การใช้คลื่นไมโครเวฟช่วยในการสกัดสารต้านมะเร็งแคมนาแคนธัลจากรากของต้นยอ

นางสาววราภรณ์ วิทยสินธนา

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MICROWAVE-ASSISTED EXTRACTION OF ANTI-CANCER DAMNACANTHAL FROM ROOTS OF *MORINDA CITRIFOLIA*

Miss Waraporn Wittayasinthana

A Thesis Submitted in Partial Fulfillment of the Requirements
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By	Miss Waraporn Wittayasinthana
Field of Study	Chemical Engineering
Thesis Advisor	Assistant Professor Artiwan Shotipruk, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master 's Degree

.....Dean of the Faculty of Engineering
(Associate Professor Boonsom Lerdhirunwong, Dr.Ing.)

THESIS COMMITTEE

..... Chairman
(Associate Professor Prasert Pavasant, Ph.D.)

..... Thesis Advisor
(Assistant Professor Artiwan Shotipruk, Ph.D.)

..... Member
(Associate Professor Muenduen PhisalaPhong, Ph.D.)

..... External member
(Assistant Professor Navadol Laosiripojana, Ph.D.)

วราภรณ์ วิทยสินธนา : การใช้คลื่นไมโครเวฟช่วยในการสกัดสารต้านมะเร็งแคมนาแคนธัล จากรากของต้นยอ. (MICROWAVE-ASSISTED EXTRACTION OF ANTI-CANCER DAMNACANTHAL FROM ROOTS OF *MORINDA CITRIFOLIA*) อ. ที่ปรึกษา: ผศ. ดร. อาทิวรรณ โชติพฤกษ์, 73 หน้า.

งานวิจัยนี้ได้ศึกษาวิธีการใช้คลื่นไมโครเวฟช่วยในการสกัดสารแคมนาแคนธัลที่มีคุณสมบัติต่อต้านมะเร็งจากรากของต้นยอ ในงานวิจัยนี้ได้ทำการศึกษาผลกระทบของขนาดตัวอย่าง เวลาและอุณหภูมิในการสกัด ชนิดของตัวทำละลาย องค์ประกอบของตัวทำละลายเอทานอล และปริมาณตัวทำละลายต่อน้ำหนักตัวอย่างราก นอกจากนี้ยังเปรียบเทียบวิธีการใช้คลื่นไมโครเวฟช่วยในการสกัดกับวิธีการสกัดอื่น ซึ่งรวมถึงวิธีการใช้คลื่นอัลตราซาวด์ช่วยในการสกัดและวิธีสกัดด้วยน้ำที่ภาวะกึ่งวิกฤต จากผลการทดลอง พบว่าการสกัดตัวอย่างรากยอขนาดเล็กจะมีประสิทธิภาพการสกัดที่ดีกว่าตัวอย่างขนาดใหญ่ และการสกัดที่อุณหภูมิ 80 องศาเซลเซียสจะสกัดปริมาณแคมนาแคนธัลได้ต่ำกว่าที่ 60 องศาเซลเซียส อาจเนื่องจากการสลายตัวของสารประกอบที่อุณหภูมิค่อนข้างสูง โดยที่อุณหภูมิการสกัดที่ 100 และ 120 องศาเซลเซียส ปริมาณแคมนาแคนธัลจะเพิ่มขึ้นเมื่อเวลาการสกัดเพิ่มขึ้นจนถึง 5 นาที ซึ่งหลังจากเวลาการสกัดที่ 5 นาที ปริมาณแคมนาแคนธัลจะลดลงเนื่องจากการสลายตัวของสารประกอบ และจากตัวทำละลายทั้งหมดที่ใช้ทดลอง (เมทานอล อะซิโตน เอทานอล และอะซิโตนไนไตรล์) อะซิโตนจะสกัดได้ดีที่สุด นอกจากนั้นยัง พบว่า ตัวทำละลายเอทานอลกับน้ำ สามารถเพิ่มประสิทธิภาพในการสกัดได้มากขึ้นเนื่องจากตัวรากเกิดการพองตัวขึ้นเนื่องจากน้ำทำให้ตัวทำละลายสามารถเข้าไปสกัดได้ดียิ่งขึ้น และสารละลายผสมนี้ยังมีความเป็นขั้วที่เหมาะสมต่อการสกัดอีกด้วย โดยในที่นี้พบว่าอัตราส่วนของปริมาตรของตัวทำละลายต่อน้ำหนักตัวอย่างรากที่เหมาะสมมีค่าเท่ากับ 100 และเมื่อเปรียบเทียบวิธีการสกัดโดยใช้คลื่นไมโครเวฟช่วยในการสกัดกับการสกัดที่ปราศจากคลื่นไมโครเวฟ พบว่าการใช้คลื่นไมโครเวฟช่วยในการสกัดจะมีประสิทธิภาพมากกว่าวิธีการสกัดอื่น เช่น การใช้วิธีการซอกต์เลต การใช้คลื่นอัลตราซาวด์ และการใช้สภาวะน้ำกึ่งวิกฤต ซึ่งจะใช้เวลาในการสกัดมากกว่าคลื่นไมโครเวฟเพื่อให้ได้ปริมาณสารสกัดใกล้เคียงกัน

ภาควิชา.....วิศวกรรมเคมี..... ลายมือชื่อนิสิต.....วราภรณ์ วิทยสินธนา.....

สาขาวิชา.....วิศวกรรมเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550.....

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WARAPORN WITTAYASINTHANA: MICROWAVE-ASSISTED EXTRACTION OF ANTI-CANCER DAMNACANTHAL FROM ROOTS OF *MORINDA CITRIFOLIA*. THESIS ADVISOR: ASST. ARTIWAN SHOTIPRUK, Ph.D., 73 pp.

This study investigated the use of microwave-assisted solvent extraction of anti-cancer damnacanthal from root of *Morinda citrifolia*. The effects of materials size, irradiation time and temperature extraction, types of solvents, ethanol composition, and ratio of liquid to sample on the percent recoveries of the extract were determined. The extraction recovery of the extract was compared with conventional methods including ultrasound and subcritical water extraction. In the study, the percent recovery of damnacanthal was found to be significantly higher for the small particle size material. At 80 °C, the percent recovery was lower than that obtained at 60 °C due to the decomposition of the compound. The highest recovery obtained at high temperatures of 100 and 120 °C was obtained after 5 minute extraction, after which the percent recovery decreased, due to the decomposition of the compound. Of all the solvent tested (acetone, acetonitrile, methanol, and ethanol), acetone gave the highest recovery. Moreover, use of ethanol-water solution as extraction solvent increased the yield of damnacanthal due to the swelling of plant tissue matrix by water and suitable polarity of the mixture, and the appropriate ratio of liquid to sample was found to be 100. When compared with other conventional extraction methods such as soxhlet extraction, ultrasound-assisted extraction, and subcritical water extraction, microwave assisted extraction was more efficient as it required shorter time to achieve the same recovery.

Department……Chemical Engineering… Student's signature Waraporn Wittayasinthana
Field of study…Chemical Engineering… Advisor's signature
Academic year…….2007……...

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CHAPTER I

INTRODUCTION

1.1 Rationale

Morinda citrifolia (Noni) is a plant which is originated in tropical Asia or Polynesia and has been used in folk remedies for more than 2000 years. All parts of this plant, which include fruits, flowers, leaves, bark, stem, and roots have been shown to contain several biological activities (Ying et al., 2002). Noni fruits and leaves can be taken as food and herbal medicines to maintain overall health and to treat various diseases. The roots of noni plants were used by Polynesians to produce yellow or red dye. But more important the roots contain medicinally active components, namely anthraquinones, which have recently been shown to process various therapeutic properties including anti-bacterial, anti-viral, and anti-cancer activities (Chan-Blanco et al., 2006). Of the antraquinones in the roots of noni, *damnacanthal* is the most important compound due to its cancer inhibiting ability (Hiramatsu et al., 1993). Thus, in this study, we are interested in investigating an effective means for extraction of damnacanthal from *Morinda citrifolia*.

Conventionally, anthraquinones can be extracted into an organic solvent in a stirred vessel or by soxhlet extraction. These techniques are simple but they require long extraction time and large amount of solvent. Alternatively, microwave-assisted solvent extraction extraction has been proposed for rapid extraction of plant material (Wang et al,. 2006). Recent studies have shown that microwave-assisted extraction (MAE) can effectively reduce solvent volume and extraction time for extraction of various plants such as *Radix puerariae* (Guo et al,. 2001), *Salvia miltiorrhiza bunge* (Pan et al., 2002), ginseng (Shu et al., 2003), *Nothapodytes foetida* (Fulzele et al,. 2005), tobacco leaves (Zhou et al., 2006), peppers (Barbero et al., 2006). As microwaves are transmitted as waves, it can penetrate biomaterials and interact with polar molecules such as water in the biomaterials to create heat. Consequently, microwaves can heat a whole material to penetration depth simultaneously and

homogeneously (Wang et al., 2006). The enhanced extraction efficiency is due to rapid heating of polar liquids in the microwave field.

In our previous studies, we have investigated various alternative methods for extraction of antioxidative anthraquinones from roots of *Morinda citrifolia* and compared the results with those obtained by conventional solvent extraction techniques. The methods investigated include subcritical water extraction (Shotipruk et al., 2004; Pongnaravane et al., 2006), ultrasound assisted extraction (UAE) (Hemwimon et al,. 2006 a), and MAE (Hemwimon et al,. 2006 b). Although our result shows that, in stead of solvent, subcritical water could be used to extract total anthraquinones from the root material, the drawback of this technique is however the high temperature employed, and the resulted diluted aqueous extract needs to be further concentrated. In addition, our subsequent study, in which the water extract was analyzed for the amount of damnacanthal, showed that at the temperature higher than 170 °C, degradation of damnacanthal was observed despite the high amount of total anthraquinones and high antioxidant activity (Anekpankul et al,. 2007). Alternatively, UAE was shown to reduce the time of extraction compared with conventional solvent extraction, however, the antioxidant activity of the extract decreased possibly due to the radical reaction within the system. On the other hand, MAE at 60 °C could reduce the time of extraction and the amount of ethanol solvent used without lowering the antioxidant acitivity of the extract (Hemwimol, 2006 b). However, the method of quantitative analysis employed in the study was spectrophotometry, which only allowed quick determination of the amount of total anthraquinones, which may not correlate well with the amount of the more important target anti-cancer compound such as damnacanthal. Therefore, in this study, we propose to determine the effect of various parameters on extraction of damnacanthal by MAE. Here, the analysis of damnacanthal will be carried out using reversed–phase high performance liquid chromatography (RP-HPLC) and the effects of various parameters of MAE such as time, temperature, types of solvents, solvent compositions, solvents to sample ratio, and particle size of the sample will be determined on the extraction efficiency. Furthermore, the results of MAE will be compared with those obtained by conventional extraction such as heating extraction, ultrasonic extraction, soxhlet extraction, and pressurized hot water extraction (PHWE).

1.2 Objectives

1.2.1 To investigate the appropriate conditions for microwave assisted solvent extraction of damnacathal from *Morinda critifolia* roots.

1.2.2 To compare the results of MAE with other methods of extraction such as heating extraction, ultrasound extraction, soxhlet extraction, and PHWE.

1.3 Working scopes

1.3.1 Investigation on the effect of temperature (60-120 °C), time of extraction (5-30 min), type of solvent (acetonitrile, methanol, ethanol, and acetone), composition of solvent (ethanol:water of 20%, 50%, and 80%), and solvent to sample or L/S (50-150 ml/g) on extraction of damnacanthal by microwave assisted extraction.

1.3.2 Comparision of the results on MAE with other methods of extraction such as heating extraction, ultrasound extraction, soxhlet extraction, and PHWE.

1.4 Expected benefits

Provide a new and efficient alternative for extraction of damnacanthal anticancer compound from the available plant, *Morinda citrifolia*.

CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

BACKGROUND

2.1 *Morinda citrifolia*

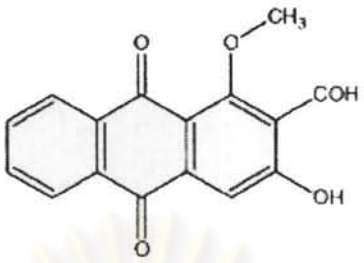
Morinda citrifolia is a tropical plant that belongs to the family Rubiaceae, which is sometimes known by different names such as Indian Mulberry, Noni, Ach, Mengkudu, Nhau, Painkiller bush, or Cheese fruit, or, in Thai, Ton Yor or Yor. It is a small tree, typically 3-10 m tall, with abundant wide elliptical leaves (5-17 cm length, 10-40 cm width). All parts of the noni plant are used for medicinal and nutritional purposes such as fruits, seeds, bark, leaves, flowers, and roots. The ripe fruits are taken to treat asthma and diabetes, and to reduce blood pressure and promote menstruation. The seeds have a purgative action, and the leaves are used to treat external inflammations and relieve pain, the bark has strong astringent properties and can treat malaria, the flower essences relieve eye inflammation, and the roots are used to produce a yellow dye and to relieve chronic diseases. One of the most important constituents responsible for the therapeutic properties of *M. citrifolia* is anthraquinones, which is found mostly in the fruits, leaves, bark, and in the highest amount in the roots of the plant (Chan-Blanco et al., 2006).

Figure 2.1 *Morinda citrifolia* plant

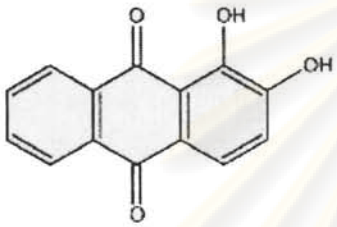
2.2 Anthraquinones

Anthraquinones are the main constituent in the root of *Morinda citrifolia*, which is consisted of several derivatives, differing in the R groups as shown in Figure 2.2. Some examples of these compounds are damnacanthal, alizarin, morindone, norindin, and etc, and the molecular structures of these compounds are shown in Figure 2.3.

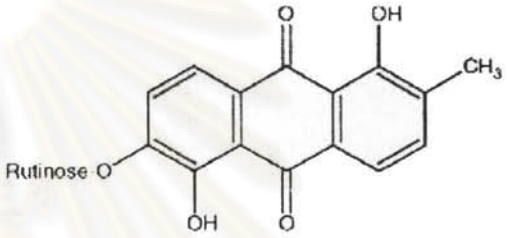
Figure 2.2 Basic structures of anthraquinones.



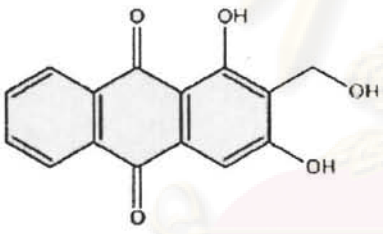
3-hydroxy-1-methoxyanthraquinone-2-carboxaldehyde (damnacanthal).



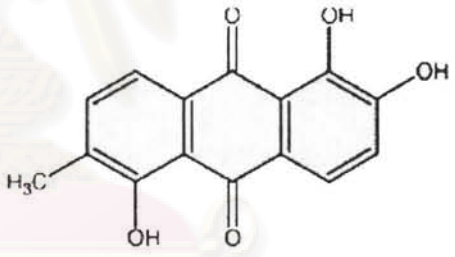
1,2-dihydroxyanthraquinone (alizarin).



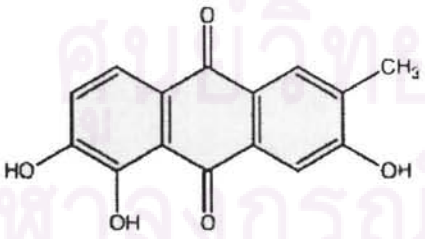
Morindine



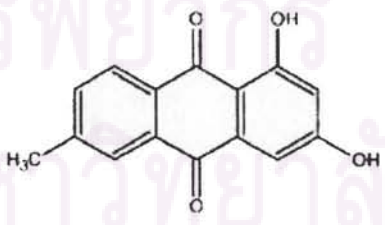
3-hydroxy-2-hydroxymethylanthraquinone (lucidin).



Morindone



2-methyl-3,5,6-trihydroxyanthraquinone



1,3-dihydroxy-6-methyl anthraquinone

Figure 2.3 Derivatives of anthraquinones (Chan-Blanco et al., 2006)

In this study, damnacanthal (3-hydroxy-1-methoxyanthraquinone-2-carboxaldehyde) is a target compound for extraction due to its important medicinal values as anticancer agent. It has been found that damnacanthal is able to inhibit the proliferation of cancerous cells with malignancies (Hiramats et al., 1993). Physical and chemical properties of damnacanthal are summarized in Table 2.2.

Table: 2.1 Properties of damnacanthal (www.alexis-biochemicals.com).

Name	3-hydroxy-1-methoxyanthraquinone-2-carboxaldehyde
Formula	$C_{16}H_{10}O_5$
Molecular weight	282.3
Solubility at 25 °C (M)	Soluble in DMSO (25 mg/ml)

2.3 Mechanism of extraction

Two mechanisms associated with solvent extraction of natural products involve mass transfer and solute solubility consideration as discussed below.

2.3.1 Factors affecting mass transfer:

Size of the particles -- When a solute dissolves, the action takes place only at the surface of each particle. When the total surface area of the solute particles is increased, the solute dissolves more rapidly. Breaking a solute into smaller pieces increases its surface area and hence its rate of solution.

Stirring -- With liquid and solid solutes, stirring brings fresh portions of the solvent in contact with the solute, thereby increasing the rate of solution.

Amount of solute already dissolved -- When there is little solute already in solution, dissolving takes place relatively rapidly. As the solution approaches the point where no solute can be dissolved, dissolving takes place more slowly.

Temperature -- For liquids and solid solutes, increasing the temperature not only increases the amount of solute that will dissolve but also increases the rate at which the solute will dissolve.

2.3.2 Factors affecting solute solubility

Molecular size -- The larger the molecule or the higher its molecular weight the less soluble the substance will be. Larger molecules are more difficult to surround with solvent molecules in order to solvate the substance. In the case of organic compounds the amount of carbon branching will increase the solubility since more branching will reduce the size of the molecule and make it easier to solvate the molecules with solvent.

Polarity -- The polarity of the solute and solvent molecules will affect the solubility. Generally polar solute molecules will dissolve in polar solvents and non-polar solute molecules will dissolve in non-polar solvents. The polar solute molecules have a positive and a negative end to the molecule. If the solvent molecule is also polar, then positive ends of solvent molecules will attract negative ends of solute molecules. This is a type of intermolecular force known as dipole-dipole interaction. Non-Polar molecules also have a type of intermolecular force much weaker but present called London Dispersion forces where the positive nuclei of the atoms of the solute molecule will attract the negative electrons of the atoms of a solvent molecule. This gives the non-polar solvent a chance to solvate the solute molecules.

Temperature -- Generally, an increase in the temperature of the solution increases the solubility of a solid solute. A few solid solutes, however, are less soluble in warmer solutions. For all gases, solubility decreases as the temperature of the solution rises.

Pressure -- For solids and liquid solutes, changes in pressure have practically no effect on solubility.

2.3.3 Solubility of solid in liquid

The solubility is a key word in the efficiency of extraction process. It is a measure of solute concentration that is in equilibrium with the solvent at a given temperature. Therefore, the most appropriate extraction solvents or mixtures of solvents should have nearly the same polarities as those of the solutes. The polarity of a solvent is specified by the dielectric constant. If it is desired to dissolve a polar compound, a relatively polar solvent that has a low dielectric constant should be used.

In general, the solute solubility depends on the interaction between the molecules of the solute and the solvent, which is dictated by the molecular structures and the activity coefficient of the solution. However, this is not always the case. Similar molecules such as phenanthrene and anthracene isomers exhibit very different solubility in benzene (20.7 and 0.81 mol%, respectively). This example demonstrates that the solubility does not only depend on the activity coefficient but also the ratio of fugacity of pure solid and the standard state fugacity according to the following equation.

$$X = \frac{f_{pure-solid}}{\gamma\, f^{o}_{subcooled-liquid}} \qquad (2.1)$$

Where X is the solubility of the solute in the solvent.

γ is the liquid-phase activity coefficient.

$f_{\text{pure solid}}$ is fugacity of solid at equilibrium.

$f_{\text{subcooled liquid}}$ is standard state fugacity taken to be that of subcooled liquid.

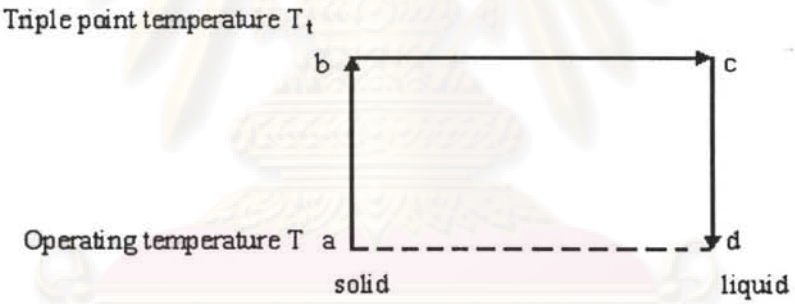


Figure 2.4 Path independence thermodynamic properties

$\Delta M_{a\to d} = \Delta M_{a\to b} + \Delta M_{b\to c} + \Delta M_{c\to d}$ (where M means any state property)!!!

The ratio of the two fugacities are relate to the change of Gibbs energy in going from the state of solid (denoted as state a) to subcooled liquid (denoted as state d) following form equation:

$$\Delta G = RT\, ln\left(\frac{f_{subcooled-liquid}}{f_{pure-solid}}\right) \qquad (2.2)$$

The change of Gibbs energy is related to the change of enthalpy(ΔH) and entropy(ΔS) by the following equation:

$$\Delta G_{a\to d} = \Delta H_{a\to d} - T\Delta S_{a\to d} \qquad (2.3)$$

To calculate $\Delta H_{a\to d}$ and $\Delta S_{a\to d}$, it is more convenient to employ thermodynamic cycle as shown in Figure 2.4. Because the enthalpy and entropy are not dependent of the path, the $\Delta H_{a\to d}$ and $\Delta S_{a\to d}$ can be calculated from a $\rightarrow$ b, b $\rightarrow$ c and c $\rightarrow$ d.

For the enthalpy change from a to d can be determined from the following equation.

$$\Delta H_{a\to d} = \Delta H_{a\to b} + \Delta H_{b\to c} + \Delta H_{c\to d} \qquad (2.4)$$

The above equation becomes

$$\Delta H_{a\to d} = \Delta_{fus} H_{at\ T_t} + \int_{T_t}^{T} \Delta C_P dT \qquad (2.5)$$

where $\Delta_{fus}H$ is the enthalpy of fusion, $\Delta C_P = C_{P\ liquid} - C_{P\ solid}$, the difference between the heat capacity of liquid and the heat capacity of solid, and T_t is the triple point temperature of the solute.

Similarly, the entropy change from a to d can be determined from the following equation

$$\Delta S_{a\to d} = \Delta S_{a\to b} + \Delta S_{b\to c} + \Delta S_{c\to d} \qquad (2.6)$$

which can be written as follows:

$$\Delta S_{a\to d} = \Delta_{fus} S_{at\ T_t} + \int_{T_t}^{T} \Delta C_P dT \qquad (2.7)$$

where $\Delta_{fus}S$ is entropy of fusion which is related to $\Delta_{fus}H$ by the following equation:

$$\Delta_{fus} S = \frac{\Delta_{fus} H}{T_t} \qquad (2.8)$$

Substituting Equations (2.3), (2.5), and (2.7) into Equation (2.2), and assuming that ΔC_P is constant over the temperature range $T \rightarrow T_t$, we obtain the following equation.

$$ln\left(\frac{f_{subcooled-liquid}}{f_{pure-solid}}\right) = \frac{\Delta_{fus} H}{RT_t}\left(\frac{T_t}{T} - 1\right) - \Delta C_P\left(\frac{T_t}{T} - 1\right) + \Delta C_P\, ln\left(\frac{T_t}{T}\right) \qquad (2.9)$$

This equation gives an expression for the ratio of the fugacities, which can be substituted (2.8) in equation (2.9) to give the expression for the solubility as follows.

$$ln\ X = -\frac{\Delta_{fus}S}{R}\left(\frac{T_t}{T}-1\right)+\Delta C_p\left(\frac{T_t}{T}-1\right)-\Delta C_p\ ln\left(\frac{T_t}{T}\right)-ln\ \gamma \qquad (2.10)$$

As an approximation, the term of ΔC_P can be neglected and it is permissible to substitute melting temperature for triple point temperature. Then, Equation (2.10) becomes:

$$ln\ X = \frac{-\Delta_{fus}S}{R}\left(\frac{T_m}{T}-1\right)-ln\ \gamma \qquad (2.11)$$

To represent the values at equilibrium or saturation, the superscript, *SAT*, is used and the equation becomes:

$$ln\ X^{SAT} = \frac{\Delta_{fus}S}{R}\left(1-\frac{T_m}{T}\right)-ln\ \gamma^{SAT} \qquad (2.12)$$

This equation shows that the solute solubility depends on temperature and intermolecular forces between solute and solvent as represented by the activity coefficient. For an ideal solution, the activity coefficient is equal to 1. For non-ideal solution, activities coefficient is not equal to 1. Many solubility estimation methods such as Robbins chart, UNIFAC model, Hansen solubility parameter, and Margules equation can be used to estimate the value of activity coefficient, and thus solubility. The knowledge of solute solubility in extraction solvents at various conditions is beneficial for the design of the process.

2.4 Microwave extraction

Microwave is a form of electromagnetic energy or electromagnetic radiation, consisting of electrical field and magnetic field. The range of the electromagnetic spectrum for microwave is between 300 MHz and 300 GHz, (Fig.2.5), but the most commonly used frequency for commercial microwave ovens is 2450 MHz, while the typical energy output range between 600 and 900 W. Some of the more common uses of microwaves include satellite communications, and industrial heating and therapeutic diathermy treatment.

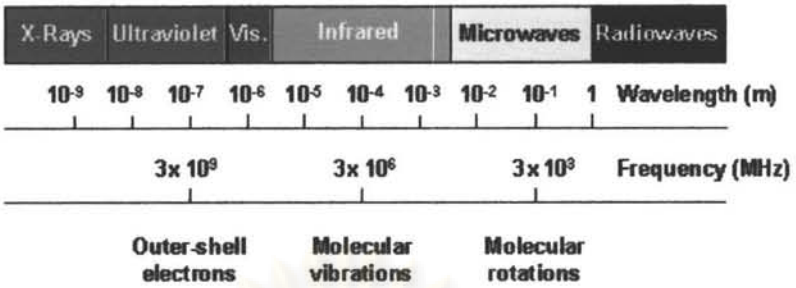


Fig 2.5 Electromagnetic spectrum. (www.anton-paar.com)

The properties of microwaves are described as being reflective, transparent and absorptive, depending on the medium through which microwave travels. The different characteristics are depicted in Figure 2.6. In polar liquids, the liquid molecules are said to be able to absorb microwave. Other non-polar liquids or solids such as glass, paper, and plastics are transparent to microwave. The opaque materials such as metals are reflective to microwave. It is only in the material that can absorb microwave, that heating occurs. Such the property of microwave leads to household microwave heating and in extraction of natural materials.

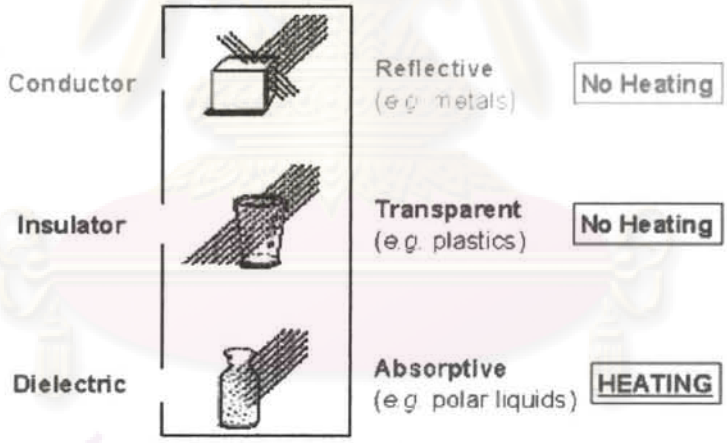


Figure 2.6 The interaction of microwaves with different materials. (www.anton-paar.com)

2.4.1 Microwave heating

Microwave heating occurs as a result of an interaction of the electrical field with a compound of interest within the microwave field a material via two specific mechanisms: dipole interactions and ionic conduction. Both mechanisms require effective coupling between components of the target material and the rapidly oscillating electrical field of the microwaves. The dipole interactions occur with polar

molecules as the polar ends of molecules try to align themselves and oscillate in step with the rapidly oscillating electrical field of microwave as shown in Figure 2.7. Collisions and friction between the moving molecules result in heating. The ionic conduction on the other hand occurs when a microwave field is applied to solution containing ions, which move as a result of changing electric field of microwave. Consequently, ions collide, and thus heat is generated. As the concentration of ions in the solution increases, more collisions occur, causing the solution to heat faster.

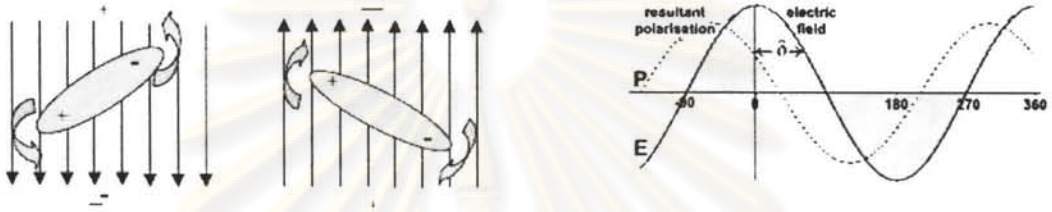


Figure 2.7 Realignment of a dipole in an electromagnetic.

The heating action of microwave has been employed in extraction of environmental samples and nutraceuticals from plant materials. Because microwave can penetrate biomaterials and interacts with polar molecules such as water or polar components of target biomolecule to create heat, it can heat the whole material to penetration depth simultaneously. Cell disruption is therefore stimulated by internal superheating, which facilitates desorption of chemicals from the matrix. As a result, MAE has been shown to increase the yield and reduce the process time compared with other conventional solvent extraction methods.

2.4.2 The Influence of Parameters on MAE.

In typical extraction, the influence of parameter such as time, temperature, microwaves power, nature of the solvent and the matrix, moisture content and the stability of compounds are important. In MAE, rapid heating is responsible for its success of MAE. This can be said to be determined by two parameters defining the dielectric properties of the solvent. The first is the dielectric constant or relative permittivity, ε', which describes the ability of the material to be polarized. It is a measure of the ability of a material to store microwave energy. The second is the dielectric loss factor,ε'', which describes dielectric response of materials in an applied microwave field. It is a measure of the efficiency in which the absorbed microwave

energy can be converted into heat inside a material when an electric field is applied. From these two parameters, a dissipation factor, δ, is defined as the ratio of the dielectric loss constant and the dielectric constant, or expressed mathematically by:

$$\delta = \frac{\varepsilon''}{\varepsilon'} \qquad (2.13)$$

In general, solvents suitable for MAE should be selected such that the dissipation factor and dielectric constants are high. Table 2.2 shows the dielectric constants and dissipation factors of some common solvents.

Table 2.2 Dielectric constants and dissipation factors of some common solvents. (Zlotorynski et al,. 1995)

Solvent	Dielectric constant (ε')	Dielectric loss factor (ε'')	Dissipation factor/ tan $\delta \times 10^{-4}$
Acetone	20.7	11.5	5555
Methanol	32.7	20.9	6400
Water	78.3	12	1570
Ethanol	24.3	6.1	2500
Hexane	1.88	0.00019	.10
Ethyl Acetate	6.02	3.2	5316

Literature reviews

In recent years, various studies have employed MAE for extraction of organic and inorganic from environmental materials and plant materials. Environmental materials examined are such as phenol, *o*-cresol, *m*-cresol, and *p*-cresol from soils (Llompart et al., 1997), wool wax from solid wool scour wastes (Carrillo et al,. 2003), endocrine disrupting chemicals in river sediments (Liu et al,. 2004), nonylphenols and phthalate esters in sediment samples (Cortazar et al., 2005), organotin compounds in fortified flour samples (Wang et al., 2006), and PCBs and CBZs in fly ash from municipal solid waste incinerators (Sun et al., 2006). In extraction of plant materials, the microwave assisted extraction was found to be more efficient than conventional solvent extraction (Pan et al., 2002; Hao et al., 2002; *Liu et al., 2004*; Fulzele et al., 2005) as it is able to significantly reduce the extraction time. The main parameters affecting the efficiency of MAE are temperature, time of extraction, type and composition of solvents. The review of previous application of MAE on extraction of environmental and plant samples is summarized in Table 2.3 and 2.4.

Table 2.3 Review of previous work of microwave-assisted extraction to organic compound from environmental material.

Author	*Material*	*Contaminants*	*Conditions*	*Analysis*	*Conclusions*
1. Llompart et al., 1997	Soils	Phenol, *o*-cresol, *m*-cresol, and *p*-cresol	**Time** 2-10 min **Temperature** 60-140°C **Solvent** solvent mixture of pyridine50-200 µl, acetic anhydride 200-1000 µl, hexane 9 ml.	GC-MS	- The optimization temperature for MAE was 130 °C in 10 ml volume of solvent (200 µl pyridine, 800 µl cetic anhydride and 9 ml of hexane). - Comparison with ultrasonic extraction procedures indicated that microwave-assisted methods gave high recoveries and shorter extraction times.
2. López-Mesas et al,. 2003	solid wool	wool wax	**Frequency & power** 2450 MHz, 700 W (10-100%) **Time** 2-15 min **Solvent** Toluene, Acetone, Hexane, and acetone/hexane.		- The optimum MAE condition was extraction of 1.25 g of sample with 90% power in 10 ml of hexane:acetone (1:1) for 8 min.

Author	*Material*	*Contaminants*	*Conditions*	*Analysis*	*Conclusions*
3. Liu et al,. 2004	Sediments	Endocrine disrupting chemicals.	**Frequency & power** 2450 MHz, 1200 W **Time** 5, 15 ,25, 40 min **Temperature** 90, 110, 130 °C **Solvent** Methanol, Acetone Ethyl acetate, Dichloromethane, Hexane.	GC-MS	- The most efficient microwave extraction of the target compounds was achieved using methanol of 110°C and 15 min of holding time.
4. Cortazar et al,. 2005	Sediments	Nonylphenols and phthalate esters	**Time** 5–25 min **Temperature** 100 °C **Solvent** Methanol, Acetone, *n*-hexane or the solvent mixture	GC–MS and HPLC–DAD-UV–FLD	- The optimum extraction was carried out at an intermediate pressure (159 kPa) with pure methanol for 15 min.

Author	*Material*	*Contaminants*	*Conditions*	*Analysis*	*Conclusions*
5. Ganeshjeevan et al,. 2005	Solid	Chlorophenols	**Frequency & power** 2450 MHz. 300 W (10–40%) **Time** 5–30 min **Solvent** Methanol and Acetone	GC with NPD	- Aqueous extractants were as efficient as organic solvents for the MAE of chlorophenols, with the added advantage of being more eco-friendly. They are suitable for vegetable and biological samples which swell in the aqueous extractant.
6. Wang et al., 2006	Flour	Oranganotin compounds (OTs)	**Frequency & power** 800W **Time** 2-10 min **Temperature** 70-120 °C **Solvent** hexane–acetic acid (80/20, v/v)	normal-phase HPLC	- The suitable MAE conditions for extraction of organotin compounds was in acetic acid– hexane (20/80, v/v) at 100 °C for 3 min.

Author	*Material*	*Contaminants*	*Conditions*	*Analysis*	*Conclusions*
7. Barriada-Pereira et al., 2006	Vegetables	Organochlorine pesticides	**Frequency & power** 800W **Time** 1 min ramp from 100 to 800W 4 min hold at 800W **Temperature** 110 °C **Solvent** hexane:acetone (50:50)	GC–ECD	- The optimum microwave extraction condition was extraction of 1.25 g of sample with 90% power in 10 ml of hexane:acetone (1:1) for 8 min .
8. Sun et al., 2006	Solid waste incinerators .	PCBs and CBzs	**Frequency & power** 2450 MHz, **Time** 15 or 20 min **Temperature** 20-200 °C **Solvent** hexane/acetone, toluene/acetone, toluene/water, toluene/methanol, toluene/dichloromethane	GC-MS	- The optimum condition was extraction in 30 ml of toluene/ acetone(1:1) or a 15-ml of toluene, with an irradiation time of 15 min. -MAE was found to have high extraction efficiency compared with that of soxhlet. Time and organic solvent consumption were reduced.

Table 2.4 Review of previous work of microwave-assisted extraction to natural products from plant.

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
1. Pan et al., 2000	Licorice root	Glycyrrhizic acid (GA)	**Frequency & power** 2450 MHz, 700W **Time** 0.5-10 min (pre-setting: 15 s power on 15 s power off 3 s power on for heating 15 s power off for cooling) **Temperature** 85 - 90°C **Solvent** Water, Ethanol, ethanol - water, Ammonia, ethanol-water- ammonia	HPLC	- Microwave was suitable for fast extraction. Appropriate MAE conditions are: times of 4–5 min, ethanol concentrations of 50–60% (v/v), ammonia concentrations of 1–2% (v/v), and liquid/solid ratios of 10:1(ml/g).
2. Pan et al., 2002	*Salvia iltiorrhiza bunge*	Tanshinones	**Frequency & power** 2450 MHz, 700 W (10-100%) **Time** 0.5-5 min (pre-setting 25 s power on , 2 s heated, 10 s power off for cooling) **Temperature** 80 °C **Solvent** Ethanol	HPLC	- MAE resulted time and high extraction efficiency.

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
3. Guo et al,. 2001	Radix puerariae	Puerarin	**Frequency & power** 2450 MHz, 700 W (10-100%) **Time** 2, 5, 8, 12, 30 min **Temperature** 85, 90, 100, 115,130, 135 °C **Solvent** Ethanol in wa(0, 30, 50, 70, 95%)	HPLC	- The main factors that affect MAE of effective constituents are microwave processing time, temperature and pressure in the sample vessel and solvent.
4. Pan et al,. 2001	*Salvia miltiorrhiza bunge* of root	Tanshinones	**Time** 0.5 – 5 min (pre-setting:25 s of power-on (80°C), 2 s of power-on for heating, 10 s of power-off for cooling) **Temperature** 80 °C **Solvent** *n*-butylacetate, ethanol, methanol, acetone, *n*-butanol , ethylacetate and tetrahydrofuran	HPLC	- Compared with the conventional methods, the MAE procedure employed provides high extraction efficiency within a short time, and is less labor intensive. - An appropriate MAE condition was ethanol concentrations of 95% (v/v), 2 min, and liquid/solid ratio of 10:1 (ml/g).

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
5. Hao et al,. 2002	*Artemisia annua* L	Artemisinin	**Frequency& power** 650 W **Time** 2, 4, 6, 8, 10,12, 14, 18 min **Solvent** Ethanol, Trichloromethane, Cyclohexane, *n*-hexane, Petroleum ether, No. 120 solvent oil and No. 6 extraction solvent oil	HPLC	- The optimal MAE condition was 12 min duration of microwave radiation, the diameter of raw materials less than 0.125 mm, the solvent to material ratio more than 11.3. -Compared with conventional extraction method, MAE used shorter time.
6. Shu et al., 2003	Ginseng root	Ginsenosides	**Frequency & power** 2450 MHz 30,150 W **Time** 1, 2, 5, 10, 15 **Solvent** Water, Ethanol	HPLC	- Microwave assisted extraction (fifteen minute with 70 % and 30 % ethanol- water solutions, 150 W) was more effective than conventional solvent extraction with 70% ethanol-water solutions.

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
7. Li et al,. 2004	*E.ulmodies*	Geniposidic chlorogenic acid	**Frequency & power** 2450 MHz, 700 W (90, 70, 50%) **Time** 10, 30, 50 s **Temperature** room temp **Solvent** Methanol, Water	HPLC	- The optimum extraction condition for geniposidic acid was 50% microwave power for 40s with 80% aqueous methanol (20ml/g) and that for chlorogenic acid was 50% microwave power for 30 s with 20% aqueous methanol (20ml/g).
8. Fulzele et al,. 2005	*Nothapodytes foetida*	Camptothecin (CPT) and 9-Me-CPT.	**Frequency & power** 100 W **Time** 3 min microwave irradiation **Temperature** 80 °C **Solvent** Methanol, Ethanol.	HPLC	- MAE was found to have higher extraction efficiency than the other extraction techniques, and thus MAE is an alternative extraction technique for speedy extraction of anti-cancer drug CPT and 9-Me-CPT of *N. foetida*.

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
9. Zhou et al., 2006	Tobacco leaves	Solanesol	**Frequency & power** 2450 MHz, 700 W **Time** 5, 10, 20, 40, 60min (pre-setting: 45 s power on 3 s heating, 9s power off for cooling) **Temperature** 60 °C **Solvent** Hexane, Ethanol, hexane:ethanol (3:1, 1:1, 1:3)	HPLC	- The optimal condition was extraction with hexane:ethanol (1:3 v/v) with 0.05 mol/l NaOH. Compared to heat reflux extraction, MAE reduced extraction time and gave higher percentage of Solanesol extracted.
10. Martino et al., 2006	*Melilotus officinalis*	coumarin, *o*-coumaric and melilotic acids.	**Frequency & power** 100 W **Time** 5, 10 min **Temperature** 50, 110 °C **Solvent** Ethanol :water	HPLC	- The optimum condition was MAE are 50% v/v aqueous ethanol, two heating cycles of 5 min, and at 50 ◦C. - MAE was a simple and rapid method suitable for extraction of coumarin and its related compounds from plant material.

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
11. Barbero et al., 2006	Peppers	Capsaicinoids	**Frequency & power** 500 W **Time** 5-30 min **Temperature** 50-200 °C **Solvent** Methanol, Ethanol, Acetone, Ethyl acetate and Water	HPLC	- The optimum condition was 125 °C, 25ml of solvent, 0.5 g of freshly triturated peppers. 5 min extraction time, employing 100% ethanol as solvent.
12. Hemwimon et al,. 2006	*Morinda citrifolia* of roots	Anthraquinones	**Frequency** 2450 MHz, **& power** 1200 W (60%) **Time** 5, 10, 15, 20 min **Temperature** 60, 80,100, 120 °C **Solvent** Acetone, Methanol, Ethanol, Acetonitrile, ethanol:water (20:80, 50:50, 80:20)	Spectro-photometer	-The appropriate condition for maximum anthraquinones with MAE was extraction with 80% ethanol, at the extraction temperature of 60 ° C, and extraction times of 30 min. -The antioxidant activity of the extracts obtained with soxhlet extraction and MAE was found to be the highest, compared with maceration and UAE.

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
13. Chen et al,. 2007	Ganoderma atrum	Total triterpenoid saponins	**Frequency & power** 2450 MHz, 800 W (100%). **Time** 20 min **Temperature** 60, 70, 78, 100, 120°C **Solvent** 95% ethanol, chloroform, ethyl acetate, n-butanol, acetone, and methylene chloride/methanol mixture (v/v, 1:1).	UV/Vis spectrophotometer	- Compared with the other techniques such as shaking extraction, heat reflux extraction, ultrasonic extraction and SFE, the MAE method employed provides high extraction efficiency in short time, and was less labor intensive. - MAE of triterpenoid saponins from Ganoderma atrum could be concluded with the best solvent of 95% ethanol, the ratio of solvent to material of 25, duration of microwave radiation of 5 min, and the extraction temperature of 90 °C.

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
14. Mauricio et al., 2007.	Soybeans	isoflavones	**Power** 500W using magnetic stirring at 50% of nominal power **Time** 10, 15, 20, 25 and 30 min. **Temperature** 50, 75, 100, 125, 150 °C **Solvent** EtOH or MeOH, with several water percentages (30–70%).	HPLC-UV	- A fast (20 min) and quantitative method was developed for the MAE of isoflavones from soybeans. - The optimized extraction condition was 0.5 g of sample extracted by 25 mL of 50% EtOH at 50 ◦C for 20 min.
15. Zigoneanu et al., 2007.	Rice bran	Vitamin E components (tocopherols and tocotrienols)	**Time** *First*, temperature increased to the set extraction temperature for 5 min, using an energy level of 800 W maximum. *Second*, hold the samples for a total period of 15 min at the extraction temperature, using an energy level of 500 W maximum. **Temperatures** 40, 60, 80, 100, and 120°C. **Solvent** isopropanol and hexane.	Normal-phase HPLC	- Isopropanol was the best solvent for the extraction of c-tocopherol and c-tocotrienol as compared with hexane for both MAE and conventional solvent extraction. -No differences in oil yield, total vitamin E, and antioxidant activity of oil was noticed between the two methods.

Author	*Material*	*Compounds*	*Conditions*	*Analysis*	*Conclusions*
16. Mao et al., 2007	*Rhodiola* L.	Salidroside and tyrosol.	**Frequency & power** 2450MHz 200, 400 and 700 W **Time** *Soaked up the solution*: 10, 30, 60, 120, 90, 120 and 150 min. *Heated by a microwave*: 1–8 min. **Solvent** Ratio of methanol-water mixture (10, 20, 30, 40, 50, 60, 80 and 90%, v/v, methanol).	HPLC	- The optimal MAE are soak time (60 min), extraction solvent volume (1 g sample, 5 mL), extract solvent composition (50% methanol/water) , microwave power (400 W) and extraction time (5 min). - The proposed MAE–HPLC–DAD is a simple, rapid and low-cost method for quantitative analysis of salidroside and tyrosol, and a potential tool for quality assessment of *Rhodiola* L. sample.

Author	*Material*	*Compounds*	*Conditions*		*Analysis*	*Conclusions*
17. Chen et al.,2008	*Herba Epimedii*	Flavonoids	**Frequency & power** **Time** **Temperature** **Solvent**	20-100 W 1-10 min 80 °C ethanol and methanol	HPLC	- The Dynamic microwave-assisted extraction (DMAE) was more effective compared with other extraction methods, RE, UE and SOX and the extraction yield obtained by DMAE is similar to that obtained by PMAE. - In the DMAE, the four flavonoids in *Herba Epimedii* would not decompose easily when increasing the extraction time.
18. Wang et al.,2008	Panax ginseng root	Ginsenosides	**Time** 2, 5, 10, 15 and 30 min **Pressure** 100, 200, 300, 400 and 500 kPa, **Solvent** methanol, 70% (v/v) ethanol–water and water.		HPLC	- Compared with soxhlet extraction, ultrasound-assisted extraction and heat reflux extraction, HPMAE has excellent advantages; such as shorter time extraction, and a higher yield. The optimization conditions of HPMAE were: 70% (v/v) ethanol–water, extraction pressure of 400 kPa, and extraction time of 10 min.

CHAPTER III

MATERIALS AND METHODS

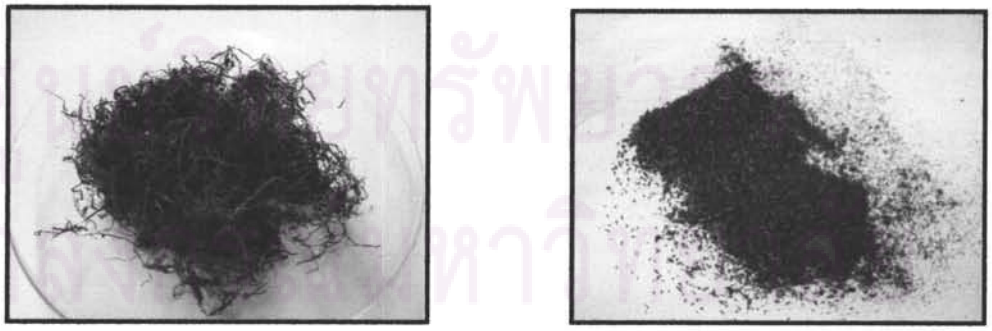
3.1 Materials

3.1.1 Reagents and standards

Morinda citrifolia plants were purchased from a market in Thailand. Standard damnacanthal was purchased from Merck, Germany. Solvent used in this study were acetonitrile (99.9%, HPLC grade), methanol (99.9%, HPLC grade), ethanol (99.9%, analytical grade), acetone (99.5%, HPLC grade), and were purchased from VWR international Ltd, UK.

3.1.2 Plant material preparation

Fresh rootlet of *Morinda citriforia* were harvested, washed, and dried completely in an oven at about 50 °C for 2 days. The dried rootlet was then ground into small sizes using two different methods, using a mortar and a pestle (0.25 mm) or a ball mill (0.02 mm).



(a) (b)

Figure 3.1 (a) Dried roots **(b)** ground dry roots of *Morinda citrifolia*.

3.2 Method

3.2.1 Microwave-assisted extraction

Microwave-assisted extraction was performed on a MARS 5 (1200 W, 2450 MHz), microwave accelerated reaction system from CEM Corp. (Mathews, NC, USA), shown in Figure 3.2. This unit was equipped with twelve 100 ml closed PEEK vessels covered with special TFM sleeves, a power sensor, a pressure sensor, a temperature sensor, and a temperature controller.

The extraction was performed by adding 0.1 g of ground dried roots with a specified amount of a solvent in three of the vessels. The vessels were placed symmetrically in the microwave field. For all experiments, 60% of power output (60% of 1200 W) was used and the ramping time for all extraction runs was 2 min. The experiments were carried out to determine the effects of extraction time (5, 10, 15, 20, and 30 min), extraction temperature (60, 80, 100, and 120 °C), type of solvent (acetonitrile, methanol, ethanol, and acetone), composition of solvent (ethanol:water of 20%, 50%, and 80%), solvent to sample or L/S (50, 80,100 and 150 ml/g) on MAE efficiency. The ranges of variables are listed in Table 3.1. After irradiation, the vessels were allowed to cool down for 5 min and the solution was filtered thorough a filter paper (Whatman, no.1 125 dia.) in order to separate it from the sample residue.

The percent recovery was taken as the fraction of damnacanthal in the root samples that was extracted. The total amount of damnacanthal in the root sample (100%) was determined by extracting the roots repeatedly 3 times in ethanol with MAE at 120 °C for 3 min at the 0.1g:10ml sample to ethanol ratio. In order to avoid degradation of the product, after each extraction the ethanol was separated from the sample, into which 10 ml volumes of ethanol fresh solvent was added, and was allowed a period of 2-3 h prior to then next MAE. This method was found to recover the highest amount of damnacanthal, and was therefore suitable for the determination of the total amount of the

compound in the sample. Each fraction of the samples was preparation by evaporated and analyzed for the amount of damancanthal by using Reversed Phase-High Performance Liquid Chromatographic (RP-HPLC).

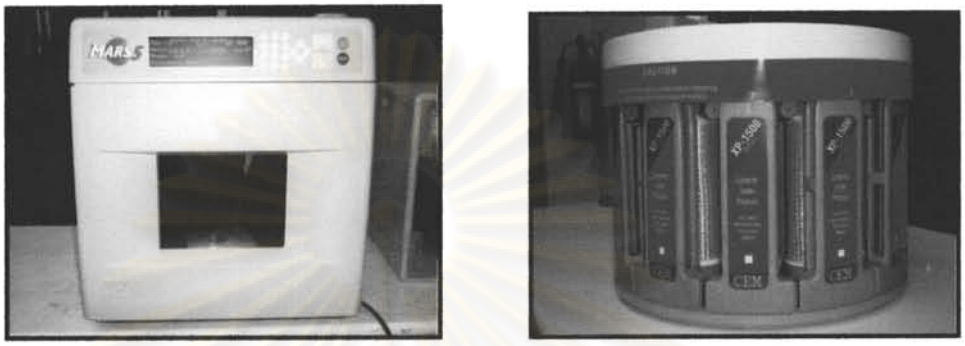


Figure 3.2 Schematic diagram of Microwave apparatus (MARS 5)

Table 3.1 Extraction conditions tested for MAE

Parameter	Condition
Time radiation (min)	5, 10, 15, 20, 30
Temperature(°C)	60, 80, 100, 120
Type of solvent	acetonitrile, methanol, ethanol, acetone
Solvent composition(ethanol:water)	20:80, 50:50, 80:20
Materials size	0.25 and 0.02 mm
Volume of liquid to sample (L/S)	50, 80, 100 and 150 ml/g

3.2.2 Heating extraction

In order to determine the amount of damnacanthal within ground dry roots of *Morinda citrifolia*, the damnacanthal were removed from the roots sample by repeated extraction in a 8.8 ml batch pressure vessel (SUS- 316 stainless steel, AKICO, Japan). The system is schematically shown in Figure 3.1 In this system, 0.08 grams of the roots sample was extracted with 8 ml of 99.9% (v/v) ethanol or 80% ethanol in water at 120°C for 30 min. After

heating, the vessels were allowed to cool down for 5 min and the solution was filtered thorough a filter paper (Whatman, no.1 125 dia.) in order to separate it from the sample residue. The extracts was then determined for the amount of damnacanthal by using RP-HPLC.

3.2.3 Soxhlet extraction

In classical soxhlet extraction, sample was placed into cellulose extraction a thimbles (Whatman, ENGLAND) and was extracted with ethanol with the approximate cycle of 7 cycles h^{-1}, until the solvent was clear or approximately 4 h. The concentration of damnacanthal in the extract was measured using RP-HPLC.

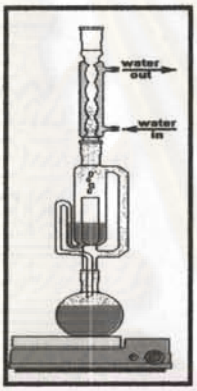


Figure 3.3 Schematic diagram of soxhlet extraction

3.2.4 Ultrasound assisted extraction

For the UAE experiments, an ultrasonic bath was used as an ultrasound source. The bath, 275DAE (Crest Ultrasonics, USA), was basically a rectangular container (23.5 cm ×13.3 cm ×10.2 cm), to which two 38.5 kHz transducers were annealed at the bottom, and the bath power rating was 270 W. Extraction was carried out at the power setting of 9 with a wattmeter energy check 3000 (Voltcraft, Germany). The extraction of damnacanthal was performed by adding the ground roots into an ethanol solvent at selected solvent to sample ratio in a 28 ml glass tube. The tube was then partially immersed into the ultrasonic bath, which contains 2.2 l of water. The bottom

of the flask was approximately 5 cm above the bottom of the bath. The solvent surface in the flask was kept at the level of the water in the ultrasonic bath. Extraction was conducted for 15, 45, and 60 min at 60 °C.

3.2.5 Analysis RP-HPLC procedure

All extracts were evaporated under vacuum to dryness, and re-dissolved in two types of solvent. First, 5 ml of acetonitrile was added to the dried sample. The clear solution was removed and the residue was then dissolved in about 10 ml of DMSO to obtain a clear solution. Both acetonitrile and DMSO fractions were filtered through a membrane filter (0.45μm, Millipore, USA) before being subjected to HPLC analysis. The RP-HPLC condition for the analysis of damnacanthal was carried out with HPLC of a pump (Prostar 240, Varian, USA), equipped with photodiode array detector (Prostar 335, Varian, USA). The analysis was carried out at room temperature using a phenomenex Luna C18, 100 A pore size, 5 μm particle size, 250mm × 4.60 mm I.D. column. The mobile phase consisted of a mixture 0.05% phosphoric acid aqueous solution (A) and acetonitrile (B). The gradient elutions employed are shown in Table 3.2. The flow rate of the mobile phase was 1 ml/min and an injection volume of 50 μL was used. The UV detection wavelength was 250 nm. Each analysis was carried out at ambient temperature. A standard calibration curve was made from a plot of peak areas versus concentrations for a series of standard solutions in appropriate solvents.

Table 3.2 Gradient elution for HPLC analysis of damnacanthal (Modified from Zhang et al., 2006).

Time (min)	0.05% phosphoric acid aqueous (A)	Acetonitrile (B)
0-20	20-28 %	80-72 %
20-30	28-45 %	72-55 %
30-55	45-10%	55-90 %

CHAPTER IV

RESULTS AND DISCUSSION

The main purpose of this study is to investigate the extraction of an anticancer anthraquinone compound, damnacanthal, from the roots of *Morinda citrifolia*. The reverse-phase high performance liquid chromatography (RP-HPLC) was employed for the quantitative analysis of damnacanthal in the extracts obtained with MAE at various conditions to determine the appropriate conditions for MAE, and to compare the results with other methods of extraction such as heating extraction, ultrasound extraction, soxhlet extraction, as well as SWE results obtained in the previous study (Anekpankul et al., 2007). In this experimental, the percent recovery was defined as follows:

$$\text{Percentre recovery} = \frac{\text{Amount of damnacanthal in extraction}}{\text{Total amount of damnacanthal in sample}} \times 100$$

4.1 Microwave assisted extraction

In this section, the results for MAE of damnacanthal are presented, whose experiments was carried out in a closed vessel at a 60% power output (60% of 1200 W). The ramping time was 2 minutes, which was followed by a specified period of extraction, and a 5 minute cool-down time. Variables investigated included the size of the root sample, irradiation time and temperature, types of solvents, solvent compositions, and liquid to sample ratio.

4.1.1. Effect of materials size

Materials size and distribution usually have a significant influence on the performance of MAE. In the study, the experiment was carried out to determine the percent recovery for samples with two different particle sizes (0.25 and 0.02 mm). The microwave extraction was carried out at 60 °C with 99.9% ethanol for various durations of 5, 10, 15, 20, 30 and 60 minutes total time, including ramping time and holding time. The results are shown in Figure 4.1. For both sizes of the materials, the percent damnacanthal recovery increased as the extraction time increased. However, the percent recovery was found to be significantly higher for the small particle size material. In small size sample, the mass transfer limitation was reduced as the larger overall surface area of the small sample allowed better contact between the root sample and the solvent. The small sample was therefore used in the subsequent experiments.

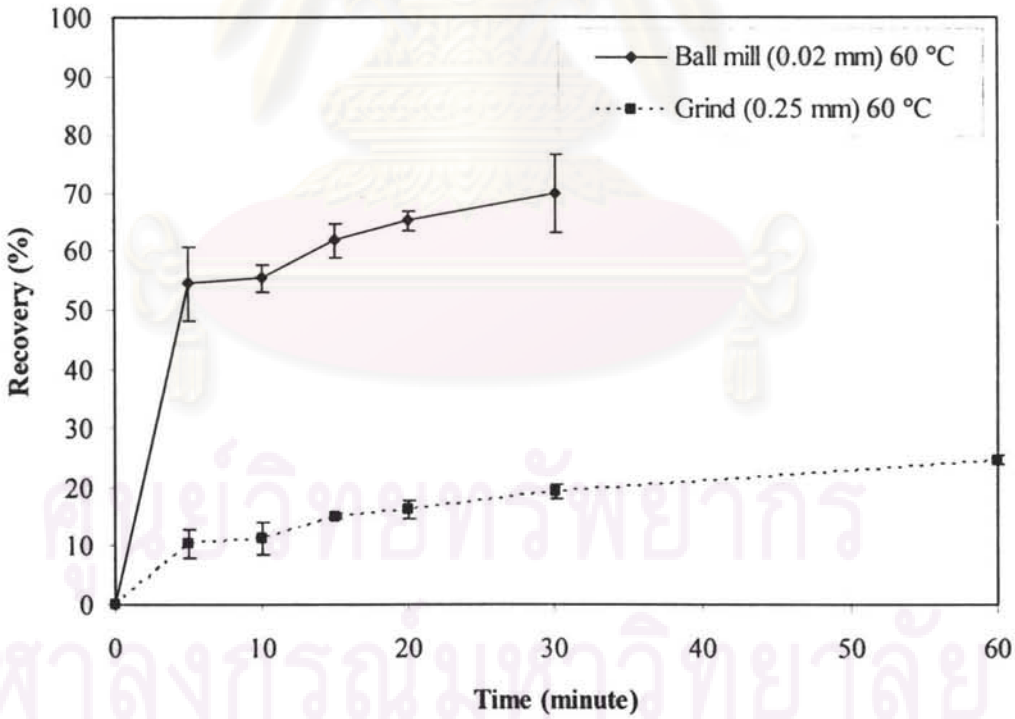


Figure 4.1 Effect of materials size on percent recovery of damnacanthal from MAE at extraction temperature: 60 °C, L/S: 100 and solvent: 99.9% ethanol.

4.1.2. Effect of irradiation time and temperature.

The effects of MAE time from 3 to 30 minutes and temperature from 60 to 120°C on percent recovery of damnacanthal were investigated and the results are shown in Figure 4.2. The extraction was performed with the small materials size (0.02 mm) using 99.9% ethanol. The MAE was carried out under pressure in a closed vessel in which the temperature of solvent can be increased above the solvent boiling temperature. The results show that at lower extraction temperatures of 60 and 80°C, the percent recovery of damnacanthal increased when the irradiation time increased from 5 to 30 minute. The percent recovery of damancanthal at 80 °C was lower than that obtained at 60 °C, possibly due to the decomposition of the compound at high temperature. At higher temperatures of 100 and 120°C, the percent recoveries increased as the irriadation time increased up to 5 minutes, at which the recoveries of 58.44 and 77.87 were obtained for extraction at 100 °C and 120 °C, respectively. The maximum recovery was obtained with MAE at 120 °C, and only 5 min irridation time. The recovery of MAE at 120 °C after 3 min irridation time can be similar to 5 min. Thus, the recovery was obtained with MAE at 120 °C, and only 3 min irridation time was required.

The higher recoveries at higher temperatures of MAE could be attributed to the increase in the solubility of the compound, caused by the increase in the molecular motion of the compounds at elevated temperature. Moreover, at high temperature, the solvent density and viscosity decrease and opening cell matrix, result in increased mass transfer of the solvent into the matrix of plant. However, as can be seen from the figure 4.2, when the irridation time further increased, the pronounced decrease in the extraction efficiency was observed, indicating that damnacanthal decomposed.

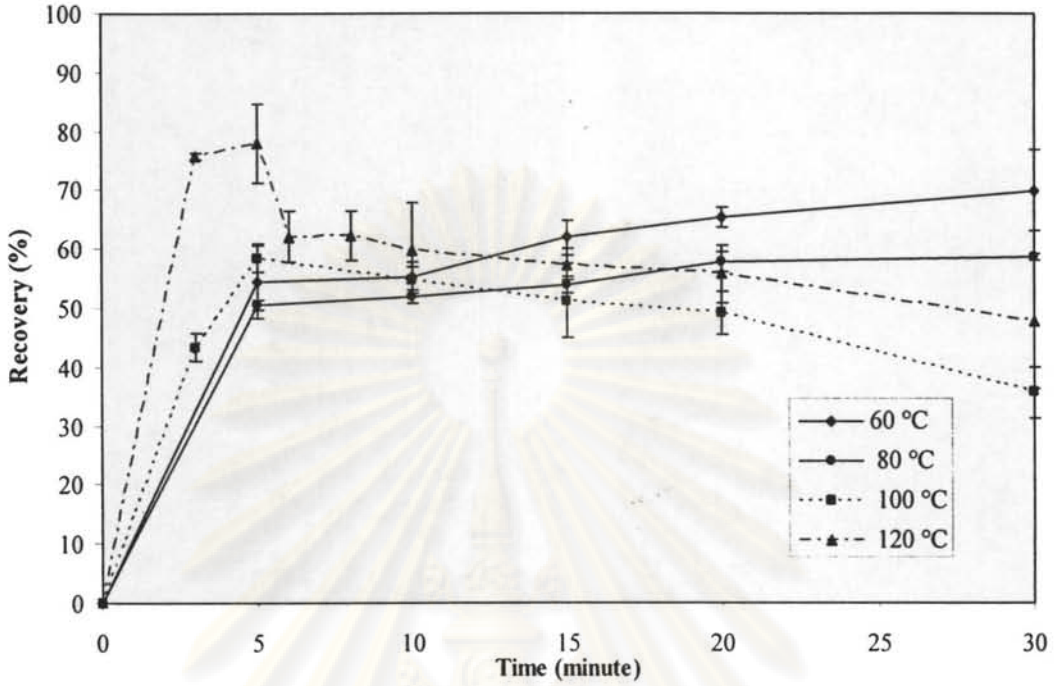


Figure 4.2 Effect of extraction temperature on extraction efficiency damnacanthal of MAE at material size 0.02 mm and L/S =100 (10 ml of 99.9% ethanol/0.1 g of sample).

4.1.3. Effect of type of solvent

In MAE, the most suitable solvent should be selected based on the capacity of the extraction solvent for absorbing and transmitting the microwave energy. In addition, the solvent should be able to dissolve the target analytes, or in other words, the polarity of the extraction solvent should match that of the target compound. In this study, the effect solvent type on the percent recovery of damnacanthal was determined. The root material of 0.02 mm average diameter was extracted with organic solvent using MAE at 120 °C for 3 min and with the liquid to sample ratio (L/S ratio) of 100 (10ml of 99.9% ethanol to 0.1 g of sample). Figure 4.3 shows the percent recovery of damnacanthal obtained by various extraction solvents (acetone, methanol, ethanol, and acetonitrile).

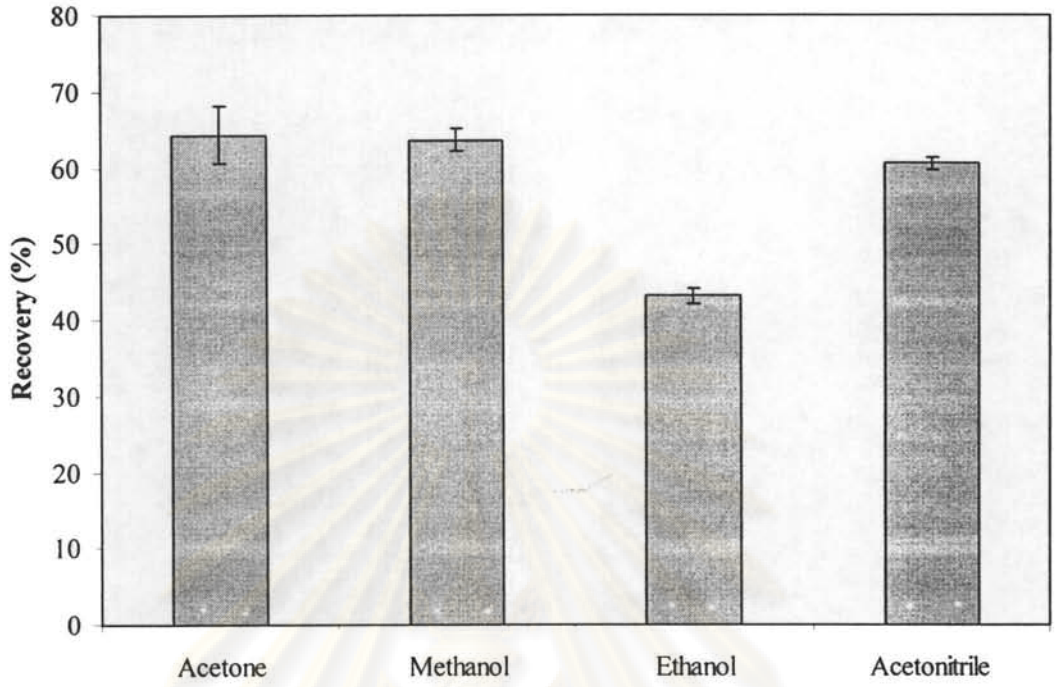


Figure 4.3 Effect of solvent type on extraction efficiency of MAE of damnacanthal. Material size: 0.02 mm, extraction temperature: 120 °C, extraction time: 3 min and L/S: 100.

In general, the ability of the solvent under microwave field to absorb and transmit the microwave energy would be determined by the two parameters defining the dielectric properties of the solvent. These are the dielectric constant (ε') and dielectric loss (ε"), whose ratio (dielectric loss to dielectric constant) defines the dissipation factor (δ). The solvent that heats up rapidly under microwave extraction usually has high dielectric constant and dielectric loss. The values for dielectric constants and dissipation factor are listed in Table 4.1. Of the solvents tested in this study, acetone and methanol have the highest values of dielectric and dielectric loss constants, thus should quickly absorb much of the microwave energy and transform it into heat. Acetonitrile and ethanol have higher dielectric constant but much lower dissipation factor compared with acetone and methanol, therefore the microwave heating rate should be lower than that of the other two solvents. The extraction result however showed that ethanol gave the lowest extraction efficiency while acetonitrile gave rather high efficiency despite the very low value of dissipitation factor. The high

efficiency of MAE with acetonitrile could be a result of its polarity (dielectric constant) that is slightly higher than ethanol, which could be more suitable for the solubilization a slightly polar compound such as damnacanthal.

Table 4.2 Dielectric constant and dissipation factor of solvents (Zlotorzynski et al,. 1995).

Type of solvents	Polarity index	Dielectric constant ε' (F/m)	Dielectric loss ε"	Dissipation factor δ
Acetone	5.1	20.7	11.5	0.5555
Methanol	5.1	32.7	15.2	0.6400
Ethanol	5.2	24.3	6.1	0.2286
Acetonitrile	5.8	37.5	2.3	0.062
Water	9	80	12	0.15

Although acetone, methanol and acetonitrile resulted in more effective MAEs than ethanol, ethanol is more widely accepted as it complies with good manufacturing practice and low toxic for nutritionally and pharmaceutics. In this study, ethanol was therefore chosen as extraction solvent. The efficiency of extraction could however be improved by adjusting the polarity by addition of water and thus, in the subsequent experiment, the effect of ethanol composition in ethanol water mixture on the extraction efficiency would be investigated. It should be noted here that the extraction efficiency (75.67%) obtained by MAE with ethanol in figure 4.2 was higher than the result shown in figure 4.3 (43.10%), obtained at the same extraction conditions (material size 0.02 mm at the extraction temperature 120 °C for 3 min), when the effect of type of solvent was studied. This was because two different sets of plant materials were used and they were prepared by using different of ball mill apparatus. The first set of sample was prepared with model (Planetary Ball Mill PM100, Retsch GmbH&CO.Kg.) ball mill and was used for the study on the effect of particle size and temperature. However, some technical difficulties was later encountered with the ball

mill, during which time, plant root samples were lost. As a result, the new set of samples needed to be prepared with different ball mill (FHM 100, Herzog) and was used for the study to determine the effect of solvent type and ethanol water composition on the percent recovery of damnacanthal. Although different samples and preparation to obtain similar size samples, using the second set would have resulted in the the same most suitable sample size (0.02 mm) and temperature (120 °C), the conditions at which further investigation were conducted.

4.1.4. The effect of ethanol-water compositions

The effect of ethanol-water composition on percent recovery of damnacanthal is shown in figure 4.4. The percent ethanol in water, 20%, 50%, 80% and 100% were used. The microwave extraction was performed with the root material of 0.02 mm diameter at the extraction temperature of 120 °C. The L/S was equal to 100 and MAE was carried out for 3 min. The results in figure 4.4 demonstrated that the addition of water into ethanol improved extraction efficiency, and the highest percent recovery of damnacanthal were obtained by using 80% ethanol in water followed by using 50% and 20% ethanol solutions. The addition of water helped increase the polarity of water and could possibly enhance swelling of root material, thus increased the contact surface area between the root materials with the solvent.

Table 4.3 Polarities of ethanol-water mixtures at various compositions (Lide et al,. 1992).

Ethanol compositions	Polarity index	Dielectric constant
100%	5.2	24.3
80%	5.96	32.126
50%	7.1	46.76
20%	8.24	65.27

The most suitable ethanol water mixture was the 80% ethanol solution which gave the dielectric constant close to that of methanol and acetonitrile, which were previously found to be suitable solvents for extraction of damnacanthal. This gave average recovery of 95%.

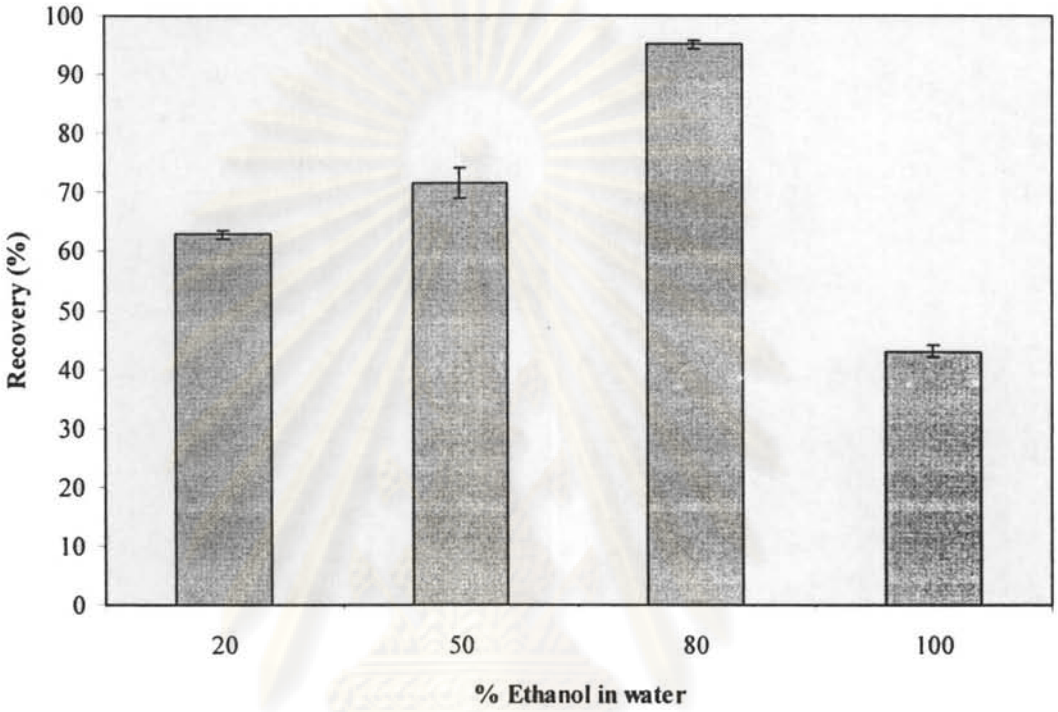


Figure 4.4 Effect of ethanol compositions on extraction efficiency of MAE of damnacanthal. Material size: 0.02 mm, extraction temperature: 120°C, extraction time: 3 min, and L/S =100.

4.1.5. The effect of liquid to sample ratio

To evaluate the effect of liquid to sample ratio (volume of extraction solvent/amount of plant sample) on the percent recovery of damnacanthal, a series of extraction using the sample mass of 0.1 g and different solvent volumes (5-15 ml) were carried out at 120 °C for 3 minutes with 80% ethanol in water. The extracted amount of damnacanthal is shown in figure 4.5, which showed that the percent recovery of damnacanthal increased with the increase in volume of solvent and reach the highest value when the liquid to solid ratio was 100, that is the solvent volume must be large enough to ensure that the sample is immersed and can be extracted effectively. However, when larger solvent volume was used (i.e. For the L/S ratio of

150), the percent recovery of damnacanthal no longer increased. Thus, the value of 100 was considered the optimal ratio of liquid to solid for the MAE process.

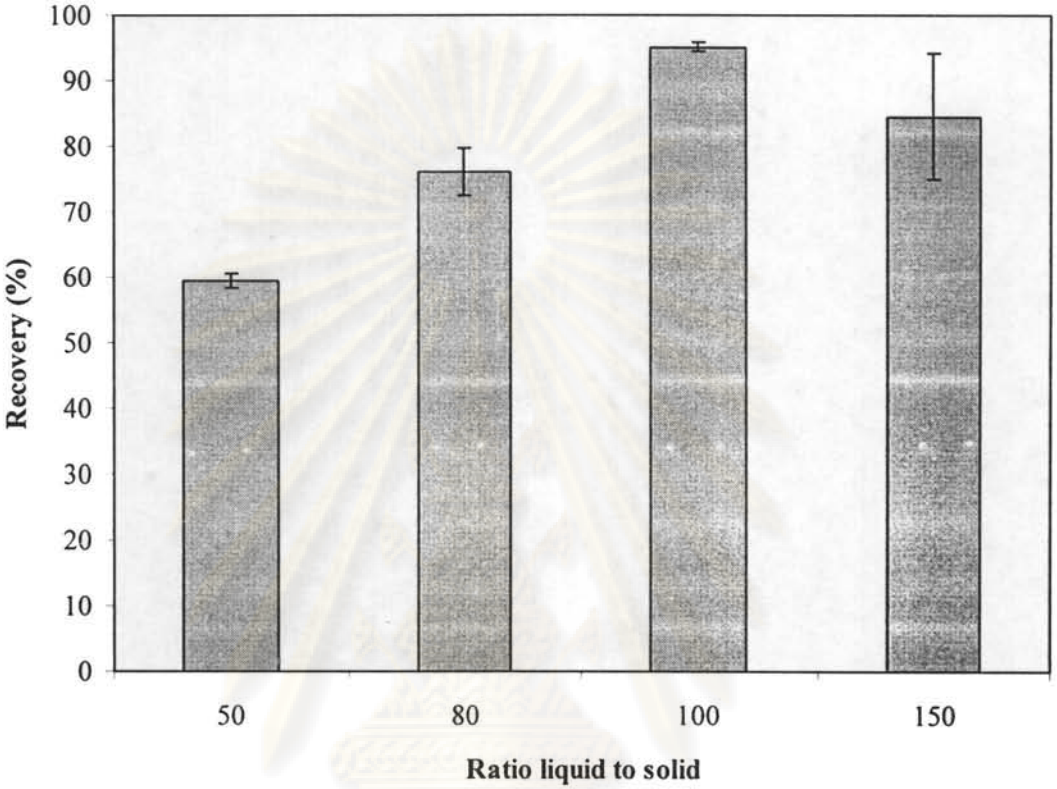


Figure 4.5 Effect of liquid to solid ratio on MAE efficiency of damnacanthal. Material size: 0.02 mm, extraction temperature: 120°C, extraction time: 3 min, and solvent: 80% ethanol in water.

4.2 Comparison of MAE with classical methods

The efficiency of extraction using microwave was compared with that of other classical methods. Table 4.3 summarizes the results.

Table 4.3 Comparison of various extraction methods.

Extraction methods	Time	Temperature	Type of solvent	Recovery (%)
Heating extraction	30 min	120 °C	Ethanol	21.15 ± 0.23
Heating extraction	30 min	120 °C	Ethanol:water (80:20)	54.64 ± 0.64
Ultrasound extraction	15 min	60 °C	Ethanol	33.54 ± 1.15
Ultrasound extraction	15 min	60 °C	Ethanol:water (80:20)	81.77 ± 3.06
Ultrasound extraction	45 min	60 °C	Ethanol	35.05 ± 1.61
Ultrasound extraction	45 min	60 °C	Ethanol:water (80:20)	88.48 ± 1.41
Ultrasound extraction	60 min	60 °C	Ethanol	43.40 ± 0.42
Ultrasound extraction	60 min	60 °C	Ethanol:water (80:20)	94.64 ± 0.25
Soxhlet extraction	4 hr	Boiling point	Ethanol	72.59 ± 2.26
Soxhlet extraction	4 hr	Boiling point	Ethanol:water (80:20)	97.22 ± 1.36
Microwave extraction	3 min	120 °C	Ethanol	43.10 ± 1.87
Microwave extraction	3 min	120 °C	Ethanol:water (80:20)	95.09 ± 0.67
Subcritical extraction	60 min	170 °C	Water	95.13 ± 2.30

For all solvent extraction methods with ethanol, 80% ethanol in water was found to give higher recovery than pure ethanol. When various extraction methods were compared using 80% ethanol in water at the extraction temperature 120°C, MAE could most efficiently extract the compound, that is, only 3 min was required to extract 95% of damnacanthal while 30 min was required for 55% recovery by heating extraction without microwave irradiance. With use of 80% ethanol solution, UAE at 60 °C could give a comparable percent recovery as MAE at 120 °C for 3 min, however UAE took considerably longer time (60 min vs 3 min). Moreover, when compared with subcritical water extraction at 170 °C for 60 min, MAE with 80% ethanol solution at 120 °C for 3 min gave comparable recovery but requried much shorter time. In addition, other advantages of MAE are the small amount of solvent and the shorter exposure time to high temperature, thus minimizing the compound degradation. From the results in this study, MAE is considered a pontential alternative because of its process simplicity and lower cost compared with subcritical water extraction and other conventional solvent extraction methods.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. Microwave-assisted extraction provides a promising alternative for extraction of the anti-cancer damnacanthal from roots of *Morinda citrifolia*.
2. The small size sample (0.02 mm) was more easily extracted than the larger size sample (0.25 mm).
3. The amount of damnacanthal in the extract increased as the temperature increased up to 60°C and at 80°C, the decomposition of damnacanthal occurred.
4. The decomposition of damnacanthal was observed at higher MAE temperature of 100 and 120°C, after the first 5 minute irriadation time.
5. The maximum recovery was obtained with 5 min irradiation time MAE at 120 °C.
6. The appropriate condition for microwave extraction was 0.02 mm size sample, extraction temperature at 120 °C, and extraction time of 3 minutes, 10 ml of 80% ethanol to 0.1 g of sample.
7. Microwave extraction gave the highest yields while requiring the shortest extraction times when compared with the other methods.

5.2 Recommendations

1. Investigation of factors affecting extraction efficiency various particle sizes from ball mill apparatus.
2. The study of effects should use experimental design and statiatic in analysis.
3. Development of the separation and purification process of the compound, damnacanthal by solid phase extraction or chromatography from the extract solution should be considered the further work.

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APPENDICES

APPENDIX A
EXPERIMENTAL DATA

A-1 Standard calibration curve of damnacanthal

Table: A-1.1 Standard calibration curve data.

Concentration of damnacanthal (mg/ml)	Area (UV detector at 250 nm)
0.004	1580566
0.005	2167358
0.01	4329081
0.02	9821437
0.03	15522303
0.04	20819257

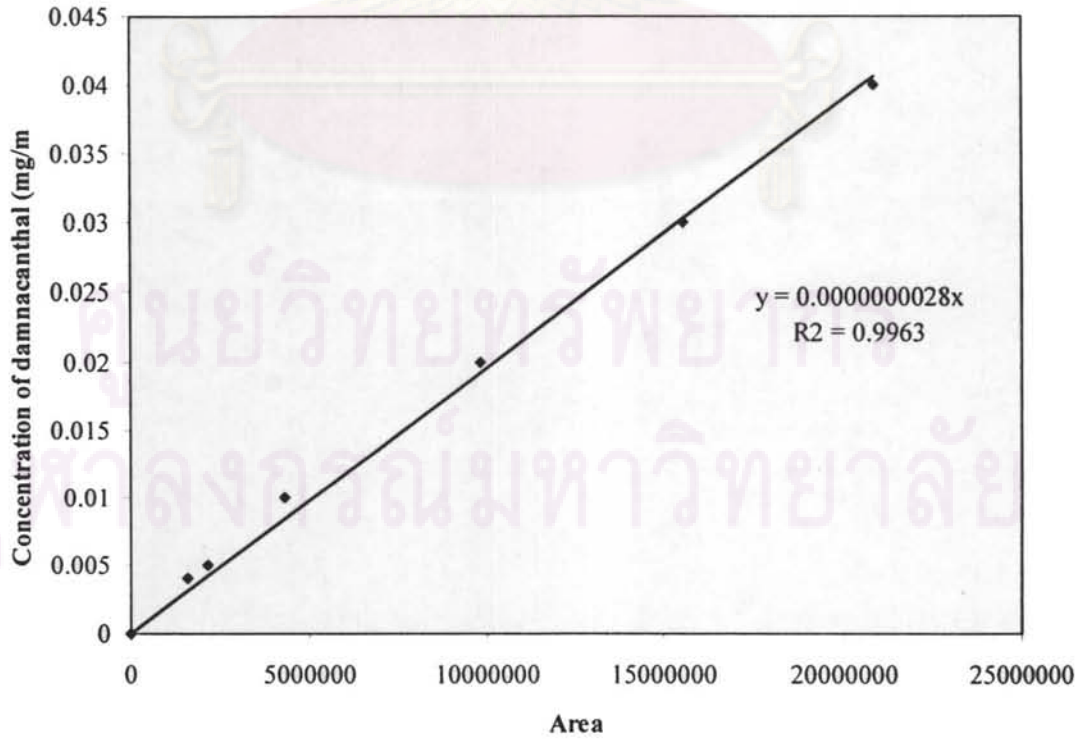

Figure A-1.2 Standard calibration curve of damnacanthal (average).

A-2 Property of *Morinda citrifolia* roots

A-2.1 Particle size analysis

Table: A.2.1 Particle size results of *Morinda citrifolia* roots.

No.	Diameter of roots (mm.)	Std.
1	0.0204	0.0005
2	0.0196	
3	0.0194	
Average	**0.0198**	

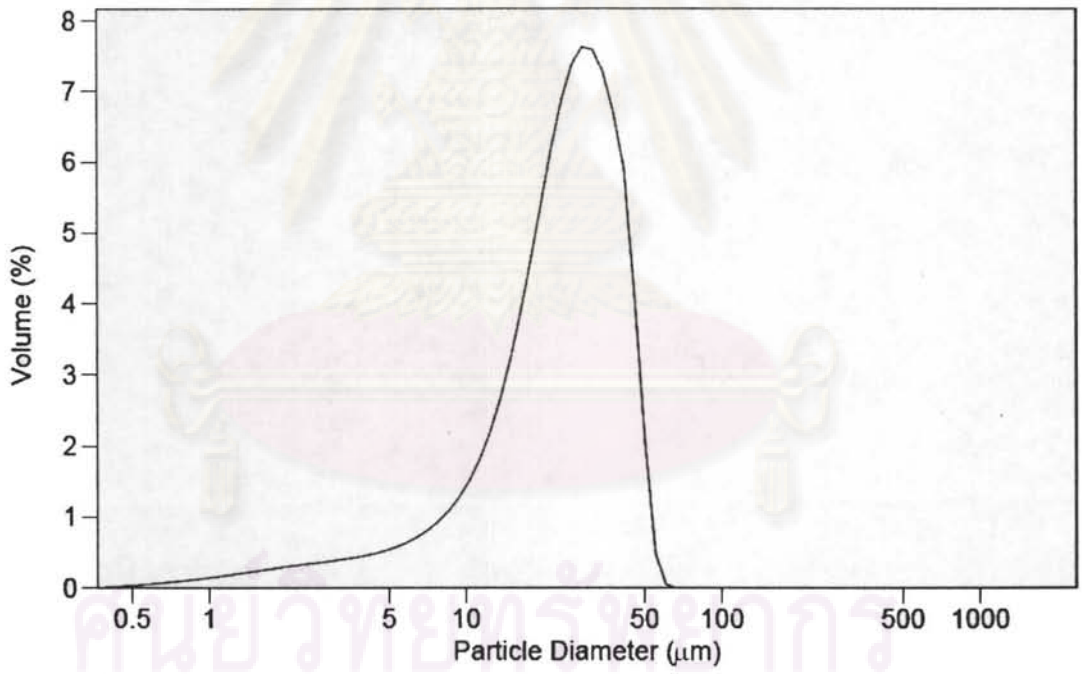

Figure A-2.1 Particle size distribution of powder of *Morinda citrifolia* roots.

Table: A.2.2 Particle size results of *Morinda citrifolia* roots.

No.	Diameter of roots (mm.)	Std.
1	0.2440	0.0056
2	0.2538	
3	0.2440	
Average	**0.2473**	

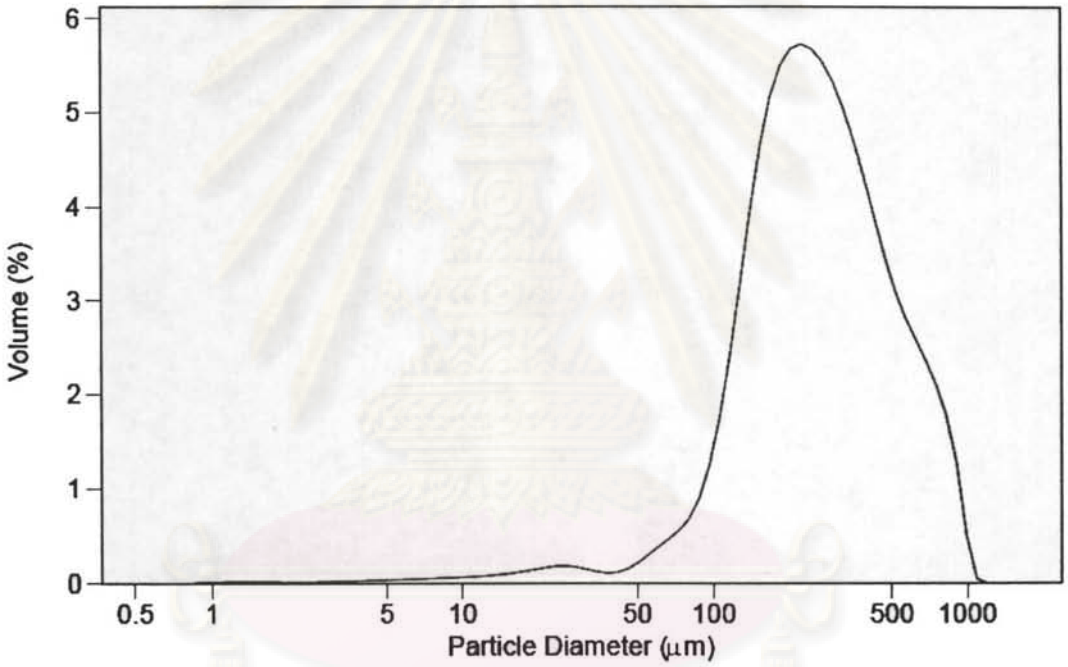

Figure A-2.2 Particle size distribution of powder of *Morinda citrifolia* roots.

APPENDIX B

EXPERIMENTAL DATA

B-1 Experimental data of damnacanthal extract with microwave-assisted extraction

Effect of material size

Table B-1.1: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 60 °C and power 60% of 1200 W and 10 ml of ethanol as solvent to 0.1g of root dries using particle sizes 0.25 mm.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
5	8.63	12.04	10.33	2.4126
10	9.37	13.24	11.30	2.7338
15	15.44	14.88	15.16	0.3979
20	15.04	17.27	16.15	1.5767
30	20.29	18.49	19.39	1.2749
60	24.11	25.27	24.69	0.8219

Table B-1.2: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 60 °C and power 60% of 1200 W and 10 ml of ethanol as solvent to 0.1g of root dries using particle sizes 0.02 mm.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
5	58.94	50.05	54.49	6.2853
10	57.06	53.81	55.44	2.2959
15	59.89	64.00	61.95	2.9057
20	66.42	64.10	65.26	1.6407
30	74.73	65.15	69.94	6.7725

Effect of irradiation time and temperature

Table B-1.3: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 80 °C and power 60% of 1200 W and 10 ml of ethanol as solvent to 0.1g of root dries using particle sizes 0.02 mm.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
5	49.99	51.06	50.52	0.7582
10	51.17	52.69	51.93	1.0762
15	52.92	54.95	53.94	1.4393
20	56.72	59.08	57.90	1.6744
30	58.31	59.12	58.72	0.5747

Table B-1.4: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 100 °C and power 60% of 1200 W and 10 ml of ethanol as solvent to 0.1g of root dries using particle sizes 0.02 mm.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
3	41.67	45.08	43.38	2.4110
5	56.85	60.03	58.44	2.2484
10	53.13	56.26	54.70	2.2125
15	55.61	46.87	51.24	6.1829
20	51.78	46.52	49.15	3.7197
30	32.65	38.61	35.63	4.2144

Table B-1.5: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 120 °C and power 60% of 1200 W and 10 ml of ethanol as solvent to 0.1g of root dries using particle sizes 0.02 mm.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
3	75.24	76.10	75.67	0.6046
5	73.11	82.62	77.87	6.7268
6	65.14	59.02	62.08	4.3230
8	65.16	59.32	62.24	4.1252
10	65.50	54.24	59.87	7.9624
15	55.56	59.21	57.38	2.5808
20	59.16	52.36	55.76	4.8114
30	55.95	39.67	47.81	11.5129

Effect of type of solvent

Table B-1.6: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 120 °C for 3 min and power 60% of 1200 W and particle sizes 0.02 mm using 10 ml of various solvent to 0.1g of root dries.

Type of solvent	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
Acetone	66.96	61.67	64.31	3.7401
Methanol	62.51	64.67	63.59	1.5302
Ethanol	42.35	43.86	43.10	1.0656
Acetonitrile	61.09	59.97	60.53	0.7885

Effect of ethanol-water compositions

Table B-1.7: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 120 °C for 3 min power 60% of 1200 W and particle sizes 0.02 mm using 10 ml of various percent of ethanol in water to 0.1g of root dries.

Ethanol in water (%)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
20	62.30	63.41	62.85	0.7853
50	69.60	73.23	71.42	2.5619
80	95.56	94.61	95.09	0.6720
100	42.35	43.86	43.10	1.0656

Effect of liquid to samples ratio

Table B-1.8: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 120 °C for 3 min power 60% of 1200 W and particle sizes 0.02 mm using various volume of 80%ethanol in water to 0.1g of root dries.

Liquid/Solid (ml/g)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
50	60.27	58.60	59.44	1.1825
80	73.63	78.79	76.21	3.6460
100	95.56	94.61	95.09	0.6720
150	91.24	77.75	84.49	9.5397

B-2 Experimental data of damnacanthal extract with heating extraction.

Table B-2.1: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 120 °C for 3 min using 8 ml of ethanol as solvent to 0.08 g of root dries.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
30	33.71	36.18	34.94	1.7453

Table B-2.2: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 120 °C for 3 min using 8 ml of 80%ethanol in water as solvent to 0.08 g of root dries.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
30	54.19	55.09	54.64	0.6372

B-3 Experimental data of damnacanthal extract with soxhlet extraction.

Table B-3.1: Recovery (%) of Damnacanthal was operating condition at extraction temperature at boiling point for 3 min using 200 ml of ethanol as solvent to 0.1g of root dries.

Time (hr)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
4	74.19	70.99	72.59	2.2631

Table B-3.2: Recovery (%) of Damnacanthal was operating condition at extraction temperature at boiling point for 3 min using 200 ml of 80%ethanol in water as solvent to 0.1 g of root dries.

Time (hr)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
4	96.26	98.18	97.22	1.3552

B-4 Experimental data of damnacanthal extract with ultrasound-assisted extraction.

Table B-4.1: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 60 °C for 3 min using 10 ml of ethanol as solvent to 0.1 g of root dries.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
15	32.72	34.35	33.54	1.1532
45	33.91	36.19	35.05	1.6099
60	43.10	43.69	43.40	0.4168

Table B-4.2: Recovery (%) of Damnacanthal was operating condition at extraction temperature at 60 °C for 3 min using 10 ml of 80% ethanol in water as solvent to 0.1 g of root dries.

Time (min)	Recovery of damnacanthal(%)			Std.
	No.1	No.2	Average	
15	83.93	79.60	81.77	3.0591
45	89.48	87.48	88.48	1.4093
60	94.46	91.42	92.94	2.1440

B-5 HPLC chromatogram of damnacanthal extract from *M. citrifolia*.

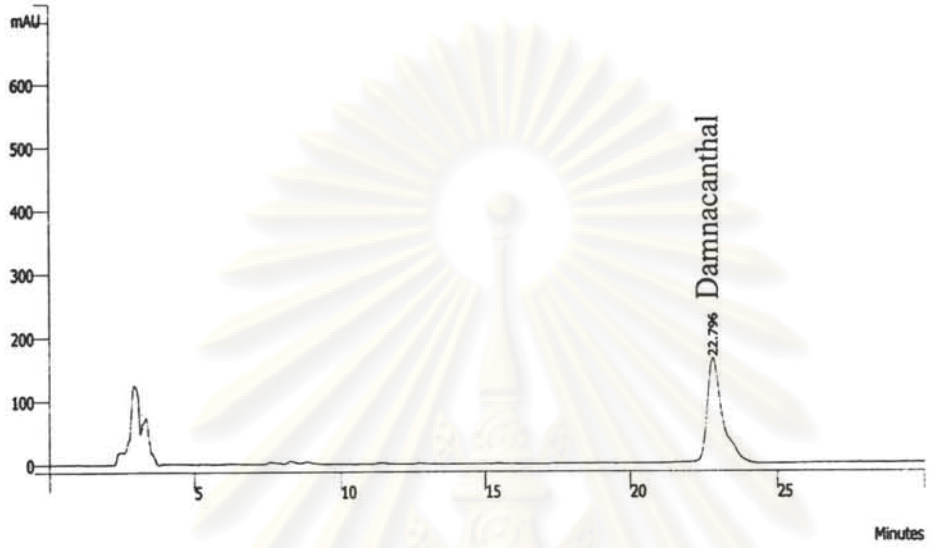

Figure B-5.1 HPLC chromatogram of damnacanthal (retention time, t_R = 22.796 min) in standard

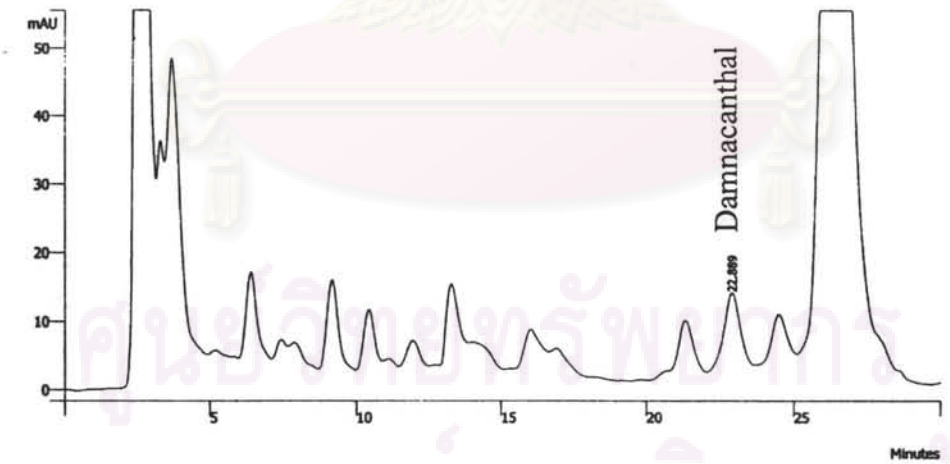

Figure B-5.2 HPLC chromatogram of damnacanthal (retention time, t_R = 22.889 min) in noni roots extracts obtained from MAE, dissolved into 5 ml of acetonitrie.

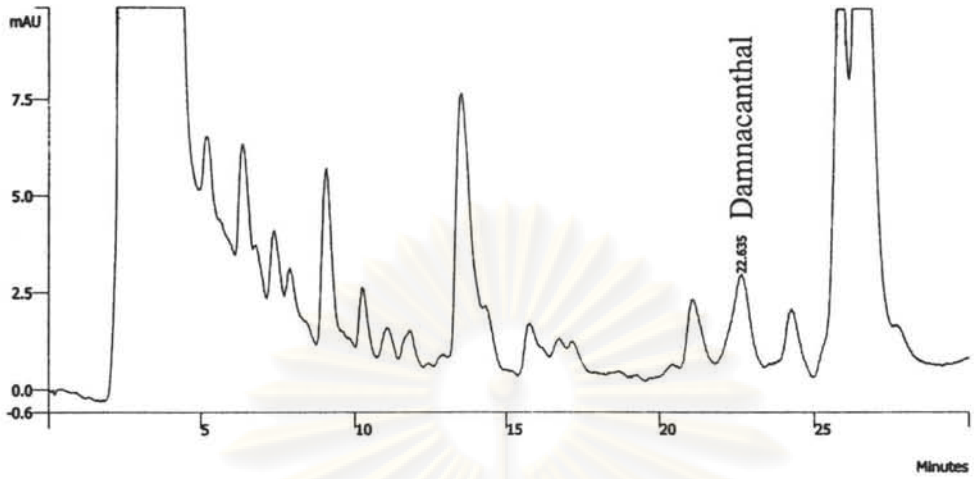

Figure B-5.3 HPLC chromatogram of damnacanthal (retention time, t_R = 22.635 min) in noni roots extracts obtained from MAE, dissolved into 10 ml of DMSO.

APPENDIX C

The 6 th National Grad Research Conference

(Grad-Research 6 th)

13-14 October 2006, Mahamakut Building,
Chulalongkorn University, Thailand

Microwave-Assisted Extraction of Antioxidative Anthraquinones from Roots of *Morinda citrifolia*

Author Waraporn Witayasinthana

Advisor Artiwan Shotipruk* Surasak Hemwimon

Affiliation Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University

*To whom correspondence should be addressed: Artiwan Shotipruk, Department of Chemical Engineering, Chulalongkorn University, Bangkok 10330, Thailand
Tel: 662-218-6868, Fax: 662-218-6877, Email: artiwan.sh@chula.ac.th

Abstract

This study demonstrated promising results for microwave-assisted extraction (MAE) of antioxidant anthaquinones from roots of a medicinally important plant, *Morinda citrifolia*. The effects of temperature, ethanol compositions, and types of solvents on the percent recovery of the extract were determined. The extraction recovery and the antioxidant activity of the extract were compared with those of the extracts obtained from the conventional methods including ultrasound-assisted extraction (UAE). The percent recovery of anthraquinones was found to increase with increasing MAE time and temperature, and was highly dependent on the type of solvents used. Among the four solvents tested (acetone, ethanol, methanol, and acetonitrile), methanol gave the highest recovery. Furthermore, it was found that the anthraquinones recovery was also affected by the amount of water present in the extraction solvent. This was due to the relative polarity and swelling of plant tissue matrix by water. To achieve the same recovery as with MAE, maceration, soxhlet extraction, and UAE required much longer time. In addition, the antioxidant activity of the MAE extract was found to be only slightly lower than that of soxhlet extraction but significantly higer than those obtained by maceration and UAE.

Key words: Microwave, extraction, *Morinda citrifolia*, anthraquinones, MAE

Introduction

Morinda citrifolia (Noni), a plant which grows prevalently in tropical region, has recently gained a great deal of interest by scientists and medical professionals due to the pharmaceutical values this plant offers. *Wang et al. (2002)* has recently published a review of Noni research, which summarizes the therapeutic effects of various compounds in this plant [1]. Traditionally, the roots of Noni plants were used by Polynesians to produce yellow or red dye, but more importantly, they are now known to contain medicinally active components, such as anthraquinones, which, due to its antioxidant activities, possess various therapeutic properties [2]. These include anti-bacterial, anti-viral, and anti-cancer activities as well as analgesic effects. This makes the compounds potentially useful in several medical applications [3-5]. Increasing number of studies is focusing on finding efficient methods for production and extraction of anthraquinones from these plants. Much of the literature also involves production of the compound in root culture of *Morinda citrifolia* [6-8]. Nevertheless, extraction of anthraquinones directly from plant roots is still more widely conducted and is conventionally performed by solvent extraction. Other techniques which include supercritical carbondioxide extraction, subcritical water extraction, ultrasonic assisted extraction (UAE), and microwave assisted extraction (MAE) have also become of interest as alternatives for the conventional methods. Among these, MAE is the simplest and the most economical technique for extraction of many plant derived compounds [9].

In the present study, MAE of *Morinda citrifolia* roots was carried out, in which the biological activity of the extract as well as the amount of anthraquinones in the extract was concerned. Firstly, the effects of MAE time, temperature, types of solvents, and solvent compositions were determined on the percent extraction of anthraquinones. The extraction efficiency obtained with MAE was then compared with that from conventional methods such as maceration, soxhlet extraction, as well as UAE. Lastly, the antioxidant activities of the extracts obtained from various methods were compared.

Materials and Methods

Preparation of plant materials and conventional solvent extraction. Fresh roots of *Morinda citrifolia* were harvested, washed, and ground in liquid nitrogen to an average size of 0.2 mm in diameter. The ground samples were oven dried at 45 ° C and kept in a dry place until use.

Two conventional solvent extraction methods were used in this study: maceration and soxhlet extraction. In maceration, 0.1g of *Morinda citrifolia* was extracted in 10 ml of organic solvent in a 125 ml flask at 25 °C and 60 °C. The maceration time of 3 days was found to be sufficient to recover all anthraquinones in the plant roots. For the purpose of comparison with MAE and UAE, other sets of maceration experiment were carried out for 0-60 min. The extract was then filtered with a filter paper (Whatman, no.1, USA). The concentration of anthraquinones was measured by a spectrophotometer. For solvent extraction using soxhlet apparatus, 2 g of root sample was placed into a thimble with 200 ml of solvent (ethanol) contained in a 250 ml round-bottom flask. Extraction was carried out for up to 4 hours.

UAE

For the UAE experiments, an ultrasonic bath was used as an ultrasound

source. The bath, 275DAE (Crest Ultrasonics, USA), was basically a rectangular container (23.5 cm × 13.3 cm × 10.2 cm), to which two 38.5 kHz transducers were annealed at the bottom, and the bath power rating was 270 W. Extraction was carried out at the power setting of 3 which was measured to be 15.7 W with a wattmeter energy check 3000 (Voltcraft, Germany). The extraction of anthraquinones was performed by adding 0.1 g of ground dried roots into 10 ml of solvent in a 28 ml glass tube. The tube was then partially immersed into the ultrasonic bath, which contains 2.2 L of water. The bottom of the flask was approximately 5 cm above the bottom of the bath. The solvent surface in the flask was kept at the level of the water in the ultrasonic bath. Extraction was conducted for up to 60 min at 60 °C. Water in the ultrasonic bath was circulated to control the temperature and to avoid the water temperature to rise, as a result of ultrasonic exposure.

MAE

MAE experiment were performed on a MARS 5 (1200 W, 2450 MHz), microwave accelerated reaction system from CEM corp. (Mathews, NC, USA) equipped with twelve100 ml closed PEEK vessels covered with special TFM sleeves, a power sensor, a temperature sensor, and a temperature controller. The extraction of anthraquinones was conducted by adding 0.1 g of ground dried roots into 10 ml of solvent in three of the vessels. The vessels were placed symmetrically in the microwave field. For all experiments, 60% of power output (60% of 1200 W) was used and the ramping time for all extraction runs was 2 minutes. Experiments were carried out to determine the effect of irradiation time (0-60min), composition of solvent (ethanol:water of 20, 50, and 80%), type of solvents (acetone, ethanol, methanol, and acetonitrile), and temperature (60-120 °C) on MAE efficiency. After microwave irradiation, the solution was filtered through a filter paper (Whatman, no.1, USA). The anthraquinones concentration was measured by a spectrophotometer.

To determine the percent recovery, the sample residue after each extraction was extracted repeatedly in three 10-ml volumes of ethanol or until the extract was clear. The sum of the amount of anthraquionones extracted by each extraction procedure (UAE, MAE, or Soxhlet), and that remained after each extraction was found to be comparable and was taken to be the total anthraquinones in the root samples (100%). The percent recovery was determined from the fraction of anthraquinones in the root samples that was extracted.

Measurement of anthraquinones concentration

The analysis method for determining the amount of anthraquinones was modified from that described by Zenk et al [7]. The concentrations of these solutions were analyzed spectrophotometrically (Genesys 20, USA) by measuring the absorbance at 435 nm, with Alizarin or 1, 2 dihydroxyanthraquinone as a standard. This was a standard approach for determination of total anthraquinones in the solution.

Antioxidant activity measurement

Antioxidant activities of anthraquinones extracts obtained using UAE, MAE, and classical extraction methods were tested and compared by measuring the ability of the extracts to scavenge the free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) *in vitro*. The assay method was modified from that described in *Ollanketo et al.* [11]. For the purpose of comparing the antioxidant activity in various extracts, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index. To find this value, the extract was diluted in series with ethanol and 2 ml of each diluted extract was added to 2 ml of 110 µM DDPH solution. The solutions were mixed using a vortex (Model ZX3, VELP, Italy) and the mixture was then incubated for 2 hours in darkness at room temperature, after which the absorbance was measured at the wavelength of 517 nm using ethanol as a reference.

IC_{50} can be found from a plot of percent in inhibition (PI) versus the concentration of anthraquinones. The values of PI can be calculated using the following equation:

$$PI\ (\%) = [1-(A_t / A_r)] \times 100 \quad (1)$$

in which A_t and A_r are absorbance of test sample and absorbance of the DPPH reference, respectively.

Results and discussion

Comparison of extraction methods

Figure 1 shows the extraction profiles for MAE in comparison with other extraction methods such as maceration and UAE. As expected, the yield of anthraquinones obtained using MAE increased with increasing times of extraction.When compared with maceration conducted at 25 °C, MAE gave considerably higher percent recovery due to the heating effect. More importantly, when compared with maceration at 60 °C, MAE still resulted in higher percent recovery after the same 60

minute extraction time. Although the solvent temperature employed in both cases was the same, microwave heating occurred at much faster rate. Because heating is known to affect the morphological changes in the plant sample matrix and thus enhances product mass transfer, faster heating in MAE should therefore be responsible for increased mass transfer, and thus anthraquinones release rate observed. Thus, in MAE, the effects of higher heating and mass transfer rates synergistically increase the rate of anthraquinones extraction. When MAE was compared with UAE (at 60 °C) in which mass transfer was enhanced by cavitational effect, MAE yet resulted in higher initial rate of extraction. The percent recoveries of the product for both methods however approached the same value after about 18 minutes. For maceration, on the other hand, one would expect to obtain the same recovery at much longer time period due to the mass transfer limitation in this system compared to MAE and UAE. The result here presented a clear advantage of MAE in shortening the time of extraction. The quality of the extract measured in terms of antioxidant activity will later be discussed.

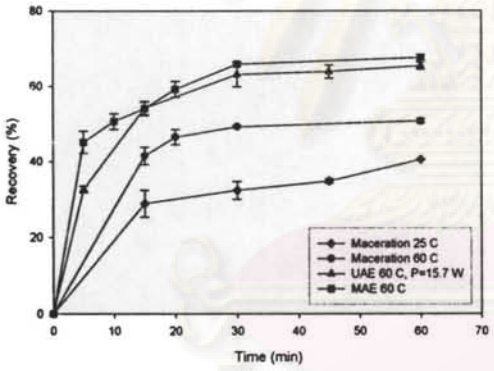

Figure 1 Effect of extraction times of maceration, UAE, and MAE on extraction efficiency.

Effect of extraction temperatures

Generally, the higher extracting temperature is profitable for extraction due to the increased solubility. In a closed microwave vessel used in this study, the temperature of the solvent could be increased above the boiling point temperature. As a result, the solubility of anthraquinones could greatly be enhanced. The effect of extraction temperature which clearly demonstrates that increasing the temperature of the solvent from 60 to 120 °C significantly increases the extraction efficiency. This is because higher temperature causes intermolecular interactions within the solvent to decrease, giving rise to higher molecular motion, and causing the solubility to increase. The increasing temperature may also cause opening cell matrix, and as a result, anthraquinones availability for extraction increases. Moreover, at high temperature, solvent viscosity decreases and the diffusivity increases, thus the efficiency of extraction increases [10, 12].

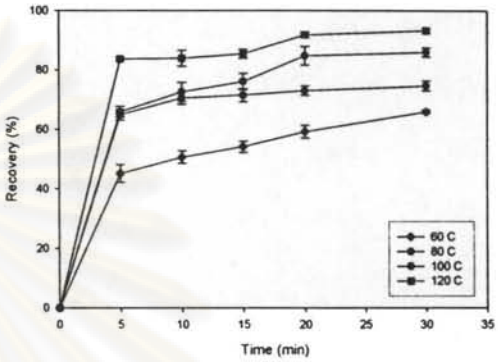

Figure 2 Effect of extraction temperature of MAE

Effect of solvent type

The effect of the type of solvents on extraction efficiency of MAE was determined at 60 ° C. The percent anthraquinones recovery after 15 minutes of MAE was compared. The results in Figure 3, plotted together with those from maceration experiments (45 minutes of extraction times), demonstrate that methanol gave the highest extraction efficiency for MAE, followed by acetone, acetonitrile, and ethanol. As seen in this figure, solvents behave differently in MAE and in conventional extraction. In maceration without microwave irradiation, the extractability of different solvents depends mainly on the solubility of the compound in the solvent, the mass transfer kinetics of the product, and the strength of the solute/matrix interactions. Under the influence of microwave, heating rate plays an important role in extraction efficiency. Thus the success of MAE is also determined by the two other parameters defining the dielectric properties of the solvent. The first is dielectric constant, or relative permittivity, ε'. This parameter describes polarizabilty of the molecule to an electric field. It is a measure of the ability of a material to store electromagnetic radiation. The second parameter is the dielectric loss factor, ε '', which measures the efficiency in which the absorbed microwave energy can be converted into heat inside a material when an

electric field is applied. From these two properties, defined another solvent dielectric property, called dissipation factor,δ,which is expressed mathematically by

$$\delta = \frac{\varepsilon''}{\varepsilon'} \qquad (2)$$

The dissipation factor is a measure of the ability of the solvent to absorb microwave energy and dissipate that energy in the form of heat. Thus, it is generally known that the solvent that heats up rapidly under microwave radiation typically have high dielectric constant and dielectric loss constant. The values for dielectric constants, dielectric loss constants, and dissipation factors determined by eq. (2) for the solvents tested in this study are listed in Table 1 [13].

Table 1 Dielectric constants and dissipation factors of different solvents [13].

Type of solvents	ε'^{a} (F/m)	ε''^{b}	tan δ^{b}
Acetone	20.7	11.5	0.5555
Methanol	32.6	15.2	0.6400
Ethanol	24.3	6.1	0.2286
Acetonitrile	37.5	2.3	0.0620
Water	78.9	12	0.1500

[a] Determined at 20 ° C
[b] at 2450 MHz

Methanol has relatively high dielectric constant and the highest dissipation factor, which means that it could absorb much of the microwave energy and transform it into heat better than the other solvents. Therefore, as was expected, the rate of microwave heating of methanol was the highest, and thus resulting in the highest extraction efficiency. Acetonitrile, on the other hand, despite its higher dielectric constant than methanol, the dissipation factor of the solvent is the lowest, the rate of heating and extraction efficiency was thus found to be relatively low. The results presented in Figure 3 also show that MAE of anthraquinones in acetone resulted in rather high extraction efficiency. Compared with ethanol, the solvent has comparable dielectric constant but has more than twice higher dissipation factor, thus acetone is expected to be more effective MAE solvent than ethanol.

From the above finding, although methanol was shown to give the highest extraction efficiency, it is highly toxic and is not practical for use in food and pharmaceutical processing. And although acetone and acetonitrile gave higher extraction MAE efficiency than ethanol, ethanol is often more preferred in practice due to its several advantages, such as being derived from natural sources and having lower cost. For these reasons, ethanol was chosen for subsequent investigation to determine the effect of different solvent to water composition on the efficiency of microwave extraction [14].

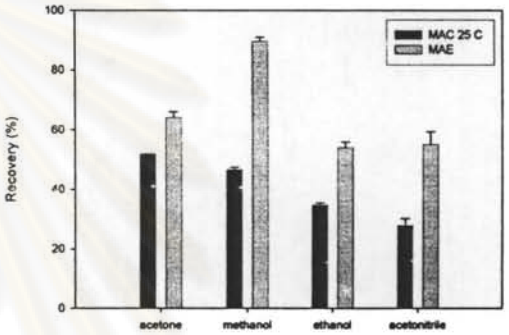

Figure 3 Effect of solvent types on extraction efficiency of 15 min MAE at 60 °C and 45 min maceration at 25 °C.

Effect of ethanol-water compositions

The effect of ethanol-water composition on percent recovery of anthraquinones is demonstrated in Figure 4 for MAE at 60 °C. From these results, it can be noted that the use of microwave clearly enhanced the product recovery across all compositions of solvents used. This was due to the increase in extraction temperature which is the key advantages of MAE. MAE in 80% ethanol in water was found to give the highest percent recovery after 15 minutes of extraction, followed by 50% ethanolic solution. In both cases, more than 90% anthraquinones could be extracted within only 15 minutes. From these results, it is clear that the addition of some amount of water enhances the extraction efficiency. One possible reason for the increased efficiency with a presence of some water might be due to the increase in swelling of plant material by water, which increased the contact surface area between the plant matrix and the solvent [14-15].

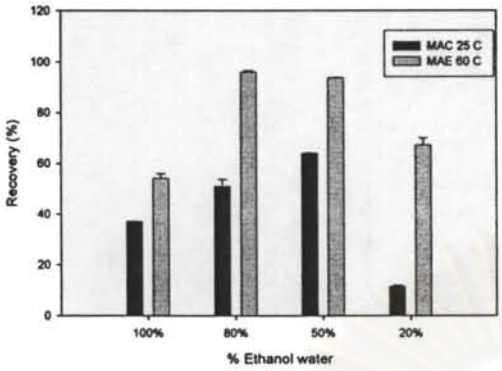

Figure 4 Effect of ethanol compositions on extraction efficiency of MAE at 60 °C at 15 minutes.

Comparison of MAE with classical methods

The efficiency of extraction using UAE and MAE was compared with that of the other classical methods. Table 2 summarizes these results. At the extraction temperature of 60 °C, UAE and MAE with pure ethanol for 30 min gave comparable recovery (approximately 65%). This was also comparable to that resulted from a 3 day maceration in pure ethanol at room temperature.

Table 2 Comparison of percent recovery and extraction time for different extraction methods.

Extraction methods	Time	Temperature	Recovery (%)
1. Maceration	60 min	60 °C	52.20±0.40
2. Maceration	60 min	Ambient	38.12±0.23
3. Maceration	3 days	Ambient	63.33 ± 2.73
4. Soxhlet	4 h	Boiling point	97.74 ± 0.31
5. UAE	60 min	60 °C	62.23 ± 0.48
6. MAE	30 min	60 °C	65.88 ± 0.60
7. MAE	15 min	60 °C	95.91 ± 0.72

1-6 [extraction EtOH]
7 [extraction in EtoH:water(80:20)]

Clearly, these findings demonstrate that UAE and MAE are promising extraction methods that offer improved efficiency by reducing the time required for extraction. When consider Soxhlet extraction for 4 hours in ethanol however, the results show that the method was able to give higher yields than UAE and MAE at 60 °C. The reason for this was due to the fact that Soxhlet extraction was carried out at the temperature closed to the boiling point of ethanol, and the plant tissues were continuously extracted with the fresh condensed solvent for extended time period. UAE and MAE on the other hand were conducted in a batch system. Nevertheless, ethanol-water mixture enhances the percent recovery to up to approximately 96%, which was comparable to using soxhlet extraction in pure ethanol. At the same temperature, UAE and MAE gave comparable recovery percentage.

Antioxidant activity

Antioxidant activity of the extracts obtained by various methods are tested and compared using 1,2-diphenyl-2-picrylhydrazyl (DPPH) radicals. The reduction of the DPPH absorbance at 517 nm after 2 hour incubation with the sample in darkness was measured. For the purpose of comparing the antioxidant activity in various extracts, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index. The IC_{50} values for various extracts are shown in Figure 5.

The IC_{50} for the extract obtained with MAE was slightly higher than that of the extract obtained with Soxhlet extraction, which means MAE extract had slightly lower antioxidant activity than that of the extract obtained with Soxhlet extraction. The degradation of the antioxidant activity observed could be resulted from the microwave radiation. Nevertheless, the extract obtained with MAE had higher antioxidant activity than those of UAE and maceration. The lower activity of the maceration extract could be resulted from extended extraction time, hence exposure to unfavorable conditions such as light and oxygen. Although UAE did not require long extraction time, it is commonly known that ultrasonication could induce free radicals formation within the liquid medium, thus causing oxidation and degradation of the anthraquinones.

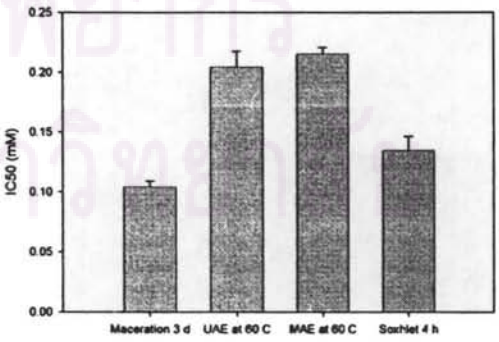

Figure 5 Antioxidant activities of extracts obtained by various extraction methods.

Conclusions

MAE gives the highest yields while requiring the shortest extraction times when compare with the other methods. The main mechanism for enhanced recovery of anthraquinones with MAE was the dipole rotation of the polar solvent in the microwave field, which was highly influenced by the solvent dielectric constant and dissipation factor. The appropriate condition for maximum anthraquinones recovery with MAE was extraction with 80% ethanol, at the extraction temperature of 60°C, and extraction times of 30 minutes. The antioxidant activity of the extracts obtained with Soxhlet extraction and MAE was found to be the highest, compared with those obtained with maceration and UAE. These results demonstrate the potential for new MAE to extract the antioxidative compounds, anthraquinones, from the roots of *Morinda citrifolia*.

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VITA

Miss Waraporn wittayasinthana was born on 16 October , 1983 in Bangkok, Thailand. She received a Bachelor's Degree of Food Engineering Department from the Faculty of Agro-industry, Chiang Mai University in 2005. After then she subsequently completed the requirements for a Master's Degree in Chemical Engineering at the Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University in 2008.