ULTRASONIC EXTRACTION OF *CLINACANTHUS NUTANS* AND ITS ANTIOXIDANT ACTIVITY

NIK FATIMA BINTI NEK KAMARZAMAN

UNIVERSITI MALAYSIA PAHANG

ULTRASONIC EXTRACTION OF *CLINACANTHUS NUTANS* AND ITS ANTIOXIDANT ACTIVITY

NIK FATIMA BINTI NEK KAMARZAMAN

A thesis submitted in fulfilment of the
requirements for completing the assessment of
Undergraduate Research Project II

Faculty of Chemical & Natural Resources Engineering

Universiti Malaysia Pahang

FEBRUARY 2013

ULTRASONIC EXTRACTION OF *CLINACANTHUS NUTANS* AND ITS ANTIOXIDANT ACTIVITY

ABSTRACT

Clinacanthus nutans is one of herb plants that contain antioxidant. This natural antioxidant can be used commercially in food as well as pharmaceutical industries. This research explored antioxidant activity of *Clinacanthus nutans* (CN) and it was extracted by using ultrasonic-assisted extraction. Antioxidant activity of CN extract was determined by using free radical scavenging activity test and ferric reducing antioxidant power (FRAP) assay. Extraction process was carried out in ultrasonic bath at 28 kHz by using different solvent to solid ratio (ranging from 5mL/g to 50mL/g), temperature (ranging from 10 to 60 ^{o}C), and sonication time (ranging from 0 min to 160 min). From result, it showed that the optimum extract of CN was obtained at 60 ^{0}C, for extraction duration of 100 minutes with 10 mL/g liquor to solid ratio. The CN extract possessed antioxidant activity by scavenging DPPH with maximum effect of 61.08 %. The scavenging ability of CN extract has potency approximately 0.07 times of ascorbic acid. The CN extract also possessed reducing power to reduce ferric to its ferrous. 100 µg/mL of CN extract had same value of absorbance as 1.74µg/mL of ascorbic acid. This result proved that ferric reducing activity of CN extract was 57 times less powerful than ascorbic acid. In conclusion, antioxidant was extracted from *Clinacanthus nutans* by using ultrasonic extraction.

PENGEKSTRAKAN *CLINACANTHUS NUTANS* MENGGUNAKAN KAEDAH ULTRASONIK DAN AKTIVITI ANTIOKSIDANYA

ABSTRAK

Clinacanthus nutans merupakan sejenis tumbuhan herba yang mengandungi antioksida. Antioksida semulajadi ini boleh digunakan secara komersial di dalam industri makanan dan farmasi. Kajian ini mengkaji aktiviti antioksida di dalam *Clinacanthus nutans* (CN) dan ia diekstrak dengan menggunakan kaedah pengekstrakan ultrasonik. Aktiviti antioksida di dalam ekstrak CN telah dikenalpasti dengan menggunakan ujian aktiviti memerangkap radikal bebas dan ujian kuasa mereduksi ferric oleh antioksida. Proses pengekstrakan dijalankan di dalam tangki ultrasonik pada frekuensi 28 kHz dengan menggunakan nisbah pelarut kepada sampel (berskala daripada 5mL/g hingga 50mL/g), suhu (berskala daripada 10 hingga 60 ºC), dan tempoh pengekstrakan (berskala daripada 0 min hingga 160 min). Hasil ujian menunjukkan bahawa extrak CN yang optimum diperolehi pada suhu 60 ^{0}C, 100 minit tempoh pengekstrakan dengan 10 mL/g nisbah pelarut kepada sampel. Ekstrak CN mengandungi aktiviti antioksida dengan 61.08 % kuasa maksima memerangkap DPPH. Keupayaan memerangkap oleh CN ini bersamaan dengan 0.07 kali keupayaan asid askorbik. Ekstrak CN ini juga mengandungi kuasa reduksi untuk mereduksi ferric kepada ferrous. 100 µg/mL ekstrak CN menghasilkan nilai penyerapan yang sama dengan 1.74µg/mL asid askorbik. Hasil kajian ini menunjukkan bahawa aktiviti mereduksi ferric oleh ekstrak CN adalah 57 kali kurang berkuasa berbanding asid askorbik. Oleh itu, dapat disimpulkan bahawa antioksida telah diekstrak daripada *Clinacanthus nutans* dengan menggunakan kaedah pengekstrakan ultrasonik.

TABLE OF CONTENTS

LIST OF FIGURES

PAGE

LIST OF TABLES

LIST OF APPENDICES

LIST OF SYMBOLS

CN	–	*Clinacanthus Nutans*
FRAP	–	Ferric Reducing Antioxidant Power
NA	–	Neuraminidase
NI	–	Neuraminidase-inhibition
DPPH	–	1,1-diphenyl-2-picryhydrazil
TPTZ	–	2,4,6-tri(2-pyridyl)-1,3,5-triazine
$Fe.Cl_3.6H_2O$	–	Iron (III) chloride hexahydrate
kHz	–	Kilo Hertz
ROS	–	Reactive oxygen species
MHz	–	Mega Hertz
min	–	Minute
ml/g	–	Mililiter per gram
^{o} C	–	Degree Celcius
µg/ml	–	Microgram per mililiter
µl	–	Microliter
mm	–	Milimeter
nm	–	Nanometer
%	–	Percentage
UV/Vis	–	Ultra violet/Visible

CHAPTER 1

INTRODUCTION

1.1 Research Background

People are exposed to wide range of diseases either it is a curable or degenerative diseases. Degenerative diseases are diseases of old age that are unavoidable. Degenerative diseases are the results of oxidative stress that occur naturally in human body. Oxidation of molecule creates cellular bi-products or free radical. Excessive amount of free radical in human body are harmful to our cellular structure because it can attack cell and cause it to become unstable. This attack will not affect only one cell but the unstable condition of this one cell will create 'chain reaction' effect which causes the cell nearby to become damaged and lead to formation of cancerous cells. Human body are exposed to free radical by many ways such as toxic medicine, exposure to pollution or radiation and the easiest way is by breaking down the food we ate.

Oxidation of molecules can be slowed down by a molecule known as antioxidant. Vitamins, minerals and compounds in food possess antioxidant activity. Therefore, having diet which rich in antioxidant will help to reduce free radical level in human body, keep it low and sustain good health. Antioxidant present naturally in fresh fruits, vegetables and whole grains. Foods that were identified to be rich in antioxidant include green leafy vegetables, citrus fruits, and green tea.

Clinacanthus nutans is a type of herb plant. This plant is very popular medicinal plant in China and Thailand. Clinical test had showed successful application of *Clinacanthus nutans* for the relief of minor inflammation of skin and insect bites (Panthong et al., 2008). Pharmacological data also had showed that this plant possess antiviral activity (Wirotesangthong et al., 2009) and antioxidant activities (Pannangpetch et al., 2007).

Extract of this plant were used to study its antioxidant activity. Ultrasonic extraction is a well-known extraction method that available in our country nowadays. Ultrasonic extraction used ultrasound which is a high frequency sound wave (usually 20 kHz to 50 kHz) for cell disruption process. There are few parameters that we need to focus to ensure the efficiency of extraction process. These parameters include extraction time, temperature, solvent to sample ratio, and nature of solvent. Ultrasonic method can help in enhancing mass transfer. Rising of interest on applying ultrasonic extraction was due to its advantage on reducing time of extraction, low consumption of energy and increasing yield (Lou et al., 2011).

There are few examples of solvent such as water, acetone, alcohol, chloroform and ether. Water is a universal solvent that used for plant extraction. Organic solvent is usually used for ultrasonic extraction compared to water because it usually produced more yield (Tiwari et al., 2011). However, usage of water as solvent will help in reducing cost. Besides that, additional step such as purification of extract was not required if water was used for extraction process. Past study showed that usage of water as solvent for extraction process gave highest yield when the samples used were young age leaves (Nantitanon et al., 2010).

1.2 Problem Statement

Natural antioxidant was broadly used in food as well as pharmaceutical industries. This antioxidant can help to reduce oxidative stress which was major cause of unavoidable degenerative diseases such as heart disease, cancer and Alzheimer. Natural antioxidant can be possible therapeutic intervention ((Pannangpetch et al., 2007).

Clinacanthus nutans was a familiar medicinal plant in China and Thailand. This plant also listed as plants that possess antioxidant activity. Study of antioxidant activity of this plant extract will enable us to use it for medicinal purposes commercially such as supplement. This research was conducted to explore antioxidant activity of *Clinacanthus nutans* by using ultrasound as the extraction method.

This research can help us in gaining antioxidant from this herb plant for medicinal purposes by using more easier and efficient method compared to other method such as Soxhlet method that already done by past researchers.

1.3 Research Objectives

1. To extract antioxidant from *Clinacanthus nutans* using ultrasonic extractor.
2. To identify the most efficient parameter for ultrasonic extraction of *Clinacanthus nutans.*
3. To study the antioxidant activity of *Clinacanthus nutans* extract using Free Radical Scavenging Activity Test and Ferric Reducing Antioxidant Power Assay.

1.4 Research Scope

First objective is to extract antioxidant from *Clinacanthus nutans* using ultrasonic extractor. In order to achieve this objective, *Clinacanthus nutans* was extracted by using an ultrasonic extractor instead of other extraction methods such as Soxhlet method or microwave- assisted extraction.

Second objective is to identify the most efficient parameter for ultrasonic extraction of *Clinacanthus nutans*. In order to achieve this objective, ultrasonic extraction was carried out by using three different parameters. These parameters

were sample to solvent ratio (15mL/g, 30mL/g and 45mL/g), temperature (20 ºC, 40 ºC and 60 ºC) and sonication time (50 min, 100 min and 150 min).

Third objective is to study the antioxidant activity of *Clinacanthus nutans* extracted using ultrasonication. In order to achieve this objective, *Clinacanthus nutans* was extracted. The extract was tested by using few tests for determination of antioxidant activity. These tests were free radical scavenging activity test and ferric reducing antioxidant power (FRAP) test.

CHAPTER 2

LITERATURE REVIEW

This chapter discussed about three major themes regarding the research topic. These themes were based on studies which related to the topic that had been done by past researchers. The first theme was about raw material which is *Clinacanthus nutans* including its plant description and medicinal values. The second theme was about antioxidant. This theme explained the definition, benefits and sources of antioxidant. Antioxidant activity of the raw material was discussed in this chapter. Meanwhile, the last theme was about the extraction process including definition of ultrasonic extraction and parameters affecting extraction process.

2.1 *Clinacanthus nutans*

2.1.1 Plant Description

Clinacanthus nutans (scientific name) was a type of herb plant. It was also known as Sabah Snake Plant or Belalai Gajah (local name), Gendis (Java), Sa-laid-pang-porn, phaya yo, phaya plongtong (Thailand). This plant came from family Acanthaceae. This plant was a local plant (Watson et al., 2008). Figure 2.1.1 below shows the picture of this plant:

Figure 2.1 *Clinacanthus nutans* plant (Watson et al., 2008)

The height of this plant usually around 2.5 metres (mature plant). It had stem wood, straight, segmented and painted green. Its leaves were lanceolate, 8-12 mm long, 4-6 mm wide, reinforced were pinnate, dark green. The plant had tubular flowers, 2-3 cm long pink. This plant can grow through stem cuttings. This plant was a very popular herb plant among traditional medical field in China and Thailand (Pieroni & Vandebroek, 2007).

2.1.2 Medicinal Values of *Clinacanthus nutans*

Clinacanthus nutans (CN) extract was identified to have antiviral activity. It has superior ability to protect mouse in neuraminidase (NA) inhibition assay and in vitro antiviral assay, against influenza virus infection. The neuraminidase-inhibition (NI) assay refers to a laboratory procedure for the detection of the NA glycoprotein subtype in influenza viruses or the NA subtype specificity of antibodies to influenza virus. (Wirotesangthong et al., 2009).

Past study stated that CN extract possesses immune response activity since it is capable to raise lymphocyte proliferation extensively and decreased activity of natural killer cells. Lymphocyte proliferation refers to measurement of the lymphocytes ability to proliferate in response to stimuli (Sriwanthana et al., 1996).

CN extract also identified to have strong anti-inflammatory activity. Anti-inflammatory activity refers to the potential of a matter or treatment that can diminish symptoms of inflammation such as pain, fever, tenderness, and swelling (Panthong et al., 2008).

Determination of antivenom activity of CN did not show any successful result but it was reported that this plant can neutralise other components in venom of snake. It has the ability to neutralise inhibitory effects of neurotoxins of *Naja naja siamensis* venom on neuromuscular transmission (Cherdchu et al., 1977).

Besides that, CN extract was identified to have antioxidant activity and defensive effect against free radical-induced haemolysis. The extract has ability to scavenge DPPH with scavenging activity of 67.65±6.59 % (Pannangpetch et al., 2007).

2.2 Antioxidant

2.2.1 Definition of Antioxidant

Antioxidant is a molecule that able to slow down or prevent oxidation process of other molecule in human body. When molecule oxidizes, they will create free radicals. Excess amount of free radical in human body will cause disturbance on our cellular structures. Free radicals will generate process that will affect the stability of our cells. This process was known as chain reaction.

Despite of its essentiality, oxygen can be defined as a highly reactive atom that has capability of becoming potentially damaging molecules normally known as "free radicals." Free radicals have ability of attacking the healthy cells of the body, then causing them to lose their configuration and function. Cell that damage as effect of free radicals appears to be a major causes to aging and degenerative diseases of aging including cataracts, cancer, cardiovascular disease, brain dysfunction, and immune system decline (Percival, 1998). Reactive oxygen species (ROS) is a term

which refers to all highly reactive, molecules that contain oxygen, including free radicals. Table below shows few types of ROS and antioxidant that can neutralize it.

Table 2.1 ROS & Corresponding Neutralizing Antioxidants (Percival, 1998)

ROS	NEUTRALIZING ANTIOXIDANTS
Hydroxyl radical	vitamin C, glutathione, flavonoids, lipoic acid
Superoxide radical	vitamin C, glutathione, flavonoids, SOD
Hydrogen peroxide	vitamin C, glutathione, beta carotene, vitamin E, CoQ10, flavonoids, lipoic acid
Lipid peroxides	beta carotene, vitamin E, ubiquinone, flavonoids, glutathione peroxidase

Once a cell in our body become unstable, they will try to attack other healthy cells to make their selves stable. This situation caused the once-healthy cells to do same thing, attacking other cells. This chain reaction will produce a lot of free radical waste products that made up from injured, broken, and deformed cells. In other words, free radical was playing an important role in formation of cancerous cells. Weaken of cell will lead to weak condition of our organs, tissues and skin. Free radical attack will lead to oxidative damage that can cause tissue and muscle degeneration (Edward, 2009).

2.2.2 Benefits of Antioxidant

Antioxidants are enclosed with a lot of benefits to our health. The benefits of antioxidants include softer and younger-looking skin, reduce weight and high blood

pressure, better resistance to colds and flu, and also energy supplier (http://www.quick-weight-loss-tips-for-women.com/benefits-of-antioxidants.html). Antioxidants provided relief from allergies, arthritis, asthma, and also menstrual symptoms. Other than that, antioxidants also improved digestion, circulation, as well as improving our sleep. Moreover, antioxidants can help in controlling diabetes.

Antioxidants can also assist for better memory and concentration. Past studies also stated that antioxidants can protect us from oxidative stress. Oxidative stress is the main cause of degenerative diseases which are the diseases of old age that were unavoidable. Antioxidants can be used to prevent many ranges of diseases from cancers to Alzheimer (http://www.antioxidants-for-health-and-longevity. com/benefits-of-antioxidants.html).

2.2.3 Sources of Antioxidant

Oxidation process occurs naturally in our body. The effectiveness of human's body natural defences against free radical and oxidative stress will be decreased as we age. Free radical is produced due to few factors such as breaking down food that we consumed, toxic medicines, smoking, sunbathing, drinking, and exposure to pollution, toxic substances or radiation.

The easiest way to reduce level of free radicals in our body, keep it at low level and keep up good health is by having diet rich in antioxidants. Vitamins,

minerals and compounds in food contain the antioxidant properties. Vitamins A, C, and E were the most well known vitamins that have antioxidants properties.

Antioxidants are naturally present in fresh vegetables, fruits, and whole grains. Bright and distinctive colours fruits and vegetables were rich in antioxidants. Food that enclosed with large amount of antioxidants includes green leafy vegetables (such as spinach), carrots, cherries, berries, grapes, apples, pears, citrus fruits, and green tea (http://www.fitday.com/fitness-articles/nutrition/vitamins-minerals/how-do-anti oxidants-benefit-me.html).

Table 2.2 Foods and Its Antioxidant (American Dietetic Association, 2010)

Food	Antioxidant Nutrients
Acorn squash, pumpkin, winter squash	Beta carotene
Apples	Catechins
Apricots, cantaloupe, peaches	Beta carotene
Beans	Catechins, vitamin E
Beets	Anthocyanins
Bell peppers	Beta carotene, vitamin C
Berries	Anthocyanins, catechins, ellagic acid (in raspberries and strawberries), resveratrol (in blueberries), vitamin C
Broccoli, greens, spinach	Beta carotene, lutein, vitamin C
Brown rice	Selenium
Carrots	Beta carotene
Chicken	Selenium
Citrus fruits	Vitamin C
Corn	Lutein
Egg	Lutein (in yolks); selenium, vitamin A
Eggplant	Anthocyanins
Tea, black or green	Catechins
Tomatoes (canned)	Lycopene, vitamin C
Watermelon	Lycopene, vitamin C
Wheat germ, whole grains	Selenium, vitamin E

2.2.4 Antioxidant Activity

Determination of antioxidant activity is an essential step in the screening process in order to get possible benefits for human's health. The determination process usually carried out by using biologically relevant assays (Wolfe et al., 2008).

There are various methods that can be used to determine the antioxidants activity. Free radical (1,1-diphenyl-2-picryhydrazil;DPPH) scavenging activity were used in past study to examine antioxidant activity of *Clinacanthus nutans* (CN). When DPPH react with free radicals scavenger, it declined the absorbance value. This method used ascorbic acid as positive control. In the past study, it was showed that CN extract had moderate scavenging activity of 67.65±6.59%. This ability was approximately 0.08 times of ascorbic acid (Pannangpetch et al., 2007).

Besides that, we can also use ferric reducing antioxidant power (FRAP) assay. FRAP assay method used ascorbic acid as reducing agent to generate standard curve. This method will reduce ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to its ferrous (Fe^{2+}-TPTZ). From past study, it was stated that CN extract capable in reducing power. CN extract had ferric reducing activity 59 times less effective than ascorbic acid. Physically, we can see presence of antioxidant by formation of intensive blue colour (Pannangpetch et al., 2007).

Table 2.2.4 below shows the antioxidant activity of few types of food that usually consumed by people.

Table 2.3 Foods and Its Antioxidant Activity (Prakash et al., 2012)

FOOD	ANTIOXIDANT ACTIVITIES) (TE/100 grams)
Red Grapes	1350
Red Cabbages	1000
Broccoli Flowers	500
Spinach	500
Green Grapes	400
Tomato	300
Green Bean	175
Green Cabbages	150
Lima Beans	1055
Red Beans	11459
Blueberries	3300
Raisins	5900
Wheat Bran	4620
Wheat Flour (refined)	600

2.3 Extraction

2.3.1 Ultrasonic Extraction

Cell membrane must be disrupted in order to extract compound that enclosed in insoluble structure. Cell disruption is a sensitive process. Good control of cell disruption is necessary in order to avoid unhindered release of intracellular products and product denaturation. Ultrasonication serves as well-controllable means for cell disruption process. Ultrasonication used high frequency sound wave (usually between 20 to 50 kHz) to propagate into liquid media. This sound wave produced microbubbles that will contract and expand alternately until it reaches resonant size.