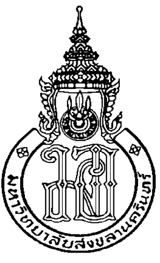

Effect of $ZnSO_4$ Fortification in Soil on Growth Characteristics, Phytochemicals and Mineral Compositions in *Mentha cordifolia* Opiz., Kitchen Mint

Timaporn Srirattanakul

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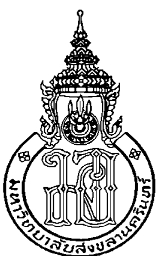

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ชื่อวิทยานิพนธ์ ผลการเสริมซิงค์ซัลเฟตในดินต่อลักษณะการเจริญเติบโต สารพฤกษเคมี และองค์ประกอบแร่ธาตุในสะระแหน่

ชื่อผู้เขียน นางสาวธิมาภรณ์ ศรีรัตนกุล

สาขาวิชา อาหารสุขภาพและโภชนาการ

ปีการศึกษา 2558

บทคัดย่อ

ภาวการณ์ขาดธาตุอาหารรองโดยเฉพาะธาตุสังกะสีเป็นปัญหาที่ยังคงรุนแรงในหลายประเทศ รายงานขององค์การอนามัยโลก ตั้งแต่ พ.ศ. 2544 ระบุว่าการขาดธาตุสังกะสีเป็นสาเหตุสำคัญ 1 ใน 5 ของการตายในประเทศกำลังพัฒนาที่ต้องบริโภคพืชและผักเป็นหลัก แต่มีแหล่งเพาะปลูกที่ไม่อุดมสมบูรณ์ มีรายงานว่าพืชที่ขาดธาตุสังกะสีจะมีอัตราการเจริญเติบโต ผลผลิตและคุณภาพที่ต่ำลง แม้ว่าประเทศไทยถูกจัดให้มีความหลากหลายทางชีวภาพของพืชในอันดับต้นๆของโลก แต่ผลจากการรวบรวมข้อมูลของ Black และคณะ (2551) ระบุว่าคนไทยมีภาวะเสี่ยงต่อการขาดธาตุสังกะสีในระดับปานกลาง จึงมีความเป็นไปได้ว่าพืชผักของไทยอาจมีปริมาณธาตุสังกะสีที่ต่ำไป ดังนั้นในการทดลองในครั้งนี้สะระแหน่สายพันธุ์ *Mentha cordifolia* Opiz. ซึ่งเป็นผักสดที่ได้รับความนิยมบริโภคใน 5 อันดับแรกจึงถูกเลือกมาเป็นกรณีศึกษา ผลการเสริมธาตุสังกะสีในรูปของซิงค์ซัลเฟตที่ระดับความเข้มข้น 0, 100 และ 200 พีพีเอ็ม ลงในดินที่ใช้ปลูกต่อลักษณะการเจริญเติบโต องค์ประกอบทางเคมี สารพฤกษเคมี การสะสมน้ำมันหอมระเหย การยอมรับทางประสาทสัมผัสของผู้บริโภคทางด้านสี กลิ่น กลิ่นรส รสชาติ ความชอบโดยรวม ปริมาณฟีนอลิก ปริมาณฟลาโวนอยด์ ฤทธิ์การต้านอนุมูลอิสระในหลอดทดลอง 3 วิธี ได้แก่ DPPH, ABTS, FRAP และฤทธิ์การต้านแบคทีเรีย ผลการศึกษาพบว่าการเสริมธาตุสังกะสีในระหว่างการปลูกสะระแหน่สามารถเพิ่มคุณค่าทางโภชนาการ สารออกฤทธิ์ทางชีวภาพและฤทธิ์การต้านอนุมูลอิสระสูงกว่าสะระแหน่ที่ไม่ได้รับการเสริมธาตุสังกะสี โดยการเสริมธาตุสังกะสีลงในดินที่ระดับความเข้มข้น 100 พีพีเอ็ม สามารถเพิ่มปริมาณโปรตีน ปริมาณคลอโรฟิลล์และปริมาณสารประกอบฟีนอลิกสูงที่สุดอย่างมีนัยสำคัญ ($p < 0.05$) ในขณะที่ความเข้มข้น 200 พีพีเอ็ม

มีการเพิ่มเพิ่มขึ้นของลักษณะการเจริญเติบโตในส่วนของใบ ลำต้น ราก รวมถึงผลผลิต การสะสมน้ำมันหอมระเหย ปริมาณสารประกอบฟลาโวนอยด์ และฤทธิ์การต้านอนุมูลอิสระอย่างมีนัยสำคัญ ($p < 0.05$) เมื่อเปรียบเทียบกับตัวอย่างอื่น ผลการตรวจสอบจุลินทรีย์ก่อโรคในอาหารพบว่ามีค่าไม่เกินมาตราฐานกรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุขในทุกชุดการทดลอง ผู้ทดสอบชิมให้คะแนนความชอบด้านกลิ่นและรสชาติในสะระแหน่ที่ได้รับการเสริมธาตุสังกะสีสูงกว่าชุดควบคุม การเสริมธาตุสังกะสีที่ระดับความเข้มข้น 100 พีพีเอ็ม ส่งผลให้ผู้ทดสอบชิมระบุว่าพืชมีกลิ่นและรสชาติที่ชัดเจนของความเป็นสะระแหน่ นอกจากนี้พบว่าในสารสกัดหยาบสะระแหน่ที่ได้รับการเสริมธาตุสังกะสีมีสารประกอบฟีนอลิกและฟลาโวนอยด์ ได้แก่ กรดโปรโตคาเทคซูอิค กรดซินามิก คาเทชิน รูติน และเควอซิทิน สูงกว่าชุดควบคุม อย่างไรก็ตามพบว่าสารสกัดหยาบสะระแหน่ด้วยแอลกอฮอล์เข้มข้นร้อยละ 95 ทั้งที่มีการเสริมและไม่เสริมธาตุสังกะสีไม่แสดงฤทธิ์การต้านแบคทีเรีย

การเพิ่มขึ้นของสารอาหารและสารออกฤทธิ์ทางชีวภาพสามารถกระตุ้นได้ด้วยการเสริมธาตุสังกะสีให้กับสะระแหน่ โดยการเสริมธาตุสังกะสีในรูปของซิงค์ซัลเฟตลงในดินระหว่างเพาะปลูกสะระแหน่ที่ระดับความเข้มข้น 100 พีพีเอ็ม เหมาะสมกับการผลิตพืชเพื่อการบริโภค ขณะที่ 200 พีพีเอ็ม เหมาะที่จะใช้ปลูกพืชเพื่อนำไปประยุกต์ใช้ในเชิงอุตสาหกรรมที่เกี่ยวข้องกับการผลิตหรือใช้ประโยชน์จากน้ำมันหอมระเหย ซึ่งเป็นอีกทางเลือกหนึ่งของการเพิ่มมูลค่าให้สะระแหน่ในอนาคต

Thesis Title Effect of $ZnSO_4$ fortification in soil on growth characteristics, phytochemicals and mineral compositions in *Mentha cordifolia* Opiz., kitchen mint.
Author Miss Timaporn Srirattanakul
Major Program Functional Food and Nutrition
Academic Year 2015

ABSTRACT

Micronutrient deficiency status especially zinc still seriously influences in many countries. Since 2002, World Health Organization reported that zinc deficiency is among the top five causes of death in many developing countries where people rely on consumption of plants and vegetables that are grown in low fertilized soil. There was a report showed that zinc deficient plant had low growth, less yield and poor quality. Eventhough, Thailand has a high diversity of plants, documentation review of Black *et al*. (2008) reported Thailand to be a moderate zinc deficiency region. This is possibly pointed out that vegetable foods consuming in Thailand contain insufficient zinc level which may relate to a low level of zinc in plantation soil. Therefore, kitchen mint *Mentha cordifolia* Opiz. which is among top 5 of fresh vegetables consumed in Thailand was selected for case study in the topic of the effect fortified zinc sulfate at concentration 0, 100 and 200 ppm into soil during plantation on growth characteristic, chemicals composition, phytochemical, oil droplet and sensory acceptability. In addition, total extractable phenolic compounds, total extractable flavonoid compounds, 3 vitro antioxidant activities determined by DPPH, ABTS and FRAP as well as antibacterial activities were monitored. The results showed that the soil fortified with $ZnSO_4$ during plantation significantly increased nutrition values, bioactive compounds and antioxidant activity in the mint compared with control sample (not fortified $ZnSO_4$). Fortification of 100 ppm $ZnSO_4$ into the soil increased protein content, chlorophyll content and total extractable phenolic compounds in the mint leave with a highest value ($p < 0.05$). While fortification 200 ppm $ZnSO_4$ into the soil significantly increased growth characteristic including leaves, stalk, root and yield, oil droplet, total extractable flavonoid compounds as well

as antioxidant activity ($p < 0.05$) compared with other samples. It was found that all mint leaf samples were absence from any pathogenic bacteria following regulation of the Department of Medical Sciences, Ministry of Public Health. In addition, the panelists also gave the odor and taste scores of the mint leaves higher than control sample. Moreover, the mint grown in the soil with fortified $ZnSO_4$ at concentration 100 ppm had a stronger odor and typical nature taste of kitchen mint. The result also showed that total extractable phenolic compounds and total extractable flavonoid compounds as protocatechuic acid, cinamic acid, catechin, rutin and quercetin in the crude extracts increased in the mint grown in the soil with fortified $ZnSO_4$ compared with the control. However, there was no antibacterial property showed in any crude mint extracted with 95% ethanol neither with nor without fortified $ZnSO_4$.

Increasing of nutrient and bioactive compounds in the mint leaves was stimulated by $ZnSO_4$ fortification in soil during plantation. Fortification 100 ppm $ZnSO_4$ was the great choice for fresh consumption based on sensory score; whereas, the plants grown in soil fortified with 200 ppm $ZnSO_4$ may be suitable for essential oil production which can be alternatively used for value added kitchen mint in the future.

ACKNOWLEDGEMENTS

I would like to express my enormous thanks and deep appreciation to my advisor, Assistant Professor Dr. Sunisa Siripongvutikorn for her keen interest on me at every stage of my research, technical suggestions with kindness. She taught valuable lessons including scientific approach, personal life as well as devotion to duty and financial support.

I also would like to thank my profound gratitude to my co-advisor, Dr. Chutha Takahashi Yupanqui for her kindness and helpful guidance throughout my study period.

During conducted field research, I am also thankful to Dr. Sureeporn Kangsanant, Dr. Tatchaporn Chaijareon, Ms. Suwanaporn Boonpeng and Mr. Piyabundh Seetapong for their field work assistant. Unforgettably, I wish to thank my friends for their kindness in fishery laboratory and functional food laboratory.

I also wish to express my thankfulness to all members of Nutraceutical and Functional Food laboratory, Fishery laboratory and Product development laboratory, Faculty of Agro-Industry, Prince of Songkla University, for their help, discussion and cheerfulness.

Lastly, and most of all, I pay my deepest gratitude to my family, including my grandmother, my mother and my father for their financial and moral support. They encouraged me a lot during the hard times. I am also thankful to my sister and my friends, who encouraged and listened to me when I had problem and disappointment.

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LIST OF ABBREVIATIONS

s	=	Second
h	=	Hour
mo	=	Mouth
min	=	Minute
kg	=	Kilogram
g	=	Gram
ml	=	Milliliter
µl	=	Microliter
ppm	=	Part per million
cm	=	Centimeter
mm	=	Millimeter
mM	=	Milli molar
µM	=	Micro molar
µm	=	Micro metre
N	=	Normality
w/v	=	Weight/Volume
v/v	=	Volume/ Volume
nm	=	Nanometer

LIST OF ABBREVIATIONS (CONTINUED)

CFU/ml	=	Colony-forming units per milliliter
MPN	=	Most probable number
am	=	Ante meridiem
pm	=	Post meridiem
°C	=	degree Celsius
WHO	=	World Health Organization
FAO	=	Food and Agriculture Organization
CIAT	=	International Center for Tropical Agriculture
IFPRI	=	International Food Policy Research Institute
IZA	=	International Zinc Association
M.	=	*Mentha*
Zn	=	Zinc
$ZnSO_4$	=	Zinc sulfate
RDAs	=	Recommended dietary allowances
MT	=	Metallothionein
NCD	=	Noncommunicable diseases
IAA	=	Indole-3-acetic acid
GA	=	Gibberellins
ABA	=	Abscisic acid

LIST OF ABBREVIATIONS (CONTINUED)

GPX	=	Glutathione peroxidase
H^+	=	Hydrogen donor
ROS	=	Reactive oxygen species
SOD	=	Superoxide dismutase
GSH	=	Glutathione
SH	=	Sulfhydryl groups
$^{\bullet}OH$	=	Hydroxyl radical
H_2O_2	=	Hydrogen Peroxide
NOX	=	NADPH oxidase
$NADPH_2$	=	Nicotinamide adenine dinucleotide hydrogen phosphate
iNOS	=	Nitric acid synthase
EOs	=	Essential oil
Hz	=	Hertz
β-	=	Beta
α-	=	Alpha
γ-	=	Gamma

Chapter 1

Introduction and review of literature

1.1 Introduction

Zinc is an essential nutrient for human life because it helps improving the immune system, increase normal growth and reduce mortality due to zinc deficiency. Zinc deficiency is among the top five risk factors of disease and death in developing countries (WHO, 2002). Though, children need Zn for growth and adults need it for normal health, this element also supports for normal growth and development from fetus, throughout adolescence (Maret and Sandstead, 2006). In addition, all DNA synthesis, neurosensory functions, diarrhea and cell-mediated immunity required Zn (Prasad *et al.*, 1992). The major source of Zn is addressed in seafood followed by red meats, cereals, dairy products and leafy vegetables (Murphy *et al.*, 1975). However, from literature review, it was found that many plants may face with zinc deficiency when low zinc or alkaline or even high acidic soil was used for planting material (Alloway, 2008). Zinc deficiency plants had poor integrity and growth performance and were low in yield and quality. On the other hand, the growth, chlorophyll, dry matter yield and essential oil production significantly increased when mint was grown in the soil supplemented with zinc (Al Ahl and Mahmoud, 2010).

Mentha cordifolia Opiz. (Family Lamiaceae) has the common name as kitchen or marsh mint. It is among the top seven vegetables consumed in Thailand, either fresh, raw, mildly cooked food or in herbal tea (Tongkhao and Mahakarnchanakul, 2013). In fact, this plant is widely grown not only in Thailand but also throughout Southeast Asian countries (Stanisavljevic *et al.*, 2012). Thai people use kitchen mint as traditional medicine to relieve gastrointestinal problem, asthma, muscle spasm and inflammation. Some scientific data have shown the beneficial effects of *M. cordifolia* including antimutagenicity (Villasenor *et al.*, 2002), analgesic, anti-nociceptive (Villasenor and Sanchez, 2009), anti-inflammatory, antioxidant activities (Poungrat *et al.*, 2010), helping to relieve colds, flu, fever, motion sickness and poor digestion problems (Nantawan and Weibiao, 2009). Phytochemicals in vegetables and herbs

have been interested mainly in preventing or delaying chronic diseases caused by oxidative stress. Ferruzzi and Blakeslee (2007) reported that therapeutic properties of chlorophyll can be summarized as being of benefit against sinusitis and skin rashes, blood purification, intestines cleaning and liver detoxification.

World Health Organization (WHO, 2002) reported that Thai people are classified as moderate zinc deficiency group even Thailand is a tropical country with a high diversity of vegetables or plants. There is hypothesized that zinc content in the soil related to agricultural products may not high enough to meet standard requirement. Al Ahl and Mahmoud (2010) reported that zinc supplementation in the soil can significantly increase the growth, dry matter yield and essential oil production in the mint. Therefore, the effect of fortified zinc sulfate in the soil on growth characteristic, phytochemical, total extractable phenolic compounds, total extractable flavonoid compounds, bioactive compounds, antioxidant and antibacterial activities as well as sensory acceptability of *Mentha cordifolia Opiz* were evaluated.

1.2 Review of literature

1.2.1 Zinc deficiency problem

Nowadays, chronic disease is the biggest events of death in the world. The leader of chronic diseases included cardiovascular disease, cancer, chronic respiratory diseases and diabetes (WHO/FAO 2003). In the studied of Yach *et al.* (1982) who reported that the number of people with mortality due to chronic disease is expected to increase continually because of consuming a high calories with low nutrients or low quality of nutrition and less fiber (Blakely *et al.*, 2012), as well as having less physical activities and staying among stress condition such as busy life, low income and high expenses. In addition, vitamin and mineral deficiencies contribute to growth retardation, morbidity, mortality, brain damage and cognitive reduction as well as develop to several diseases (FAO/WHO, 2004) when people do not consume enough of the specific types of vitamin and mineral for a period of time. As known that all trace minerals promote overall health, growth and development and help various chemical reactions able to take place in the body. The four main vitamin and mineral deficiencies of public health significance include iron deficiency, vitamin

A deficiency, iodine deficiency and zinc deficiency (WHO, 2006). Recently, zinc deficiencies likely have a large effect on the global burden of diseases (Figure 1) (Kumssa *et al*., 2015).

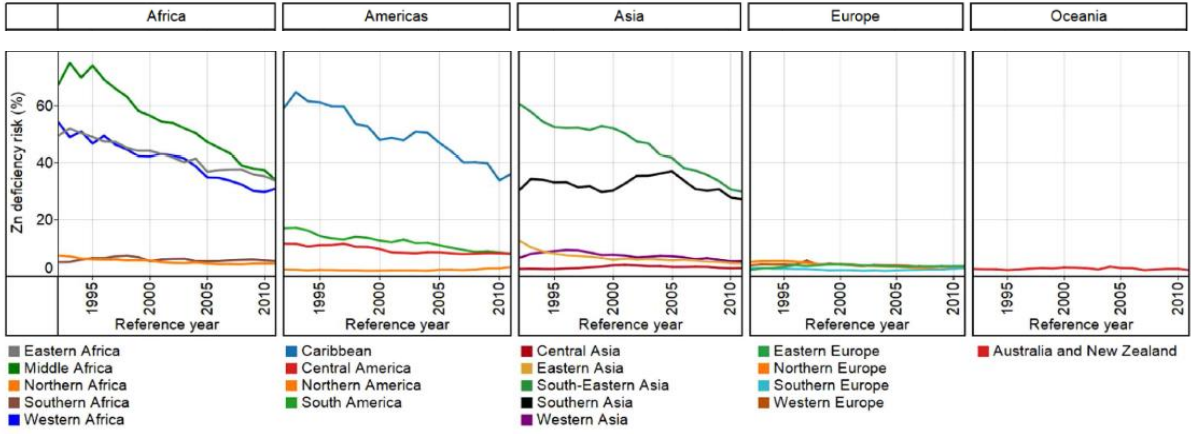

Figure 1. Regional temporal trends in population weighted mean zinc deficiency risk (%) between 1992 and 2011

Source: Kumssa *et al*. (2015)

World Health Organization (WHO, 2002) reported that Thai people are grouped of moderate zinc deficiency and the latest of Thai national nutrition survey also reported the highest prevalence of stunting, a potential sign of Zn deficiency in children found in the southern region of Thailand (Pinkaew *et al*., 2013).

1.2.2 Important of zinc for human life

1.2.2.1 Role of zinc in human

Zinc, an essential trace element, is essential for various metabolisms in all biological objects (Roohani *et al*., 2013). Under physiologic condition, Zn is relatively nontoxic, in addition zinc is classified as an ideal element to participate in catalytic, structural and cellular regulatory functions (Mccall *et al*., 2000). More than 100 specific enzymes from all six enzyme-classes including oxido-reductases, transferases, hydrolases, lysases, isomerases, and ligases such as RNA polymerase, alcohol dehydrogenase, carbonic anhydrase, and alkaline phosphatase require zinc element for their catalytic functions (Russell *et al*., 2002; Broadley *et al*.,

2007). Zinc may serve activity to these enzymes as an electron acceptor and facilitates the folding of proteins into three-dimensional configurations, thus enabling their biologic activity. Finger-like motifs of zinc is the folding form of zinc chelation with the amino acids including cysteine and histidine (Townsend *et al*., 2003). Whenever, zinc is removed from the catalytic site, activity of enzyme is lost. In addition, various processes are regulated by zinc including expression of the metallothionein gene, apoptosis (or programmed cell death) and synaptic signaling (Maret *et al*., 2003).

1.2.2.2 Zinc food sources

A wide variety of foods contain zinc particularly oysters (Brown *et al*., 2001). In addition, beans, nuts and certain types of seafood such as crab and lobster, whole grains, fortified breakfast cereals, green leafy vegetables and dairy products also provide fair zinc content (Rink and Gabriel, 2000) as shown in Table 1.

1.2.2.3 Recommended intake of zinc

Intake recommendation for zinc value can be modified by age, sex and eating habit as shown in Table 2.

1.2.2.4 Zinc absorption

Zinc is released from food as free ions during digestion. This ion may then bind to ligands in the intestinal lumen before be absorbed by transcellular in the small intestine. Zinc is transported into the enterocyte by ZnT4 which is member of ZRT, IRT-like protein (ZIP) family (Liuzzi and Cousins, 2004). Thereafter, Zn will be absorbed by metallothionein in intestine cell and be circulated to other organs (Tuerk and Fazel, 2009).

1.2.2.5 Zinc deficiency

Deficiency of zinc is a critical nutritional effect and health problem around the world (CIAT/IFPRI, 2002). In addition, zinc deficiency often occurs from consuming less zinc content foods. Zinc deficiency syndromes relate to metabolic or genetic malfunctions causing infection, immune disorder, growth retardation, poor

pregnancy, oligospermia, diarrhea, skin problem, taste abnormalities, emotional disorders, hyperammonaemia and illness as well as death (Salgueiro *et al*., 2001).

Table1. Zinc content in some selected food sources

Food Source	**Milligrams (mg / serving)**	**Percent DV***
Oysters, cooked, breaded and fried, 85.05 g (3 ounces)	74.0	493
Beef chuck roast, braised, 85.05 g (3 ounces)	6.0	47
Crab, Alaska king, cooked, 85.05 g (3 ounces)	6.5	43
Beef patty, broiled, 85.05 g (3 ounces)	5.3	35
Breakfast cereal, fortified with 25% of the DV for zinc, 26 g (¾ cup serving)	3.8	25
Lobster, cooked, 85.05 g (3 ounces)	3.4	23
Pork chop, loin, cooked, 85.05 g (3 ounces)	2.9	19
Baked beans, canned, plain or vegetarian, 127 g (½ cup)	2.9	19
Chicken, dark meat, cooked, 85.05 g (3 ounces)	2.4	16
Yogurt, fruit, low fat, 226.8 g (8 ounces)	1.7	11
Milk, low-fat or nonfat, 244 g (1 cup)	1.0	7
Almonds, dry roasted, 28.35 g (1 ounces)	0.9	6
Peas, green, frozen, cooked, 80 g (½ cup)	0.5	3
Flounder or sole, cooked, 85.05 g (3 ounces)	0.3	2

***DV** = Daily Value. DVs were developed by the U.S. Food and Drug Administration to help consumers compare the nutrient contents of products within the context of a total diet. The DV for zinc is 15 mg for adults and children age 4 and older. Food labels, however, are not required to list zinc content unless a food has been fortified with this nutrient. Foods providing 20% or more of the DV are considered to be high sources of a nutrient.

Source: Rink and Gabriel (2000)

Table 2. Recommended dietary allowances (RDAs) for zinc for human body

Age	Sex	Reference body weight (kg)	Upper intake levels for zinc (mg/day)	Mixed or refined vegetarian diets (mg/day)	Unrefined cereal based diets (mg/day)
0-6 months	M+F	-	4	-	-
6-11 months	M+F	9	5	4	5
1-3 years	M+F	12	7	3	3
4-8 years	M+F	21	12	4	5
9-13 years	M+F	38	23	6	9
14-18 years	M	64	34	10	14
14-18 years	F	56	34	9	11
Pregnancy (14-18 years)	F	-	34	11	15
Lactation (14-18 years)	F	-	34	10	11
> 19 years	M	65	40	13	19
> 19 years	F	55	40	8	9
Pregnancy (> 19 years)	F	-	40	10	13
Lactation (> 19 years)	F	-	40	9	10

Remark: M = male, F = female

Source: Hotz and Brown (2004)

1.2.2.6 Zinc toxicity

Individuals may be exposed to high intakes of zinc, either through supplemental zinc or by contact with high environmental zinc. Toxicity symptoms of zinc overload include nausea, vomiting, epigastric pain, diarrhea, lethargy and fatigue (Tuerk and Fazel, 2009). For example, approximately, 225–450 mg of zinc is known to produce immediate vomiting in adults. In addition, short-term exposure to very high levels of contaminant zinc (> 300 ppm) caused acute gastroenteritis. Chronic overdosage of zinc, in the range of 100-300 mg zinc/day for adults, may induce copper deficiency (Walker and Black, 2004) and alter the immune response and serum lipoprotein levels (Fosmire, 1990). Toxicity of zinc also can increase the synthesis of retinyl ester hydrolase and acyl CoA: retinol *O*-acyl-transferase activities, a critical step in the metabolic pathway of vitamin A that is well-established in the visual cycle in the retina of the eye (Clugston and Blaner, 2014).

1.2.2.7 Zinc and antioxidant

Zinc acts as an antioxidant facilitator by different mechanisms. First, iron (Fe) and copper (Cu) ions are inactivated by zinc competition for binding to cell membranes and proteins, displacing these redox active metals, which catalyze the production of hydroxyl radical ($^{\bullet}OH$) from hydrogen peroxide (H_2O_2). Second, zinc protects oxidation process in bio-molecules by binding to their sulfhydryl groups (SH) (Powell, 2000). Third, zinc has been proven to increasing of antioxidant protein, molecules, and enzymes including glutathione (GSH), catalase, and superoxide dismutase (SOD) but inhibits the activities of oxidant-promoting enzymes such as inducible nitric acid synthase (iNOS) and NADPH oxidase (NOXes) and reducing the generation of lipid peroxidation products (Bao *et al*., 2013). Fourth, Zn improves the expression of a metal-binding protein named metallothionein (MT) which is very rich in cysteine and is an excellent excavanger of $^{\bullet}OH$ ions (Prasad, 2014).

1.2.3 Important of zinc for plant

1.2.3.1 Zinc absorption in plants

Zn as available form of Zn^{2+} is primarily taken up from the soil by plants. Zn^{2+} in the soil soluble is up taken into plant root surface by passive transport and inside the root cell by active transports by the carrier site. However, during Zn^{2+} is absorbed, there are several minerals or metals also be taken up. A schematic of Zn uptake and transport of heavy metals in plants through metal transporters was shown in Figure 2.

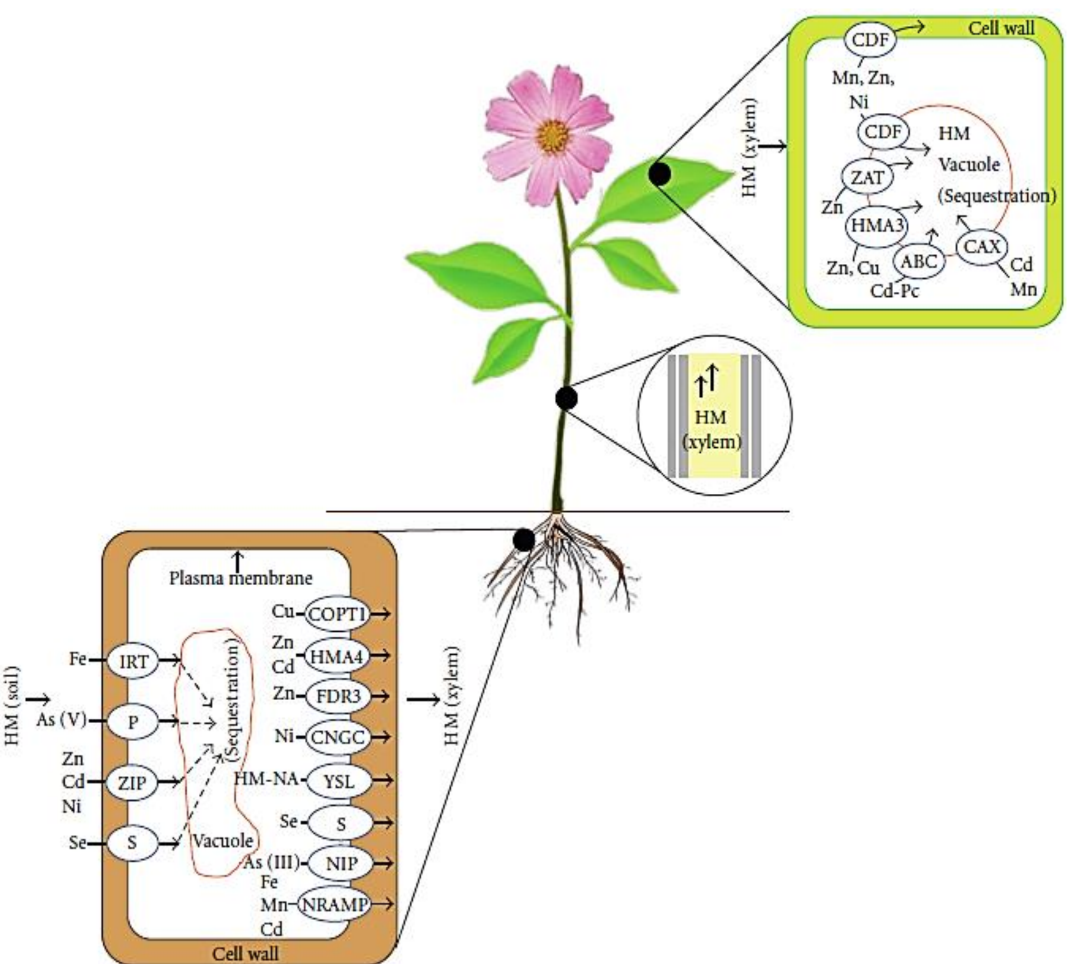

Figure 2. A schematic of Zn uptake and transport of heavy metals in plants through metal transporters

Source: Hossain *et al*. (2012)

1.2.3.2 Zinc deficiency

Zinc deficiency in plants is ubiquitous and normally occurs in a wide range of growth conditions including cold and warm climates, drained and flooded area, and in acid and alkaline soils (Guerinot and Eide, 1999). Lindsay (1972) listed eight major factors affected to zinc availability and likely to be related to deficiency as shown in Figure 3 and can be occurred at different degree of severity and combinations. Severe Zn deficiency symptoms include impaired stem elongation leading to stunting of plants, root apex necrosis, heterogeneous or interveinal chlorosis, necrotic spots on leaves, bronzing of leaves, rosetting of leaves, leaf dwarfing, malformed leaves, development of reddish-brown or bronze tints, and inward curling of leaf lamina (Alloway, 2008; IZA, 2015). Zinc deficiency also leads to biochemical and histological changes in plants including synthesis of impaired protein, increasing of peroxidase activity, and declining of auxin level (Alloway, 2008).

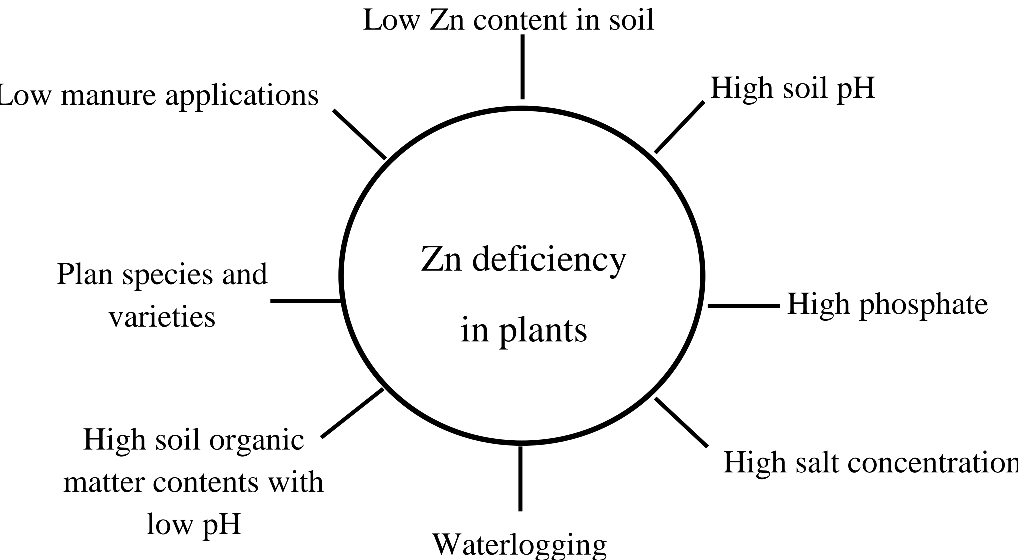

Figure 3. Schematic diagram of the causes of zinc deficiency in crops
Source: Lindsay (1972)

1.2.3.3 Zinc toxicity

The threshold of Zn toxicity varies among plant species, time of exposure to Zn stress and composition of the nutrient growth medium. Photosynthesis is strongly influenced in plants when exposed to excess heavy metals. High Zn concentrations can cause negative effect and phytotoxicity in plants. The level of zinc in mint leaves should be above 20 ppm to avoid zinc deficiency (Heap, 2005).

Generally, plants have their own process to normalize the stress of zinc either deficiency or overload manners (Reichman, 2002). When zinc is fortified into the plants during growing, some bioactive compounds such as phenolics, chlorophyll and essential oils have been reported to generate or release (Sangwan *et al*., 2001; Sakihama *et al*., 2002) to regulate zinc overload (Michalak, 2006). Besides, some scientific data stated the protecting zinc toxicity by an increasing of some phytochemicals which relate to antioxidant, anti-inflammatory and anticancer properties (Rahman, 2007; Brambilla *et al*., 2008).

1.2.3.4 Zinc biofortification in plant

Recently, zinc deficiency is the most common trace element problem. Though, taking commercial zinc supplements are generally recommended (WHO, 2001), the supplement can also be easily toxic to human body when large amount of Zn is consumed. In addition, zinc supplements are produced in various forms which are associated with various technical problems such as improper selection of matrix, the unbalancing of inhibitors and enhancer, supplement stability, acceptability and high price (Hurrell, 1997; Singh *et al*., 2016). Thus, it is believed that zinc obtaining from food is more safe and beneficial for consumption as well as a great perception of taking its as food not medicine forms. Therefore, to increases zinc content, biofortification is a great arrangement of the zinc deficiency solving.

Actually, different zinc compounds used as fertilizers were depicted in Table 3. However, $ZnSO_4$ is the most used form of biofortification because of great solubility and low-price (IZA, 2015). Fortification of $ZnSO_4$ into soil have been reported in many plants including *Lepidium Sativum* L. (Saba *et al*., 2000), citrus fruit (Tagliavini *et al*., 2001), Gladiolus (Lahijie, 2014), rice (Boonchuay *et al*., 2013),

wheat (Velu *et al.*, 2014), canola (Malakouti, 2015). However, there is no scientific information of using Zn biofortification in Thai herbs yet.

Table 3. Zinc fertilizers and typical zinc contents

Compound	Formula	Zinc content (%)
Zinc sulphate monohydrate	$ZnSO_4 \cdot H_2O$	36
Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	22
Zinc oxysulphate	$ZnO \cdot ZnSO_4$	20-50
Zinc oxide	ZnO	50-80
Zinc carbonate	ZnCO3	50-56
Zinc phophate	$Zn_3(PO_4)_2$	50
Zinc nitrate	$Zn(NO_3)_2 \cdot 3H_2O$	23
Sodium zinc EDTA	NaZnEDTA	9-13
Sodium zinc HEDTA	NaZnHEDTA	6-10

Source: IZA (2015)

1.2.3.5 Meaning of biofortification

Fortification is the technique of deliberately increasing the content of an essential micronutrient, including vitamins and minerals in foods so as to improve the nutritional quality and provide a public health benefit with minimal risk to health. Biofortification is the process aimed to increase nutrient levels in crops during plant growth rather than through manual means during processing which may be different to implement and regulate (WHO, 2015).

1.2.4 *Mentha cordifolia* Opiz. (Kitchen mint)

1.2.4.4 Kitchen mint

Mentha cordifolia Opiz. ex Fresen belongs to family Labiatae (mint family), genus *Melissa*. Its common name is kitchen mint, marsh mint and in Thai name called as Sa-ra-nae-suan, Ton-namman-mong, Hom-duan, Sa-nae (Figure 4). The commonly used part of this plant is leaf.

Figure 4. Kitchen mint

1.2.4.5 Basic nutrition

Nutritional value of edible portion of peppermint (*Mentha piperata L.*) (Institute of Nutrtion, Mahidol University, 1998) and spearmint (*Mentha spicata*) (Padmini *et al*. 2010; Sulieman *et al*. 2011) were depicted in Table 4.

Table 4. Nutrient value of fresh mint

Nutrient		Unit
Proximate		
Energy	47*	Calorie/100 g fresh wt.
Protein	3.7*, 1.75***	g/100 g fresh wt.
Fat	0.6*, 2.20***	g/100 g fresh wt.
Carbohydrate	6.8*, 10.39***	g/100 g fresh wt.
Fiber	1.2*, 6.2***	g/100 g fresh wt.
Ash	3.48***	g/100 g fresh wt.
Moisture	76.01***	g/100 g fresh wt.

Table 4. Nutrient value of fresh mint (continued)

Nutrient		Unit
Minerals		
Calcium	400*, 255.95**, 130***	ppm
Magnesium	3.90**	ppm
Sodium	147.57**, 72***	ppm
Potassium	15.56**, 240***	ppm
Iron	48*, 2.03**, 20.5***	ppm
Phosphorus	70*	ppm
Copper	0.88**	ppm
Selenium	0.26**	ppm
Chromium	0.19**	ppm
Cobalt	0.25**	ppm
Zinc	0.79**	ppm
Vitamins		
Vitamin B1	0.13*	mg/100 g fresh wt.
Vitamin B2	0.29*	mg/100 g fresh wt.
Vitamin B3	0.7*	mg/100 g fresh wt.
Vitamin C	88*	mg/100 g fresh wt.
Beta-carotene	538.35*	mg/100 g fresh wt.

Source: * Peppermint , Institute of Nutrition, Mahidol University (1998)

** Spearmint , Padmini *et al*. (2010)

*** Spearmint , Sulieman *et al*. (2011)

1.2.4.6 Bioactive compounds of kitchen mint

1.2.4.6.1Essential oil

For more than thousand years, plants and their derivatives including essential oils (EOs) have been reported in folk medicine for inhibiting or retarding the growth of pathogen including bacterial, yeast and mold (Burt and Reinders, 2003). In nature, EOs play various important roles including protecting agent, attracting matter and repelling compounds (Nazzaro *et al.*, 2013). The identified compounds by GC-MS in the extracts of *Mentha spicata* (Padmini *et al.*, 2010), *Mentha piperita* (Vienna *et al.*, 2005, Sokovic *et al.*, 2009), *Mentha cordifolia* (Chopra, 1964, Zhou, Chou, 1995 and Tibaldi *et al.*, 2012) were shown in Table 5. Chemical structures of the major constituents of mint oil were dipited as Figure 5.

Table 5. Some chemical compounds found in mint oil plants

Chemical constituents	*Mentha spicata*	*Mentha piperita*	*Mentha cordifolia*
Menthone	√ [a]	√ [c, d]	√ [e]
Isomenthone	√ [a]	√ [c]	-
Cyclodecadiene	√ [a]	-	-
Dodecatriene	√ [a]	-	-
n-decanoic acid	√ [a]	-	-
3,7,11,15-tetramethyl-2-hexadecenol	√ [a]	-	-
Phytol	√ [a]	-	-
Octadecanol	√ [a]	-	-
1,2-benzenedicarboxylic acid	√ [a]	-	-
Menthol	-	√ [c, d]	-
Methyl acetate	-	√ [c, d]	√ [e]
Menthofuran	-	√ [c]	-
Cineole	√ [b]	√ [c]	√[b]
Limonene	√ [c]	√ [c]	√ [b, e]

Table 5. Some chemicals found in mint oil plants (continued)

Chemical constituents	*Mentha spicata*	*Mentha piperita*	*Mentha cordifolia*
Ocimene	-	-	√ [e, f]
Carvone	√ [c]	√ [c, d]	√ [b, e, f]
L-menthol	-	-	√ [e, f]
Cadinene	-	-	√ [f]
P-cymene	-	-	√ [e, f]
Neral	-	-	√ [e, f]
Piperitenone oxide	√ [b]	-	√ [b, f]
Trans-piperitenone oxide	-	-	√ [b, f]
Piperitol	-	-	√ [e, f]
α-pinene	-	-	√ [e, f]
Dihydrocarveol acetate	-	-	√ [f]
Termolene	-	-	√ [f]
Pulegone	√ [c]	-	-
Trans-carveol	√ [c]	-	√ [g]
Trans-carvyl acetate	√ [c]	-	√ [g]
Coumarin	√ [c]	-	√ [e]
Dihydrocarvone	√ [c]	-	√ [e, g]
β-pinene	√ [c]	-	√ [b, e, f]

Remark: √ = found

- = not found

Source: [a] Padmini *et al*. (2010), [b] Sharopov *et al*. (2012), [c] Vienna *et al*. (2005), [d] Sokovic *et al*. (2009), [e] Tibaldi *et al*. (2012), [f] Chopra (1964) and [g] Zhou and Chou (1995)

Carveol Benzenemethanol β-bourbonene

Phytol

Piperitone Eugenol Menthol

Figure 5. Structures of major constituents of mint oil from various different species
Source: Kumar *et al.* (2011)

Mint contains numerous volatile compounds and other substances which have been reported to provide a potent of antioxidant, antibacterial, antiviral, antimicrobial, and antifungal properties (Burt, 2004; Kordali *et al.*, 2005; Kumar *et al.*, 2011). Essential oil of mint was reported to use in cancer treatment (Sylvestre *et al.*, 2006), food preservation and aromatherapy (Prabuseenivasan *et al.*, 2006; Kumar *et al.*, 2011) as well as general antimicrobial activity. Menthol, a secondary alcohol producing from peppermint has been shown to have antiseptic, analgesic, and cooling effects (Carretero, 2002). In addition, many secondary metabolites found in essential oil related to plantation conditions. Some researcher mentioned that giving excess water at an appropriate time increased more oil yield. Numerous studies reported that optimum oil yield obtained from the highest levels of irrigation (Mitchell *et al.*, 1992; Mitchell *et al.*, 1993; Westcott, 1995). However,

there were some documents addressed that excess of irrigation exhibited lower mint oil yields (Mitchell *et al*., 1993).

Phytochemicals are generally found in all fruits and vegetables whether yellow, orange, red, green, white, and purple which are classified as carotenoids, phenolic, alkaloids, nitrogen-containing compounds and organ sulfur compounds (Figure 6). However, the most studied of the phytochemicals are focused on the phenolics and carotenoids (Liu, 2004).

1.2.4.6.2Phenolics

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and generally are categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Figure 6). Phenolics are the products of secondary metabolism found in plants, involving essential functions in the reproduction and the growth of the plants; acting as defense mechanisms against pathogens, parasites, and predators, as well as protecting from oxidative damage (Dillard and German, 2000). Therefore, beside to their roles in plants, phenolic compounds have been claimed to provide human health benefits associated with reducing risk of some chronic diseases. Inaddition, it was found that proline biosynthesis is coupled to pentose-phosphate pathway, driving the synthesis of $NADPH_2$ and sugar phosphates for anabolic pathways, including phenolic and antioxidant response pathways (Shetty and Wahlqvist, 2004) as shown in the Figure 7.

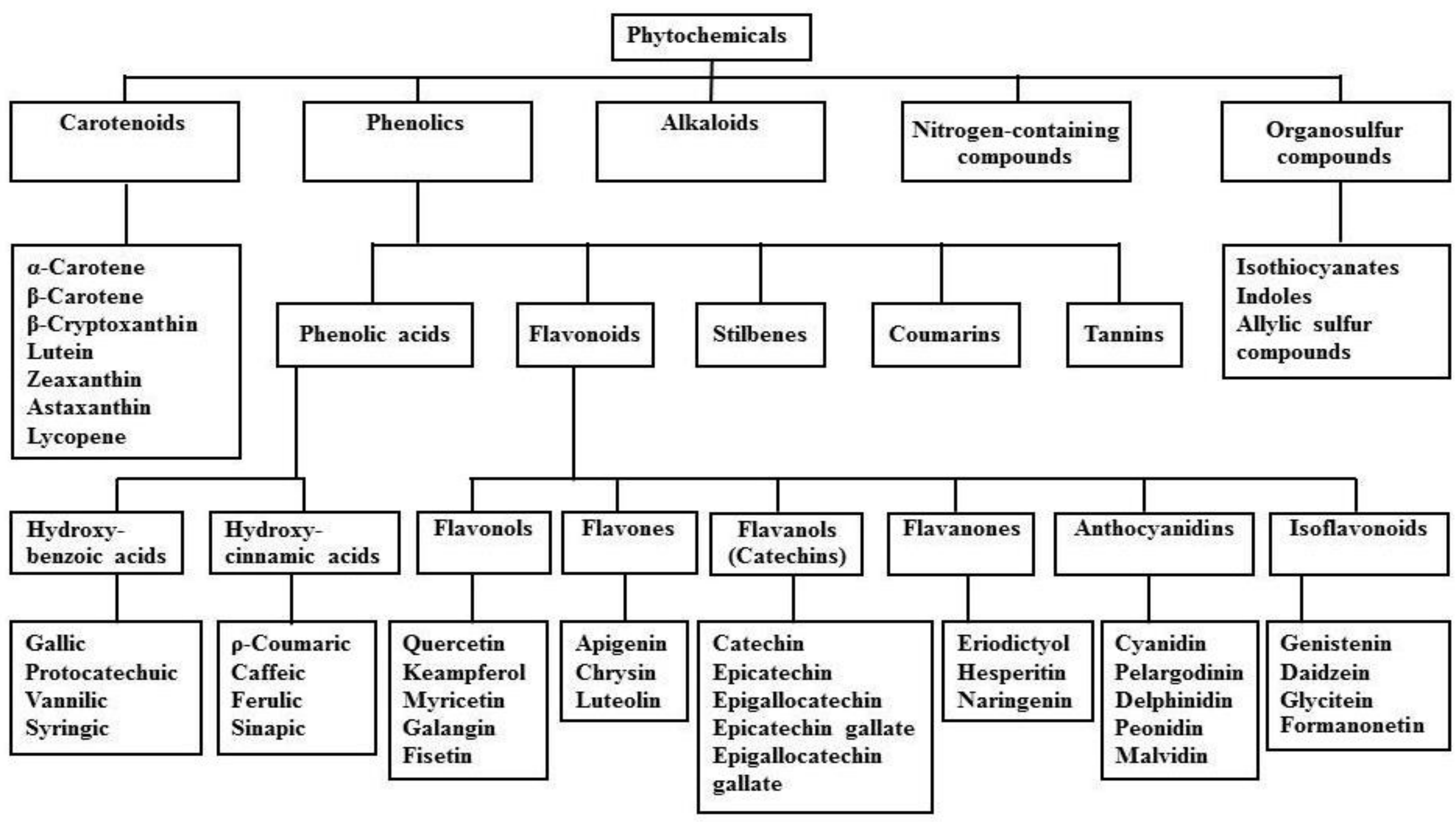

Figure 6. Classification of dietary phytochemicals

Source: Liu (2013)

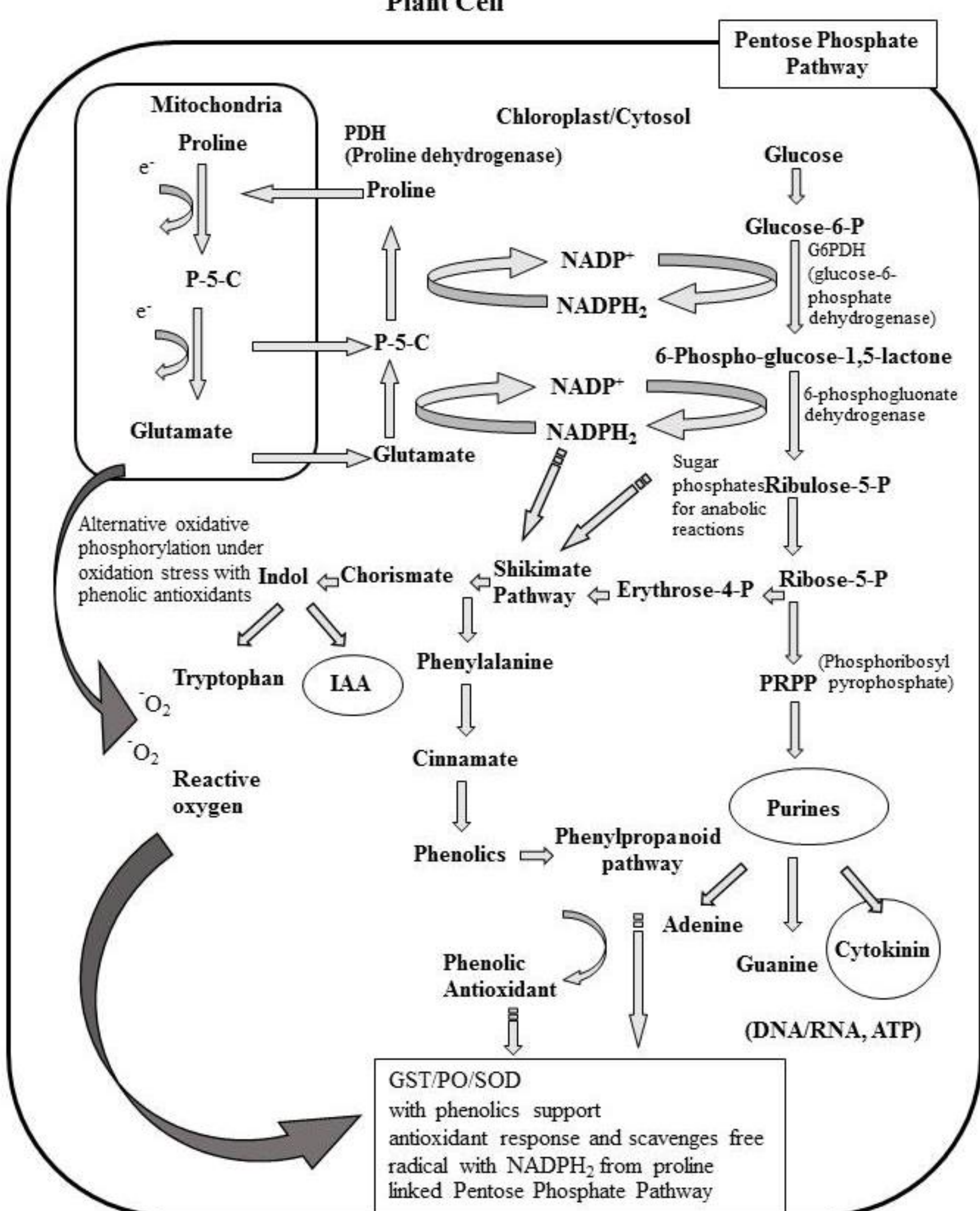

Figure 7. Original proposed model for the role of proline-linked pentose phosphate pathway in regulating phenolic biosynthesis (Abbreviations: P5C; pyrroline-5-carboxylate, IAA; indole acetic acid, GST; Glutathione-s-transferase, PO; peroxidase, SOD; superoxide dismutase)

Source: Shetty and Wahlqvist (2004)

1.2.4.3.3Flavonoids

Flavonoid, a subclass of phenolic compounds, providing antioxidant activity, has been identified in fruits, vegetables, other plant foods. These compounds have been claimed to reducing of the risk of major chronic diseases. The typical flavonoids are consisting of two aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocycle ring or C ring (Figure 8). Differences in the generic structure of the heterocycle C ring classify them as flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavonoids (Figure 9). In addition, flavonoid synthesis pathways were dipited in Figure 10.

Figure 8. The structure of flavonoids

Source: Liu (2004)

Flavonoids

Flavanols

Epicatechin
Catechin
Epigallocatechin gallate

Flavonols

Quercetin
Myrestin
Kaempferol

Flavanones

Naringenin
Hesperetin
Eriodictyol

Flavones

Apigenin
Luteolin

Anthocyanidines

Cyanidin
Malvedin
Delphinidin
Pelargonidin

Figure 9. The generic structure of flavonoids

Source: Liu (2004)

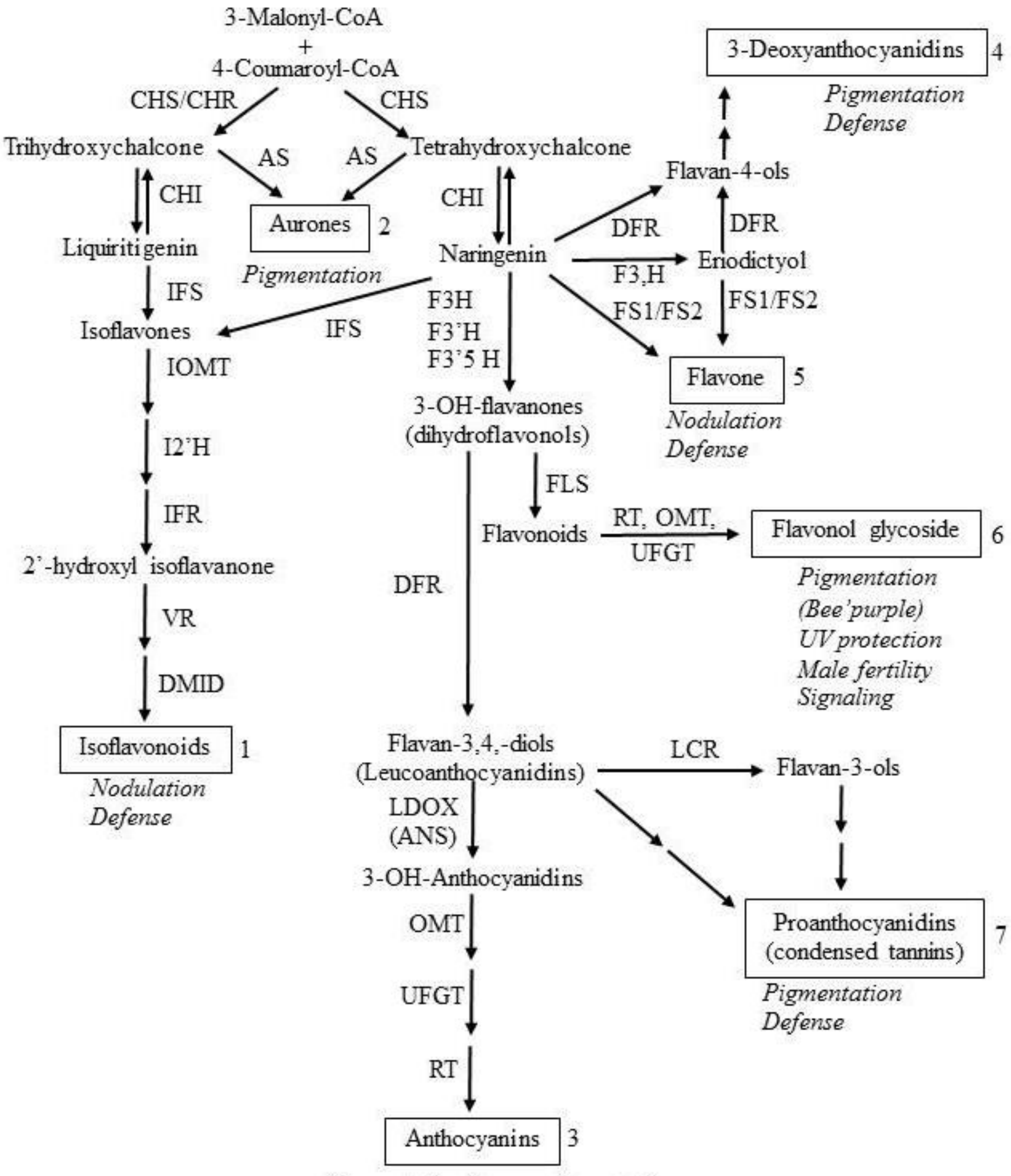

Figure 10. Phenylalanine synthesis

Source: Tohge *et al*. (2013)

1.2.4.4 Antibacterial action of mint extract

The main active compounds that can affect the antibacterial found in various plants are essential oil (EOs). EOs consist of a wide variety of secondary metabolites including primary terpenoids, monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20) as well as a variety of acids, alcohols, aldehydes, aliphatic hydrocarbons, acyclic esters (Burt and Reinders, 2003; Chorianopoulos *et al*., 2008). As known that Gram-positive bacteria are more sensitive to EOs compared with Gram-negative bacteria due to differences structures in the cell walls (Trombetta *et al*., 2005). Generally, cell wall of Gram-positive bacteria contains peptidoglycan approximately 90%–95% and the rest are teicoic acid and proteins as shown in (Figure 11). The structure of the Gram-positive bacteria cell wall allows hydrophobic molecules to easily penetrate the cells and act on both the cell wall and within the cytoplasm. On the other hand, the cell wall of Gram-negative bacteria is more complicated even thinner (2-3 nm thick) than that of Gram-positive bacteria (20-40 nm thick) (Hoiczyk and Hansel, 2000). In addition, Gram-negative bacteria contain an outer membrane (OM) which lies outside of the thin peptidoglycan layer. The presence of an OM is used to distinguish Gram-negative from Gram-positive bacteria. Since OM consists of a double layer of phospholipids that is linked to inner membrane by lipopolysaccharides (LPS), while the peptidoglycan layer is covered by an OM that contains various proteins as well as LPS. LPS consists of lipid A, the core polysaccharide, and the O-side chain, that permits Gram-negative bacteria to be more resistant to EOs and other natural extracts with antimicrobial activity. Small hydrophilic solutes are able to pass through the OM via abundant porin proteins that serve as hydrophilic transmembrane channels, and this is an explanation whether Gram-negative bacteria are not relatively rensitive to hydrophobic antibiotics and toxic substances.

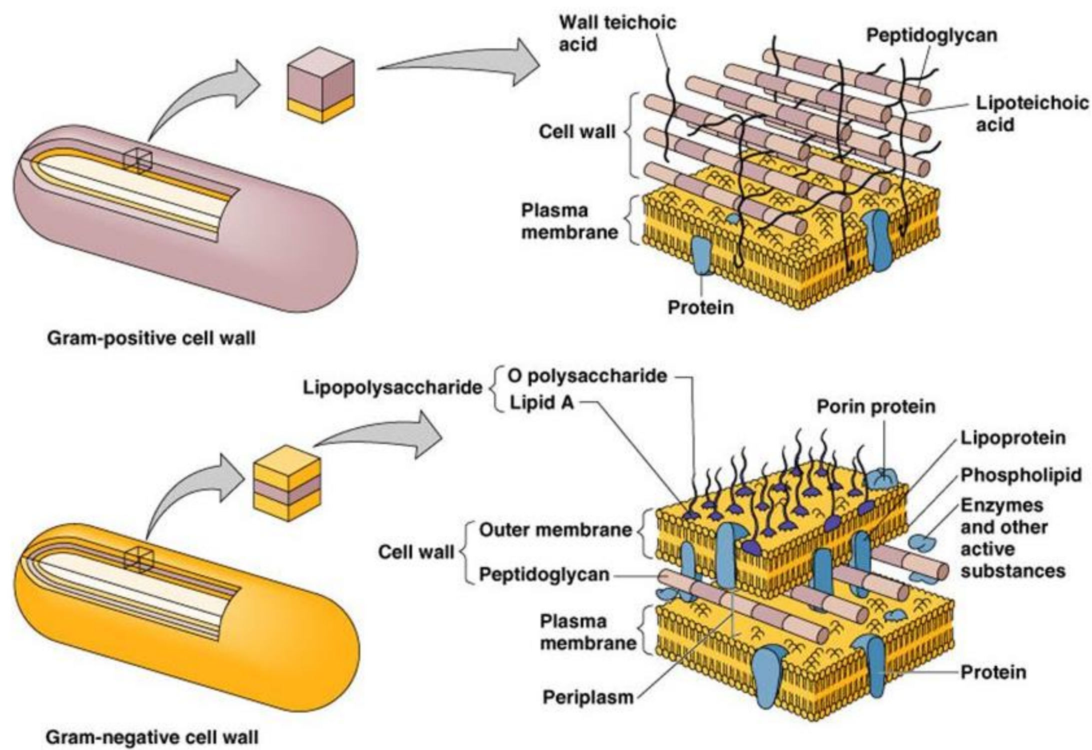

Figure 11. Schematic of the envelopes of Gram-positive and Gram-negative bacteria
Source: Crawford (2016)

Both EOs and/or their components exhibit the antibacterial action based on their chemical composition, amount and/or position of functional groups and concentration. In fact, EOs at low level can inhibit microorganism by interrupting enzymes related to the energy production, while proteins denaturation is occurred when apply EOs was applied at with high concentration (Tiwari *et al*., 2009) thus can affect the variety targets of antimicrobial activity. The compounds present in the EOs effect on physiological changes, cell lysis and death of microorganism involve various actions including interfering with proteins in the cell wall, the destabilization of the phospholipid bilayer, the destruction of the plasma membrane function and composition. The cell lysis and eventually death are occurred via muti mechanism including the loss of vital intracellular component, suppressing electron transport for energy generation and destroying the proton motive force, protein translocation and synthesis of cellular components (Figure 12) (Turina *et al*., 2006). As known that the natural extracts containing a wide range of substances possess antibacterial activity

via muti pathways. In addition, different biochemical and structural mechanisms are involved (Carson *et al*., 2002) at multiple sites within the cell and on the cell surface.

The EOs found in *M. cordifolia* were carveol (13.76%), benzenemethanol (8.11%), β-bourbonene (5.74%), phytol (4.92%), piperitenone (3.54%), eugenol (3.55%), piperitenone oxdie (3.14%) and menthol (0.3%) identified by GC-MS (Pudpila, 2011). In addition, terpenoids in EOs of *M. cordifolia* are terpenes with added oxygen molecules or methyl groups or hydroxyl group (Caballero *et al*., 2003). The antimicrobial property of most terpenoid compounds involved their hydroxyl groups which are important functional groups for expression their microorganism inhibition.

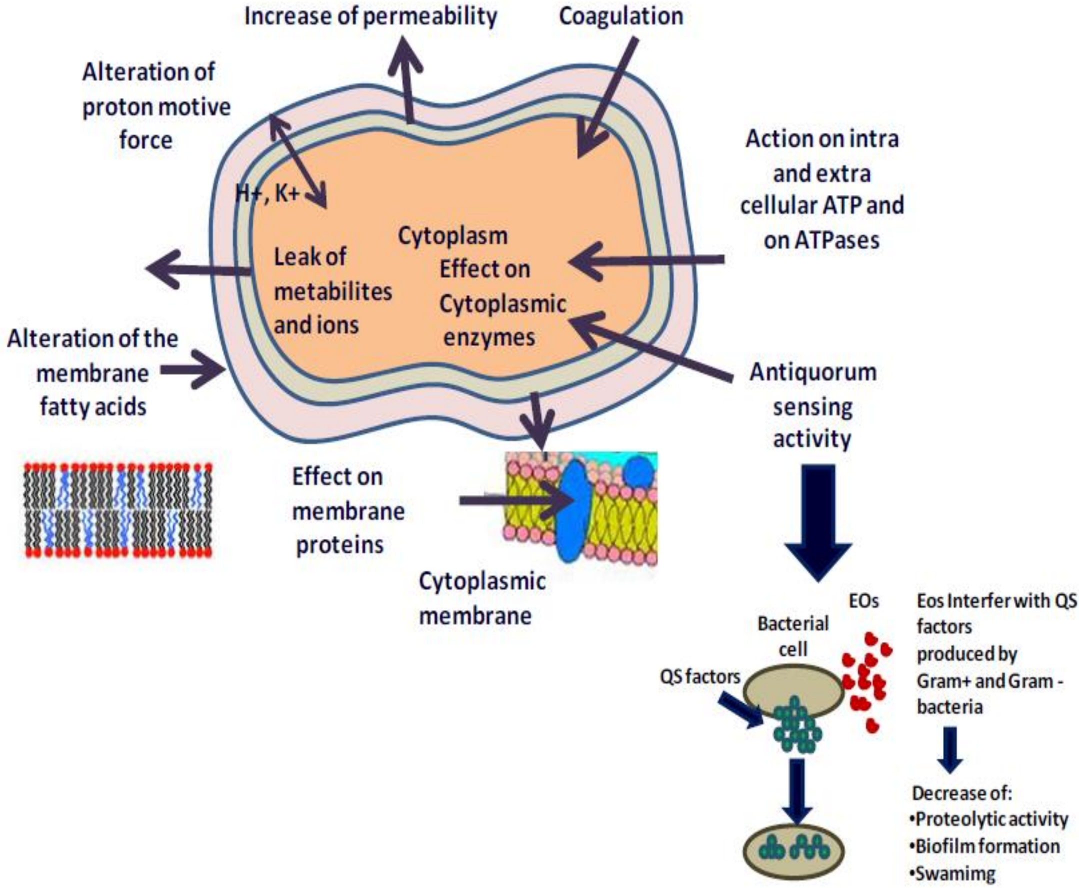

Figure 12. Mechanism of action and target sites of the essential oils on microbial cells

Source: Nazzaro (2013)

1.3 Objectives

1.3.1. To increase zinc concentrations in the soil for *M. cordifolia*, kitchen mint plantation.

1.3.2. To evaluate growth characteristics, some phytochemical and mineral contents in *M. cordifolia*, kitchen mint as affected by Zn fortification in the soil**.**

1.3.3. To evaluate antioxidant and antibacterial activities as well as sensory acceptability of *M. cordifolia*, kitchen mint as affected by soil fortification with Zn.

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Chapter 2

Increasing of bioactive compounds in *Mentha cordifolia* Opiz., kitchen mint via $ZnSO_4$ biofortification during plantation

2.1 Abstract

Plants generally need both macronutrients and micronutrients for their growth. One of the important micronutrients for plant is zinc. Zinc is essential in every life including plants by supporting the enzymatic process and many biochemical reactions. When plants received zinc in an inadequate amount, this leads to the destruction of biochemical reaction and is related to inhibition of plant growth. Therefore, yield of the plant is relatively low. It is interesting that some scientific evidence has shown a positive relation between zinc intake and the amount of essential oil and yield of plants. *Mentha cordifolia* Opiz., kitchen mint, is one of the top seven vegetables consumed in Thailand. Increases of some essential minerals in plants or biofortification during plantation have resulted in increases in essential oil and chlorophyll. The bioactive compounds of essential oil provide antibacterial, in addition, chlorophyll can be utilized for against sinusitis, purifying the blood, anti-organism, cleaning toxins and intestines. Therefore, this work aimed to evaluate the effect of fortifying zinc into the plantation soil on growth characteristic, essential oil droplet and total quality of *Mentha cordifolia* Opiz. The results showed that growth characteristics increased with increasing of Zn concentration. The sizes of mint leaves were bigger, the stalks were plumper and the lengths of the roots were longer although not significantly different, and the production of essential oil significantly increased. The proximate composition contents including protein, fat, ash and fiber of plants grown in fortified $ZnSO_4$ soil, increased compared to the control. The leaves obtained from 100 ppm $ZnSO_4$ treatment possessed the highest chlorophyll content, related to lowest in a* value. In general, the kitchen mint fortified with 100 ppm $ZnSO_4$ seemed to be the most acceptable one when determined for color and sensory attributes; whereas, it was found that 200 ppm $ZnSO_4$ treatment

showed the highest production of essential oil and lower number of microorganisms. In conclusion, soil fortified with $ZnSO_4$ at 100 ppm during plantation increased greenness, chlorophyll content and consumer acceptability of the mint leaves. Darker and larger size of oil droplet was found in the mint leaves obtained from plant grown in soil fortified with 200 ppm $ZnSO_4$.

2.2 Introduction

All living things including plants, animals and humans require zinc (Zn) for their growth and reproductive stages. Zn deficiency in humans inhibits physical growth and the immune system and its deficiency is among the top 5 causes of death and disease in many developing countries (WHO, 2002). Actually, human body needs Zn for normal growth and development from fetus throughout adolescence (Maret and Sandstead, 2006; Gao *et al.*, 2015; Murni *et al.*, 2015; Jiang *et al.*, 2016). For example, DNA synthesis, neurosensory functions and cell-mediated immunity all require Zn (Prasad *et al.*, 1992; Aper and Merkx, 2016; Gok *et al.*, 2016; Piechal *et al.*, 2015). Oyster and other meat products as well as a fortified cereal have been claimed to have a high Zn content (Murphy *et al.*, 1975; Rainbow *et al.*, 2015; Owaid *et al.*, 2015). From literature review, it was found that many plants may lack of Zn when low zinc soil or alkaline soil was used for plantation material. Zn deficient plants had poor integrity and growth performance and were low in yield and quality (Alloway, 2008). On the other hand, growth, dry matter yield and essential oil production significantly increased when mint was grown in the soil supplemented with zinc (Misra and Sharma, 2015; Al Ahl and Mahmoud, 2010).

Mentha cordifolia Opiz. (Family Lamiaceae), common name as kitchen or marsh mint, is among the top seven vegetables consumed in Thailand, either raw, in mildly cooked food or in herbal tea. In fact, this plant is easily grown not only in Thailand but also throughout Southeast Asian countries (Hasan *et al.*, 2012; Khempaka *et al.*, 2013; Tongkhao and Mahakarnchanakul, 2013). Thais use kitchen mint as traditional medicine to relieve gastrointestinal problem, asthma, muscle spasm and inflammation. Some scientific data have shown the biological effects of *M. cordifolia* such as antimutagenicity (Villasenor *et al*., 2002), analgesic, anti-

nociceptive (Villasenor and Sanchez, 2009), anti-inflammatory, antioxidant activities (Poungrat *et al*., 2010), helping to relieve colds, flu, fever, motion sickness and poor digestion problems (Nantawan and Weibiao, 2009). Ferruzzi and Blakeslee. (2007) reported that therapeutic properties of chlorophyll which is usually found in green vegetables can be summarized as being of benefit against sinusitis and skin rashes, purifying the blood and the organism, cleaning toxins and the intestines and detoxification of the liver.

Although Thailand is a tropical country and provides a diverse variety of vegetables or plants, the World Health Organization (WHO, 2002) reported that Thai people have moderate Zn deficiency. It is hypothesized that Zn content in the plantation soil is not high enough. Therefore, this present work aimed to fortify zinc into the plantation soil and monitor growth characteristics, some proximate compositions and sensory acceptability of *Mentha. Cordifolia* Opiz.

2.3 Materials and methods

2.3.1 Chemicals and media

Chemicals used were analytical grade. Zinc sulfate, potassium sulfate, copper sulfate, purified acid sand, calcium carbonate and boric acid were purchased from Ajax Finechem, Apdivision of Nuplex Industries (Aust) Pty Ltd, New Zealand. Sulfuric acid, sodium hydroxide and hydrochloric acid, sodium chloride, nitric acid, hydrogen peroxide was obtained from Merck KGaA, Thailand. Methyl red, bromocresol green, ethanol, acetone and petroleum ether were procured from LAB-SCAN, Ireland. Sodium sulfate anhydrous, L-ascorbic was purchased from QReC, New Zealand. All media for microbiological analysis were analytical grade including plate count agar, potato dextrose agar, EC broth, lauryl sulphate tryptose broth and triple sugar iron agar purchased from Merck KGaA. Buffered peptone water, eosin methylene blue agar, selenite cysteine broth, lactose broth, brilliant green agar, Baird Parker medium, tetrathionate broth base, triple sugar iron agar, lysine iron agar, XLD agar and hektoen enteric agar were purchased from Difco Michigan, U.S.A.

2.3.2 Experimental plots

A field experiment was conducted in May, 2014 at the Faculty of Agro-Industry, Prince of Songkla University. The experiment pot was divided into three rows, eleven columns with different treatments. Each row was separated from each other for 1 m width and column length with 30 cm to ensure no mix-up as shown in Figure 13.

Figure 13. The nursery and experimental plots for kitchen mint plantation

2.3.3 Soil preparation for kitchen mint plantation

Containers of diameter 19 cm and depth 13 cm were used for planting. Soil mixed with 0, 100 and 200 ppm $ZnSO_4.7H_2O$ was separately put into the containers. All soil samples were subjected to analysis for mineral contents and physicochemical characteristics such as organic matter, pH, cation exchange capacity, electrical conductivity and N, available P and K.

2.3.4 Seeding preparation

The kitchen mint seeds were soaked in tap water at a ratio of 1:2 (seed : water) overnight. The floating seeds were discarded and the same amount of soaked vital seeds were planted into the separately prepared soil. The puncture plastic sheet was used to cover the soil for controlling soil humidity. Soils were sprayed with

250 - 400 ml of water twice a day (morning at 8.00 - 8.30 am. and afternoon at 4.00 - 4.30 pm.) for 3 mo. During the seeding process, the growth of the tagged leaf (length and width) and plant height were measured as shown in Figure 14.

Figure 14. Plantation plots and position of the vital seed plants were placed

2.3.5 Harvesting and analysis

Plants aged 3 mo were harvested, then leaves, stalks and roots were cut and separated. Each plant part was washed with tap water and drained before being weighed and counted. The leaves were then subjected to quality analyses.

2.3.6 Proximate composition

The leaf samples were analyzed for the contents of moisture, ash, fat and protein according to the method of AOAC (1999) and fiber according to AOCS (1993). The determinations were run in triplicate and standard deviations were calculated for the difference.

2.3.7 Determination of macro and micro minerals

Samples were digested with nitric acid and hydrogen peroxide and filtered through Whatman No.1 filter paper. The final volume was adjusted to 100 ml using distilled water. After that the samples were analyzed using Inductively Couple Plasma-Optical Emission Spectrometer (ICP-OES) method.

2.3.8 pH and total titratable acidity

Ten gram of each sample was homogenized in 40 ml distilled water for 2 min with homogenizer and the pH was measured at room temperature by pH meter (Docu-pH Meter, Satorius, Germany). Titratable acidity was carried out using AOAC (1999) method. Briefly, the homogenized sample was prepared similar to those for pH measurement then filtered by Whatman No.4 filter paper. Thereafter 5 ml of supernatant was added with 25 ml of distilled water, followed by 3 drops of phenolphthalein indicator then titrated with 0.1N NaOH to reach the endpoint at pH 8.2 with pale pink color. The results were expressed as L-ascorbic acid equivalents.

$$\% \text{ acid} = \frac{(V) \times (N) \times (MW.) \times 100}{\text{Sample of volume (ml)} \times 1000}$$

Where; V = Sodium hydroxide solution used for titration (ml)

N = Normality of sodium hydroxide solution

Mw. = Molecular weight of L-ascorbic acid (176.14)

1000 = Factor relating mg to grams

2.3.9 Color value

The tagged leaf was measured for color value as *L**, *a** and *b** using colorimeter (Hunter lab, Model Color Quest XT, United State). The color value was expressed as Commission International de I' Eclairage (CIE) Lab coordinates where L^* represents the luminosity (0 = black, 100 = white), a^* the redness ($a^* > 0$) or greenness ($a^* < 0$) and b^* the blueness ($b^* > 0$) or yellowness ($b^* < 0$).

2.3.10 Determination of chlorophyll (a,b) and total chlorophyll

The sample was analyzed by AOAC (2000) method. Briefly, 3 g of sample was ground with calcium carbonate and purified acid sand, which helped increase the extraction of chlorophyll, and then mixed with 10 ml of 80% acetone. Then sample was vacuum filtered through Whatman No.1 filter paper using Buchner funnel porcelain. The residue material was reextracted with the same condition until

the green color of the residue on filter paper disappeared. All of the extracts were pooled then excess sodium sulfate anhydrous was added to absorb water before reparation of the chlorophyll by filtering. The final volume was adjusted to 100 ml with absolute acetone. Chlorophyll a and b were measured with a spectrophotometric absorbance at 665 and 649 nm, respectively. The concentration of both chlorophyll forms were calculated in units of mg/L according to the following equations:

$$\text{Chlorophyll a} = 11.63\text{Abs.}_{665} - 2.39\text{Abs.}_{649}$$

$$\text{Chlorophyll b} = 20.11\text{Abs.}_{649} - 5.18\text{Abs.}_{665}$$

$$\text{Total chlorophyll} = 17.72\text{Abs.}_{649} + 6.45\text{Abs.}_{665}$$

2.3.11 Determination of oil droplet

Fresh leaf sample from each treatment was cut cross-sectionally to view the oil droplet by digital photography using a Nikon Alphaphot 2 YS2 microscope.

2.3.12 Microbiological quality

2.3.12.1 Yeast and Mold

Ten grams of each sample was blended with 90 ml of 0.1% sterilized peptone water to obtain 10^{-1} dilution before being diluted to appropriate dilution (10^{-2} -10^{-6}). The appropriate dilutions were carried out and plated on the PDA then incubated at 30 $\pm$ 2 °C for 24-48 h. Microbial counts were expressed in colony froming unit per gram (BAM, 2001).

2.3.12.2 Total viable count

Ten grams of each sample was prepared as mentioned in yeast and mold counts. The appropriate dilutions were taken to plate on the PCA then incubated at 35 $\pm$ 2 °C for 24-48 h. Microbial counts were expressed in colony froming unit per gram (BAM, 2001 in Chapter 3).

2.3.12.3 *Escherichia coil*

Ten grams of each sample was blended with 90 ml of 0.1% sterilized peptone water to obtain 10^{-1} dilution before being diluted to appropriate dilution (10^{-2} -10^{-3}). Each serial dilution 1 ml at 10^{1} to 10^{3} was added into 10 ml of lauryl sulphate tryptose (LST) broth containing Durham tube inoculated LST tubes were incubated at 35 $\pm$ 2 °C for 24-48 h. Durham tube having air bubble as examined and recorded at 24 h. Gas-negative tubes were re-incubated for an additional 24 h, then determined and recorded at 48 h. All presumptive positive (gas) tubes were confirmed test and checked using *E. coli* in eosin methylene blue (EMB) agar. Typical *E. coli* colony were tesed using IMViC reaction (BAM, 2002 in Chapter 4) was determined using 5 tube most probable number (MPN) and expressed as MPN/g.

2.3.12.4 *Staphylococcus aureus*

Ten grams of each sample was blended with 90 ml of 0.1% sterilized peptone water to obtain 10^{-1} dilution before being diluted to appropriate dilution (10^{-2} -10^{-6}) then 1 ml of each dilution was inoculated into 3 tube of TSB containing 10% NaCl and 1% sodium pyruvate and incubated the tubes at 35 $\pm$ 2 °C for 48 h. One-loopful from each tube showing growth (turbidity) was picked up and streaked on the properly dried surface plate of Baird Parker medium and incubated 48 h at 35 $\pm$ 2 °C. Each plate showing growth, at least 1 colony suspected to be *S.aureus* was transferred to BHI broth. Procedure for identification and confirmation of *S.aureus* and reported as MPN/g was followed BAM, 2001(in Chapter 12).

2.3.12.5 *Salmonella* spp.

Ten-gram of each sample was blended with 90 ml of tryptic soy broth (TSB), for 2 min to obtain the homogenized mixture. One-loopful of the incubated sample was streaked on XLD and HE agar for 48 h at 35 $\pm$ 2 °C then the typical colony was taken to triple sugar iron agar (TSI) and lysine iron agar (LIA) determine followed by BAM, 2001 (in Chapter 5).

2.3.13 Sensory acceptability

Thirty blinded volunteers comprising graduate students and technicians from the Department of Food Technology, Prince of Songkla University, who were familiar with consuming fresh vegetables were recruited for sensory evaluation. The panelists were asked to evaluate the attribute preferences in color, odor, taste, flavor and overall acceptability of each sample using a nine-point hedonic scale, from “1-dislike very much” to “9 - like very much”.

2.3.14 Statistical analysis

This experiment was run in a completely randomized design (CRD). Data were analyzed as mean $\pm$ standard deviation (SD) using one way analysis of variance (ANOVA) then mean values were compared by Duncan’s multiple range test (DMRT) at a significance level of $p < 0.05$.

2.4 Results and discussions

2.4.1 Chemical composition and element in the soil sample

Initial values of moisture content, total nitrogen, organic matter, available P (Avail. P), cation exchange capacity (C.E.C), pH, electric conductivity (EC), aluminium (Al), Zn, potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) in the soil were shown in Table 6. It pointed out that the soil used in this experiment was very good fertility based on organic matter > 4.5, available P> 45, C.E.C. > 30 and K > 120 (Land Development Department, 1980).

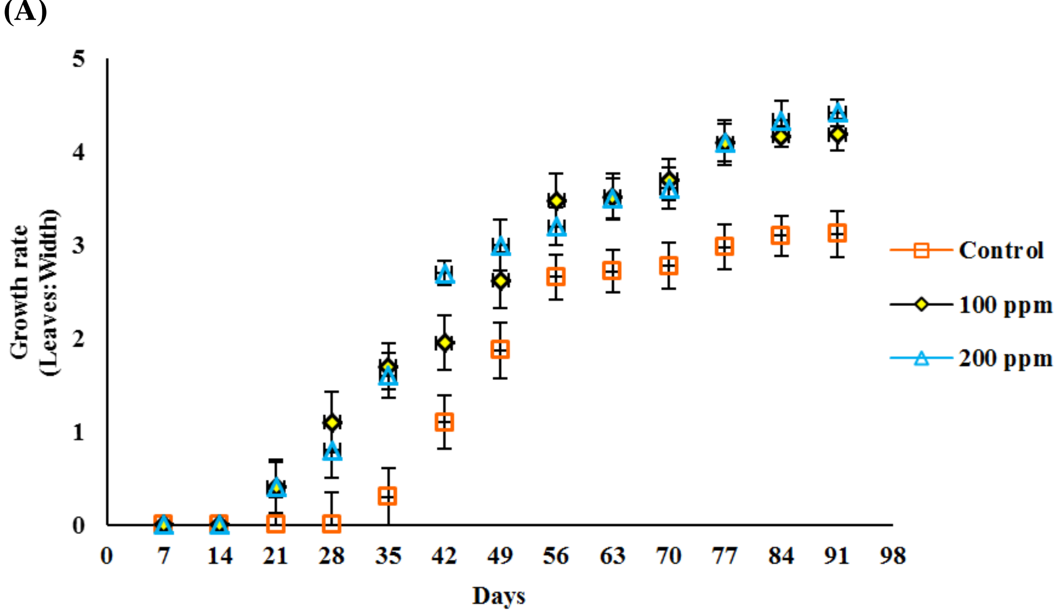
Table 6. Chemical composition and some elements of the used soil

Sample	Parameters	Quantity	Unit
Soil (Initial)	Moisture Content	21.07	g/100g
	Total nitrogen	0.47	g/100g
	Organic matter	11.11	g/100g
	Available P	146.65	mg/kg
	Cation exchange capacity (CEC)	31.96	Meq/100g
	pH	7.3	-
	Electric conductivity (EC)	355	µS/cm
	Al	0.06	Meq/100g
	K	1,142.22	mg/kg
	Ca	11,739.68	mg/kg
	Mg	1,859.05	mg/kg
	S	682.12	mg/kg
	Zn	31.43	mg/kg
Fortified with 100 ppm $ZnSO_4$	Zn	46.07	mg/kg
Fortified with 200 ppm $ZnSO_4$	Zn	50.84	mg/kg

2.4.2 Growth rate of each part of kitchen mint grown in soil fortified with $ZnSO_4$ at different concentrations

In general, growth of plant leaves and stalks of all treatments were similar as s-shape with certain of lag time (Figure 15, Figure 16). The lag time or slow growth in the earlier stage was explained by reconditioned phenomenon that normal seed need to absorb water enough for enzyme stimulation to germinate (Dangsawas, 2001). Thereafter, the plant grew very fast as experimental phase because of photosynthesis of seedling. However, the growth rate of the plant grown in fortified $ZnSO_4$ soil was higher than that of control sample. It pointed at that fortification $ZnSO_4$ into the soil improved plant growth in leaf and stalk parts. This may due to absorbed Zn helped chorismate synthesis which is a common precursor molecule for the tryptophan pathway. In fact, tryptophan is a precursor of indole-3-3 acetic acid (IAA) (Glick *et al*., 2007; Korasick *et al*., 2013). Glick *et al*., (2007) summarized that cell division and other development of plants involve with IAA, gibberellins (GA) and abscisic acid (ABA).

(A)



(B)

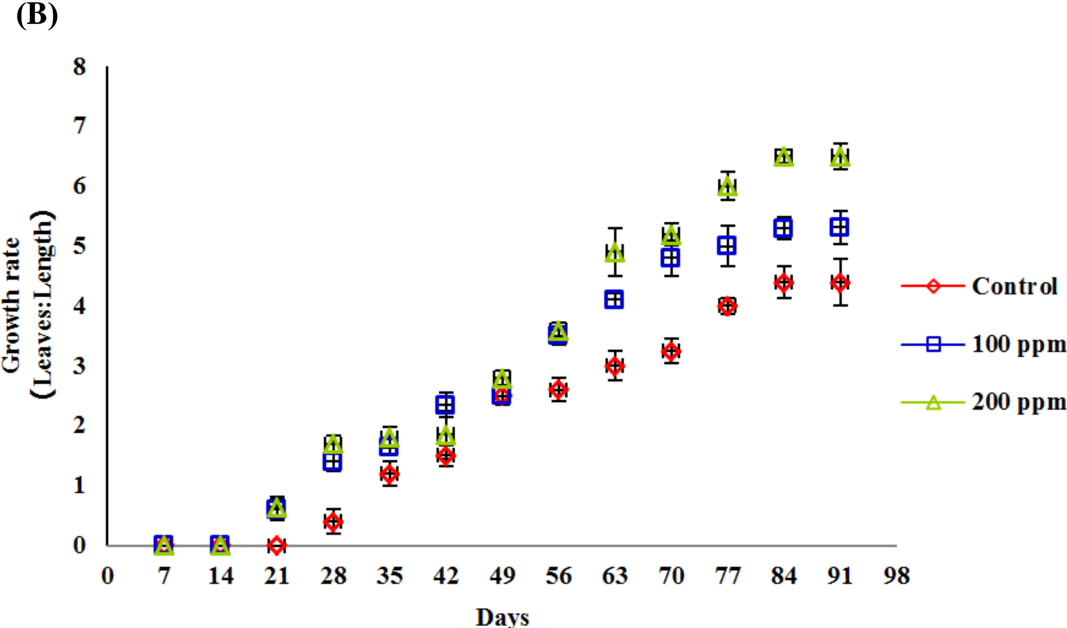
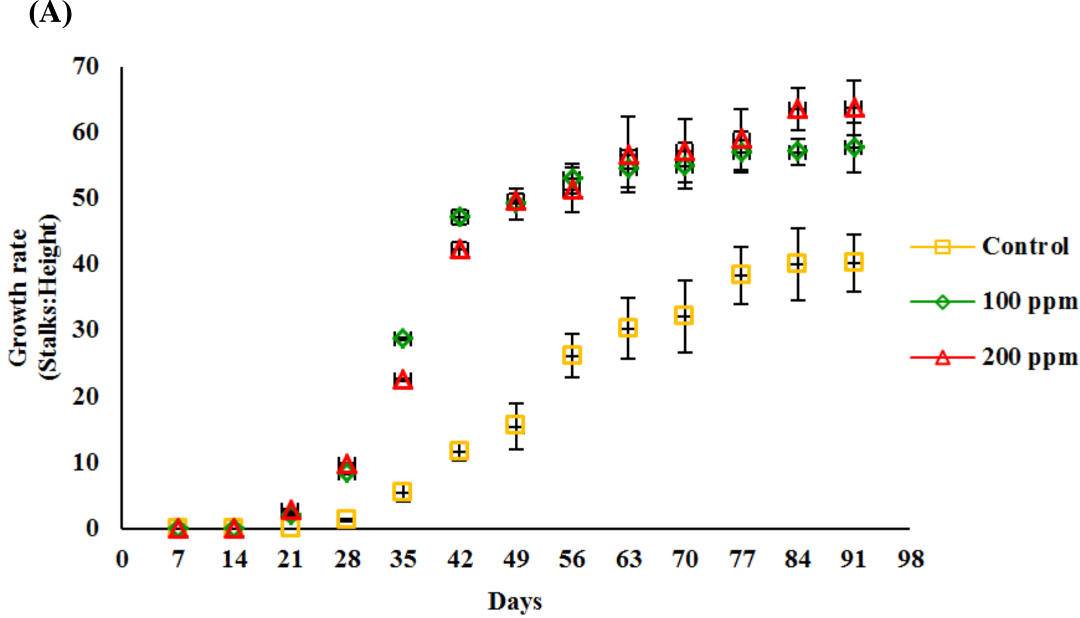


Figure 15. The relationship between Zn concentrations and leaves width (A) and length (B) of kitchen mint during plantation



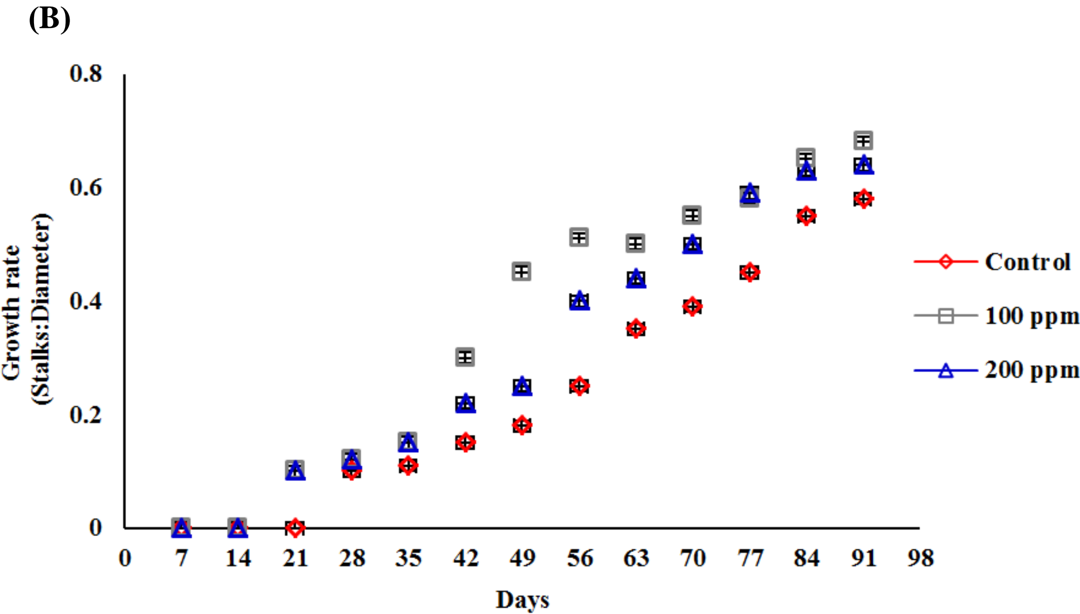


Figure 16. The relationship between Zn concentrations and stalk height (A) and diameter (B) of kitchen mint during plantation

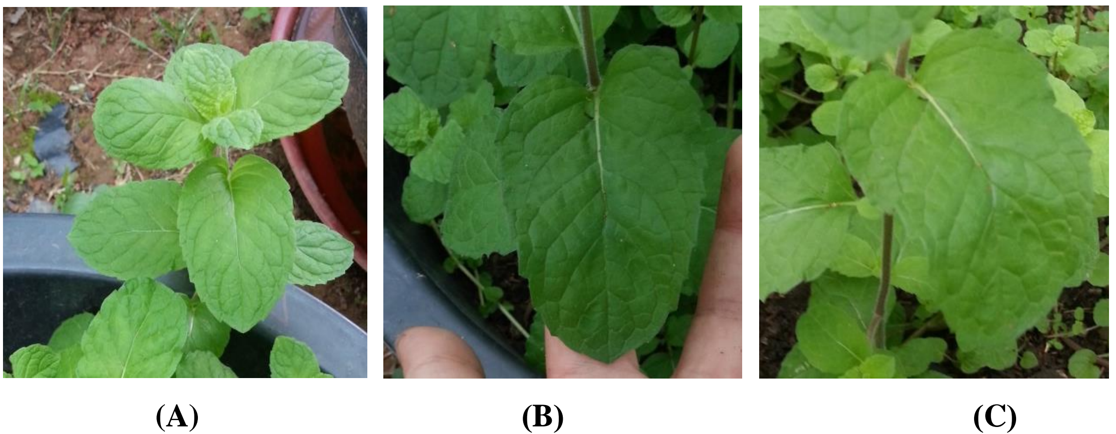
2.4.3 Effect of fortified $ZnSO_4$ into the soil on growth characteristic and yield of kitchen mint

According to Table 7 with increased concentrations of $ZnSO_4$, the size of the kitchen mint leaves were bigger (Figure 17), the stalks were plumper and the roots were longer, although the last was not significant. In fact, Zn is required for the synthesis of tryptophan which is a precursor of Indole-3-3 acetic acid (IAA), plant hormone, that stimulates cell division and stretching the cells (Ulmasov *et al.*, 1997). This may explain the bigger size of leaves, stalk and longer size of root mint when the plant was grown in fortified $ZnSO_4$ soil. However, it was noticed that mint grown in fortified $ZnSO_4$ soil produced a few bushes compared with the control sample (Figure 18). Therefore, yield of leaves, stalk and roots (fresh basis) of the mint planted in fortified soil did not increase much. This indicated that a certain amount of Zn increased division and elongation of plant cell leading to an increase of plant part size but not an increase in plant propagation as presented in picture A1 (Appendix A). However, during plantation, it was noticed that the mint grown in the soil fortified with 100 ppm $ZnSO_4$ was much invaded with worms even though all plant samples were grown in the same nursery. Hassan. (2010) reported that some plants produced specific secondary metabolites including phenolic compounds which act as insect attractants. This may be the reason for no significant increase in leaf yield in mint grown in soil fortified with 100 ppm $ZnSO_4$ though its photosynthesis determined by chlorophyll content was the highest. The results showed that 200 ppm of $ZnSO_4$ fortification increased zinc content in plants up to 47.44% as shown in Table 7. According to $ZnSO_4$ content in the plant leaves, it was found that the percentage increase of Zn tended to be slower when $ZnSO_4$ at 200 ppm was applied. It indicated that the plant started to saturate with Zn at this level and may begin to give rise to toxicity.

Table 7. Effect of $ZnSO_4$ fortification on growth characteristic and yield of kitchen mint parts and zinc level in kitchen mint leaves

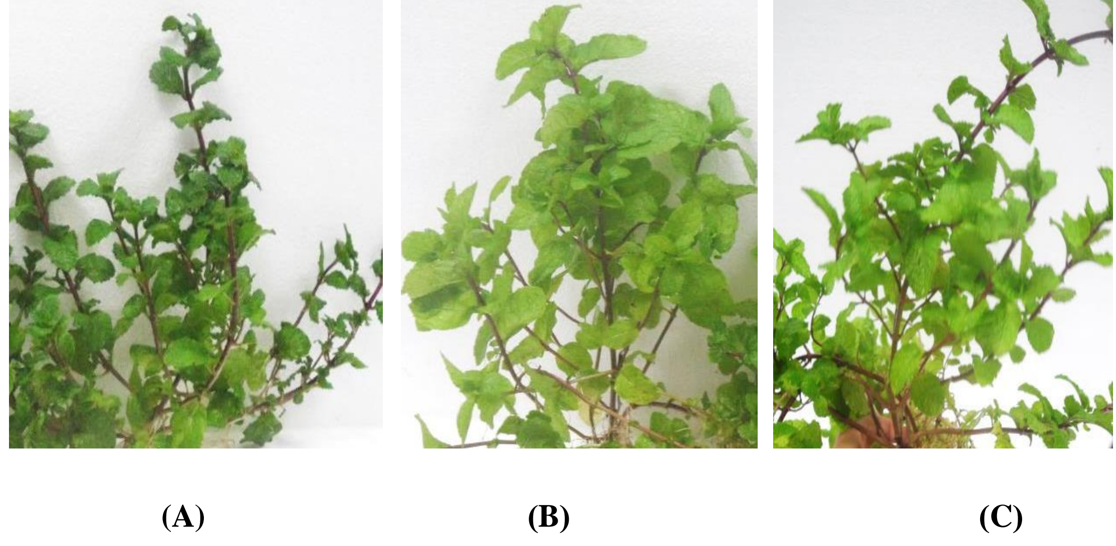
	Leaves			Stalks			Roots		Zn in Kitchen mint (ppm)	Zn increase (%)
Sample	**Width (cm)**	**Length (cm)**	**Yield (%)**	**Height (cm)**	**Diameter (cm)**	**Yield (%)**	**Length (cm)**	**Yield (%)**		
Control	3.14 ± 0.25^{c}	4.41 ± 0.39^{c}	42.89	40.43 ± 6.29^{b}	0.60 ± 0.01^{c}	39.25	43.12 ± 4.45^{ns}	17.86	32.390	0
100 ppm	4.19 ± 0.27^{b}	5.31 ± 0.27^{b}	43.07	57.76 ± 5.80^{a}	0.69 ± 0.01^{a}	38.12	49.40 ± 6.89^{ns}	18.81	44.821	38.38
200 ppm	4.47 ± 0.24^{a}	6.46 ± 0.21^{a}	44.31	63.77 ± 7.10^{a}	0.65 ± 0.01^{b}	37.27	50.10 ± 7.03^{ns}	18.42	47.755	47.437

Remark a-c, Means within columns with different letters are significantly different ($p < 0.05$). Each value was expressed as the mean $\pm$ standard deviation (n = 7)



(A) **(B)** **(C)**

Figure 17. The leaves of kitchen mint fortified with $ZnSO_4$ at different concentrations
(A): Control, (B): 100 ppm $ZnSO_4$ fortification
(C): 200 ppm $ZnSO_4$ fortification



(A) **(B)** **(C)**

Figure 18. The bush of kitchen mint fortified with $ZnSO_4$ at different concentrations
(A): Control, (B): 100 ppm $ZnSO_4$ fortification
(C): 200 ppm $ZnSO_4$ fortification

2.4.4 Effect of fortified $ZnSO_4$ on minerals of the kitchen mint

From the result, there were 4 different groups of mineral changing patterns; (1) consisted of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) and copper (Cu) content which increased and reached the peak when the plant was grown in 100 ppm $ZnSO_4$ fortified soil, (2) was calcium (Ca) and chromium (Cr) which decreased and lowest at 100 ppm $ZnSO_4$ fortification before increased afterword, (3) was cadmium (Cd) which kept constant and (4) consisted of Zn and selenium (Se) which increased as increased of $ZnSO_4$ fortification (Table 8). It pointed out that the first group and second one had a change as close and open bell shape, respectively. It indicated that the plant responded to both groups as a cycle. It meant that the minerals in group one were competitors of minerals in group two (Dangsawas, 2001). Kramer and Clemens, (2005); Sinclair and Kramer, (2012) reported that plants have mechanism to uptake and utilize as well as storage metals in order to their homeostasis. In addition, it was found that increasing of $ZnSO_4$ did not increase Cd in plant leaves. This may due to influence of *At*HMA_3 (*Arabidopsis thaliana*): heavy metal associated gene in the plant which is an important gene for Cd detoxification (Mills *et al*., 2005). Since Cd is not an essential element and toxic to almost living things including plants then they may have specific mechanism to not absorb this toxic heavy metal for protecting themself from Cd toxicity. There were some scientific data reported that both macronutrients and micronutrients exhibited less absorption when Zn level was high (Reichman, 2002; Alloway, 2008; Mousavi *et al*., 2012). The result also slowed that both Zn and Se increased as increased $ZnSO_4$ fortification. This was postulated that an increase of Se which is a part of glutathione peroxidase (GPX), an enzyme involved in the protection of tissues against oxidative stress which may induce by excess or overload Zn content. However, the documentary is not a clear conscience and needs further study.

In addition, it was found that Zn content was highest in roots followed by leaves and stalks, respectively. This result was in agreement with finding of Gupta *et al*. (2016) who reported that, roots contained higher zinc concentration than the aerial parts (leaves and shoots) because this is an indirect mechamism for plant to protect the metal toxicity. In addition, Chuan *et al*. (2016) reported that Zn uptake was highly

accumulated in a root tissue. Zinc translocation to leaf tissue exhibited a dose dependent relationship with both root and Zn levels in soil. Furthermore, the accumulation of heavy metals in plants is extremely complex in nature as various biotic and abiotic factors may likely to influence the mechanisms of phytoremediation.

Table 8. Effect of $ZnSO_4$ fortification on minerals of kitchen mint parts

Part	Parameter	Control	100 ppm	200 ppm
Leaves	(g/kg) N	52.00 ± 0.02^{c}	81.00 ± 0.01^{a}	64.00 ± 0.01^{b}
	P	51.00 ± 0.01^{b}	74.00 ± 0.02^{a}	53.00 ± 0.01^{b}
	K	253.00 ± 0.01^{b}	334.00 ± 0.03^{a}	241.00 ± 0.04^{c}
	Mg	23.00 ± 0.00^{b}	24.00 ± 0.00^{a}	21.00 ± 0.00^{c}
	Ca	136.00 ± 0.02^{a}	119.00 ± 0.00^{b}	138.00 ± 0.01^{a}
	(mg/kg) Se	1.15 ± 0.00^{c}	1.27 ± 0.00^{b}	2.62 ± 0.01^{a}
	Cd	0.09 ± 0.02^{ns}	0.09 ± 0.01^{ns}	0.09 ± 0.01^{ns}
	Cr	1.15 ± 0.00^{b}	0.98 ± 0.01^{c}	1.17 ± 0.00^{a}
	Cu	13.37 ± 0.04^{b}	14.90 ± 0.07^{a}	12.62 ± 0.01^{c}
	Zn	32.39 ± 0.01^{c}	44.82 ± 0.01^{b}	47.71 ± 0.04^{a}
Stalks	Zn	19.86 ± 0.02^{a}	19.56 ± 0.05^{a}	32.92 ± 0.01^{b}
Roots	Zn	31.35 ± 0.81^{c}	72.35 ± 0.16^{b}	108.37 ± 0.23^{a}
		1.15 ± 0.00^{b}	0.98 ± 0.01^{c}	1.17 ± 0.00^{a}

Remark a-c, Means within rows with different letters are significantly different ($p<0.05$). Each value was expressed as the mean ± standard deviation (n = 3).

2.4.5 Effect of fortified $ZnSO_4$ on chemical composition of the kitchen mint leaves

Moisture content of the mint leave samples was between 86.40 and 86.66 % as shown in Table 9. The leaves obtained from 100 ppm $ZnSO_4$ fortification had the highest protein content followed by 200 ppm $ZnSO_4$ and control, respectively. Lin and Aarts (2012) reported the highest increase of amino acid content such as alanine, aspartic acid valine, lysine, arginine, cysteine, glycine proline, glutamic acid in barley seed when the plantation soil was added with Zn at 1.6 ppm. However, the protein content in the barley seed was decreased when the plant was grown in 3.2 ppm Zn fortified soil. In addition, when the soil was more fortified with $ZnSO_4$, the fat, ash and fiber contents in the leaves were higher, with the highest in the leaves from 200 ppm fortification. Kalaikandhan *et al.* (2014) and Auld (2001) reported that synthesis of protein, nucleic acid, growth substances, chlorophyll, lipid and secondary metabolites were increased via Zn assistance. In addition, it was noticed that mint grown in soil fortified with 200 ppm $ZnSO_4$ had a different mint odor that was strongly pungent and not the typical good smell of kitchen mint. Not surprisingly, total carbohydrate in control leaves was highest, which was a typical characteristic of normal vegetables which mainly contain carbohydrate (Lintas, 1992).

As known that pH of a plant reflects the acidic and basic components found in the plant, mainly ascorbic acid, phenolic compounds and essential oil. In fact, acidity of plant materials reflects the amounts of weak organic acid, mainly ascorbic acid and others such as citric acid and phenolic compounds (McKay and Blumberg, 2006). The more acidity, the lower the pH value of sample, as found particularly in the low buffering capacity material as leafy vegetable (Lange *et al.*, 1989). Mint leaves from the fortified 100 ppm $ZnSO_4$ group showed the highest pH and the lowest total titratable acidity (Table 9). This may due to two possible reasons; (a) D-glucose, a form of carbohydrate produced in the mint was less, and/or (b) ascorbic acid content was highly used for controlling normal functions in the mint grown in fortified soil with 100 ppm $ZnSO_4$. Rai *et al.* (2013) reported that plants exposed to polluted air possessed higher vitamin C compared with non-polluted ones due to a stress-response mechanism. Moreover, Burt (2004) stated that plants having

high essential oils were classified as a low acid material because pH of the oils was about 6.7-7.3.

Table 9. Effect of $ZnSO_4$ fortification on chemical composition, pH and total titratable acidity (TA) of the fresh kitchen mint leaves

Parameter	Control	100 ppm	200 ppm
Moisture (%)	86.66 ± 0.03^{a}	86.40 ± 0.07^{b}	86.66 ± 0.05^{a}
Protein (%)	3.34 ± 0.09^{c}	5.05 ± 0.04^{a}	4.03 ± 0.05^{b}
Fat (%)	2.11 ± 0.04^{c}	2.86 ± 0.04^{b}	3.00 ± 0.02^{a}
Ash (%)	2.19 ± 0.03^{c}	2.33 ± 0.10^{b}	2.47 ± 0.02^{a}
Fiber (%)	1.43 ± 0.01^{c}	1.44 ± 0.06^{b}	1.52 ± 0.02^{a}
Carbohydrates (%)	4.30 ± 0.08^{c}	1.95 ± 0.03^{a}	2.33 ± 0.04^{b}
pH	6.56 ± 0.02^{c}	6.75 ± 0.02^{a}	6.64 ± 0.01^{b}
Total titratable acidity (%)*	1.07 ± 0.08^{a}	0.75 ± 0.04^{b}	0.80 ± 0.08^{ab}

Remark a-c, Means within rows with different letters are significantly different ($p < 0.05$). Each value was expressed as the mean $\pm$ standard deviation (n = 3). *: the value of total titratable acidity was expressed as unit of L-ascorbic acid/L.

2.4.6 Effect of $ZnSO_4$ on color values and chlorophyll content in kitchen mint

The color value in the CIE system as L*, a*, b* and -a/b of the leaves at base, middle and the apex of both sides, upper and lower from different $ZnSO_4$ fortification concentration were shown in Table 10. Results indicated that the upper side of the leaf at base and apex position from the sample fortified with 100 ppm $ZnSO_4$ had the highest green (a* value) followed by the control and 200 ppm $ZnSO_4$ fortification samples, respectively. This might be related to the right concentration of Zn aiding the synthesis of chlorophyll as show in Table 11. There were some scientific data supported that Zn helped increase chlorophyll production (Daughtry *et al.*, 2000). Recently, Rastgoo *et al.*, (2014) reported that Zn stimulated proline linked pentose phosphate pathway while proline helped phytochelatin synthesis and enhanced metal tolerance in plants. Fortification of 200 ppm $ZnSO_4$ in the soil resulted in a decreased greenness of the plant possibly due to (1) replacing of Mg^{+2} in chlorophyll by the other ions including Zn (Wakao *et al.*, 1996; Vargas and Lopez, 2002; Petrovic *et al.*, 2006; Tanaka and Tanaka, 2006) (2) reduction of enzyme involving chlorophyll synthesis (Reinbothe and Reinbothe, 1996; Hortensteiner and Krautler, 2011; Richter and Grimm, 2014; Chibuike and Obiora, 2014) and (3) toxicity of excess Zn though the symptom in the plant was still not clear (Vinod *et al.*, 2012). Fontes and Cox (1995) reported that in common plants the leaves were brown or had brown spots when soil contained more than 150-300 ppm Zn. However, the a level of Zn deficiency or overload in plants still needs further investigation, because each plant may respond to Zn content differently. It was found that though -a/b value of all kitchen mint leaves were not significant different, the -a/b value of each sample had a good relationship with chlorophyll content.

Table 10. Effect of $ZnSO_4$ fortification on color value of the kitchen mint leaves

Sample	Position	Upper side				Lower side			
		L*	a*	b*	-a/b	L*	a*	b*	-a/b
Control	leave base	67.52+0.85^{b}	-5.72±0.14^{a}	16.32±0.44^{a}	0.35±0.15^{ns}	71.62±0.44^{b}	-5.10±0.06^{b}	16.28±0.14^{a}	0.31±0.0^{ns}
	leave mid	67.65±0.91^{b}	-5.38±0.53^{a}	15.83±0.20^{b}	0.34±0.17^{ns}	69.30±0.56^{b}	-5.04±0.35^{b}	16.50±0.37^{b}	0.30±0.0^{ns}
	leave apex	64.85±0.89^{b}	-5.20±0.01^{a}	13.40±0.50^{b}	0.39±0.23^{ns}	68.87±0.05^{a}	-4.83±0.92^{b}	14.87±0.31^{c}	0.32±0.3^{ns}
100 ppm	leave base	63.61±0.63^{c}	-6.63±0.13^{c}	16.95±0.46^{a}	0.39±0.17^{ns}	66.21±0.63^{c}	-5.51±0.40^{c}	15.08±0.75^{c}	0.37±0.18^{ns}
	leave mid	64.70±0.42^{c}	-6.57±0.05^{b}	16.37±0.07^{a}	0.40±0.0^{ns}	63.16±0.16^{c}	-5.31±0.07^{c}	14.42±0.15^{b}	0.37±0.05^{ns}
	leave apex	64.13±0.60^{b}	-6.30±0.10^{b}	17.08±0.43^{a}	0.37±0.17^{ns}	66.02±0.64^{b}	-5.23±0.22^{c}	17.87±0.98^{a}	0.29±0.38^{ns}
200 ppm	leave base	68.92±0.40^{a}	-5.39±0.16^{b}	14.67±0.10^{b}	0.37±0.03^{ns}	75.30±0.65^{a}	-4.94±0.06^{a}	15.76±0.19^{b}	0.31±0.07^{ns}
	leave mid	73.15±0.42^{a}	-5.31±0.03^{a}	14.68±0.10^{c}	0.36±0.04^{ns}	73.11±0.47^{a}	-4.94±0.09^{a}	14.72±0.20^{b}	0.34±0.06^{ns}
	leave apex	71.21±0.27^{a}	-5.21±0.06^{a}	17.07±0.14^{a}	0.31±0.04^{ns}	68.87±0.05^{a}	-4.46±0.19^{a}	16.80±0.36^{b}	0.27±0.09^{ns}

Remark a-c, Means within columns with different letters are significantly different ($p<0.05$). Each value was expressed as the mean ± standard deviation (n = 10).

Table 11. Effect of $ZnSO_4$ fortification on chlorophyll of the kitchen mint leaves

Sample	Chlorophyll a (mg/1,000ml)	Chlorophyll b (mg/1,000ml)	Total Chlorophyll (mg/1,000ml)
Control	1083.11 ± 0.36^{b}	419.43 ± 0.31^{b}	1502.55 ± 0.49^{b}
100 ppm	1195.78 ± 0.36^{a}	506.36 ± 0.30^{a}	1700.91 ± 0.66^{a}
200 ppm	1004.14 ± 0.04^{c}	413.40 ± 0.32^{c}	1417.30 ± 0.08^{c}

Remark a-c, Means within columns with different letters are significantly different ($p<0.05$). Each value was expressed as the mean ± standard deviation (n = 3).

2.4.7 Effect of $ZnSO_4$ on hoard essential oil of kitchen mint leaves

The essential oil determination of the plant leaves from digitalized photography using the Nikon Alphaphot2 microscope at 40×10, 100-120V, 50/60 Hz, 0.8 A is shown in Figure 19. The result showed that essential oil drop of the control leaves was smallest while, the largest size and darker color of essential oil droplet was found in the leaves obtained from the mint grown in soil fortified with 200 ppm $ZnSO_4$. The larger size and darker color of essential oil droplet from the leaves obtained from fortified 200 ppm $ZnSO_4$ may be a good explanation of antimicrobial activity to be mentioned later. In addition, the participants noticed that the mint grown in the 200 ppm fortified $ZnSO_4$ had a strange flavor, tasted like burning, had a pungent smell and gave less cool feeling probably due to quality (darker color) and quantity (bigger size) of oil drop as shown in the Figure 19 (c).

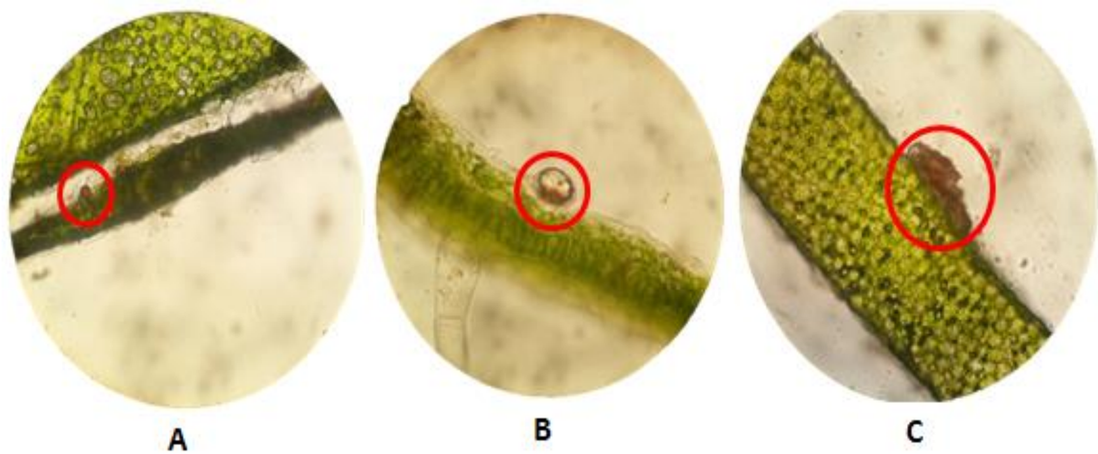


Figure 19. The essential oil drop of kitchen mint fortified with Zn at different concentrations
(A): Control, (B): 100 ppm Zn fortification
(C): 200 ppm Zn fortification

2.4.8 Effect of $ZnSO_4$ on microbiological test in kitchen mint

Generally, major groups of microorganisms found in common fresh kitchen mint are bacteria, yeasts and molds. However, species and a number of microorganisms depend on many factors such as raw material, farm management, season, climate, harvesting techniques and the management after harvesting such as washing, cutting and storage (APO, 2006; Stopforth *et al.*, 2010; FDA, 2015). It was found that the control sample contained the highest number of total viable count of 7.63×10^4 CFU/g, yeast and mold of 4.9×10^2 CFU/g, followed by the sample obtained from 100 ppm and 200 ppm $ZnSO_4$ fortification, respectively, although the differences were not significant (Table 12). The possible reason for the leaves obtained from 200 ppm $ZnSO_4$ fortification kitchen mint containing fewer microorganisms than others was that Zn induced a higher essential oil content which exhibited an antimicrobial property (Misra and Sharma, 2003; Kalemba and Kunicka, 2003; Marichali *et al.*, 2016; Ahmad, 2016). However, the number of *Staphylococcus aureus* (*S. aureus*) which was found only in small number and was not significantly different in each sample while the number of *Escherichia coli* (*E. coli*) in the sample

obtained from 200 ppm $ZnSO_4$ fortification was smallest. There was no *Salmonella* spp. detected in any sample. Actually, *E.coli* and *S.aureus* contamination indicated the poor microbiological quality of water supply during plantation, harvesting and post harvesting and as well as poor personal sanitation (Stanisavljevic *et al.*, 2012). The result pointed out that $ZnSO_4$ at 200 ppm for mint plantation possesse a significant effect on *E.coli* count. Many researchers reported that *E.coli* was highly sensitive to essential oil such as menthol (Moleyar and Narasimham, 1992), cineole (Pattnaik *et al.*, 1997), limonene, carvone and pulegone (Oumzil *et al.*, 2002). Generally, total viable count, yeast and mold, *Salmonella* spp., *S.aureus* and *E.coli* of all treatments in this experiment followed the fresh vegetable for exporting standard which were not higher 1×10^6 CFU/g, 1×10^4 CFU/g, not detected, 100 CFU/g and 100 MPN/g respectively (Department of Medical Sciences, 2007).

Table 12. Effect of $ZnSO_4$ fortification on microbiological test of the kitchen mint leaves

Sample	Yeast and Mold CFU/g	TPC CFU/g	*Salmonell* Spp. CFU/g	*S. aureus* MPN/g	*E.coli* MPN/g
Control	4.9×10^2	7.63×10^4	ND	9.2$\pm$0.02	9.2$\pm$0.02
100 ppm	4.76×10^2	6.57×10^4	ND	9.2$\pm$0.01	9.2$\pm$0.02
200 ppm	3.2×10^2	5.17×10^4	ND	9.2$\pm$0.02	3.6$\pm$0.01

Each value was expressed as the mean $\pm$ standard deviation (n = 3). ND: Not detected

2.4.9 Effect of $ZnSO_4$ on sensory acceptability in kitchen mint

Sensory acceptability of kitchen mint grown in the soil fortified with zinc sulfate at different concentration evaluated by 30 panelists using the 9-point hedonic scale was showed in Table 13. The result showed that overall and taste attributes of the leaves with fortified 100 ppm $ZnSO_4$ and 200 ppm $ZnSO_4$ were significantly higher than those of controls at $p < 0.05$. However, no significant differences in color and flavor characteristics were detected from any sample. The odor score of the sample with 100 ppm $ZnSO_4$ fortification was significantly higher than the control but not in 200 ppm $ZnSO_4$ fortification. However, some panelists suggested that the kitchen mint grown in the soil fortified with 100 ppm $ZnSO_4$ gave a stronger and clearer natural odor compared with control sample. In addition the panelists also commented on the sharp taste and burning sensation of leaves obtained from 200 ppm $ZnSO_4$ fortification while the leaves at 100 ppm $ZnSO_4$ fortification possessed a significant cool taste like the characteristic taste of menthol (Ahijevych and Garrett, 2004; Patel *et al.*, 2007; Lindsey *et al.*, 2006; Mundel and Jones, 2010; Moghtader, 2013) (presented in form A1 Appendix A). It was pointed out that sharp taste and burning sensation may relate to some different compounds appearing in mint when the plant was grown in the soil fortified with 200 ppm $ZnSO_4$. This needs to be further investigated.

Table 13. Effect of $ZnSO_4$ fortification on sensory acceptability of the kitchen mint leaves

Sample	Color	Odor	Taste	Flavor	Overall
Control	7.58 ± 0.86^{ns}	6.77 ± 1.14^{b}	6.46 ± 0.95^{b}	6.81 ± 0.75^{ns}	6.69 ± 0.97^{b}
100 ppm	7.77 ± 0.71^{ns}	7.38 ± 0.98^{a}	7.31 ± 0.68^{a}	7.19 ± 0.75^{ns}	7.27 ± 0.87^{a}
200 ppm	7.73 ± 0.83^{ns}	7.15 ± 0.97^{ab}	6.96 ± 0.96^{a}	7.08 ± 0.98^{ns}	7.27 ± 1.04^{a}

Remark a-c, Means within columns with different letters are significantly different ($p<0.05$). Each value was expressed as the mean ± standard deviation (n = 30).

2.5 Conclusion

In conclusion, $ZnSO_4$ fortification in soil can increase the level of zinc content in kitchen mint leaves significantly with improvement of growth characteristics, yield, protein and fat content as well as the consumer acceptability score. The fortification with $ZnSO_4$ 100 ppm of the mint leaves showed the highest chlorophyll content. However, darker and larger size of oil droplet was found in the mint leaves obtained from plant grown in soil fortified with 200 ppm $ZnSO_4$. The plant grown with fortified 100 ppm $ZnSO_4$ was the optimum choice based on sensory score and suitable for consumption; whereas plants grown in soil fortified with 200 ppm $ZnSO_4$ may be suitable for essential oil production.

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Chapter 3

Effect of zinc biofortification on phenolic, flavonoid accumulation and antioxidant activity of kitchen mint during plantation

3.1 Abstract

Increasing of zinc biofortification in plant is a good recommended method for producing phytochemicals and secondary metabolites. The present experiment aimed to evaluate the effect of biofortification of $ZnSO_4$ at 0, 100 and 200 ppm on phytochemical, total extractable phenolic compounds using gallic acid, Trolox and L-ascorbic acid as standards and total extractable flavonoids compounds using quercetin and catechin as standards. Radical scavenging activities were measured using DPPH, ABTS and FRAP assays. Some bioactive profiles were determined using HPLC technique as well as antibacterial activity determination was also monitored. It was found that the ethanol plant extracts exhibited alkaloids, tannins, cardiac glycosides, flavonoids, phenols, terpenoids and steroids. Total extractable phenolic compounds were the highest in the mint leaves grown in soil fortified with 100 ppm of $ZnSO_4$, meanwhile significantly increasing of flavonoid and the highest antioxidant activity were presented in the sample obtaining from 200 ppm of $ZnSO_4$ fortification. Though, active compounds matching of the extract using HPLC technique did not completely identify, suspected flavonoids compounds was higher in the sample grown in 200 ppm of $ZnSO_4$ fortified soil compared to sample grown in 100 ppm of $ZnSO_4$ fortification. However it was disappointed that there was no antibacterial activity when tested with *E. coli*, *S. typhi*, *S. aureus* and *P. acne*.

3.2 Introduction

With busy life, less exercise, imbalance diet with high fat and carbohydrate particular sugar, less fiber together with high stress lead to increasing of noncommunicable diseases (NCD) (WHO, 2002). An imbalance metabolism occurs in human body starting from high oxygen consumption in living cell until leads to over accumulation of reactive oxygen species (ROS) including superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($HO^{\bullet}$) and non-free radical such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Halliwell *et al*., 2009). ROS can easily induce peroxidation of membrane lipids causing lipid oxidation (Halliwell and Chirico, 2016), damage essential biomolecules including nucleic acids, lipids, proteins and carbohydrates as well as DNA. Those causes can bring malfunction or mutation of various cells and/or organelles (Moller *et al*., 2007). Reducing ROS or antioxidant agent is hot issue addressed worldwide. In addition, natural product producing from plants as known as phytochemicals has been intensively studied because of its safety, affordable cost and available market (Dillard and German, 2000). In order to increase phytochemicals, various techniques have been used such as tissue cultures (Hussain *et al*., 2012), mineral biofortification (Nasiri *et al*., 2010) breeding and genetic modification (Zamir, 2001). Zinc is an essential mineral in all living cells; microbial, plants and animal. Insufficient intake of Zn, human body will suffer from skin problem, hair loss and muscle weakness (King *et al*., 2000). In point of plant physiological functions, inadequate of Zn leads to a decrease number of tillers, increases chlorosis and smaller leaves, prolongs crop maturity period, spikelet sterility and lowers quality of harvest products (Hafec *et al*., 2013). Alloway (2008) asserted that Zn fortification enhanced various biological compounds such as flavonoid, phenolic, chlorophyll, menthol, essential oil, protein in mint family and citrus during plantation.

Mentha cordifolia Opiz or kitchen mint belongs to genus *Mentha* and family Lamiaceae. It is a top five vegetables consuming in Thailand (Tongkhao and Mahakarnchanakul, 2014) and be used as folk medicine since ancient times for the treatment of heart burns, indigestion, colic, flatulence, coughs and flu, nausea, irritable bowel syndrome, herpes, and certain skin infections including acne (Rios and

Recio, 2005). The aim of this study was to investigate the effect of zinc fortification into soil on phenolic compounds and flavonoids compounds, as well as antioxidant activity of the kitchen mint during plantation.

3.3 Material and methods

3.3.1 Reagents and culture media

All chemicals used, were of analytical grade. 2,2-diphenyl-1-picryhydrazyl radicals (DPPH), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,4,6-tripyridyl-*s*-triazine (TPTZ) were purchased from Sigma Chemical Co. (Germany). Trolox, pyrogallol, protocatechuic acid, *p*-coumaric acid, vanillic acid, gallic acid, caffeic acid, ferulic acid, syringic acid, apigenin, ellagic, quercetin, catechin and chlorogenic acid were obtained from Sigma (USA). Ascorbic acid from Fluka, potassium persulfate, sodium nitrite, aluminium chloride and ferric chloride hexahydrate from Ajax Finechem, while sodium hydroxide from Apdivision of Nuplex Industries (Aust) Pty Ltd, New Zealand were used. Folin-Ciocalteu's phenol reagent was purchased from Merck Millipore (Darmatadt, Germany). HPLC grade of menthol, 2-propanol and acetonitrile were used for some active compounds profiling with HPLC technique. All media for microbiological analysis were analytical grade such as brain heart agar (BHA), brain heart infusion broth (BHI) and buffered peptone water purchasing from Difco Michigan, U.S.A. Ampicillin and tetracycline were used as standard drugs and purchased from Oxoid, England.

3.3.2 Bacterial cultures

The four strains of bacteria were selected and used as test organisms including *Escherichia coli*, *Salmonella typhimurium*, *Staphlococcus aureus* from Songklanagarind hospital and *Propionibacterium acne* DMST 14916 from Department of Medical Sciences.

3.3.3 Preparation of mint extract

Mint was planted in experiment pot with diameter 19 cm and 13 cm depth containing soil fortified with 0, 100 and 200 ppm $ZnSO_4$ for 3 mo in a nursery of Faculty of Agro-Industry, Prince of Songkla University. After harvested plants, the leaves were washed with running tap water, drained on the sieve for 5 min before taken to absorb with facial tissue to remove excess water. Thereafter, 1 kg of plant leaves from each group was blended with 4,000 ml of ethanol and stirred at room temperature 28 $\pm$ 2 °C for 24 h, then filtered with chess cloth and centrifuged at 8000×g. The clear supernatant was collected and dried using a rotary evaporator for 42 °C for 1 hr. The sample with dark-brown color and viscous was subjected to solid content analysis then kept in dark bottle and stored at 4 °C during analysis.

3.3.4 Phytochemicals screening

Mint extract was screened for the main phytochemical compounds followed standard procedures as described by Sofowara (1993) and Harborne (1973) with some modifications. The results were recorded as absence or presence with low, medium, or high intensity. The screening test was detected in various group as followed:

3.3.4.1 Test for alkaloids

Three-four drop of each extract was added with 3- 4 drop of Dragendorff's reagent and Mayer's reagent then gently shaken. An orange and creamy white precipitate indicated the presence of tannins.

3.3.4.2 Test for tannin I

Two-ml of each extract was added with a few drops (3- 4) of 1 % ferric chloride then gently shaken. A bluish-black or brownish-green precipitate indicated the presence of tannins.

3.3.4.3 Test for tannin II

Two-ml of each extract was added with 1 ml of lead subacetate ($C_4H_{10}O_8Pb_3$) then gently shaken. A bluish-black or brownish-green precipitate indicated the presence of tannin.

3.3.4.4 Test for cardiac glycoside I

Each extract 0.5 ml was mixed with 0.5 ml of ethanol and evaporated in evaporating dish. Thereafter, a few drops (2) of Ray mond's reagent and Kedde's reagent were added before added with 2 drop of alcohol potassium hydroxide. After, the mixture was gently shaken a brown color indicated the presence of cardiac glycoside.

3.3.4.5 Test for cardiac glycoside II

Each extract, 0.5 ml, was mixed with 0.5 ml of ethanol then 1 ml of glacial acetic acid, 10 % ferric chloride and 1 ml concentrated sulfuric acid were followed added. A reddish brown coloration of the inter face was formed to show positive results for the presence of cardiac glycoside.

3.3.4.6 Test for saponin

One ml of the extract was added with 1 ml of distilled water then shaken vigorously for 30 s. Persistence of foams and duration was the indicator for saponin**.**

3.3.4.7 Test for flavonoids I

One ml of the extract was added in to 1 ml of 10 % lead acetate then gently shaken. A muddy brownish coloration was observed indicating the presence of flavonoids.

3.3.4.8 Test for flavonoids II

One ml of the extract was added into to 1 ml of 10 % ferric chloride before gently shaken. The wooly brownish signified the presence of flavonoids.

3.3.4.9 Test for phenols

Two-ml of the extract was warmed at 45-50 °C then 2 ml of 3% ferric chloride was added. The color changed to blue or green indicating the presence of phenols.

3.3.4.10 Test for terpernoids

Five-ml of each sample extract was mixed with 2 ml of chloroform, and 3 ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

3.3.4.11 Test for sterols

Two-ml of each sample extract was mixed with 2 ml concentrated sulfuric acid. A reddish brown in samples indicated steroids.

3.3.5 Determination of total extractable phenolic compounds

The total extractable phenolic compounds were determined according to the Folin-Ciocalteu method (Herald *et al.*, 2012). The reaction was performed in the 96-well plates started from mixing 75 µl deionized water with the appropriate extract concentration, 25 µl then mixed with 25 µl of Folin-solution (1:1, DI: Folin-Ciocalteu) and followed with 100 µl of sodium carbonate (Na_2CO_3) at concentration 75 g/1,000ml. After 90 min of incubation in the dark at room temperature, the absorbance was measured at 765 nm by spectrophotometric microplate reader. The total extractable phenolic compounds was expressed as µg of gallic acid, L-ascorbic acid and Trolox equivalent/mg of dry weight of the sample, using an equation of each calibration curve ranged from 20 to 50, 40-120 and 200-450 µg/ml ($y = 0.0087x - 0.0752$; $R^2 = 0.995$, $y = 0.0087x - 0.0752$; $R^2 = 0.995$, $y = 0.0087x - 0.0752$; $R^2 = 0.995$), respectively.

3.3.6 Determination of total extractable flavonoid compounds using quercetin as standard

Total extractable flavonoid compounds were determined by the aluminum calorimetric method (Yang *et al.*, 2011) using quercetin as the reference standard. Briefly, the test sample of ethanol solution 150 µl was mixed with 2% w/v $AlCl_3$ in 96 well plates. After 15 min of incubation in the dark at room temperature, the absorbance was measured at 435 nm by spectrophotometric microplate reader. The total extractable flavonoid compounds was expressed as µg of quercetin equivalent/mg of dry weight of the sample, using an equation obtaining from calibration curve ranged from 40 to 140 µg/ml ($y = 0.0041x - 0.0126$; $R^2 = 0.995$).

3.3.7 Determination of total extractable flavonoid compounds using catechin as standard

Total extractable flavonoid compounds were determined by the aluminum calorimetric method (Herald *et al.*, 2012) using catechin as the reference standard. Briefly, 10 µl of $NaNO_3$ (50g/1,000ml) was added to 100 µl distilled water and then mixed with 25 µl of appropriate extract concentration. After incubation in the dark at room temperature for 5 min, 15 µl of $AlCl_3$ (100 g/1,000ml) was added to the mixture, followed by 50 µl of NaOH (1 mol/1,000ml) and 50 µl deionized water. The absorbance was measured at 510 nm by spectrophotometric microplate reader. The total extractable flavonoid compounds was expressed as µg of catechin equivalent/mg of dry weight of the sample, using an equation obtaining from calibration curve ranged from 240 to 840 µg/ml ($y = 0.0007x + 0.0337$; $R^2 = 0.9974$).

3.3.8 Determination of antioxidant activity

The standard curve for DPPH, ABTS and FRAP assays was prepared using pyrogallol, protocatechuic acid, *p*-coumaric acid, vanillic acid, gallic acid, L-ascorbic acid, caffeic acid, ferulic acid, sringic acid, Trolox, apignin, ellagic, quercetin, catechin and chlorogenic acid. The activity was expressed as µmol equivalent of each standard used as mentioned/ g sample.

3.3.8.1 DPPH radical-scavenging activity (DPPH assay)

DPPH scavenging activity was determined by the modified method of Brand-Williams *et al*. (1995). The mint extract at appropriate concentration, 100 µl was added with 100 µl of 0.2 mM 2,2-diphenyl-1-picryhydrazyl radicals (DPPH) in 95% ethanol. The mixture was kept at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a spectrophotometer.

3.3.8.2 ABTS radical-scavenging activity (ABTS assay)

ABTS assay was determined by the modified method of Re *et al*. (1999). The ABTS working solution included 4 mM 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2.5 mM potassium persulphate as ratio of 1:1 were mixed at room temperature then stand for 16 h in dark. After 16 h, ABTS solution was mixed with DI at ratio 1:14 then measured at 734 nm using a spectrophotometer with to obtain OD with 1.1 $\pm$ 0.02. The extracts, 20 µl was added with 280 µl of ABTS solution. The mixture was mixed at room temperature in the dark for 2 h before measured absorbance at 734 nm using a spectrophotometer.

3.3.8.3 Ferric reducing antioxidant power assay (FRAP assay)

FRAP Assay was determined by the modified method of Benzie and Strain (1996). The FRAP working solution included 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ), 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 300 mM acetate buffer pH 3.6 as ratio of 1:1:10 (v/v/v). The extracts, 30 µl was added with 270 µl of FRAP reagent. The mixture was react at room temperature in the dark for 30 min than the absorbance was measured at 595 nm using a spectrophotometer.

3.3.9 Preparation of sample and standard solution for phytochemical profiling using HPLC technique

Three-mg of crude extract was dissolved with 3 ml of acid methanol (62.5% methanol: 6 M HCl, ratio 4:1 v/v) and stand at 70 °C for 2 h. Stock standard solutions of 14 standard phenolics and flavonoids such as pyrogallol, protocatechuic acid, (+)-catechin, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, rutin, ferulic acid, quercitrin, quercetin, cinamic acid, apigenin and kempferol were prepared at 0.5 mg/ml (500 ppm) with HPLC grade methanol. Working solutions were prepared from the stock solution of each standard at appropriate methanol dilution.

3.3.10 High performance liquid chromatographic analysis for phytochemicals

Phytochemicals were analyzed by a high performance liquid chromatograph (Agilent Technologies 1200 series) equipped with a Zorbax Eclipase XDB-C18 column (4.6×150 mm, inner diameter 5 mm; Agilent Technologies). The DAD detector was applied to scan the phytochemical of interest to ascertain their within a range of 210 to 366 nm. A gradient solvent system was employed with 0.1% Trifluoroacetic acid (TFA) (solvent A), 100% acetonitrile (solvent B). The elution profile was the following proportions (v/v) of solvent B: 0.00-5.00 min, 0-5%; 5.00-10.00 min, 5-10%; 10.00-15.00 min, 10-10%; 15.00-20.00 min, 10-15%; 20.00-30.00 min, 15-25%; 30.00-35.00 min, 25-25%; 35.00-40.00 min, 25-50%; 40.00-50.00 min, 50-80%; 50.00-60.00 min, 80-80%; 60.00-70.00 min, 80-100%. The column set at 40 °C was flushed with a flow rate of 0.8 ml/min. All the prepared solutions were filtered through 0.22 μm membranes and the mobile phase was degassed before injection on to HPLC. Chromatographic was identified and confirmed by comparison of retention time and spectrum with standards.

3.3.11 Determination of antibacterial activity

The disc-diffusion assay as described by Rasoanaivo and Ratsimamanga-Urverg (1993) was used to determine the growth inhibition of bacterial by the crude extract. Stock cultures of *E.coli*, *S.typhimurium*, *S. aureus* were grown in nutrient broth at 37 °C for 18 h and *P. acne* was grown in nutrient broth at 37 °C for 48 h in anaerobic. Final bacterial concentrations were 10^8–10^9 cfu/ml. 0.1 ml of each the inoculum was spread on Mueller-Hinton agar (MHA). Sterile filter paper (Whatman No. 1, diameter 6 mm) impregnated with 20 µl of the extracts and ethanol 40% at a concentration of 20 µl/disc as a negative control were put on the plate inoculated with each test micro-organism and incubated at 37 °C for 18 h for *E.coli*, *S.typhimurium*, *S. aureus* and 48 h for *P. acne* in anaerobic jar. The diameter of the clear zone shown on plates was measured using calipers and expressed in millimeters as its antibacterial activity. Standard drug; ampicillin and tetracycline at concentration 30 and 10 µg/disc, respectively were used as references.

3.3.12 Statistical analysis

Completely randomized design (CRD) was used throughout this experiment. Data was subjected to analysis of variance (ANOVA) and mean comparisons were performed using the Duncan's multiple-range tests. Differences between means were considered significant when $p < 0.05$.

3.4 Results and discussion

3.4.1 Phytochemical screening

The result showed that the mint leaves contained alkaloid, tannin, cardiac glycoside, flavonoid, phenol, terpenoid and steroid but not saponin (Table 14). In addition, it was found that intensity of alkaloid and terpenoid obtained from 200 ppm of $ZnSO_4$ fortification of the mint leaves was higher than others. However, cardiacs glycoside in control sample was the highest. Though, screening of phytochemicals as described conditions did not provide clealy result of bioactive

compounds, it was used as a guide line to focus and follow biological activity and bioactive compound profiling using HPLC technique further.

Table 14. Effect of different levels of $ZnSO_4$ fortification on phytochemical profiling *M. cordifolia* during plantation

Phytochemical	**Sample**		
	Control	**$ZnSO_4$ 100 ppm**	**$ZnSO_4$ 200 ppm**
Alkaloid	+	++	+++
Tannin (I,II)	+	++	++
Cardiac glycoside (I,II)	+++	+	+
Saponin	-	-	-
Flavonoid (I,II)	+	++	++
Phenols	+	+	+
Terpenoid	+	+	++
Steroid	+	+	+

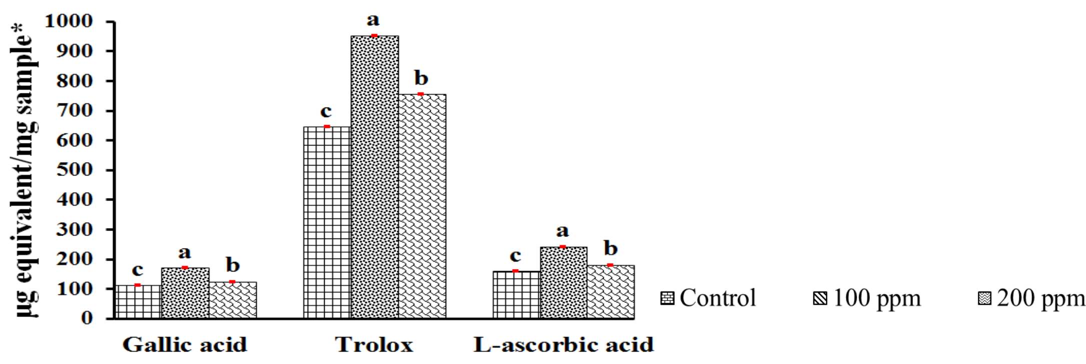
- means absent, + means present with low intensity, ++ means present with medium intensity, +++ means present with high intensity, control: control kitchen mint,100 ppm: kitchen mint fortified with 100 ppm $ZnSO_4$, 200 ppm: kitchen mint fortified with 200 ppm $ZnSO_4$.

3.4.2 Total extractable phenolic compounds and total extractable flavonoid compounds

The result revealed that the grown in 100 ppm $ZnSO_4$ fortified soil yielded the highest total extractable phenolic compounds determined by Folin-Ciocalteu and using gallic acid, Trolox and L-ascorbic acid (AA) as standard (Figure 20, a). On the other hand, it was found that total extractable flavonoids compounds in the sample

grown in 200 ppm $ZnSO_4$ fortified soil was the highest (Figure 20, b). A significantly increased total extractable phenolic compounds in the leaves obtain from 100 ppm $ZnSO_4$ fortified soil during plantation indicated the increasing of phytochemicals (Figure 21) may due to Zn facilitated enhancing secondary metabolic substances during plant growth. In addition, it was noticed that the plants grown in 100 ppm $ZnSO_4$ fortified soil was heavy invaded with caterpillar even it was also experimented in the same nursery with other treatments (Figure 16). This observation pointed out that at certain $ZnSO_4$ fortified soil supported higher growth leading to increasing specific compounds as insect attractants (Mandel *et al*., 2010). Dixon and Paiva (1995) reported that secondary metabolite product as phenolic compound were significantly increased when plant was invaded by insect. Therefore, increasing of total extractable phenolic compounds in the plant leaves grown in 100 ppm $ZnSO_4$ fortified soil should derive from 2 pathways; (1) $ZnSO_4$ enhanced secondary metabolite product and (2) plant produced more phenolic compound to cure the injured cell from caterpillar invasion. However, an increasing of total extractable flavonoids compounds with high value in the leaves obtained from 200 ppm $ZnSO_4$ fortified soil may indicate the transformation of simple phenolic acid to be complex phenolic compounds as flavonoids (Wadhwa *et al*., 2014). The increasing of flavonoids in this experiment particularly in the sample fortified with 200 ppm $ZnSO_4$ may be due to defense mechanism of plant to decrease toxicity of zinc (Emamverdian *et al*., 2015; Symonowicz and Kolanek, 2012). Mourato *et al*. (2015) stated that flavonoids responded to metal chelating property. In addition, Poschenrieder (2006) reported that metal chelated flavonoids provided better antioxidant. It was hypothesized that flavonoids containing in the mint grown with fortified $ZnSO_4$ particularly at 200 ppm may in the Zn chelated flavonoids leading to increasing antioxidant activity which expressed in antioxidant assay.

(a)



(b)

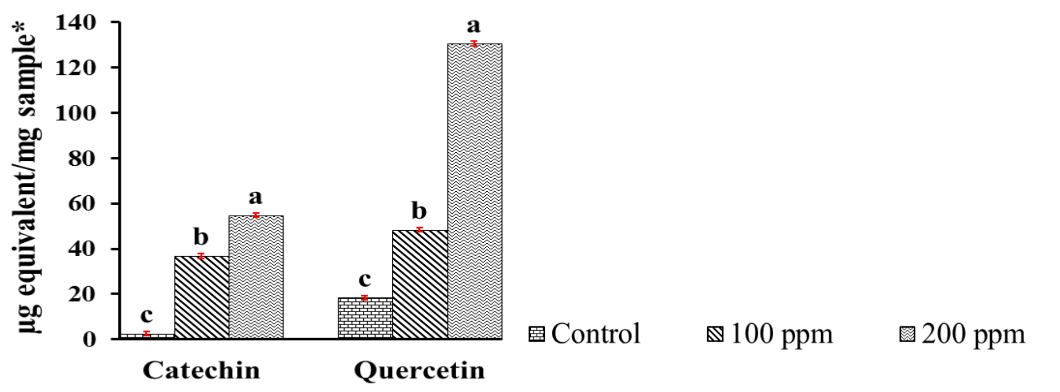


Figure 20. Total extractable phenolic compounds (a) and total extractable flavonoids compounds (b) of kitchen mint fortified with different $ZnSO_4$ concentration. Each value was expressed as the mean $\pm$ standard deviation (n = 3). Different little letters indicate significant differences ($p < 0.05$). * Each value was expressed as the µg equivalent of standard/mg sample, control: control kitchen mint, 100 ppm: kitchen mint fortified with 100 ppm $ZnSO_4$, 200 ppm: kitchen mint fortified with 200 ppm $ZnSO_4$.

Figure 21. Caterpillar invasion of the plant grown in 100 ppm $ZnSO_4$ fortified soil

3.4.3 Antioxidant activity

The equation of each different standard antioxidant, used concentration and R^2 of each antioxidant assay determined by DPPH, ABTS and FRAP were presented in Table B1 (Appendix B). Based on used concentration of each standard, it was found that antioxidant activity (Table 15) well exhibited its antioxidant property in DPPH ABTS and FRAP assay because of high amount of OH group, small size and low hindrance structure (Chen *et al*., 2015). In general, it was found that standard concentration used in DPPH was lowest compared with other assays. It revealed that DPPH radical was easily to react with antioxidant particularly with smaller molecule. In addition, it was discovered that pyrogallol was the greatest antioxidant in all test antioxidant assays. Tian and Schaich (2013) concluded that antioxidant property of each standard depended on (1) size, smeller size easier reaction, (2) OH attached position which *ortho* is better for deliberating H^+ or electron other position compared with *para* and *meta* (Chen *et al*., 2015); (Bendary *et al*., 2013), (3) amount of OH groups which antioxidant activity seemed to increase as OH group increase (Bentes *et al*., 2011); (Simic *et al*., 2007), (4) solvent used for both stock preparations as radicals and standard which facilitated to appropriate polarity, (5) appropriate standard concentration, (6) assay used including DPPH ABTS and FRAP assay. This result showed that type and concentration of used standard were significant and needed to state in the experiment for better understanding. Surprisingly, it was found that using AA was not a good standard for ABTS activity in the experiment. However, Tian and Schaich (2013) stated that ascorbic acid exhibited fair antioxidant activity when determined by ABTS assay. This difference may due to solvent used, radical concentration, time for reaction and analytical methods. Therefore, it is difficult to directly compare the result of each laboratory without using the same standard procedure. Tian and Schaich (2013) stated that even the same method used, the result of each laboratory station may not well correlate when protocol did not standardize. Therefore, comparing antioxidant activity from the different methods may not reflect actual result.

The higher fortified $ZnSO_4$, the higher antioxidant activity (Figure 22, Figure 23 and Figure 24). It was hypothesized that at higher $ZnSO_4$ content, 200 ppm was too high until the plant started to stress leading to production of specific substances as essential oil, tannin, flavonoids to protect plant cells from stressor (Hussain *et al*., 2012). The results revealed that overall all antioxidant activities determined as DPPH, ABTS and FRAP assay of the leaves obtained from 200 ppm $ZnSO_4$ fortified soil were highest compared with other assays may mainly due to flavonoid activity. As know that flavonoids exhibited electron transfer property higher than H^+ donor (Han *et al*., 2012). It was also noticed that the stems were apparently deeper in purple which related to flavonoid compounds at 200 ppm compared to other samples as shown in the Figure 25.

Table 15. Antioxidant activity (IC_{50}) of each used standard determind as DPPH, ABTS and FRAP assay

Standard	Antioxidant activity IC_{50} (µmol/sample)		
	DPPH activity	**ABTS activity**	**FRAP activity**
Pyrogallol	1.54	1.33	7.35
Gallic acid	1.91	3.73	7.81
Chlorogenic acid	5.68	23.44	125
Caffeic acid	5.10	18.52	111.11
Protocatechuic acid	6.85	25.64	111.11
Ferulic acid	41.66	33.33	20.83
Trolox	13.89	107.143	27.78
L-ascorbic acid	6.76	25.64	16.67
Syringic acid	35.71	35.71	30.30
p-coumaric acid	100	24.39	125
Vanillic acid	300	166.67	300
Ellagic	3.14	22.73	19.23
Quercetin	3.05	35.71	83.33
Apignin	250	17.42	100
Catechin	3.57	17.54	8.20

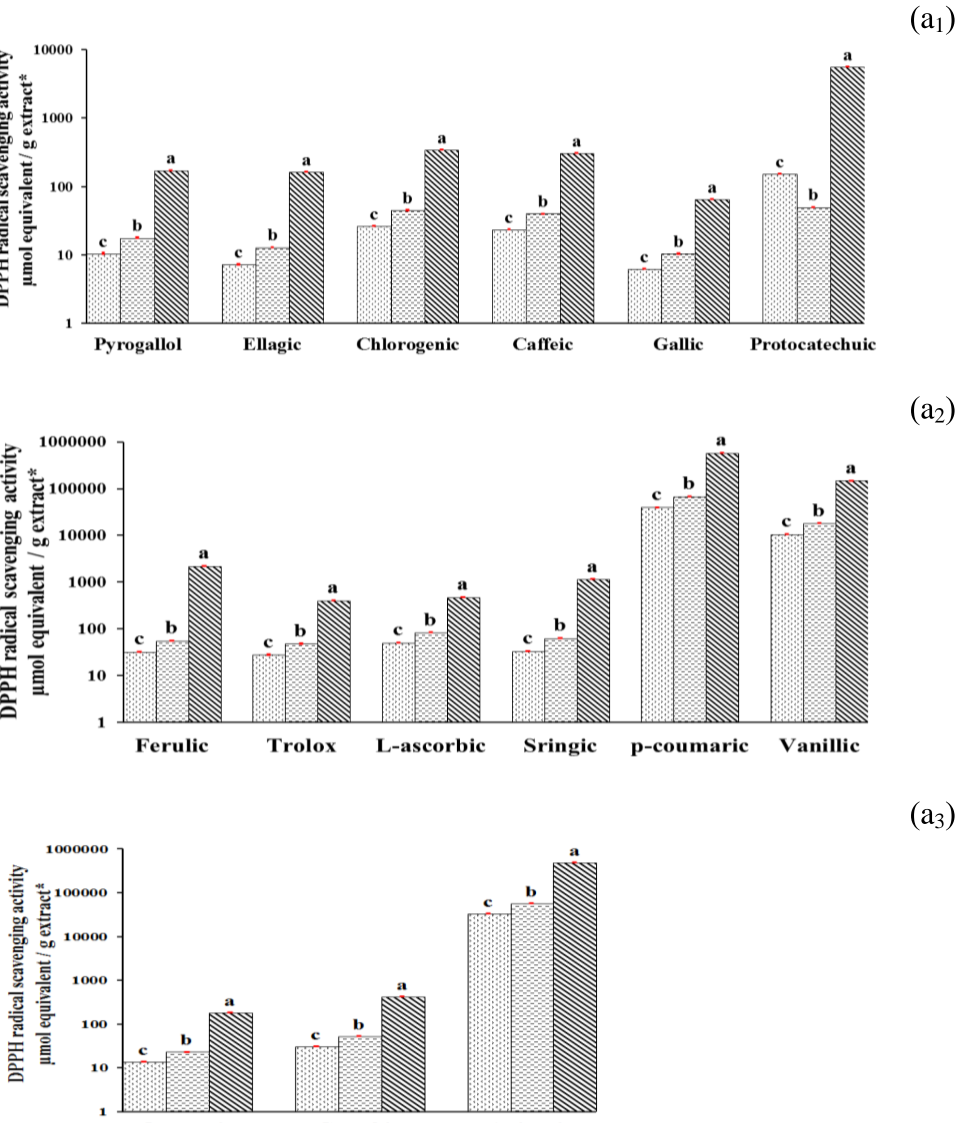


Figure 22. Antioxidant activity: DPPH (a_1-a_3) of kitchen mint fortified with different $ZnSO_4$ concentrations. Each value was expressed as the mean ± standard deviation (n = 3). Different little letters between treatments indicate significant differences ($p < 0.05$). * Each value was expressed as the µmol equivalent of standard/g sample, control: control kitchen mint, 100 ppm: kitchen mint fortified with 100 ppm $ZnSO_4$, 200 ppm: kitchen mint fortified with 200 ppm $ZnSO_4$.

(a_1): means phenolic standards with high activity (>5-1,000 µmol/g),

(a_2): means phenolic standards with high activity (>10-100,000 µmol/g),

(a_3): means flavonoid standards with high activity (>10-100,000 µmol/g)

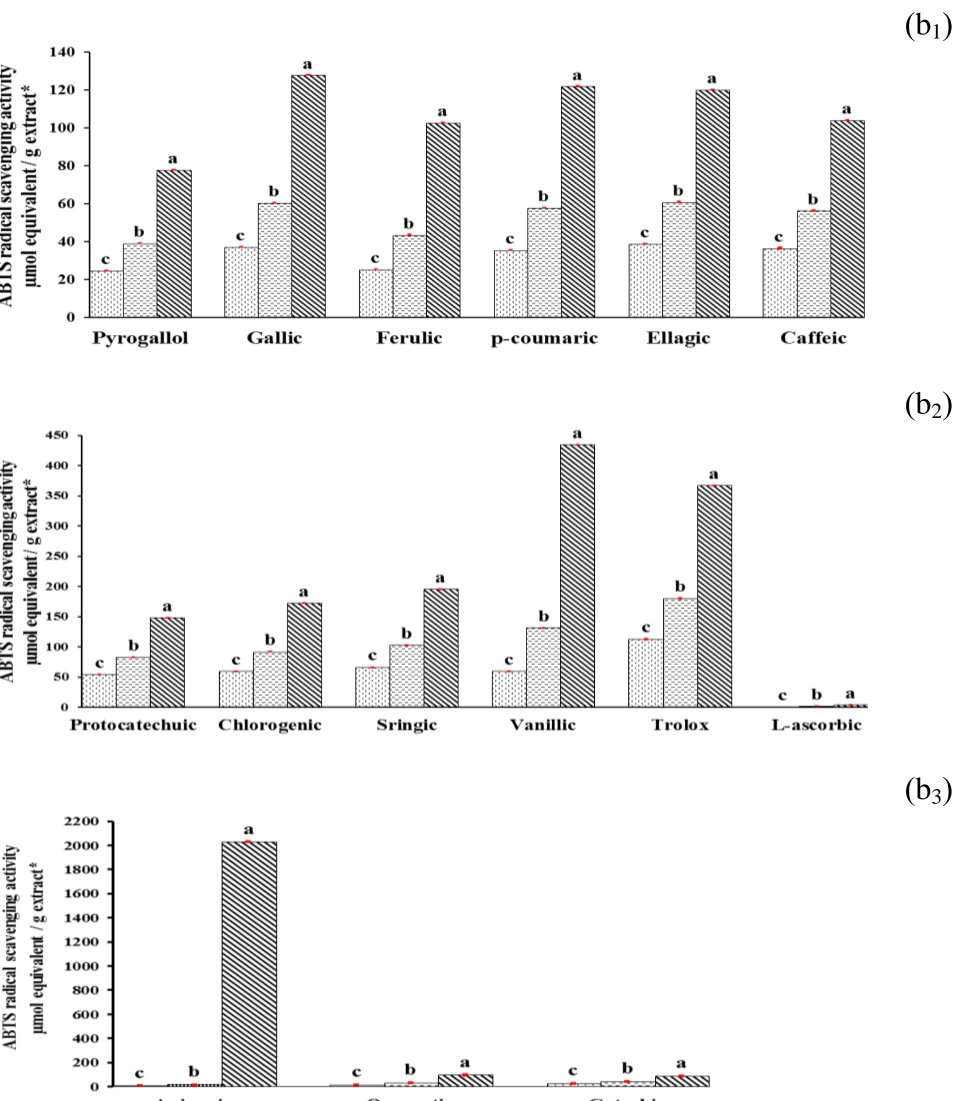


Figure 23. Antioxidant activity: ABTS (b_1-b_3) of kitchen mint fortified with different $ZnSO_4$ concentrations. Each value was expressed as the mean $\pm$ standard deviation (n = 3). Different little letters between treatments indicate significant differences ($p < 0.05$). * Each value was expressed as the µmol equivalent of standard/g sample, control: control kitchen mint, 100 ppm: kitchen mint fortified with 100 ppm $ZnSO_4$, 200 ppm: kitchen mint fortified with 200 ppm $ZnSO_4$.

(b_1): means phenolic standards with high activity (>20-130 µmol/g),

(b_2): means phenolic tandards with less activity (1-450 µmol/g),

(b_3): means flavonoid standards with less activity (>5-2,000 µmol/g)

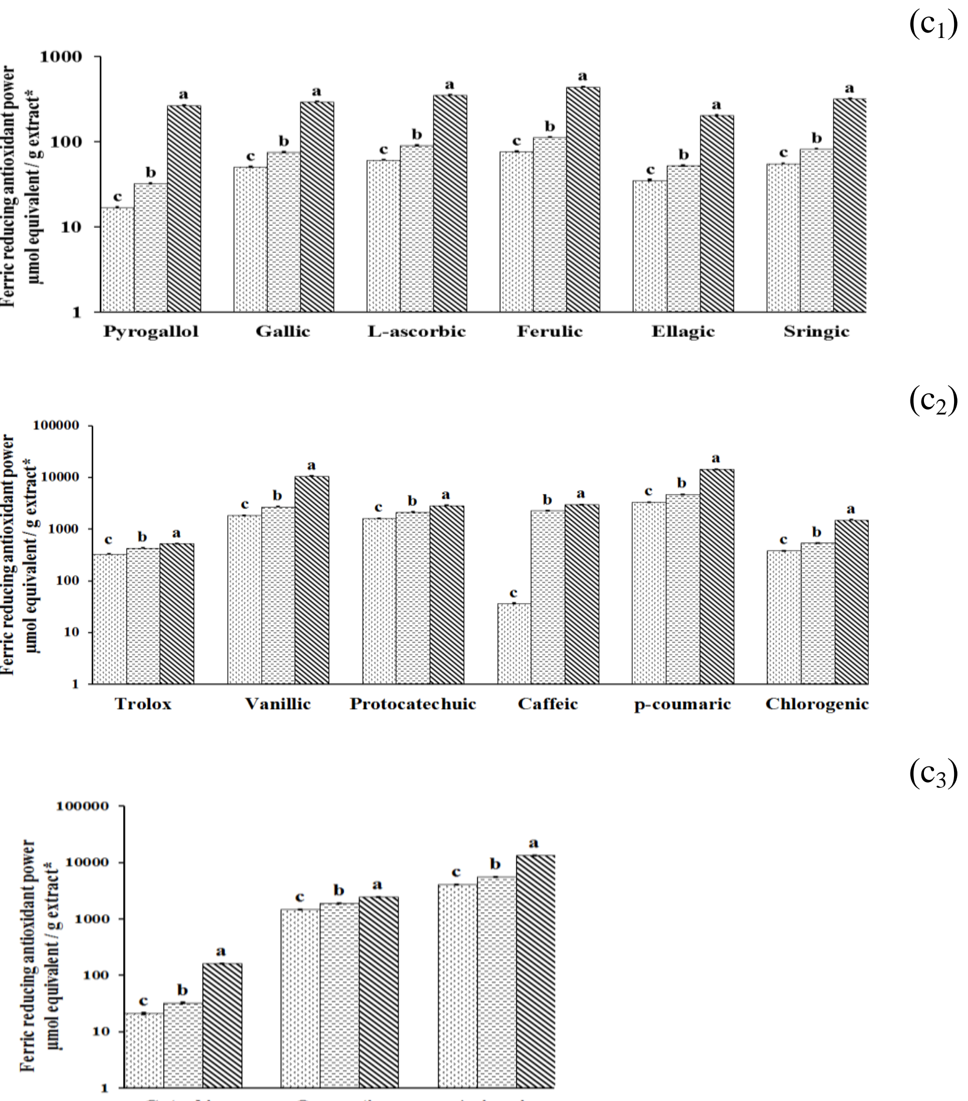


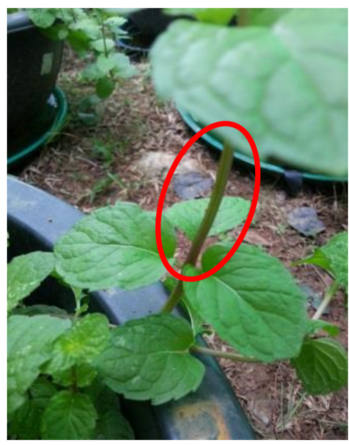
Figure 24. Antioxidant activity: FRAP (c_1-c_3) of kitchen mint fortified with different $ZnSO_4$ concentrations. Each value was expressed as the mean $\pm$ standard deviation (n = 3). Different little letters between treatments indicate significant differences ($p < 0.05$). * Each value was expressed as the µmol equivalent of standard/g sample, control: control kitchen mint, 100 ppm: kitchen mint fortified with 100 ppm $ZnSO_4$, 200 ppm: kitchen mint fortified with 200 ppm $ZnSO_4$.

(c_1): means phenolic standards with high activity (>10-500 µmol/g),

(c_2): means phenolic standards with high activity (>10-10,000 µmol/g),

(c_3): means flavonoid standards with high activity (>10-10,000 µmol/g)

(a): control kitchen mint,


(b): kitchen mint fortified with 100 ppm $ZnSO_4$,

(c): kitchen mint fortified with 200 ppm $ZnSO_4$

Figure 25. The stem color of the kitchen mint grown in fortified and unfortified $ZnSO_4$ soil

3.4.4 Bioactive compounds by HPLC analysis

In fact, it was found that it could not separate the peak in the crude extract which was partial hydrolyzed with acid methanol and without further heating as shown in the Figure 26. It was believed that, crude sample contained both bound and free from of phenolic compound (Proestos and Komaitis, 2013). Therefore, to separate each active compound, acid hydrolysis with heating was applied. The phenolic compounds were determined at 267 nm while the flavonoid compounds were determined at 320 nm (flavones) and 366 nm (flavonoles), respectively. The identified each active compound was based on combination of retention time, spike and spectral matching. In addition, standard agent was spited into the sample to verify the peak height, at the same wavelength. HPLC chromatograms of standard and crude extract were presented in Figure 27. The result revealed that all samples contained both phenlic compound as ferulic acid (Figure 27) and flavonoids including quercitrin and kempferol (Figure 28). However, it was found that the peak hight of protocatechuic acid, cinamic acid, rutin, quercetin and apignin of fortified sample increased as increased $ZnSO_4$ content in the soil.

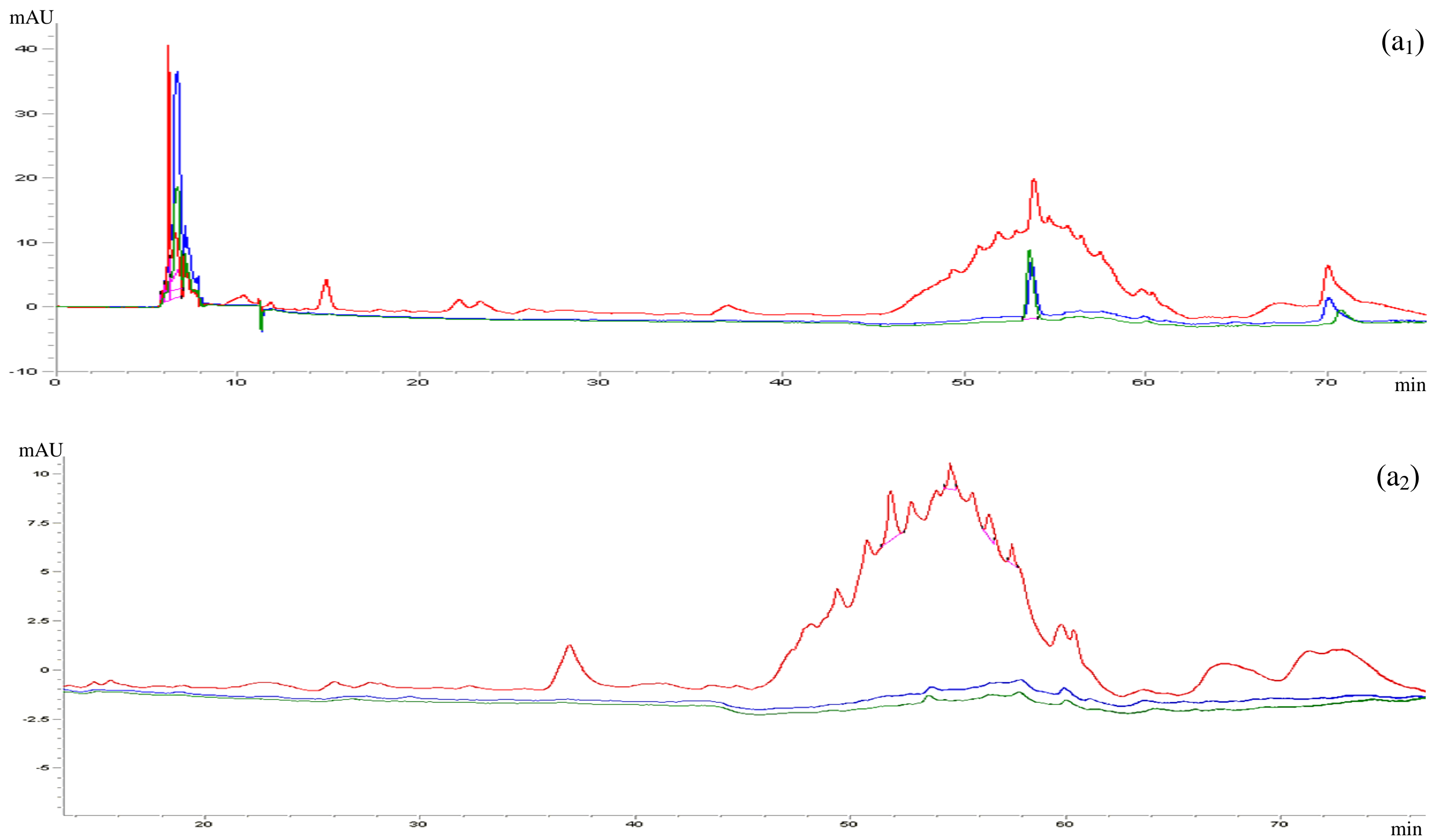


Figure 26. Typical HPLC chromatogram of crude extract partial hydrolyzed with acid methanol at 280 nm (a_1) and 366 nm (a_2) where; ▬ : control kitchen mint, ▬ : kitchen mint fortified with 100 ppm $ZnSO_4$, ▬ : kitchen mint fortified with 200 ppm $ZnSO_4$

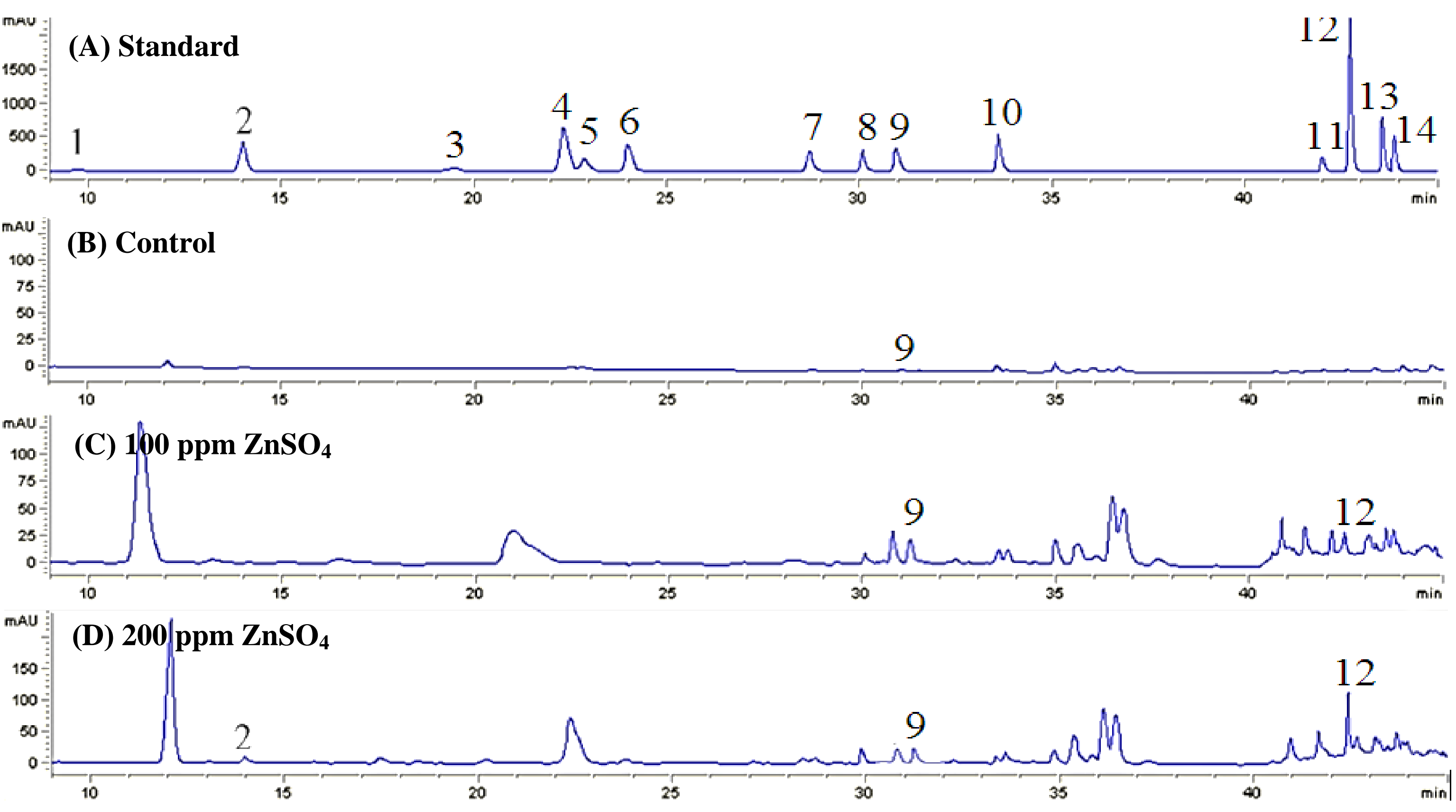


Figure 27. Typical HPLC chromatogram of standard at 267 nm (A) where; 1. pyrogallol, 2. protocatechiuic acid, 3. catechin, 4. vanillic acid, 5. caffeic acid, 6. syringic acid, 7. *p*-coumaric acid, 8. rutin, 9. ferulic acid, 10. Quercitrin, 11. quercetin, 12. cinamic acid, 13. apigenin, 14. kempferol, (B): control kitchen mint, (C): kitchen mint fortified with 100 ppm $ZnSO_4$, (D): kitchen mint fortified with 200 ppm $ZnSO_4$

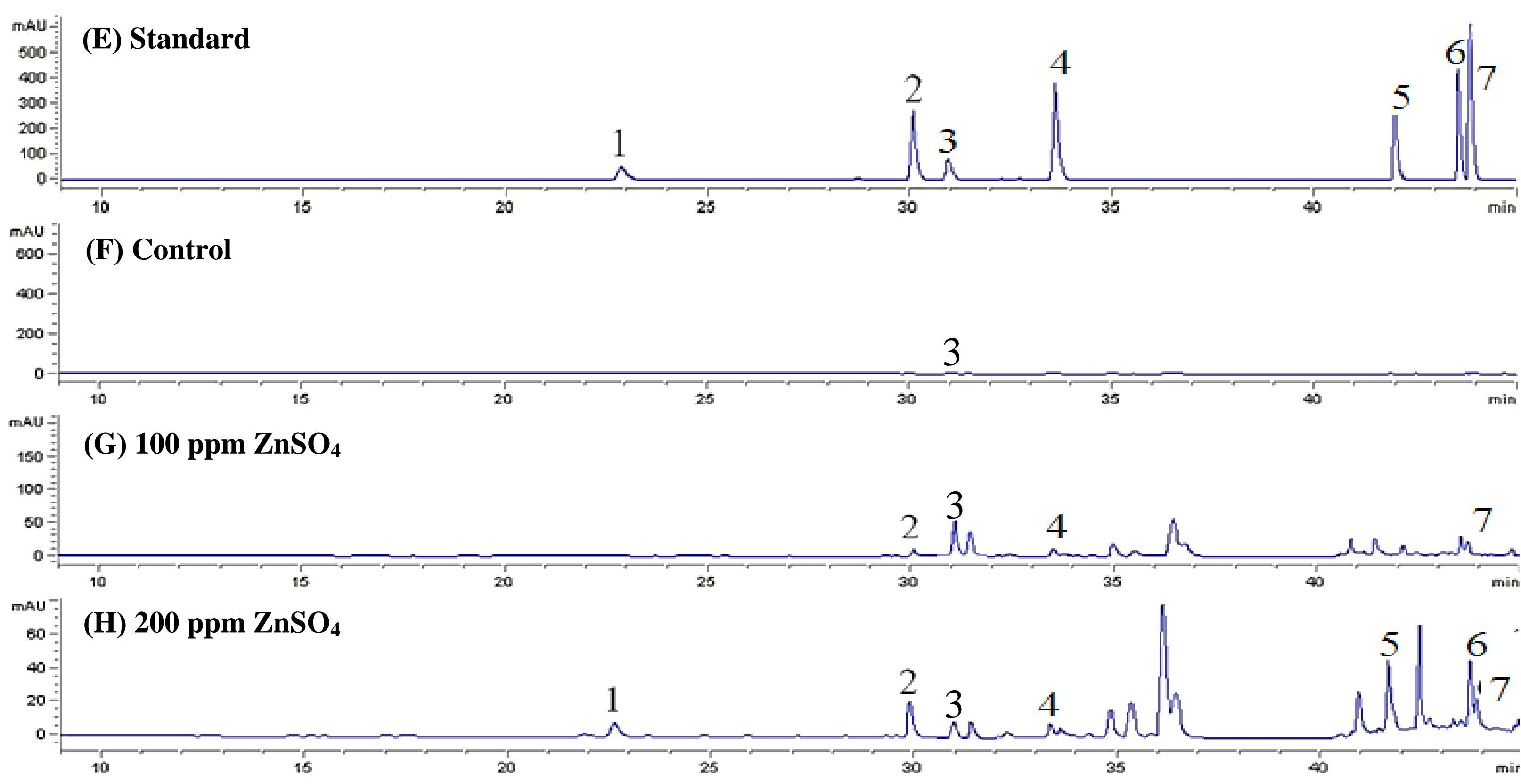


Figure 28. Typical HPLC chromatogram of standard at 366 nm (E) where; 1. vanillic acid, 2. rutin, 3. ferulic acid, 4.quercitrin, 5. quercetin, 6. apigenin, 7. kempferol, (F): control kitchen mint, (G): kitchen mint fortified with 100 ppm $ZnSO_4$, (H): kitchen mint fortified with 200 ppm $ZnSO_4$

3.4.5 Antibacterial activity

The antibacterial effect of mint extracts were tested against two Gram positive (*Staphylococcus aureus* and *Propionibacterium acnes*) and two Gram negative (*Escherichia coli* and *Salmonella typhimurium*) bacterial using disc diffusion method at concentration of 5 µg/disc to 500 µg/disc were presented in Table 16. The results showed that the extracts did not show zone inhibition on any test organism. This may due to used crude extract containing less essential oil including pulegone, piperitenone oxide, limonene, isomenthone, linalool, 1,8-cineole,beta-pinene, alpha-pinene, gamma- terpinene, camphene, neomenthol (Mohkami *et al*., 2014) menthol (Pudpila *et al*., 2011) which have been reported to relate to antibacterial property. In addition, it was assumed that active compounds responding to bacterial inhibition may bigger than pore size of the disc (Jiang, 2011). Jiang (2011) reported that though disc diffusion has been used for antimicrobial screening in many plants, its disadvantage was penetration through the paper disc. The standard drug ampicillin 30 µg/disc tested on *S. aureus*, *P. acnes*, *E. coli* and *S. typhi* showed different inhibitory activity against with inhibition zone diameters ranging from 11.56, 11.82, 26.5 and 29.49 mm, respectively. While tetracycline 10 µg/disc tested on *E. coli, P. acnes*, *S. aureus* and *S. typhi* showed the inhibition on those tested organism as no inhibition zone, zone with but clear, 18.48 and 23.41 mm, respectively.

In theory, flavonoids and phenolic compounds can act as antibacterial agent against many pathogenic bacterias such as *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, and *Staphylococcus epidermis* (Karimi *et al*., 2012). It pointed out that antimicrobial activity may not successful detection if concentration and method were not appropriated uses.

Table 16. Antibacterial activity kitchen mint fortified with Zn compared to standard drug

Bacterial	Control	Mint fortified with Zn extract			Standard drug	
	40%EtOH (mm)	Control (mm)	100 ppm (mm)	200 ppm (mm)	Ampicillin 30 µg/disc (mm)	Tetracycline 10 µg/disc (mm)
Gram positive						
Staphylococcus aureus	0	0	0	0	11.56	18.48
Propionibacterium acnes	0	0	0	0	11.82	23.41
Gram negative						
Escherichia coli	0	0	0	0	26.5	0
Salmonella typhimurium	0	0	0	0	29.49	Zone but not clear

Remark: 0 means no zone to detect

3.5 Conclusion

It could be concluded that fortification of $ZnSO_4$ at 100 and 200 ppm into plantation soil significantly affected to qualitative phytochemical, the total extractable phenolic compound, total extractable flavonoid contents and antioxidant activity. Phytochemical profiling showed that alkaloids and terpenoids in the sample obtaining from 200 ppm $ZnSO_4$ fortification were higher than that of control but cardiacs glycosides in the control group was the highest. In addition, there was no saponin detected in all samples. The crude extract obtained from 100 ppm $ZnSO_4$ provided the highest total extractable phenolic content while the extract from 200 ppm $ZnSO_4$ fortification soil showed the highest total extractable flavonoid and antioxidant activity. In addition, it was found that pyrogallol exhibited the highest ability even higher gallic acid which is universal standard used for both total extractable content and antioxidant activity when determined by DPPH, ABTS and FRAP assays. However there was no antibacterial activity detected in the all extracts.

3.6 References

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Chapter 4

Conclusion and suggestion

4.1 Conclusion

According to the results, it was found that $ZnSO_4$ fortification into soil can increase the level of zinc content in kitchen mint leaves significantly. The highest of chlorophyll and total extractable phenolic compound contents were found in the mint grown in the soil fortified with 100 ppm $ZnSO_4$. Total extractable flavonoid contents, essential oil and antioxidant activity were the highest in the mint grown in 200 ppm $ZnSO_4$ soil. Mint grown in soil fortified with $ZnSO_4$ at 100 and 200 ppm significantly different in qualitative phytochemicals, the total extractable phenolic compound, total extractable flavonoid contents, antioxidant activity and bioactive compounds. There was no detectable antibacterial activity in all mint samples. Ferulic acid was a major compound found in all treatments. Addition, quercetrin and kempferol were major flavonoids which highest increased in the mint grown in 200 ppm $ZnSO_4$. Mint grown in 100 ppm $ZnSO_4$ fortified soil is great idea for daily consumption as vegetables while the plant grown in 200 ppm $ZnSO_4$ soil is recommended for essential oil production and/or specific purposes.

4.2 Suggestion

4.2.1 Antioxidative and anti-inflammatory activities using cell line and animal trial as well as clinic trial should further study.

4.2.2 $ZnSO_4$ fortification should try on other plants for better sensory acceptability and functional property.

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List of Publication

Srirattanakul, T., Siripongvutikorn, S. and Sae-Wong, C. 2015. Increasing of bioactive compounds in *Mentha cordifolia* Opiz., kitchen mint via $ZnSO_4$ biofortification during plantation. Funct. Food Health Dis. 6: 279-290.

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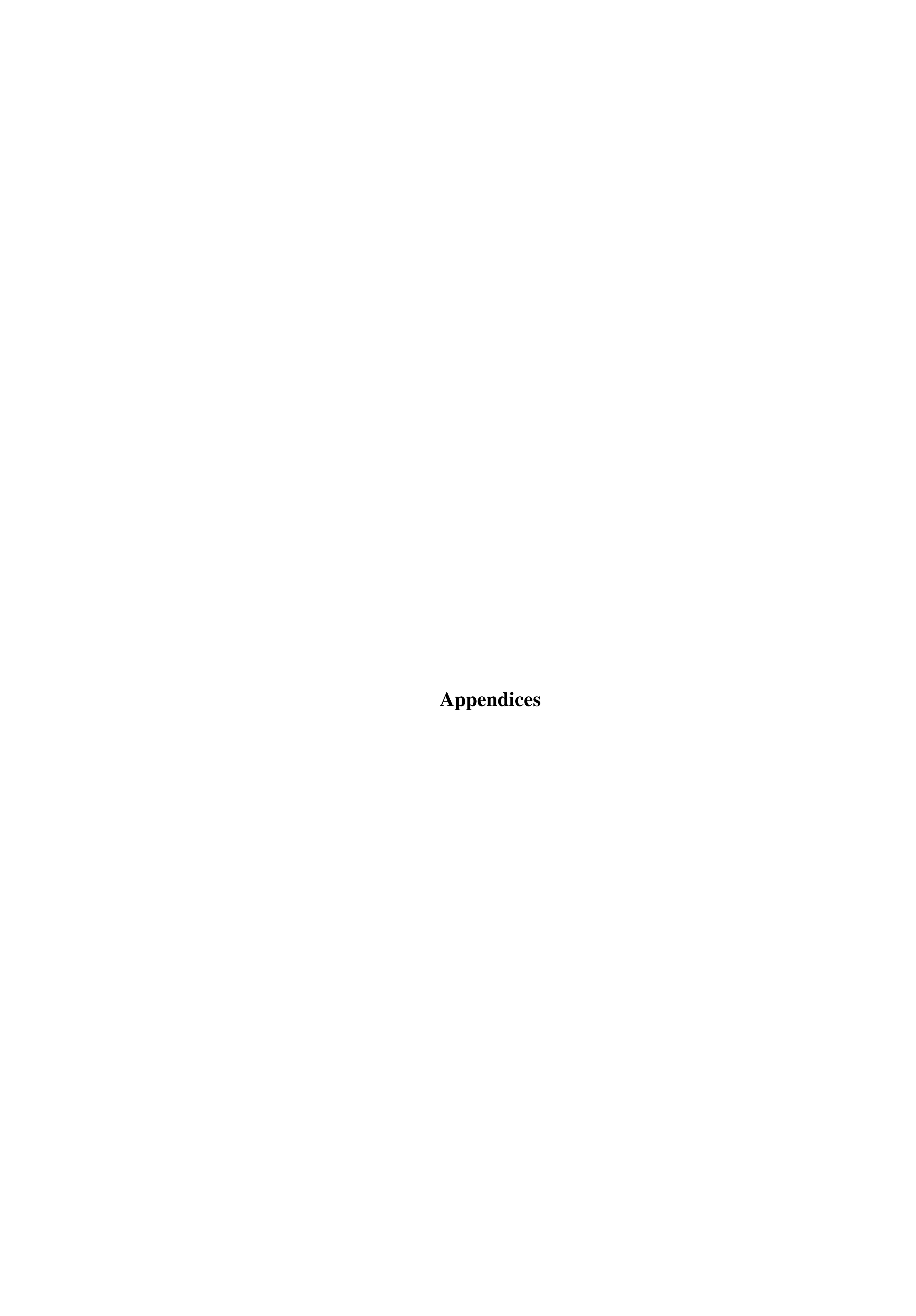


Appendices

Appendix A

Control **100 ppm $ZnSO_4$** **200 ppm $ZnSO_4$**

Figure A1. Fortification $ZnSO_4$ on growth characteristic of kitchen mint

Form A1. The consumer acceptability of kitchen mint

Direction: Please, determine the sample as in the order and write the score of each sample where

1 = dislike very much	2 = dislike much	3 = dislike moderately
4 = slightly dislike	5 = neither like nor dislike	6 = slightly like
7 = like moderate	8 = like much	9 = like very much

Note: Use scissors to cut the leaves for obtaining the odor of mint leaves

The result of sensory score obtaining from some panelists given the comment(s)

1. Naree

Appearance	sample		
	121	234	321
Color สี	6	6	6
Flavor กลิ่นรส	5	4	4
Taste รสชาติ	5	4	4
Odor กลิ่น	6	4	4
Overall acceptability ความชอบโดยรวม	5	5	5

Comment:

234 ขมและมีรสเผ็ด

321 มีรสเผ็ดมาก

2. Aob

Appearance	sample		
	121	234	321
Color	7	9	7
Flavor	8	9	7
Taste	7	7	6
Odor	7	7	6
Overall acceptability	7	8	7

Comment:

[illegible] ระหว่างตัวอย่าง 234 [illegible]

3. Worrapanit Ch.

Appearance	sample		
	121	234	321
Color	8	8	8
Flavor	8	3	6
Taste	8	4	6
Odor	8	4	7
Overall acceptability	8	6	7

Comment:

121 กลิ่น mint ชัดเจนสุด

4. Sirintip

Appearance	sample		
	121	234	321
Color	9	9	9
Flavor	9	6	7
Taste	9	6	6
Odor	9	6	6
Overall acceptability	9	6	7

Comment:

121

5. Preeya Hmadhlu

Appearance	sample		
	121	234	321
Color	๘	๙	๙
Flavor	๗	๙	๗
Taste	๗	๘	๗
Odor	๘	๗	๗
Overall acceptability	๘	๘	๗

Comment:

ชอบ 234 มากที่สุด เพราะ กลิ่นแรงสุด

6. Chairat

Appearance	sample		
	121	234	321
Color	8	8	8
Flavor	8	6	4
Taste	8	6	4
Odor	~~8~~ 7	8	4
Overall acceptability	8	6	4

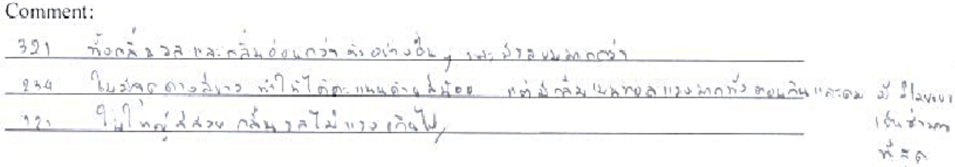
Comment:

321 ไม่ กลิ่นไม่ชัดเจน รสขม

7. Snimsa

Appearance	sample		
	121	234	321
Color	8	7	7
Flavor	8	7	5
Taste	8	8	5
Odor	8	7	6
Overall acceptability	8	7	5

Comment:



Appendix B

Table B1. The used concentration, equation and R^2 of each standard antioxidant

Standard	DPPH activity	ABTS activity	FRAP activity
L-ascorbic acid	concentration 20-60 µM equation $y = 0.0074x - 0.0971$; $R^2 = 0.9959$	concentration 200-600 µM equation $y = 0.1127x + 0.1314$; $R^2 = 0.9978$	concentration 10-120 µM equation $y = 0.003x$; $R^2 = 0.9866$
Phenolic compounds			
Pyrogallol	concentration 1-13 µM equation $y = 0.0162x + 0.0346$; $R^2 = 0.9924$	concentration 10-100 µM equation $y = 0.0067x + 0.0327$; $R^2 = 0.9919$	concentration 10-150 µM equation $y = 0.0034x + 0.032$; $R^2 = 0.9987$

Table B1. The used concentration, equation and R^2 of each standard antioxidant (continued)

Standard	DPPH activity	ABTS activity	FRAP activity
Phenolic compounds			
Protocatechuic acid	concentration 5-30 µM equation $y = 0.0073x + 0.0043$; $R^2 = 0.9941$	concentration 50-150 µM equation $y = 0.0039x - 0.0517$; $R^2 = 0.9992$	concentration 500-1000 µM equation $y = 0.0009x - 0.3253$; $R^2 = 0.9967$
p-coumaric acid	concentration 400-1000 µM equation $y = 0.000005x + 0.0141$; $R^2 = 0.9594$	concentration 25-125 µM equation $y = 0.0041x + 0.0669$; $R^2 = 0.9936$	concentration 500-1000 µM equation $y = 0.00008x - 0.0206$; $R^2 = 0.9975$

Table B1. The used concentration, equation and R^2 of each standard antioxidant (continued)

Standard	DPPH activity	ABTS activity	FRAP activity
Phenolic compounds			
Vanillic acid	concentration 400-800 µM equation $y = 0.00002x + 0.0057$; $R^2 = 0.9889$	concentration 100-300 µM equation $y = 0.0009x + 0.2305$; $R^2 = 0.9914$	concentration 400-1000 µM equation $y = 0.0001x$; $R^2 = 0.9801$
Gallic acid	concentration 3-11 µM equation $y = 0.0524x - 0.0667$; $R^2 = 0.9976$	concentration 10-100 µM equation $y = 0.0039x + 0.0683$; $R^2 = 0.9856$	concentration 10-120 µM equation $y = 0.0036x$; $R^2 = 0.9842$

Table B1. The used concentration, equation and R^2 of each standard antioxidant (continued)

Standard	DPPH activity	ABTS activity	FRAP activity
Phenolic compounds			
Caffeic acid	concentration 1-35 µM equation $y = 0.0098x - 0.0063$; $R^2 = 0.9969$	concentration 25-125 µM equation $y = 0.0054x + 0.0265$; $R^2 = 0.996$	concentration 500-1000 µM equation $y = 0.0009x - 0.3501$; $R^2 = 0.9967$
Ferulic acid	concentration 10-200 µM equation $y = 0.1289\ln(x) - 0.2496$; $R^2 = 0.9837$	concentration 25-125 µM equation $y = 0.0045x + 0.1234$; $R^2 = 0.9061$	concentration 10-120 µM equation $y = 0.0024x$; $R^2 = 0.9771$

Table B1. The used concentration, equation and R^2 of each standard antioxidant (continued)

Standard	DPPH activity	ABTS activity	FRAP activity
Phenolic compounds			
Sringic acid	concentration 25-150 µM equation $y = 0.0021x + 0.0963$; $R^2 = 0.9955$	concentration 50-200 µM equation $y = 0.0028x - 0.0041$; $R^2 = 0.9992$	concentration 10-150 µM equation $y = 0.0033x$; $R^2 = 0.9999$
Trolox	concentration 20-90 µM equation $y = 0.0072x + 0.0111$; $R^2 = 0.9985$	concentration 200-500 µM equation $y = 0.0014x + 0.0454$; $R^2 = 0.997$	concentration 90-150 µM equation $y = 0.0064x - 0.4905$; $R^2 = 0.9968$

Table B1. The used concentration, equation and R^2 of each standard antioxidant (continued)

Standard	DPPH activity	ABTS activity	FRAP activity
Phenolic compounds			
Ellagic	concentration 400-900 µM equation $y = 0.000006x + 0.0129$; $R^2 = 0.922$	concentration 10-150 µM equation $y = 0.0044x + 0.0234$; $R^2 = 0.9939$	concentration 500-1000 µM equation $y = 0.0052x$; $R^2 = 0.9916$
Chlorogenic acid	concentration 1-20 µM equation $y = 0.0159x + 0.0678$; $R^2 = 0.9944$	concentration 25-125 µM equation $y = 0.0032x - 0.0121$; $R^2 = 0.9935$	concentration 25-150 µM equation $y = 0.0008x - 0.3149$; $R^2 = 0.9995$

Table B1. The used concentration, equation and R^2 of each standard antioxidant (continued)

Standard	DPPH activity	ABTS activity	FRAP activity
Flavonoid compounds			
Apignin	concentration 1-20 μM equation $y = 0.0164x - 0.0009$; $R^2 = 0.9958$	concentration 25-125 μM equation $y = 0.1857\ln(x) - 0.3367$; $R^2 = 0.9945$	concentration 400-800 μM equation $y = 0.0001x - 0.0568$; $R^2 = 0.9904$
Quercetin	concentration 1-25 μM equation $y = 0.007x - 0.0028$; $R^2 = 0.9984$	concentration 25-125 μM equation $y = 0.0042x + 0.2026$; $R^2 = 0.9903$	concentration 10-100 μM equation $y = 0.0012x - 0.4015$; $R^2 = 0.9896$

Table B1. The used concentration, equation and R^2 of each standard antioxidant (continued)

Standard	DPPH activity	ABTS activity	FRAP activity
Flavonoid compounds			
Catechin	concentration 1-30 µM equation $y = 0.0088x + 0.0074$; $R^2 = 0.9992$	concentration 50-150 µM equation $y = 0.0057x + 0.0481$; $R^2 = 0.9927$	concentration 600-1000 µM equation $y = 0.0061x + 0.0144$; $R^2 = 0.9929$