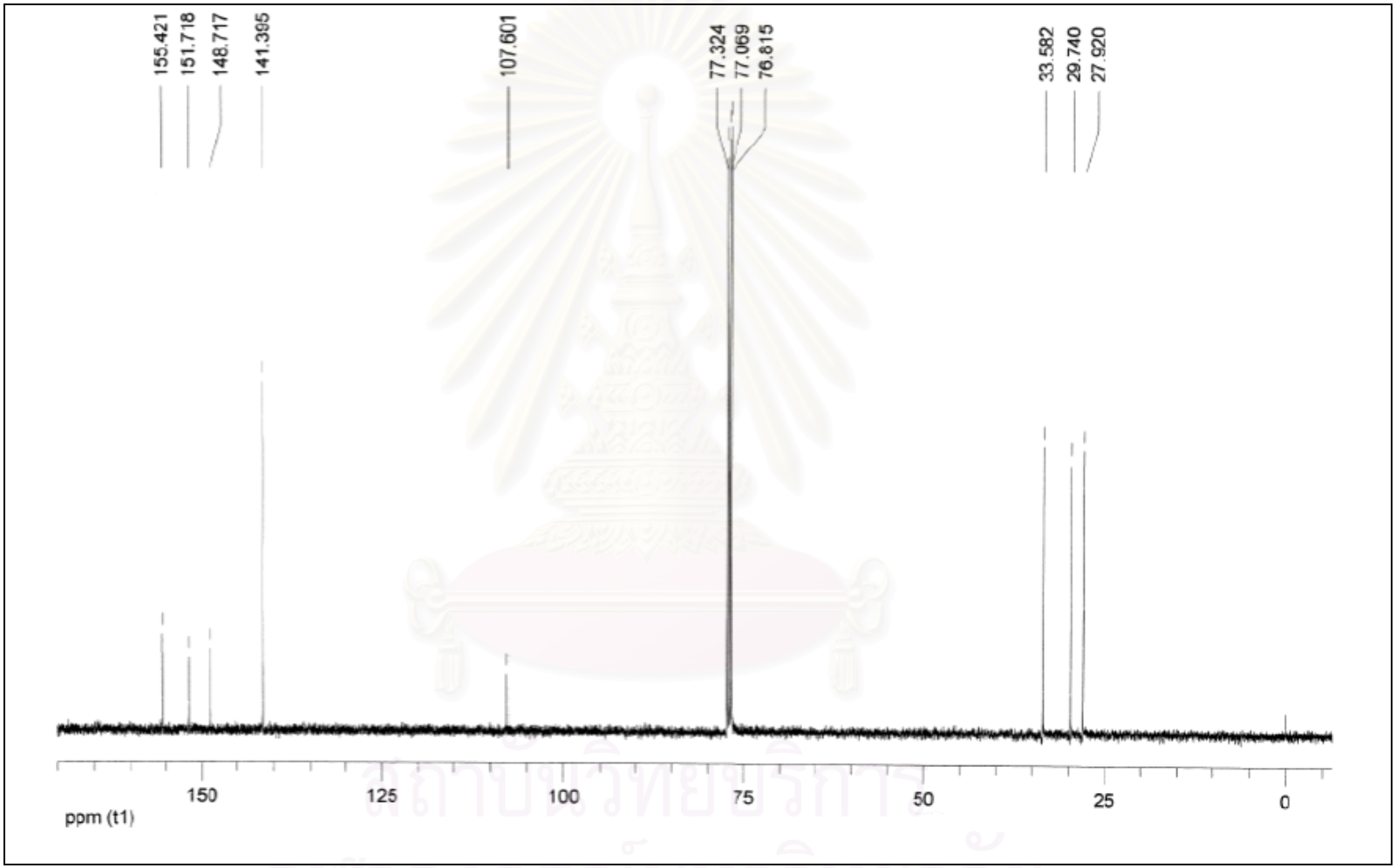

Fig. 3.22 ^{1}H-NMR spectrum of caffeine

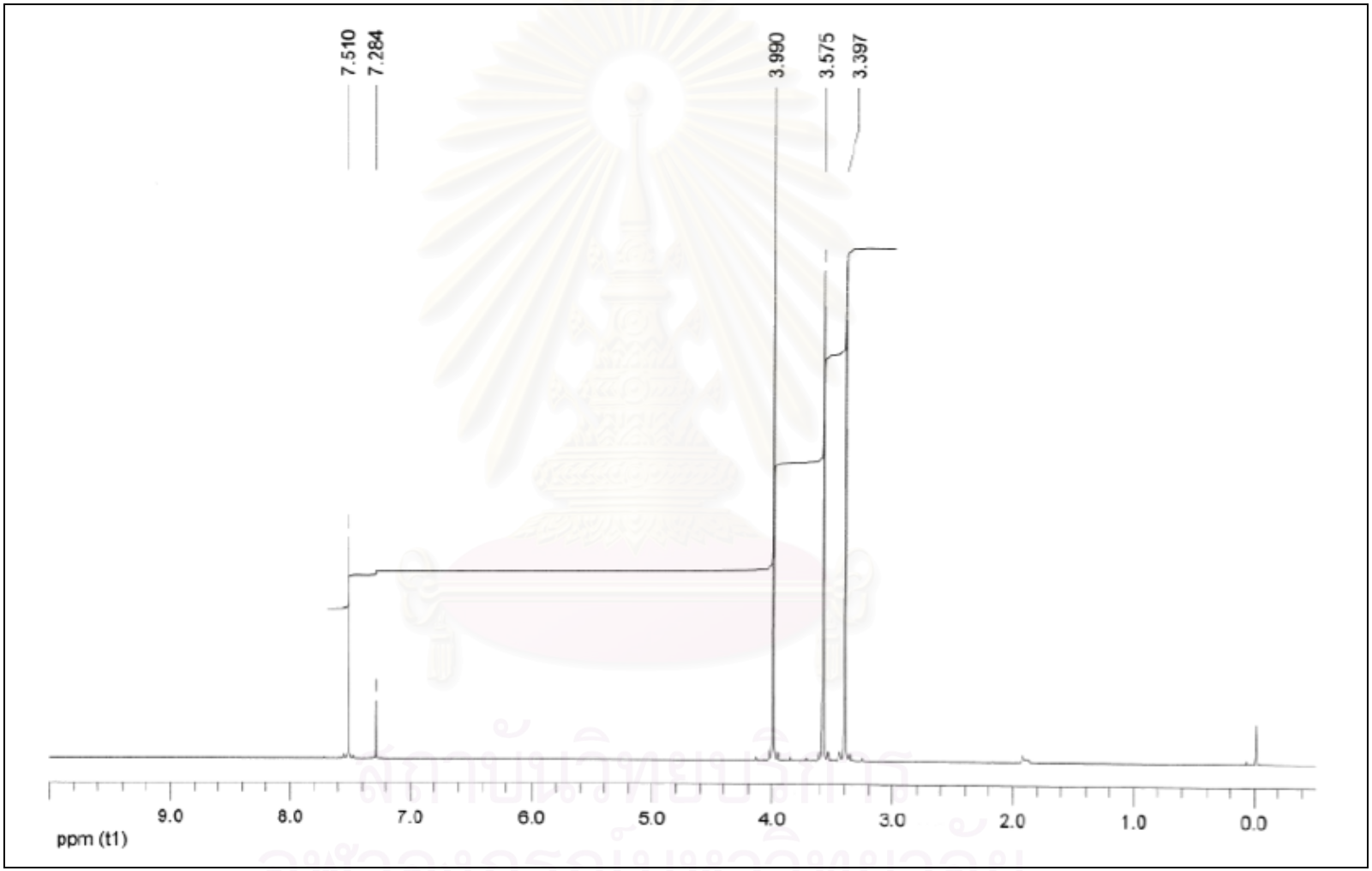

Fig. 3.23 ^{13}C-NMR spectrum of caffeine

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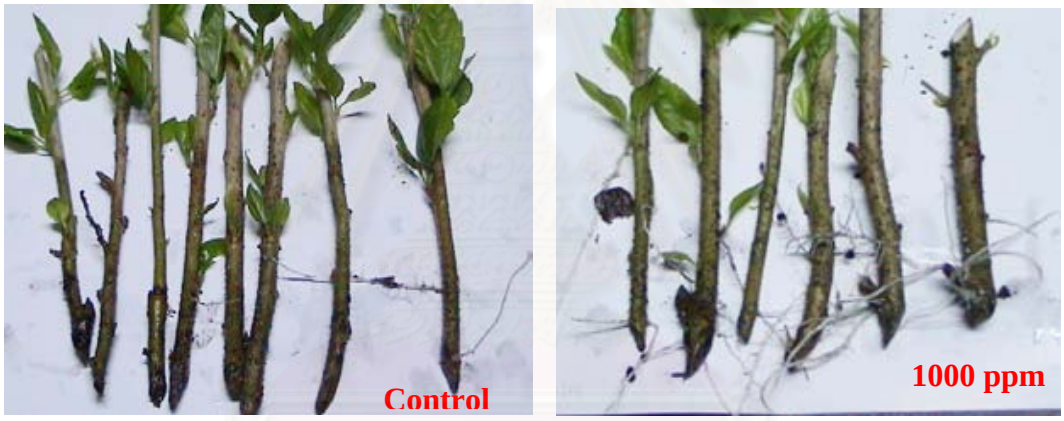
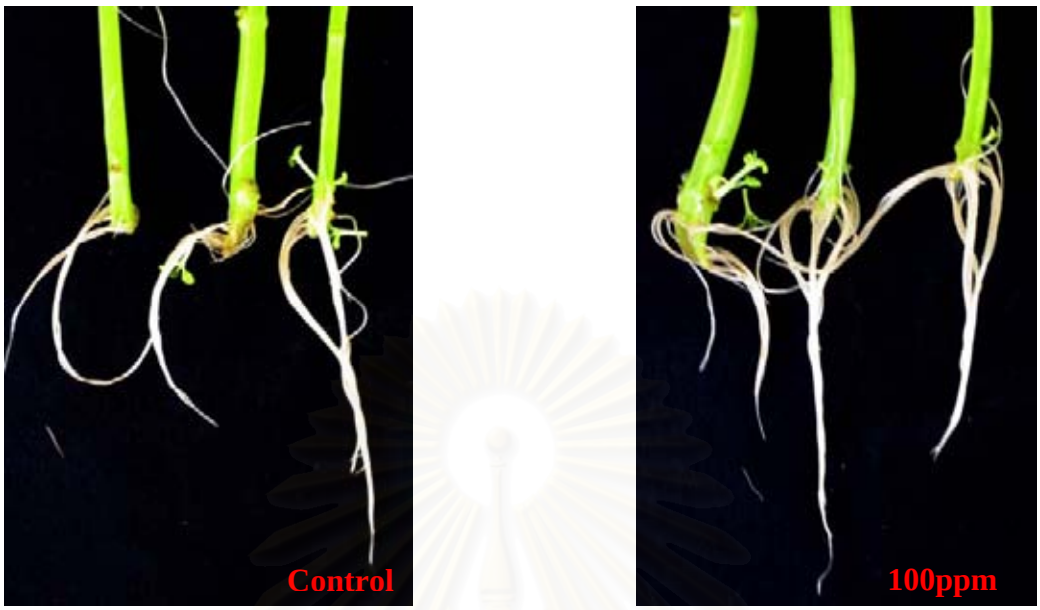
Table 3.21 The average fresh and dry weight of the root of substance of roasted coffee

Substance	Dry weight (g)			
	1000	100	10	1
Chlorogenic acid (Ch)	1.431	1.611	1.602	1.692
Theophylline (Tp)	1.431	2.259	2.070	1.701
Theobromine (Tb)	1.512	1.917	2.340	1.746
Trigonelline (Tr)	1.341	2.151	1.953	1.998
Caffeine (Ca)	1.602	1.971	2.619	1.782



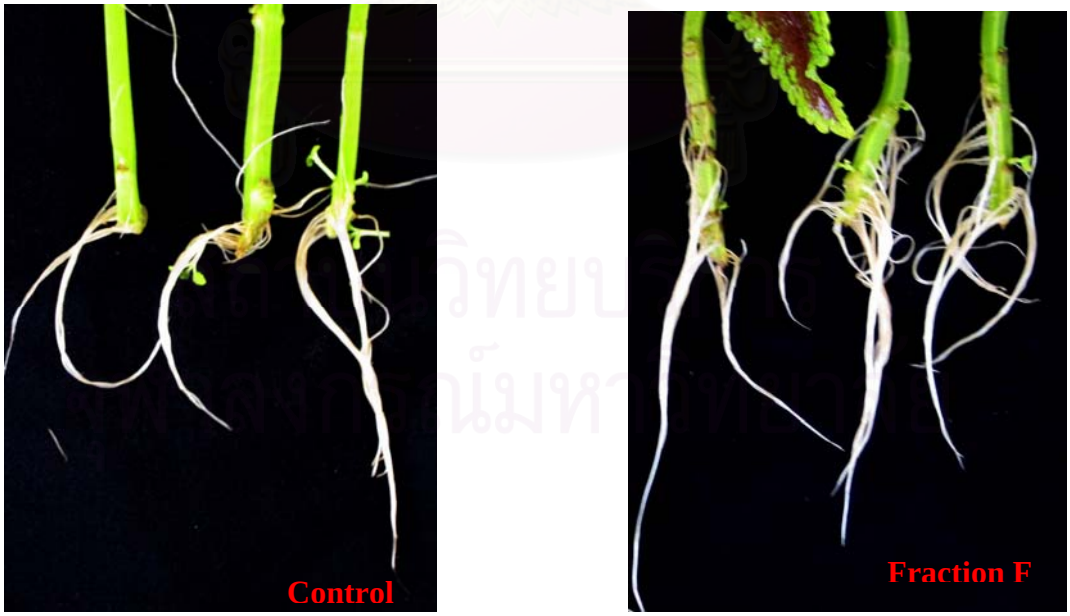
(a) **(b)**

Figure 3.24 Stem cutting of *H. rosa-sinensis* after treatment with the ethanolic extract (a) control (b) at 1000 ppm



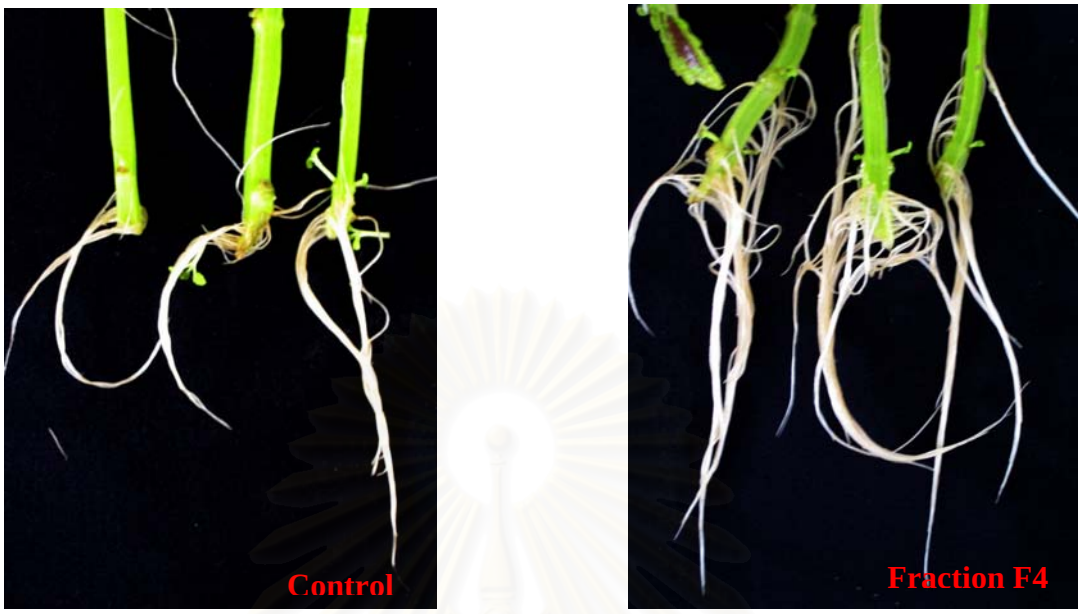
(a) **(b)**

Figure 3.25 Stem cutting of *C. atropurpureus* after treatment with the ethanolic extract (a) control (b) at 100ppm



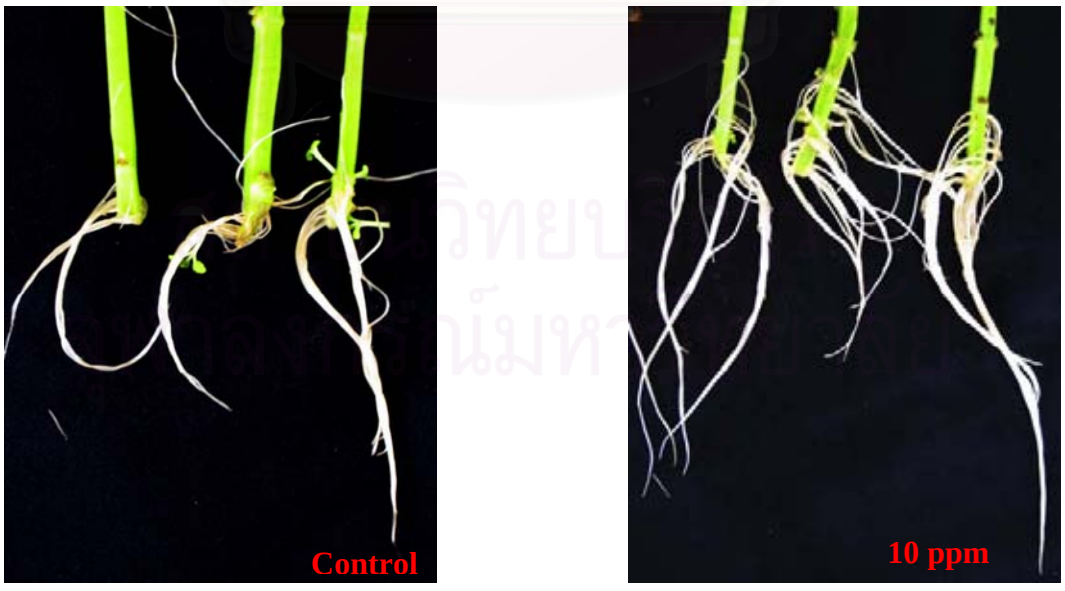
(a) **(b)**

Figure 3.26 Stem cutting of *C. atropurpureus* after treatment with subfraction (a) control (b) Fraction F



(a) **(b)**

Figure 3.27 Stem cutting of *C. atropurpureus* after treatment with fraction
(a) control (b) Fraction F4



(a) **(b)**

Figure 3.28 Stem cutting of *C. atropurpureus* after treatment with caffeine
(a) control (b) at 10 ppm

(a)

(b)

Figure 3.29 Stem cutting of *H. rosa-sinensis* after treatment with caffeine (a) control (b) at 100ppm

VITA

Mr. Nattakorn Puengyen was born on July 19, 1979 in Bangkok, Thailand. He obtained a Bachelor Degree of Science (Environmental) from Faculty of Science, Silpakorn University in 2000. Since 2002, he has been a graduate student studying Biotechnology as his major course to Chulalongkorn University. During he studying in Master's degree, he was obtained a scholarship support form the Graduate School of Chulalongkorn University.