Doctoral Dissertation

Biological Activities and Environmental Interactions of Momilactones A and B, and Phytochemicals in Rice

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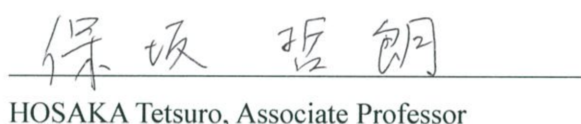

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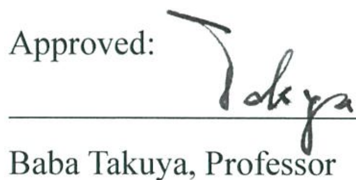
Approved:  Date: February 22. 2019

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ABSTRACT

Momilactones A and B (MA and MB, respectively) are phytoalexins and plant growth inhibitors available in rice husk. The isolation and purification of these two compounds are complicated, laborious, and ineffective. Thus, the known biological properties of MA and MB have been limited only to allelopathy, antioxidant, antifungal, and antimicrobial activities. The present study was conducted to establish a protocol to simplify and optimize quantities of MA and MB by using combinations of ethyl acetate (EtOAc), distilled water, methanol (MeOH), temperature, and pressure for extractions. The chemical components, antioxidant, and antimicrobial activities in rice husk were thus examined. By using a temperature at 100 °C combined with EtOAc and MeOH 100%, MA and MB were enriched to much greater quantities than in non-treated rice husk. Treatment with either ethyl acetate (100 °C, 1 h) or distilled water (100 °C, 2 h) combined with MeOH 100% provided maximum yields of MA (51.54–58.76 µg/g dry weight (DW)) and MB (102.23–104.43 µg/g DW). Because the melting points of MA and MB were 234–236 °C and 240 °C, respectively, the treatment at 100 °C may effectively increase the quantities of MA and MB. The enrichment increased the amounts of MA and MB purified by column chromatography by 5 and 15 times, respectively. The use of either only distilled water or MeOH $\leq$ 50% at any temperature could not successfully isolate both MA and MB. Although the treatments that afforded maximum yields of MA and MB were not proportional to total contents of phenolics, flavonoids, or antioxidant and antimicrobial activities, the extractions established by this research were useful to utilize rice husk as a source of antioxidants and antimicrobial agents. The optimization of MA and MB yields aids simpler and more productive purification of these two compounds and contributes to the search for further biological activities of MA and MB.

Although investigations on phytochemicals in rice plant parts and root exudates have been extensively conducted, the chemical profile of essential oil (EO) and its potent biological activities are not well understood. In this study, chemical compositions of rice leaf EO and *in vitro* biological activities were investigated. From 1.5 kg fresh rice leaves, an amount of 20 mg EO was obtained by distillation and analyzed by gas chromatography-mass spectrometry (GC-MS), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI). This revealed the presence of twelve

volatile constituents, of which methyl ricinoleate (27.86%) was the principal compound, followed by palmitic acid (17.34%), and linolenic acid (11.16%), while 2-pentadecanone was the least (2.13%). Two phytoalexin momilactones A and B were identified for the first time in EO by using ultra-performance liquid chromatography coupled with electrospray mass spectrometry (UPLC/ESI-MS). The amounts of each were low (9.80 and 4.93 ng/g fresh weight, respectively for MA and MB). The assays of DPPH (IC_{50} = 73.1 μg/mL), ABTS (IC_{50} = 198.3 μg/mL), FRAP (IC_{50} = 700.8 μg/mL) and β-carotene oxidation (LPI = 79%) revealed that EO possesses excellent antioxidant activity. The xanthine oxidase assay indicated that the antihyperuricemia potential was at a moderate level (IC_{50} = 526.0 μg/mL) compared with the standard allopurinol (IC_{50} = 21.5 μg/mL). The EO exerted potent inhibition on the growth of *Raphanus sativus*, *Lactuca sativa*, and two noxious weeds *Echinochloa crus-galli* and *Bidens pilosa*, but in contrast, the growth of rice seedlings was promoted. Among the examined plants, growth of *E. crus-galli* root was the most inhibited, suggesting that constituents found in EO may potential be used for the control of the problematic paddy weed *E. crus-galli.* It was found that the EO of rice leaves contains rich phytochemicals, which are potent as antioxidants and in gout treatment, as well as weed management. The findings of this study highlight the potential value of rice leaves to provide extra benefits for rice farmers in developing countries.

The compounds secreted by plant roots have been documented to serve important roles as chemical attractants and repellants in the rhizosphere, the narrow zone of soil or wet soil immediately surrounding the root system. In this study, we investigated the allelochemical interaction of rice (*Oryza sativa*) and weed (*Monochoria vaginalis* and *Eleocharis*) plants in paddies and examined their growth inhibitory property from wet soil extracts. The results of field study showed that rice and weed exerted strong mutual inhibition, as their growth parameters were reduced by 2.47–42.22%. However, the slight inhibitory effects between tested weeds was observed to be 1.59–15.55%. Although the TPC and TFC of aqueous extracts from studied plants decreased (40% and 5%) significantly when they were interfered by their neighboring species, that of EtOAc extracts increased (43% and 9%). The exudation of two well-known phytoalexin compounds from rice momilactones A and B was measured to be 13% and 2% in field 1 (herbicide application), 15% and 5% in field 2 (non-herbicide application) using the chromatographic analyses HPLC and UPLC. Interestingly, the more abundant field contained a small amount of momilactones (75.6 ng/g fresh weigh (FW)). In the various wet soil extracts from studied fields, the growth of *L. sativa* was the most suppressed, whilst that of *Oryza sativa* was the

least influenced. The abandoned field showed slight inhibition on test plant species. Findings in this study suggest that the chemicals exudate from rice and paddy weeds-including phenolic acids, flavonoids, and momilactones-inhibited successive plants and can be stored in wet soil for a long time.

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CHAPTER I. GENERAL INTRODUCTION

1.1. Background

1.1.1. Rice Husk

Rice husk or rice hull is the thin coating on the seeds or grains of rice. The material components of rice husk are silica and lignin, which protect the grain during the development and majority stages of rice production. Each kilogram of milled rice produces about 0.28 kg of rice husk as a byproduct (Chandler *et al*., 1979; Gariboldi *et al*., 1984; Juliano *et al*., 1991). According to Natarajan *et al*. (1998), rice husk accounts for approximately 20% of paddy rice weight.

Globally, the total rice production in 2018 was 661 million tons, thus, rice milling generated 132 million tons of rice husk (Ryan *et al*., 2011). Rice husk is mostly considered as a waste product and therefore usually either burned in the open or dumped on wasteland. Recently, rice husk has found to have a high calorific value (3410 kcal/kg), which can be utilized as a renewable fuel (Demirbaş *et al*., 2001). However, rice husk is difficult to decompose and does not burn easily with open flame unless air is blown through it (Lim *et al*., 2012). Rice husk has high silica (SiO_2) content, which makes it slow to decompose when disposed. It is highly tolerant to moisture penetration and fungal decomposition (Gewona *et al*., 2013). The handling of rice husk is therefore difficult.

Rice husk is produced centrally at rice mills and has low moisture content, since the paddy is dried to 14% or less before milling (Ali *et al*., 1976, 1980a, b, 1982). The disadvantage is that rice husk has very low density and therefore transport over a long distance is expensive (Anthoni *et al*., 1982). The most widespread use of rice husk is, therefore, as a fuel. This is especially true where a need exist close to the rice mill. Depending on the energy conversion efficiency, the final product can be either white rice husk ash or black carbonized rice husk (Andreoli *et al*., 2000).

However, rice husk maybe also used for some non-energy purposes. Because it is easily accessible and inexpensive, small amounts of rice husk have always been used for

various purposes, including energy generation, bricks production, in steam engines and gasifiers to power rice mills, and to generate heat for rice dryers (Adam *et al*., 2010). Furthermore, its high silica content makes it an excellent material for the steel and concrete industries. Also, to a smaller extent, rice husk can be used as a soil conditioner for activated carbon and as an insulator (Bansal *et al*., 2009). More recently, the creation of electrical power on a small to medium scale, up to 5 MW has, been piloted throughout Asia with some promising approaches, but also some demonstrated limitations (Busca *et al*., 2008). Their failure was mostly due to feedstock supply problems once the formerly free waste rice husk becomes a tradeable commodity, and due to logistical problems and the high cost when transport distances become too long (Chang *et al*., 1997).

1.1.2. Plant Secondary Metabolites of Rice Husk

Over the last 50 years, thanks to the development of biochemical technology and the rise of molecular biology, scientists have clearly demonstrated that plant secondary metabolites play a major role in the adaptation of plants to their environment (Bourgaud *et al*., 2001). These molecules have been described as being antibiotic, antifungal, antiviral, and antibacterial to protect plants from pathogens (phytoalexins) and also antigerminative or toxic for other plants (allelopathy). Moreover, they constitute the necessary UV absorbing compounds for preventing severe leaf damage from sunlight. They also interact with animals, such as insects (antifeeding properties) or even cattle-avoiding being fed on during foraging by creating smelly compounds or toxic substances (Akula and Ravishankar, 2011).

Secondary metabolites are chemicals produced by plants for which their role does not contribute to the growth, photosynthesis, reproduction, or other “primary” functions. It is estimated that there are more than 200,000 known secondary metabolites in plants, representing a vast reservoir of diverse “secondary” functions (Fraire and Balderas, 2013). Due to their diverse potential biological activities, humans use many of these compounds to produce medicines, flavorings, recreational drugs, etc. Based on their structure and biosynthetic origin, plant secondary metabolites can be grouped into three main classes: (i)

flavonoids and allied phenolic and polyphenolic compounds, (ii) terpenoids (made from mevalonic acid, composed almost entirely of carbon and hydrogen), and (iii) nitrogen-containing alkaloids and sulphur-containing compounds.

Plants produce a large variety of secondary compounds containing a phenol group. These phenolic compounds are synthesized via two different routes: the shikimate pathway and the acetate-malonate pathway, and thus represent a heterogeneous group. The shikimate pathway participates in the synthesis of most plant phenolics, whereas the malonate pathway is of less significance in higher plants, although it is an important source of phenolic products in fungi and bacteria (Taiz and Zeiger, 2002). Phenolic compounds are classified into several groups, including anthocyanins, the pigment that attracts animals; flavonoids, compounds that serve as ultraviolet light protectants; isoflavonoids (phytoalexins), the compounds that act as antifungal and antibacterial defenses; lignin, the phenolic macromolecule involved in mechanical support and protection; and tannins, polymeric phenolic compounds that function as feeding deterrents to herbivores.

Phenolics are characterized by having at least one aromatic ring with one or more hydroxyl groups attached. More than 8,000 phenolic structures have been reported and they are widely dispersed throughout the plant kingdom. Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large, complex tannins and derived polyphenols. They can be classified based on the number and arrangement of their carbon atoms and are commonly found conjugated to sugars and organic acids (Crozier *et al*., 2008). Phenolics have been found containing various physiological functions, such as stress response, protective properties, and allelopathic interaction during the growth of plants. In addition, these compounds also possess many different biological activities, including antiartherogenic, anti-inflammatory, antimicobial, antiallergenic, antithrombotic, antioxidation, cardioprotective, and vasodilative effects (Balasundram *et al*., 2006).

1.1.3. Momilactones A and B

The name momilactone is a combination of words. In Japanese, *momi* mean rice husk and the chemical structures MA and MB come from the *lactone* group (Figure 1) (Fukuta *et*

al., 2007). Many plants are found to secrete a wide range of compounds into the rhizosphere, and they change the chemical and physical properties of the rhizosphere soil. This affects the associated community of microbes, fungi and plants (Anitha *et al*., 2010). The secretion of compounds such as allelochemicals from plants inhibits the germination and growth of neighboring plants, and their ability to compete effectively for resources (Ansaldi *et al*., 2002).

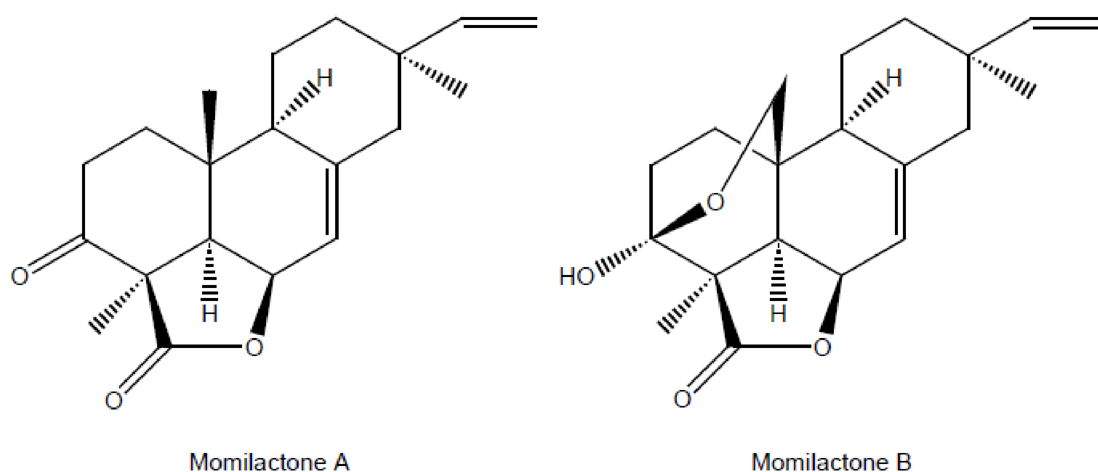


Figure 1. Chemical structures of momilactone A (MA) and momilactone B (MB).

The negative impacts of commercial herbicide use on the environment make it desirable to diversify weed management options (Appanna *et al*., 1995). Many investigations have attempted to exploit the allelopathy of plants for weed control purposes in a variety of agricultural settings (Arora *et al*., 2001). Rice has also been extensively studied for its allelopathy potential as part of a strategy for sustainable weed management; examples include breeding allelopathic rice strains (Feng *et al*., 2006).

Large field screening programs and laboratory experiments in many countries have proved that rice is allelopathic and releases allelochemicals into its rhizosphere (Chou *et al*., 1976; Chung *et al*., 2001; Bi *et al*., 2007). Some compounds, such as phenolic acids, fatty acids, phenylalkanoic acids, hydroxamic acids, terpenes, and indoles, have been identified as potential rice allelochemicals (Garrity *et al*., 1992; Huang *et al*., 1997; Ebana *et al*., 2001; He *et al*., 2004).

1.1.4. Momilactones Released into the Environment

Several putative allelochemicals, such as *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, and ferulic acid, have been found in extracts of rice leaf and straw, and in soil-decomposed straw (Kuwatsuka and Shindo, 1973; Chou and Lin, 1976; Chou and Chiou, 1979). These compounds are released from living rice plants and act as allelochemicals by inhibiting the growth of neighboring plants. Momilactone B is released into the neighboring environment of rice throughout its life cycle. The rate of MB release from rice increases until flowering initiation and then decreases (Hisashi Kato-Noguchi *et al*., 2003). The release level of MB may depend on the production level of MB in seedlings, which may affect the allelopathic potential of these rice cultivars as a growth inhibitor. Release levels of MB and its effectiveness as a growth inhibitor suggest that it may play an important role in rice allelopathy.

Studies regarding momilactone and rice husk are limited. No study has attempted to use temperature, extracting solvents, and pressure to optimize the yield of MA and MB. However, few studies have investigated different extracting solvents, temperatures, and pressures on the optimization of chemical components, and antioxidant and microbial activities of rice husk. There are very few studies about the enzyme activity of MA and MB, but no report about antihyperuricemia activity of MA and MB. Studies are yet to investigate the biological essential oil from rice leaf or the environmental interaction between the rice plant and weeds.

1.2. Research Objectives

- To isolate and identify bioactive compounds from rice husk.
- To explore the potential use of rice husk in different extracting solvents, temperatures, and pressures; optimization of chemical component extraction, antioxidant and antimicrobial activities.
- To evaluate antihyperuricemia activity of MA and MB isolated from rice husk.
- To investigate and biologically evaluate essential oil from rice leaf.
- To investigate environmental interaction between rice and weeds

CHAPTER II.
EFFICACY FROM DIFFERENT EXTRACTIONS FOR CHEMICAL PROFILE AND BIOLOGICAL ACTIVITIES OF RICE HUSK

2.1. Introduction

Rice husk is an agricultural waste that farmers have experienced difficulties in disposing of in large quantities. The annual output of rice husk worldwide is about 120 million tons (Abbas *et al.*, 2010). Rice husk is traditionally disposed of in landfills, and has recently been used for electricity generation, but simultaneously a large number of greenhouse gases are being produced (Li *et al.*, 2011). Rice husk contains a high percentage of silica (15-20%) (Lu *et al.*, 2008) and lignin (20%) (Lu *et al.*, 2008), which cause obstacles in processing and exploiting potential uses of rice husk. Others are cellulose (35%), hemicellulose (25%), crude protein (2%), and ash (17%) (Lu *et al.*, 2008), thus it is a challenge to develop rice husk to be a value-added byproduct, as it has hard surface, high silica content, small bulk density, and is not easily fermented by bacteria. In addition, the emission of rice husk ash has received social criticisms and complaints because of the associated carcinogenic and bio-accumulative effects (Li *et al.*, 2011). Rice husk does not show a remarkable commercial interest, and its price is very low (30-40 euro/ton in Europe) (Salanti *et al.*, 2010), and 25 USD/ton in India (Bhattacharrya *et al.*, 2014). Rice husk has elevated ash and lignin contents; thus, it is not appropriate to use as animal feed raw material. Rice husk has been reported to be a potential source for bioethanol production (Abbas *et al.*, 2010); furfural, one of the top value bio-based chemicals, has been recently synthesized from rice husk (Li *et al.*, 2011; Delbecq *et al.*, 2016). However, the manufactured price appears to be difficult for consumption in markets because of its non-competitive price, as compared to another renewable source.

Rice husk can be burned under controlled conditions to achieve a large amount of silica (approximately 95% of the total ash content), and is applicable in building materials, adsorbent phase for the treatment of waste water, solid phase for supported enzymes, and filters (D'Souza *et al.*, 2002; Krishnani *et al.*, 2008). Recent efforts have used H_2SO_4 (Lu *et al.*, 2008; Zhang *et al.*, 2010), $ZnCl_2$ and H_3PO_4 (Kennedy *et al.*, 2004; Kalderis *et al.*, 2008), and KOH (Jain *et al.*, 1995) and NaOH (Guo *et al.*, 2003) as the pre-treatment

chemicals to prepare D-xylose and activated carbon in rice husk. However, in view of the environmental perspective, the use of these chemicals is not preferred. The establishment of a safe, convenient, and effective extraction to utilize the potential of rice husk is required.

There are several well-known biochemicals in rice husk such as momilactones which play a role as plant growth inhibitors (Kato *et al.*, 1973, 1977, 2008; Fukuta *et al.*, 2007) and are involved in drought and salinity tolerance in rice (Xuan *et al.*, 2016). 4-Hydroxybenzoic acid and *trans*-4-hydroxycinnamic acid were identified from rice husk and showed antimicrobial potential (Cho *et al.*, 1998). Glycosyl flavonoid (Ramarathnam *et al.*, 1988, 1989) and phytic acid (Wu *et al.*, 1999) in Katakuhara cutivar, and 2,3,6-trimethylanisole (anisole); *m*-hydroxybenzaldehyde; 4-hydroxy-3-methoxybenzaldehyde (vanillin); and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) in wild rice have been reported as antioxidants in rice husk. The polysaccharide fraction was useful for various applications, such as adhesives, films, and biofuel production (Saha *et al.*, 2005). Rice husk has been described as exhibiting weed-suppressing abilities (Xuan *et al.*, 2003, 2005; Khanh *et al.*, 2005).

In order to explore further potential use of rice husk and provide a positive measure to reduce the environmental problems caused by this rice by-product, this study was conducted to examine total phenolic content (TPC), total flavonoid content (TFC), antioxidant (DPPH radical scavenging activity, β-carotene bleaching method, and reducing power), and antimicrobial (*Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Bacillus subtilis,* and *Proteus mirabilis*) activities of rice husk. In order to enhance the commercial value of rice husk, different extracting solvents, temperatures, and pressures on optimization of chemical components, and antioxidant and microbial activities were investigated.

2.2. Materials and Methods

2.2.1. Preparation of Rice Husk and Extracts

Rice husk (subtype: Japonica; var. Koshihikari) were collected from rice mills in Saijo area, close to Hiroshima University, Higashi Hiroshima, Japan, in August 2017. An amount of 200 g dried rice husk was used in each of the extraction methods as described in Table 1. Briefly, experiments were designed by immersing samples in a volume of 1 L of different solvents (MeOH 10, 30, 50, 70, 100% and distilled water, respectively) under various temperature conditions (room temperature, 100 °C, boiled or dried, 1-4 h) and pressure (120 kPa). Each extract was concentrated under reduced pressure at 30 °C. The obtained crude extracts were dissolved in distilled water and successively partitioned with hexane and

ethyl acetate. The hexane supernatants were removed, and all the ethyl acetate extracts (M1-M26) were kept in the dark at 5 °C for further analysis.

Table 1. Yields of the ethyl acetate (EtOAc) extracts

Methods	Codes	EtOAc Crude Extract (g)
MeOH 100%	M1	0.500 ± 0.004
MeOH 70%	M2	0.460 ± 0.040
MeOH 50%	M3	0.120 ± 0.010
MeOH 30%	M4	0.097 ± 0.007
MeOH 10%	M5	0.099 ± 0.001
Distilled water (room temperature)	M6	0.040 ± 0.001
Distilled water (100 °C)	M7	0.012 ± 0.001
Distilled water (100 °C, 30 min) + MeOH 100%	M8	0.067 ± 0.004
Distilled water (100 °C, 30 min) + EtOAc 100%	M9	0.500 ± 0.030
Distilled water (100 °C, 1 h) + MeOH 100%	M10	0.446 ± 0.020
Distilled water (100 °C, 2 h) + MeOH 100%	M11	0.500 ± 0.009
Distilled water (100 °C, 3 h) + MeOH 100%	M12	0.500 ± 0.020
Distilled water (100 °C, 4 h) + MeOH 100%	M13	0.500 ± 0.010
Dried (100 °C, 1 h) + MeOH 100%	M14	0.450 ± 0.030
Dried (100 °C, 1 h) + MeOH 70%	M15	0.400 ± 0.020
Dried (100 °C, 1 h) + MeOH 50%	M16	0.168 ± 0.020
Dried (100 °C, 1 h) + MeOH 30%	M17	0.058 ± 0.001
Dried (100 °C, 1 h) + MeOH 10%	M18	0.193 ± 0.030
Dried (100 °C, 1 h) + distilled water (room temperature)	M19	0.162 ± 0.040
Dried (100 °C, 1 h) + distilled water (100 °C)	M20	0.620 ± 0.040
Dried (100 °C, 1h), distilled water (100 °C, 1 h) + MeOH 100%	M21	0.314 ± 0.010
Dried (100 °C, 2h), distilled water (100 °C, 2 h) + MeOH 100%	M22	0.500 ± 0.030
Dried (100 °C, 3h), distilled water (100 °C, 3 h) + MeOH 100%	M23	0.600 ± 0.020
Dried (100 °C, 4h), distilled water (100 °C, 4 h) + MeOH 100%	M24	0.400 ± 0.020
Distilled water (100 °C, 120 kPa) + MeOH 100%	M25	0.400 ± 0.020
Dried (100 °C, 120 kPa) + MeOH 100%	M26	0.500 ± 0.050

Values are means ± SD (standard deviation) (n = 3).

2.2.2. Determination of Total Phenolic Content (TPC)

The total phenolic content assay was conducted using the Folin-Ciocalteu method as described in previous studies (Minh *et al.*, 2016, 2017) with minor modifications. An aliquot of 200 µL of each sample (500 µg/mL) was mixed 1.0 mL of a 10-fold diluted Folin-Ciocalteu's reagent in distilled water and 0.8 mL of 7.5 wt% aqueous sodium carbonate solution, respectively. The obtained solutions were incubated in the dark for 30 min at room temperature. Blanks were prepared by adding 99.8% MeOH in the same manner with samples. The absorbance was measured at 765 nm using a HACH DR/4000U spectrophotometer (HACH Company, Loveland, CO, USA). The TPC evaluation was then assessed on the basis of a standard calibration curve (r^2 = 0.9933) using gallic acid as a standard (10, 25, 50, and 100 µg/mL). The TPC value was expressed as mg of gallic acid equivalent per gram dry weight (mg GAE/g dry weight (DW)).

2.2.3. Determination of Total Flavonoid Content (TFC)

The total flavonoid content in EtOAc extracts was determined spectrophotometrically using the method described by Quettier-Deleu *et al*. (2000). A volume of 0.5 mL of sample extract was mixed with 0.5 mL of 2% aluminum chloride-methanol solution. MeOH (99.8%) was used as the blank in this assay. After 15 min at room temperature, the mixtures were measured at the absorbance of 430 nm using a spectrophotometer (HACH Company, Loveland, CO, USA). The TFC was expressed as mg of rutin equivalent per gram dry weight (mg RE/g DW).

2.2.4. Antioxidant Properties

2.2.4.1. DPPH Radical Scavenging Activity

The antioxidant activity of the EtOAc extracts was determined according to the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method described by Elzaawely and Tawata (2012). Briefly, an aliquot of 0.5 mL sample extracts was mixed with 0.25 mL of 0.5 mM DPPH and 0.5 mL of 0.1 M acetate buffer (pH 5.5). The mixtures were incubated for 30 min in the dark, at room temperature. The absorbance was read at 517 nm using a spectrophotometer (HACH DR/4000U-Hach company, Loveland city, Colorado, USA). The control used in this assay was DPPH (1000 µg/mL) in a test tube and the blank was 99.8% MeOH (1 mL). The percentage of the DPPH radical scavenging activity was calculated using the following formula:

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

Where A_0 = absorbance of control, A_1 = absorbance of sample. The results were interpreted as 50% inhibition concentration (IC_{50}) values expressed in µg/mL (concentration in parts per million). The lower IC_{50} value indicates higher DPPH radical scavenging activity. All measurements were performed in triplicate.

2.2.4.2. Determination of Antioxidant Activity with the β-Carotene Bleaching Test

The β-carotene bleaching assay was performed as described by Xuan *et al.* (2018) with some modifications. An amount of 2 mg of β-carotene/linoleic acid was initially prepared by dissolving in 10 mL of chloroform. An aliquot of the β-carotene solution (1.5 mL) was added to 20 mg of linoleic acid and 200 mg of Tween-40. After chloroform was evaporated under vacuum at 45 °C, an amount of 50 mL pure oxygenated water was added and shaken vigorously to form an emulsion. A methanolic solution of 0.12 mL of sample extracts was mixed with 1 mL of the emulsion. Methanol was used as a control. The solutions were incubated at 50 °C and recorded at 492 nm using a spectrophotometer (HACH DR/4000U-Hach company, Loveland city, Colorado, USA). All extracts were measured at zero time and every 30 min up to 180 min. The assay was carried out in triplicate for each extract. Lipid peroxidation inhibition (LPI) was calculated using the following formula:

$$\text{LPI} (\%) = A_1/A_0 \times 100$$

Where A_0 = absorbance value measured at zero time for the test sample, A_1 = corresponding absorbance value measured after incubation for 180 min. Higher LPI value shows the higher antioxidant activity.

2.2.4.3. Reducing Power

The reducing power was determined following the method described in a previous study (2017). Various concentrations of each extract (1 mL) or BHT (with concentrations 25, 50, 100 and 250 µg/mL in MeOH) were mixed with 2.5 mL phosphate buffer (0.2M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (10 g/L). The obtained mixture was incubated at 50 °C for 30 min, followed by adding 2.5 mL trichloroacetic acid (100 g/L). The obtained mixture as centrifuged at 4,000 rpm for 10 min. Finally, the upper layer (0.5 mL) was mixed with 0.5 ml deionized water and 0.5 mL $FeCl_3$ (1 g/L). The absorbance was measured at 700 nm; higher absorbance indicates higher reducing power. The absorbance ability of the reaction mixture is directly proportional to the reducing power.

2.2.5. Antimicrobial Activity Test

2.2.5.1. Nutrient Agar Powder Preparation

An amount of 4.5 g of agar powder and 6 g of LB broth (Lennox) were dissolved in 300 mL of distilled water and sterilized by autoclaving for 20 min in 121 °C. After that, about 20 mL LB agar was poured into each sterilized petri plate (86.5 mm diameter × 14.5 mm height). After cooling down to 55 °C, all agar petri dishes were stored at 4 °C in the dark for further experiments.

2.2.5.2. LB Medium Preparation

Two grams of LB broth were dissolved in 200 mL distilled water. The obtained mixture was sterilized for 20 min by autoclave at temperature 121 °C. The solution was cooled to 55 °C and placed at temperature 4 °C in the dark for further experiments.

2.2.5.3. Antimicrobial Test

Antimicrobial activity of the sample extracts was evaluated using the disk diffusion agar method described by Fukuta *et al*. (2007). In this experiment, the LB broth medium was used to grow the bacteria for 24 h at 37 °C. The final population was standardized to be 1.26×10^8 CFU/mL (*E. coli*), 5.2×10^6 CFU/mL (*K. pneumoniae*), 1.8×10^6 CFU/mL (*L. monocytogenes*), 6.0×10^6 CFU/mL (*B. subtilis*), and 2.2×10^6 CFU/mL (*P. mirabilis*). An amount of 0.1 mL of the bacteria suspension was spreader evenly on each plate filled with the LB agar. After that, filter paper dishes (6 mm diameter) impregnated by 20 µL of each sample extract (with a concentration 100 mg/mL in MeOH) were laid on the surface of LB agar plates. After 24 h of incubation at 37 °C, the inhibition zone was measured. Ampicillin and streptomycin (0.03 mg/disc) were used as standards in this assay.

2.2.6. Chemical Constituents Identification by Gas Chromatography-Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC), and Electrospray Ionisation Mass Spectromety (EI-MS)

The chemical components of M1 to M26 were determined by using a GC-MS system. An aliquot of 1 µL sample dissolved in methanol was injected into the GC-MS system (JMS-T100 GCV, JEOL Ltd., Tokyo, Japan). The column was DB-5MS and was 30 m in length, 0.25 mm internal diameter, and 0.25 µm in thickness (Agilent Technologies, J & W Scientific Products, Folsom, CA, USA.). Helium was chosen as the carrier gas, and the split ratio was 5:1. The method to operate GC oven temperatures was maintained, as the initial

temperature was 50 °C without hold time, the programmed rate was 10 °C min^{-1} up to a final temperature of 300 °C with 20 min of hold time. The injector and detector temperature were set at 300 °C and 320 °C, respectively. The mass range scanned from 29-800 amu. The control of the GC-MS system and the data peak processing were carried out using JEOL's GC-MS Mass Center System Version 2.65a. Compounds with peak areas > 0.3% in the GC-MS analysis was determined as principal substances.

The gradient liquid chromatographic system (model LC-10A series; Shimadzu, Tokyo, Japan) included two LC-10AD pumps controlled by a CMB-10A interface module, a model 7725i manual injector valve (Rheodyne) equipped with a 20 mL sample loop, and a multi-dimensional UV-VIS detector (model SPD-10A). Data were collected and analyzed using a class LC-10. The work station was equipped with an HP-DeskJet printer. The method involved the use of a Waters Spherisorb S10 ODS2 column (250 × 4.6 mm, I.D., 10μ) and binary gradient mobile phase profile. The extraction efficiency, peak purity, and similarity were validated using a photo diode array detector, and a mobile phase consisting of 0.1% TFA in acetonitrile: water (70:30, *v*/*v*). The mobile phase was filtered through a 0.45 μm Millipore filter and degassed by sonication for 30 min. The flow rate was adjusted to 4 mL min^{-1} with a run time of 50 min. Injection volume was adjusted to 10 μL and detection was made at 210 nm**.** MA and MB were identified by HPLC and their peak areas were recorded. The presence of MA and MB were confirmed by an EI-MS system. MA: ESI^{+}: 315 $[M+H]^{+}$ ($C_{20}H_{27}O_3$); ESI^{-}: 313 $[M-H]^{-}$($C_{20}H_{25}O_3$); HRMS 315.1959 $[M+H]^{+}$ (calc for $C_{20}H_{27}O_3$, 315.1960). MB: ESI^{+}: 331 $[M+H]^{+}$ ($C_{20}H_{27}O_4$); ESI^{-}: 329 $[M-H]^{-}$ ($C_{20}H_{25}O_4$); HRMS 330.1905 $[M+H]^{+}$ (calc for $C_{20}H_{27}O_4$, 315.1909).

2.2.7. Statistical Analysis

The data were analyzed by one-way ANOVA using the Minitab 16.0 software for Window. Upon significant differences, means were separated using Tukey's test at $p < 0.05$ with three replications and expressed as means ± standard deviation (SD) ($n = 3$).

2.3. Results

2.3.1. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The contents of total phenolics and flavonoids in EtOAc extracts prepared from rice husk varied from 63.8 to 5962.3 mg GAE/g DW, and from 21.8 to 571.6 mg RE/g DW, respectively (Table 2). The extraction of M7 (distilled water only, at 100 °C) yielded the greatest TPC, followed by M6 (distilled water only at room temperature), whereas M7 was

the most excellent in providing the maximum amount of TFC, followed by M17 (dried at 100 °C, 1h, combined with MeOH 30%), and M6. The M17 method was potent in both TPC and TFC, and DPPH scavenging activity. The results suggest that the highest amount of phenolic and flavonoid was detected in M7.

2.3.2. Antioxidant Activity by the DPPH Radical Scavenging Assay

The DPPH radical scavenging activity of rice husk extracts are shown in Table 2 and is exhibited in IC_{50} value, of which, the smaller values indicate greater activity. Table 4 showed that the DPPH radical scavenging activity in terms of IC_{50} values were varied among extractions. The extraction of M7 (distilled water only, at 100 °C) was found to yield the greatest TPC, followed by M6 (distilled water only at room temperature), whereas M7 was the most excellent in providing the maximum amount of TFC, followed by M17 (dried at 100 °C, 1h, combined with MeOH 30%), and M6. The M17 method was potent in both TPC and TFC, and DPPH scavenging activity. The M20 (dried and then boiled at 100 °C for each 1 h) resulted in the highest DPPH scavenging activity, followed by M3, M4, M6, and M17 (Table 2). However, the IC_{50} values of these extractions, including M20, were not statistically different.

2.3.3. Antioxidant Activity by β-Carotene Bleaching Method

The antioxidant activity and the percentage lipid peroxidation inhibition (% LPI) results of the different methods are presented in Figure 2a, b. It was observed that the presence of antioxidants in different extractions prepared from rice husk reduced the oxidation of β-carotene (Figure 2a; Table S1). The LPI values varied from 67% to 86% (Figure 2b). Consequently, M10 (distilled water (100 °C, 1 h) + MeOH 100%) shown the highest percent of LPI (86%) meaning that it had the strongest antioxidant activity, following by M22 (dried (100 °C, 2h) and distilled water (100 °C, 2 h) + MeOH 100%) (85%) (Figure 2b). Most of EtOAc extract inhibits β-carotene ranging from 76% to 82%, contrarily, M7 (distilled water (100 °C) (67%) was lower in antioxidant activity (Figure 2b; Table S1). However, there was not much difference among extracting solvents in the β-carotene bleaching method and lipid peroxidation inhibition.

2.3.4. Reducing Power

In the assay of the reducing power (Figure 3; Table S2), the yellow color of the test changes to green depending on the reducing power of the test specimen or capacity of each compound. The presence of substances (i.e., antioxidants) reduces Fe^{3+}/ferricyanide iron

complex form. In this study, the reducing powers of rice husk extracts and BHT increased with the concentration of tested samples, of which M6 and M20 were the most potent, whereas no significant difference among other extractions was found.

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity of rice husk in different extraction

Methods	Codes	TPC (µg GAE/g)	TFC (µg RE/g)	DPPH IC_{50} (µg/mL)
MeOH 100%	M1	63.8 ± 7.2 n	25.8 ± 2.0 jk	437.3 ± 18.8 b
MeOH 70%	M2	131.4 ± 19.4 mn	34.7 ± 3.9 jk	240.3 ± 11.0 d
MeOH 50%	M3	1128.0 ± 80.2 e	177.9 ± 8.5 d	79.1 ± 1.5 h
MeOH 30%	M4	1894.9 ± 129.1 d	123.6 ± 2.3 e	63.9 ± 2.6 h
MeOH 10%	M5	764.8 ± 3.6 f	62.8 ± 8.1 gh	310.7 ± 22.6 c
Distilled water (room temperature)	M6	5029.8 ± 116.2 b	229.2 ± 11.1 c	56.7 ± 1.1 h
Distilled water (100 °C)	M7	5962.3 ± 123.9 a	571.6 ± 18.5 a	98.0 ± 2.8 gh
Distilled water (100 °C, 30 min) + MeOH 100%	M8	225.5 ± 9.4 ijklmn	60.4 ± 5.5 gh	200.3 ± 7.4 de
Distilled water (100 °C, 30 min) + EtOAc 100%	M9	180.2 ± 21.0 klmn	38.3 ± 5.8 hijk	247.7 ± 22.6 cd
Distilled water (100 °C, 1 h) + MeOH 100%	M10	211.7 ± 10.0 jklmn	47.9 ± 2.6 ghij	216.2 ± 1.6 de
Distilled water (100 °C, 2 h) + MeOH 100%	M11	316.9 ± 17.0 hijkl	96.8 ± 16.8 f	154.6 ± 10.5 efg
Distilled water (100 °C, 3 h) + MeOH 100%	M12	254.3 ± 25.8 ijklm	68.3 ± 12.5 g	220.9 ± 41.4 de
Distilled water (100 °C, 4 h) + MeOH 100%	M13	158.3 ± 19.4 lmn	28.1 ± 3.1 jk	246.3 ± 23.0 cd
Dried (100 °C, 1 h) + MeOH 100%	M14	179.0 ± 19.1 klmn	57.1 ± 4.5 ghi	238.2 ± 7.2 d
Dried (100 °C, 1 h) + MeOH 70%	M15	185.7 ± 10.1 jklmn	35.3 ± 1.6 jk	163.7 ± 9.5 efg
Dried (100 °C, 1 h) + MeOH 50%	M16	391.9 ± 46.8 hi	43.2 ± 1.8 hijk	422.7 ± 15.4 b
Dried (100 °C, 1 h) + MeOH 30%	M17	3879.3 ± 16.3 c	308.4 ± 17.5 b	83.3 ± 10.3 h
Dried (100 °C, 1 h) + MeOH 10%	M18	584.1 ± 78.6 g	109.9 ± 4.6 ef	684.0 ± 43.2 a
Dried (100 °C, 1 h) + distilled water (room temperature)	M19	392.4 ± 88.8 hi	38.4 ± 1.4 hijk	214.5 ± 2.7 de
Dried (100 °C, 1 h) + distilled water (100 °C)	M20	353.9 ± 23.0 hij	21.8 ± 0.9 k	54.7 ± 2.5 h

Dried (100 °C, 1 h), distilled water (100 °C, 1 h) + MeOH 100%	M21	266.2 ± 9.3 hijklm	46.7 ± 3.7 ghijk	203.6 ± 8.7 de
Dried (100 °C, 2 h), distilled water (100 °C, 2 h) + MeOH 100%	M22	341.7 ± 13.5 hijk	101.7 ± 6.2 ef	183.5 ± 73.5 def
Dried (100 °C, 3 h), distilled water (100 °C, 3 h) + MeOH 100%	M23	239.3 ± 22.8 ijklm	56.3 ± 3.8 ghi	189.9 ± 22.6 de
Dried (100 °C, 4 h), distilled water (100 °C, 4 h) + MeOH 100%	M24	175.7 ± 18.5 klmn	29.6 ± 1.5 jk	251.0 ± 6.7 cd
Distilled water (100 °C, 120 kPa) + MeOH 100%	M25	333.3 ± 20.4 hijk	62.4 ± 3.9 gh	167.1 ± 4.9 efg
Dried (100 °C, 120 kPa) + MeOH 100%	M26	430.0 ± 27.0 gh	93.7 ± 5.7 f	113.7 ± 20.8 fgh
	BHT	-	-	9.3 ± 1.1 i

Values represent means ± SD (standard deviation). Values with similar letters in each column are not significantly different ($p < 0.05$) ($n = 3$). -: measurement was not conducted; BHT: butylated hydroxytoluene; TPC: total phenolic content; TFC: total flavonoid content

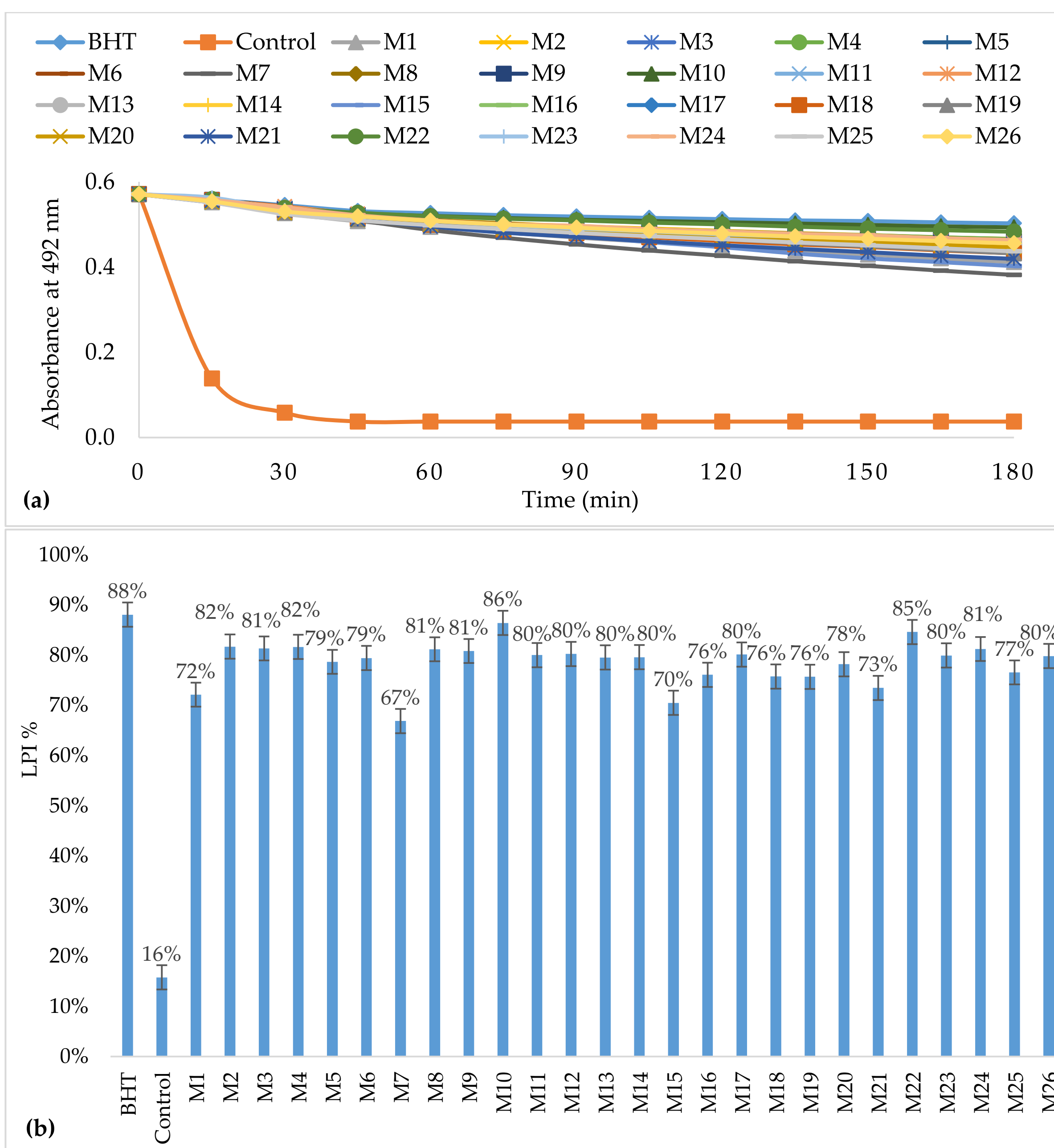


Figure 2. Antioxidant activities measured by β-carotene bleaching method (**a**) and their lipid peroxidation inhibition (%LPI) (**b**); Control (MeOH); BHT: butylated hydroxytoluene.

2.3.5. Antimicrobial Activity

The antimicrobial activities of different extractions on growth of five bacteria including *E. coli*, *K. pneumoiae*, *L. monocytogenes*, *B. subtilis*, and *P. mirabilis* are shown in Table 3. It was found that the antimicrobial activity varied among bacteria and extractions. As compared to the standards including ampicillin and streptomycin, the inhibitory level can be ranked as follows: *E. coli* > *B. subtilis* > *K. pneumoniae* and *L. monocytogenes* > *P.*

mirabilis. Among the individual bacteria, the strongest antimicrobial activity on *B. subtilis* was observed in M3-M5, M9, M13, M15, M17-18, and M24 which showed the suppressive level was similar to that of streptomycin. In the case of *L. monocytogenes*, M19-M20, M5, and M18 were the extractions with the strongest inhibition. In *K. pneunomiae*, M4 and M18 were the best candidates to inhibit growth of the bacteria, but they were statistically similar to M2-M3, M10, M12-M13, M17, M19, M23-M24, and M26 (Table 3). In *E. coli,* while the M21 was shown to be the least effective, the other extractions showed similar antimicrobial activity. In *P. mirabilis*, extractions M9, M11-13, M18, M20, and M22 exerted maximum inhibition.

Compared among extractions, M18 was the most potent and showed the strongest inhibition against emergence of all studied bacteria, followed by M3, M4, M21, and M13 (Table 3). M1, M22, M23, and M25 showed the least effectiveness as they showed strong antimicrobial activity with only one bacteria species, whereas the activity of the M14 remained unknown, as it did not show strong inhibition on *L. monocytogenes*, *B. subtilis*, and *P. mirabilis*, whilst the examination on *E. coli* and *K. pneumoniae* was not conducted (Table 3).

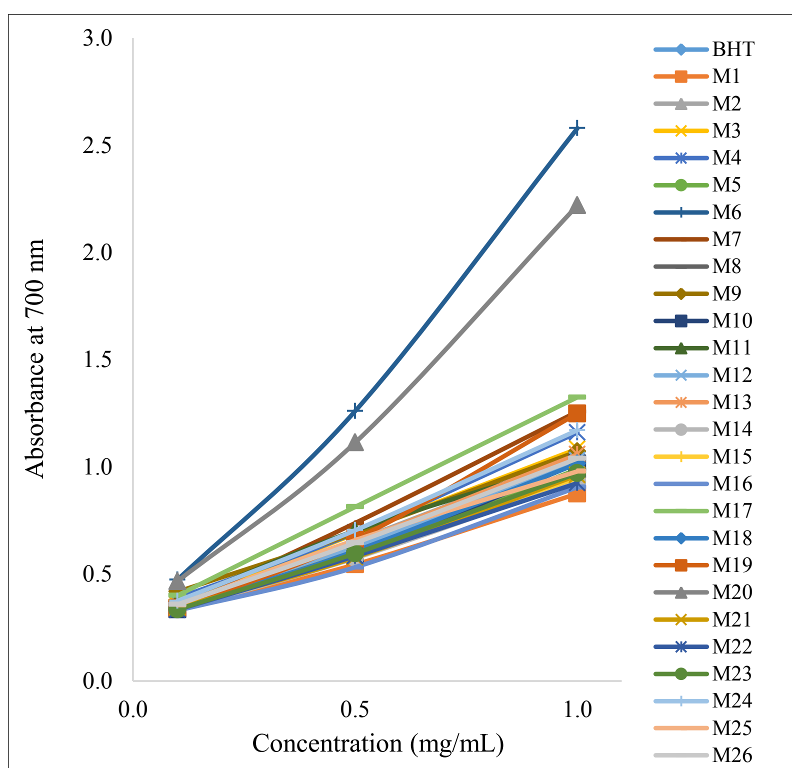


Figure 3. Reducing power activity of rice husk in different extraction and standard antioxidant. BHT: butylated hydroxytoluene.

2.3.6. Identification Compound from each Ethyl Acetate Extractions

By GC-MS, major compounds in rice husk relevant to different extracts were identified and showed in Table 4. They included cis-11-octadecenoic acid methyl ester, momilactone A, momilactone B, n-hexadecanoic acid methyl ester 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol, methyl (2E)-3-(4-hydroxyphenyl)-2-propenoate, *trans-p*-coumaric acid, 3-hydroxy-4-methoxybenzoic acid, L-lactic acid, butanoic acid, catechol, hexanoic acid, 4-hydroxybenzenepropanoic acid, phenol, and dihydrobenzofuran. These chemicals belonged to momilactones, phenolic acids, phenols, and long-chain fatty acids. It was found that the presence of chemicals varied among extracting protocols, of which cis-11-octadecenoic acid methyl ester, 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol, and *trans-p*-coumaric acid accounted for greater peak areas as compared to other putative constituents. The peak areas of momilactones A and B (MA and MB, respectively) were lesser than these compounds. However, MA and MB were not detected in the extracts with only distilled water or MeOH $\leq$ 50% at any temperature (M3-5; M6-7; M16-20). Cis-11-octadecenoic acid methyl ester was found in M1, 2, 11, 12, 15, 18, 22, 23, whilst 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol and *trans-p*-coumaric acid were detected in M3,4, M8-14, M16,17, M21, and M24-26 (Table 4).

Table 3. Antimicrobial activity of different extraction on five bacteria

Methods	Codes	Zone of inhibition (mm)				
		E. coli	*K. pneumoniae*	*L. monocytogenes*	*B. subtilis*	*P. mirabilis*
MeOH 100%	M1	9.7 ± 1.5 cdef	7.7 ± 1.2 cd	7.3 ± 0.6 h	6.3 ± 0.6 h	-
MeOH 70%	M2	10.3 ± 0.6 cdef	8.3 ± 1.5 cd	9.3 ± 0.6 fgh	8.3 ± 0.6 defgh	-
MeOH 50%	M3	9.0 ± 1.0 cdef	9.3 ± 1.2 cd	12.3 ± 7.6 cde	10.7 ± 0.6 bcde	7.3 ± 1.6 fg
MeOH 30%	M4	11.7 ± 0.6 cd	10.3 ± 0.6 c	12.3 ± 7.6 cde	9.7 ± 0.6 bcdef	7.3 ± 0.6 fg
MeOH 10%	M5	11.0 ± 1.7 cd	6.3 ± 0.6 d	13.7 ± 7.6 c	10.0 ± 1.0 bcde	7.3 ± 1.6 fg
Distilled water (room temperature)	M6	10.0 ± 1.7 cdef	6.3 ± 0.6 d	9.7 ± 7.6 efgh	9.0 ± 1.0 cdefg	7.3 ± 1.6 fg
Distilled water (100 °C)	M7	-	-	-	-	7.3 ± 0.6 fg
Distilled water (100 °C, 30 min) + MeOH 100%	M8	8.3 ± 1.5 cdef	6.3 ± 0.6 d	9.3 ± 0.6 fgh	8.3 ± 0.6 defgh	-
Distilled water (100 °C, 30 min) + EtOAc 100%	M9	12.0 ± 1.7 c	-	8.3 ± 9.6 gh	9.7 ± 0.6 bcdef	10.0 ± 1.0 cdef
Distilled water (100 °C, 1 h) + MeOH 100%	M10	9.3 ± 1.5 cdef	6.7 ± 1.2 cd	8.7 ± 7.5 fgh	8.3 ± 0.6 defgh	7.3 ± 1.6 fg
Distilled water (100 °C, 2 h) + MeOH 100%	M11	12.3 ± 2.1 c	-	7.3 ± 0.6 h	6.7 ± 1.2 gh	9.7 ± 2.6 cdef
Distilled water (100 °C, 3 h) + MeOH 100%	M12	7.7 ± 2.1 def	7.3 ± 0.6 cd	10.0 ± 1.0 defgh	9.3 ± 0.6 cdef	10.7 ± 2.6 cde
Distilled water (100 °C, 4 h) + MeOH 100%	M13	10.3 ± 0.6 cdef	8.3 ± 1.2 cd	10.3 ± 9.6 defg	10.3 ± 0.6 bcde	9.7 ± 0.2 cdef
Dried (100 °C, 1 h) + MeOH 100%	M14	-	-	7.3 ± 0.6 h	6.3 ± 0.6 h	8.7 ± 0.5 efg
Dried (100 °C, 1 h) + MeOH 70%	M15	9.0 ± 0.0 cdef	-	8.3 ± 0.6 gh	10.7 ± 0.6 bcde	-
Dried (100 °C, 1 h) + MeOH 50%	M16	10.0 ± 1.7 cdef	-	9.7 ± 6.6 efgh	7.3 ± 0.6 fhg	6.3 ± 1.6 g
Dried (100 °C, 1 h) + MeOH 30%	M17	10.7 ± 1.5 cde	7.0 ± 1.0 cd	11.3 ± 6.6 cdef	10.3 ± 0.6 bcde	6.3 ± 1.6 g
Dried (100 °C, 1 h) + MeOH 10%	M18	9.3 ± 1.5 cdef	10.3 ± 1.5 c	13.7 ± 1.6 c	11.3 ± 0.6 bc	11.7 ± 1.6 cde
Dried (100 °C, 1 h) + distilled water (room temperature)	M19	10.0 ± 1.0 cdef	7.3 ± 1.5 cd	16.7 ± 8.6 b	-	8.3 ± 1.6 efg

Dried (100 °C, 1 h) + distilled water (100 °C)	M20	9.3 ± 1.2 cdef	6.3 ± 0.6 d	17.7 ± 0.6 b	8.7 ± 1.2 defgh	10.7 ± 1.6 cde
Dried (100 °C, 1 h), distilled water (100 °C, 1 h) + MeOH 100%	M21	6.3 ± 0.6 f	6.3 ± 0.6 d	12.7 ± 8.6 cd	7.3 ± 0.6 fhg	8.3 ± 0.6 efg
Dried (100 °C, 2 h), distilled water (100 °C, 2 h) + MeOH 100%	M22	9.7 ± 0.6 cdef	-	8.7 ± 1.6 fgh	-	12.3 ± 0.5 c
Dried (100 °C, 3 h), distilled water (100 °C, 3 h) + MeOH 100%	M23	6.7 ± 1.2 ef	8.3 ± 0.6 cd	8.3 ± 8.6 gh	7.7 ± 0.6 efgh	9.0 ± 1.0 defg
Dried (100 °C, 4 h), distilled water (100 °C, 4 h) + MeOH 100%	M24	8.3 ± 0.6 cdef	10.0 ± 1.0 cd	10.3 ± 9.6 defg	9.7 ± 0.6 bcdef	8.7 ± 0.6 efg
Distilled water (100 °C, 120 kPa) + MeOH 100%	M25	9.0 ± 1.7 cdef	-	7.3 ± 9.6 h	6.3 ± 0.6 h	9.0 ± 1.0 defg
Dried (100 °C, 120 kPa) + MeOH 100%	M26	7.7 ± 0.6 def	9.3 ± 1.2 cd	8.3 ± 0.6 gh	6.3 ± 0.6 h	-
Methanol	MeOH	-	-	-	-	-
Ampicillin	Amp	34.0 ± 1.0 a	44.3 ± 3.2 a	24.7 ± 3.1 a	17.3 ± 1.5 a	43.3 ± 1.5 a
Streptomycin	Str	19.0 ± 1.0 b	15.3 ± 0.6 b	18.0 ± 1.7 b	12.0 ± 1.0 b	29.3 ± 1.5 b

Values represent means ± SD (standard deviation). Values with similar letters in a column are not significantly different ($p < 0.05$); -: not measured

Table 4. Principal compounds identified in rice husk

Methods	Codes	Major Constituents	Retention Times (min)	Peak Area (%)
MeOH 100%	M1	OA, MA, MB, HaM	18.42, 18.70, 15.04, 16.73	19.26, 0.33, 0.98, 1.97
MeOH 70%	M2	OA, MA, MB, HaM	18.42, 18.70, 15.04, 16.73	22.04, 0.53, 1.05, 1.85
MeOH 50%	M3	HM, PM, *p*C	14.78, 14.90, 15.25	27.76, 18.41, 9.45
MeOH 30%	M4	HM, HmA, *p*C	14.78, 12.81, 15.25	28.22, 9.95, 31.24
MeOH 10%	M5	LA, HmA, BA	3.63, 12.81, 3.00	45.68, 12.82, 7.28
Distilled water (room temperature)	M6	BA, Ca, HA	3.00, 7.85, 5.01	19.54, 9.53, 15.08
Distilled water (100 °C)	M7	BeA, P, Ca	13.56, 4.74, 7.85	21.22, 10.32, 19.04
Distilled water (100 °C, 30 min) + MeOH 100%	M8	HM, *p*C, MA, MB	14.78, 15.25,18.70, 15.04	21.49, 12.09, 1.21, 3.03
Distilled water (100 °C, 30 min) + EtOAc 100%	M9	HM, *p*C, MA, MB	14.78, 15.25,18.70, 15.04	24.71, 19.01, 1.12, 2.18
Distilled water (100 °C, 1 h) + MeOH 100%	M10	HM, *p*C, MA, MB	14.78, 15.25,18.70, 15.04	28.28, 8.43, 2.36, 0.71
Distilled water (100 °C, 2 h) + MeOH 100%	M11	HM, OA, MA, MB	14.78, 18.42, 18.70, 15.04	20.26, 18.78, 3.71, 7.33
Distilled water (100 °C, 3 h) + MeOH 100%	M12	HM, OA, MA, MB	14.78, 18.42, 18.70, 15.04	14.06, 14.07, 0.55, 1.53
Distilled water (100 °C, 4 h) + MeOH 100%	M13	HM, *p*C, MA, MB	14.78, 15.25, 18.70, 15.04	33.78, 31.49, 1.55, 3.12
Dried (100 °C, 1 h) + MeOH 100%	M14	HM, *p*C, MA, MB	14.78, 15.25, 18.70, 15.04	26.88, 8.26, 4.11, 8.09
Dried (100 °C, 1 h) + MeOH 70%	M15	HaM, OA, MA, MB	16.73, 18.42, 18.70, 15.04	19.54, 42.11, 1.82, 3.16
Dried (100 °C, 1 h) + MeOH 50%	M16	HM, *p*C	14.78, 15.25	37.53, 7.71
Dried (100 °C, 1 h) + MeOH 30%	M17	Be, HM, *p*C	8.21, 14.78, 15.25	15.17, 20.49, 17.55
Dried (100 °C, 1 h) + MeOH 10%	M18	OA, HaM	18.42, 16.73	16.93, 8.99
Dried (100 °C, 1 h) + distilled water (room temperature)	M19	BA, P	3.00, 4.74	54.35, 16.22
Dried (100 °C, 1 h) + distilled water (100 °C)	M20	BA, BeA	3.00, 13.56	54.8, 8.01

Dried (100 °C, 1 h), distilled water (100 °C, 1 h) + MeOH 100%	M21	HM, *p*C, MA, MB	14.78, 15.25, 18.70, 15.04	50.85, 24.01, 1.12, 1.07
Dried (100 °C, 2 h), distilled water (100 °C, 2 h) + MeOH 100%	M22	HM, OA, MA, MB	14.78, 18.42, 18.70, 15.04	21.82, 21.96, 1.26, 2.88
Dried (100 °C, 3 h), distilled water (100 °C, 3 h) + MeOH 100%	M23	HaM, OA, MA, MB	16.73, 18.42, 18.70, 15.04	12.13, 17.72, 2.21, 4.48
Dried (100 °C, 4 h), distilled water (100 °C, 4 h) + MeOH 100%	M24	HM, *p*C, MA, MB	14.78, 15.25, 18.70, 15.04	23.05, 20.13, 0.98, 3.01
Distilled water (100 °C, 120 kPa) + MeOH 100%	M25	HM, *p*C, MA, MB	14.78, 15.25, 18.70, 15.04	43.85, 29.00, 2.02, 3.05
Dried (100 °C, 120 kPa) + MeOH 100%	M26	HM, *p*C, MA, MB	14.78, 15.25, 18.70, 15.04	52.46, 15.75, 0.22, 3.08

MA and MB were identified by HPLC and confirmed by EI-MS; Other compounds were identified by GC-MS.

Abbreviations: OA: cis-11-octadecenoic acid methyl ester, MA: momilactone A, MB: momilactone B, HaM: n-hexadecanoic acid methyl ester, HM: 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol, PM: methyl (2E)-3-(4-hydroxyphenyl)-2-propenoate, *p*C: *trans*-*p*-coumaric acid, HmA: 3-hydroxy-4-methoxybenzoic acid, LA: L-lactic acid, BA: butanoic acid, Ca: catechol, HA: hexanoic acid, BeA: 4-hydroxybenzenepropanoic acid, P: phenol; Be: dihydrobenzofuran.

2.4. Discussion

Rice husk, an agricultural waste, is abundantly available in rice producing countries. It is also of high calorific value, making it a good source of renewable energy (Lim *et al.*, 2012). Except for important sustainable energy value, the search for phytochemicals which may be exploited for herbal drug preparations on rice husk has been conducted sporadically (Rajanna *et al.*, 2015; Herna'ndez *et al.*, 2014). Momilactones A (MA) and B (MB) were isolated from rice husk and found to be correlated to the drought and salinity tolerance capacities (Xuan *et al.*, 2016); these compounds concentrated on plan growth, pathogen, and antioxidant capacity (Fukuta *et al.*, 2007), and cytotoxic and antitumor activity (Kim *et al.*, 2007). Both MA and MB further were found from many other rice cultivars (Kim *et al.*, 2007; Mennan *et al.*, 2012). However, this study is the initial step to observe that rice husk, especially the EtOAc extract, contains rich antioxidants that should be exploited. M7 (distilled water only, at 100 °C) was found to yield the greatest TPC and TFC. M6 (distilled water only at room temperature) and M20 (dried and then boiled at 100 °C for each 1 h) were shown to be strongest in antioxidant capacity. M18, which was the most potent, showed a strong inhibition against emergence of all studied bacteria (temperature 100 °C + 10% MeOH).

Annual worldwide production of rice husk is about 120 million tons, of which the amounts from China, India, Indonesia, Bangladesh, Vietnam, Thailand, Philippines, and Japan were 35.3, 25.5, 10.5, 7.6, 6.9, 5.2, 4.6, 2.7, and 2.2 million tons, respectively (Abbas *et al.*, 2010). Remarkable production of rice husk outside of Asian countries took place in Brazil (2.3 million tons) (Abbas *et al.*, 2010). This causes serious problems for disposal and results in environmental problem (Anda *et al.*, 2015). Because rice husk has 15-20% silica, cellulose (35%), hemicellulose (25%), ash (17%), and only 2% protein, it is difficult to exploit it as a valuable product (Lu *et al.*, 2008; Salanti *et al.*, 2010). The use of chemicals in pre-treatment H_2SO_4 (Li *et al.*, 2011; D'Souza *et al.*, 2002), $ZnCl_2$ and H_3PO_4 (Zhang *et al.*, 2010; Kalderis *et al.*, 2008), and KOH (Kennedy *et al.*, 2010) and NaOH (Jain *et al.*, 2010), for decomposing rice husk is convenient, but these solvents are toxic for humans and the environment. Thus, the use of temperature, pressure, EtOAc, MeOH, and water in this study are more ecofriendly. At an industrial scale, MeOH could be replaced by C_2H_5OH which is much safer for humans. The optimization of antioxidant and antimicrobial activities needs only a minimum of 10% MeOH, therefore it turns to be safe as this amount can be easily evaporated during processing.

This study revealed that major constituents in rice husk included momilactones A and B, phenols, phenolic acids, and long-chain fatty acids, although their presences varied among extracting protocols (Table 4). There were 3 constituents, including cis-11-octadecenoic acid methyl ester, 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol, and *trans*-*p*-coumaric acid, which obtained the maximum peak areas as compared to other compounds. MA and MB were also the major chemicals but appeared with lesser peak areas and they were not detected in the extracts with either solely distilled water or MeOH ≤ 50% at any temperature. Among them, MA and MB and *trans*-*p*-coumaric acid have been known as bioactive compounds, thus their existence may help to increase the value of rice husk. In this study, because many identified can neither successfully be purified nor purchased, the peak areas were used to compare the efficacy of different extracting protocols. The use of standards of these identified constituents for quantification will help determine with more accuracy the quantities of chemicals obtained in rice husk, that need further elaboration.

Findings of this study suggest that the quantity of paddy rice by-product's waste can be reduced when rice husk can be exploited for medicinal and pharmaceutical purposes, as it is revealed to possess rich and safe antioxidants, and antimicrobials. The use of temperature, extracting solvents, and pressure, as shown in this study, to optimize the antioxidant and antimicrobial capacities is useful to apply at an industrial scale. The search for novel bioactive compounds in rice husk will also help to foster the valuable use of the rice by-product.

2.5. Conclusions

This study indicated that the extracts prepared from rice husk contained potent amounts of phenolic and flavonoid contents. Rice husk also showed promising antioxidant and antimicrobial activity. The use of temperature at 100 °C for > 2 h, combined with either EtOAc or 10% MeOH can optimize the chemical components and antioxidant and antimicrobial capacities of rice husk.

2.6. Supplementary Data

Table S1. β-carotene bleaching inhibiting activity

Time	Absorbance at 492 nm												
	0 (min)	15 (min)	30 (min)	45 (min)	60 (min)	75 (min)	90 (min)	105 (min)	120 (min)	135 (min)	150 (min)	165 (min)	180 (min)
BHT	0.571	0.559	0.545	0.532	0.527	0.522	0.519	0.516	0.513	0.510	0.508	0.505	0.503
Control	0.571	0.138	0.058	0.037	0.037	0.037	0.037	0.037	0.037	0.037	0.037	0.037	0.037
M1	0.571	0.552	0.526	0.508	0.494	0.482	0.469	0.459	0.449	0.439	0.429	0.420	0.412
M2	0.571	0.556	0.534	0.521	0.511	0.503	0.496	0.491	0.485	0.480	0.475	0.470	0.466
M3	0.571	0.558	0.534	0.519	0.508	0.500	0.494	0.488	0.483	0.478	0.473	0.468	0.464
M4	0.571	0.558	0.534	0.518	0.508	0.500	0.495	0.489	0.484	0.479	0.475	0.470	0.466
M5	0.571	0.558	0.540	0.517	0.506	0.495	0.487	0.480	0.473	0.467	0.461	0.455	0.449
M6	0.571	0.558	0.543	0.525	0.511	0.501	0.492	0.485	0.479	0.470	0.465	0.459	0.453
M7	0.571	0.556	0.534	0.510	0.487	0.469	0.453	0.439	0.427	0.414	0.403	0.392	0.382
M8	0.571	0.557	0.538	0.522	0.510	0.503	0.495	0.489	0.484	0.479	0.474	0.468	0.463
M9	0.571	0.557	0.537	0.522	0.510	0.499	0.493	0.487	0.482	0.477	0.471	0.467	0.461
M10	0.571	0.561	0.534	0.526	0.520	0.515	0.511	0.508	0.505	0.502	0.499	0.496	0.493
M11	0.571	0.558	0.540	0.520	0.505	0.494	0.489	0.483	0.477	0.471	0.468	0.463	0.457
M12	0.571	0.557	0.542	0.525	0.509	0.499	0.496	0.487	0.481	0.474	0.468	0.462	0.458
M13	0.571	0.557	0.539	0.520	0.507	0.498	0.491	0.484	0.478	0.473	0.467	0.460	0.454
M14	0.571	0.558	0.535	0.518	0.508	0.498	0.491	0.483	0.478	0.471	0.466	0.459	0.454
M15	0.571	0.552	0.532	0.511	0.498	0.481	0.472	0.459	0.447	0.432	0.420	0.412	0.402
M16	0.571	0.557	0.536	0.515	0.501	0.489	0.480	0.471	0.463	0.455	0.448	0.441	0.434
M17	0.571	0.558	0.538	0.519	0.506	0.497	0.490	0.484	0.478	0.473	0.468	0.463	0.457
M18	0.571	0.557	0.532	0.513	0.500	0.488	0.479	0.470	0.461	0.453	0.447	0.439	0.432
M19	0.571	0.554	0.528	0.512	0.502	0.492	0.483	0.474	0.466	0.456	0.449	0.440	0.432
M20	0.571	0.552	0.527	0.511	0.499	0.490	0.484	0.477	0.470	0.463	0.458	0.452	0.446
M21	0.571	0.555	0.534	0.512	0.495	0.481	0.471	0.461	0.451	0.443	0.435	0.427	0.419
M22	0.571	0.560	0.539	0.528	0.520	0.514	0.510	0.504	0.500	0.495	0.490	0.487	0.483
M23	0.571	0.563	0.534	0.519	0.509	0.499	0.491	0.484	0.477	0.472	0.467	0.462	0.456
M24	0.571	0.557	0.540	0.522	0.510	0.501	0.497	0.489	0.484	0.478	0.474	0.468	0.464
M25	0.571	0.551	0.525	0.510	0.498	0.489	0.480	0.473	0.466	0.458	0.451	0.444	0.437
M26	0.571	0.554	0.530	0.519	0.509	0.500	0.493	0.485	0.479	0.472	0.467	0.461	0.456

Table S2. EtOAc extracts, and BHT measured by reducing power method

Extraction	Absorbance at 700 nm		
	0.1 (mg/mL)	0.5 (mg/mL)	1.0 (mg/mL)
MeOH		0.373±0.003	
BHT	0.373±0.008	0.617±0.003	0.924±0.004
M1	0.335±0.005	0.543±0.003	0.860±0.004
M2	0.373±0.008	0.666±0.003	1.048±0.004
M3	0.356±0.013	0.607±0.003	0.962±0.004
M4	0.416±0.012	0.702±0.003	1.085±0.004
M5	0.369±0.011	0.703±0.003	1.022±0.004
M6	0.336±0.002	0.642±0.003	1.023±0.004
M7	0.344±0.011	0.649±0.003	1.063±0.004
M8	0.333±0.004	0.584±0.003	0.939±0.004
M9	0.334±0.004	0.593±0.003	0.958±0.004
M10	0.374±0.003	0.705±0.003	1.145±0.004
M11	0.335±0.005	0.571±0.003	0.928±0.004
M12	0.333±0.003	0.613±0.003	0.956±0.004
M13	0.354±0.004	0.684±0.003	0.977±0.004
M14	0.357±0.009	0.635±0.003	1.033±0.004
M15	0.356±0.015	0.690±0.003	1.090±0.004
M16	0.386±0.006	0.770±0.003	1.198±0.004
M17	0.335±0.010	0.578±0.003	1.001±0.004
M18	0.472±0.009	1.228±0.003	2.478±0.004
M19	0.352±0.007	0.720±0.003	1.268±0.004
M20	0.334±0.003	0.582±0.003	1.020±0.004
M21	0.328±0.002	0.529±0.003	0.911±0.004
M22	0.399±0.009	0.811±0.003	1.358±0.004
M23	0.341±0.006	0.614±0.003	1.021±0.004
M24	0.341±0.001	0.640±0.003	1.249±0.004
M25	0.465±0.003	1.192±0.003	2.174±0.004
M26	0.342±0.004	0.588±0.003	0.964±0.004

CHAPTER III.
MOMILACTONES A AND B: OPTIMIZATION OF YIELDS FROM ISOLATION AND PURIFICATION

3.1. Introduction

Rice husk are an agricultural waste that farmers have experienced difficulties disposing of in large quantities. However, rice husk is reported to be an effective source for weed management in crops (Khanh *et al.*, 2015; Xuan *et al.*, 2003, 2015). Among plant growth inhibitors that have been detected in rice husk, such as phenolic acids, fatty acids, phenylalkanoic acids, hydroxyamic acids, terpenes, and indoles, momilactones A (MA) and B (MB) have showed a particular potency for controlling weed emergence (Lee *et al.*, 2002; Kato-Noguchi *et al.*, 2010). Trials carried out in fields, greenhouses, and laboratories showed that MA and MB were promising for weed management (Kato-Noguchi *et al.*, 2008a, b; Chung *et al.*, 2006; Toyomatsu *et al.*, 2008). Chemical structures of MA and MB are shown in Figure 1.

MA and MB were first identified and isolated from rice husk and it was reported that they caused inhibition on germination and root elongation of rice seedlings (Kato *et al.*, 1973, 1977; Takahashi *et al.*, 1976). Momilactone is the combination of two words: momi means rice husk in Japanese, and the chemical structures of MA and MB are lactone (Figure 1). The two compounds were also found in rice leaves and straws and documented as phytoalexins (Cartwright *et al.*, 1981; Kodama *et al.*, 1988; Fukuta *et al.*, 2007). Several analytical methods to identify and quantify MA and MB by reverse phase-high performance liquid chromatography (RP-HPLC) (Kim *et al.*, 2007), thin layer chromatography and flame ionization detection(TLC/FID) (Saha *et al.*, 1981), HPLC-MS-MS (Lee *et al.*, 1999), thin layer chromatography (TLC) (Nozaki *et al.*, 2007), chromatography mass spectrometry in the selected ion monitoring mode (GC-MS-SIM) (Kodama *et al.*, 1988), and gas chromatography - mass spectrometry (GC-MS) (Chung *et al.*, 2006; Xuan *et al.*, 2016); The uses of cold percolation and extracting solvents of either methanol (MeOH) or MeOH-H2O (8:2 w/w) to isolate MA and MB have been reported (Kato *et al.*, 1977; Takahashi *et al.*, 1976; Chung *et al.*, 2015). However, the actual yields of MA and MB purified by column chromatography were low (MA: 0.8-15 μg/g; MB: 0.5-10 μg/g husks) (Kato *et al.*, 1973;

Takahashi *et al.*, 1976; Chung *et al.*, 2015). Therefore, methods to enrich and optimize quantities of MA and MB in rice husk are needed.

Although phenolics play an important role in rice allelopathy (Li *et al.*, 2010), MA and MB showed a particular mode of action on plant inhibition (Kato-Noguchi *et al.*, 2013). MA and MB have been found only in rice plants and rice exudates, but they were also observed in the moss Hypnum plumaeforme (Nozaki *et al.*, 2007; Kato-Noguchi *et al.*, 2009), although the two plants are taxonomically quite distinct (Kato-Noguchi *et al.*, 2009). Besides allelopathy, MA and MB were recently reported to correlate to drought and salinity tolerance in rice (Xuan *et al.*, 2016). MA and MB have been found in rice leaves, husks, straws, and root exudates (Kodama *et al.*, 1988; Lee *et al.*, 1999; Kato-Noguchi *et al.*, 2013; Grosso *et al.*, 2015; Cartwright *et al.*, 1977). The gene involved in the biosynthesis of MA has been identified in *Echinochloa crus-galli* (Guo *et al.*, 2017). Contents of MA and MB depended on rice varieties, growing stage, and extraction methods (Kato-Noguchi *et al.*, 2003, 2009, 2010; Xuan *et al.*, 2016). The use of high temperature in extraction, combined with relevant extracting solvents may effectively enrich yields of natural products (Kato-Noguchi *et al.*, 2009).

In this study, the effectiveness of different extractions by using combination of distilled water, MeOH, and ethyl acetate (EtOAc) combined with boiling and heating conditions, and pressure to optimize yields of MA and MB was examined.

3.2. Materials and Methods

3.2.1. Preaparation of Rice Husk and Extracts

An amount of 30 kg dried rice husk (subtype: Japonica; var. Koshihikari) were collected from rice mills in Saijo area, close to Hiroshima University, Higashi Hiroshima, Japan, in August 2017. The rice husk was ground to a fine powder and immersed in MeOH 100% for 2 weeks at room temperature. Then, this was concentrated under vacuum to produce an extract for isolation and purification of MA and MB.

3.2.2. Extraction and Isolation of MA and MB

The MeOH extract was diluted with MeOH again and mixed with activated charcoal (2 h) to remove chlorophylls and odors and filtered through a celite bed. By this step, no absorption of MA and MB was detected in the activated charcoal (data not shown). It was kept in a refrigerator for 4 h (5 °C) to crystallize fat and filtered through filter papers. The supernatant was concentrated by a rotary evaporator. The obtained solid matrix was

dissolved in 300 mL distilled water and sequentially partitioned with hexane, EtOAc. The yields of hexane (40 g), EtOAc (350 g), and water (80 g). The EtOAc extract was subjected to a normal-phase column chromatography [silica gel (70-230 mesh ASTM); LiChroprep RP-18 column (40-63 μm)] to yield 60 fractions, of which fractions 1-10 in hexane, fractions 11-20 in hexane:EtOAc (9.5:0.5), fractions 21-30 in hexane:EtOAc (9:1), fractions 31-50 in hexane:EtOAc (8:2), fractions 51-60 in hexane:EtOAc (7:3). After examination by TLC, fractions 21-30, were further subjected to a column chromatography over silica gel with chloroform: methanol, (99:1) to yield a mixture of MA and MB. These mixtures were further purified by column chromatography over silica gel with chloroform: methanol (99.8:0.2; 99.6:0.4; 99.4:0.6; 99.2:0.2; 99.0:1.0) to successfully purify MA (350 mg) and MB (200 mg).

3.2.3. Reagents and Analytical Instruments

The solvents used for extraction of the rice husk were of analytical grade and purchased from Junsei Chemical Co., Ltd., Tokyo, Japan. The solvents used for HPLC analysis of the extracts as well as the standard chemicals were of HPLC grade and procured from Merck, Tokyo, Japan. The standard MA and MB were isolated in our laboratory as mentioned above at purity of 98% and 97%, respectively. The solvents were filtered through a 0.45 μm Millipore membrane (Millipore, Billerica, MA, USA) before injection into the HPLC. Digital melting point apparatus from Sonar India was used to determine melting points, whereas a Rudolf autopol model polarimeter was used for measurement of the optical rotations. Pre-coated TLC plates of thickness 0.25 mm and column chromatography filled with silica gel of 70-230 mesh ASTM and LiChroprep RP-18 (40-63 mm) were procured from Merck (Darmstadt, Germany). Visualization of the TLC spots was displayed by 5% H_2SO_4 in ethanol spray reagent. Both ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Brucker DRX-500 model spectrometer (Bruker India Scientific Pvt. Ltd., New Delhi, India) operating at 500 and 125 MHz, respectively. The NMR spectra were obtained in deuterated chloroform using tetramethylsilane (TMS) as an internal standard. The Fast Atom Bombardment Mass Spectroscopy (FABMS) data were recorded on a JEOL SX- 102 spectrometer (JEOL USA Inc., Peabody, MA, USA) and electrospray ionization mass (ESI) in direct mass analysis of high performance liquid chromatography - photodiode array - mass spectrometry detectors (HPLC-PDA-MS) spectrometer (Shimadzu Corporation, Kyoto, Japan) and high resolution mass spectroscopy (HRMS) was measured on Agilent technology 6545Q-TOF LC/MS (5301 Stevens Dreek Blvd. Santa Clara, CA, USA). Infrared

spectroscopy was recorded on a Fourier Transform Infrared (FT-IR) spectrophotometer Shimadzu 8201 PC (4,000-400 cm^{-1}) (Shimadzu Cooporation, Kyoto, Japan).

3.2.4. NMR Data of MA and MB

MA*:* Colourless crystalline compound; R_f 0.48 ($CHCl_3$:MeOH; 9.5:0.5); m.p. 234-236 °C; IR ν_{max}: 2936, 1766, 1698, 1637, 1390, 1188, 990, 908; ^{1}H NMR ($CDCl_3$; 500 MHz): δ 1.90 (m, H_2-1α), 2.59-2.63 (m, H-2), 2.31 (d, *J* = 5.0, H-5), 4.84 (t, *J* = 5.0, H-6), 5.70 (d, *J* = 5.0, H-7), 1.74-1.80 (m, H-9, H-11α), 1.32 (m, H_2-11β), 1.56-1.62 (m, complex, H_2-1β, H_2-12), 2.20, 2.19 (d, *J* = 12.5, H_2-14), 5.84 (d d, *J* = 17.0, 11.0, H-15), 4.97, 4.93 (d d, *J* = 17.0 & 1; 10.0 & 1, H-16), 0.88 (s, H-17), 1.52 (s, H-18), 0.98 (s, H-20). ^{13}C NMR ($CDCl_3$; 125 MHz): δ 34.89 (C-1), 31.21 (C-2), 205.20 (C-3), 53.57 (C-4),46.46 (C-5), 73.17 (C-6), 114.03 (C-7), 148.96 (C-8), 50.18 (C-9), 32.46 (C-10), 23.99 (C-11), 37.24 (C-12), 40.13 (C-13), 47.53 (C-14), 148.03 (C-15), 110.17 (C-16), 21.80 (C-17), 21.47(C-18), 174.32 (C-19), 21.96 (C-20); HPLC-PDA-MS ESI^+: 315 $[M + H]^+$ ($C_{20}H_{27}O_3$); ESI^-: 313 $[M - H]^-$ ($C_{20}H_{25}O_3$); HRMS 315.1959 $[M + H]^+$ (calc for $C_{20}H_{27}O_3$, 315.1960). (Compare NMR data with previous literature (Kato *et al.*, 1973, 1977; Cartwright *et al.*, 1981; Kim *et al.*, 2007)).

MB: Colourless crystalline compound; R_f 0.42 ($CHCl_3$:MeOH; 9.5:0.5); m.p. 240 °C; IR ν_{max}: 2920, 1737, 1662, 1637, 1461, 1296, 992, 916; ^{1}H NMR ($CDCl_3$; 500 MHz): δ 1.99 (m, H-1α), 2.13-2.06 (m, complex H-2, H-14), 2.20 (dd, *J* = 6.5, 2.0, H-5), 4.97 (t, *J* = 4.5, H-6), 5.68 (d, *J* = 5.0, H-7), 1.72-1.64 (m, H-9, H-11α), 1.30 (m, H-11β), 1.56-1.51 (m, complex, H-1β, H-12), 5.82 (dd, *J* = 17.0, 11.0, H-15), 4.93 (d d, *J* = 10.0 & 1, H-16), 0.87 (s, H-17), 1.43 (s, H-18), 3.58, 4.07 (dd, 9.0, 3.1 7 9.0, 3.5). ^{13}C NMR ($CDCl_3$; 125 MHz): δ 28.81 (C-1), 26.44 (C-2), 96.60 (C-3), 50.35 (C-4), 42.97 (C-5), 73.76 (C-6), 114.00 (C-7), 146.70 (C-8), 44.68 (C-9), 30.74 (C-10), 24.79 (C-11), 37.22 (C-12), 39.99 (C-13), 47.42 (C-14), 148.83 (C-15), 110.23 (C-16), 21.86 (C-17), 18.99 (C-18), 180.48 (C-19), 72.72 (C-20); HPLC-PDA-MS ESI^+: 331 $[M + H]^+$ ($C_{20}H_{27}O_4$); ESI^-: 329 $[M - H]^-$ ($C_{20}H_{25}O_4$); HRMS 330.1905 $[M + H]^+$ (calc for $C_{20}H_{27}O_4$, 315.1909). (Compare NMR data with previous literature (Kato *et al.*, 1973, 1977; Cartwright *et al.*, 1981; Kim *et al.*, 2007)).

3.2.5. RP-HPLC and Separation Conditions

The gradient liquid chromatographic system (model LC-10A series; Shimadzu, Tokyo, Japan) included two LC-10AD pumps controlled by a CMB-10A interface module, a model 7725i manual injector valve (Rheodyne) equipped with a 20 mL sample loop, and a multi-dimensional UV-Vis detector (model SPD-10A) (Shimadzu Europe, Duisburg, Germany). Data were collected and analyzed using a class LC-10. The work station was equipped with an HP-DeskJet printer (Shimadzu Europe, Duisburg, Germany). The method involved the use of a Waters Spherisorb S10 ODS2 column (250 × 4.6 mm, I.D., 10μ) and binary gradient mobile phase profile. The extraction efficiency, peak purity and similarity were validated using a photo diode array detector, and a mobile phase consisting of 0.1% TFA in acetonitrile: water (70:30, *v*/*v*). The mobile phase was filtered through a 0.45 μm Millipore filter and degassed by sonication for 30 min. The flow rate was adjusted to 4 mL min^{-1} with run time of 50 min. Injection volume was adjusted to 10 μL and detection was made at 210 nm**.** Linearity was observed in the range of 10 to 250 μg/mL, with correlation coefficients of MA and MB were 0.9936 and 0.9928, respectively**.** Detection limit of MA was 0.9552 ng/mL and quantitation limit were 3.1840 ng/mL. Detection limit of MB was 1.008 ng/mL and quantitation limit were 3.3617 ng/mL.

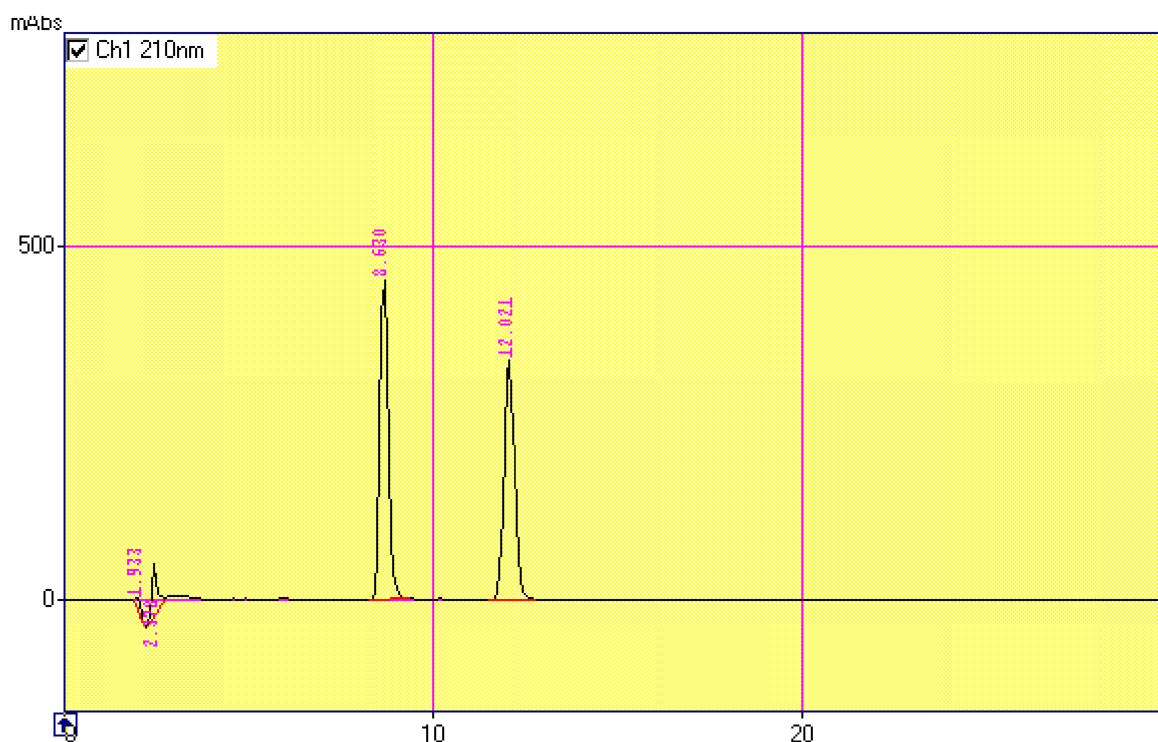


Figure 4. HPLC chromatogram for momilactone A (12.021 min)
and momilactone B (8.630 min) measured at 210 nm.

3.2.6. MA and MB Enrichment

An amount of 200 g of the dried rice husk were immersed in 1 L solvent and treated by different extractions as shown in Table 5, of which a different dilution of MeOH (10%, 30%, 50%, 70% and 100%) was conducted for 1 week (M1, M2, M3, M4 and M5, respectively). In similar manner, methods M6 and M7 included distilled water at room temperature and 100 °C, respectively (Table 5). In the methods M8-M13, distilled water (100 °C) was used in different durations (30 min, 1 h, 2 h, 3 h and 4 h, respectively), and then immersed in either MeOH 100% (M8, M10, M11, M12 and M13) or EtOAc (M9) for 1 week (Table 5). Various dilutions of MeOH (100%, 70%, 50%, 30% and 10%) conducted in room temperature and boiled distill water were tested (M14-M20). Then the samples were dried by an oven at 100 °C (SANYO Laboratory Convection Oven MOV-212F D, Sanyo Electric, Osaka, Japan) for 1 h (Table 5). The methods M21-M24 included the samples dried at 100 °C and boiled distilled water in many durations (1-4 h), then placed in MeOH 100% (Table 5). The pressure (120 kPa) was applied, combined with either distilled water or dried (M25 and M26, respectively), followed by using MeOH 100% (Table 5). The obtained crude extracts were concentrated under reduced pressure at 30 °C and dissolved in 100 mL distilled water and successively partition in hexane and ethyl acetate. The hexane supernatant was removed, and the ethyl acetate extract was kept in the dark at 5 °C for further analysis.

Table 5. Yields of ethyl acetate (EtOAc) extract, MA and MB in different extractions

Methods	Codes	EtOAc Crude Extract (g)	MA (µg/g DW)	MB (µg/g DW)
Controls (standards MA and MB by CC)	M0	0.17	1.20 ± 0.05 j	0.70 ± 0.03 h
MeOH 100%	M1	0.50	22.78 ± 0.19 ef	42.80 ± 8.76 cdef
MeOH 70%	M2	0.46	38.35 ± 3.08 c	67.81 ± 4.76 bc
MeOH 50%	M3	0.12	nd	nd
MeOH 30%	M4	0.10	nd	nd
MeOH 10%	M5	0.10	nd	nd
Distilled water (room temperature)	M6	0.04	nd	nd
Distilled water (100 °C)	M7	0.01	nd	nd
Distilled water (100 °C, 30 min) + MeOH 100%	M8	0.67	18.54 ± 1.77 fg	63.80 ± 5.33 cd
Distilled water (100 °C, 30 min) + EtOAc 100%	M9	0.50	21.27 ± 1.38 f	49.63 ± 2.49 cde
Distilled water (100 °C, 1 h) + MeOH 100%	M10	0.45	28.83 ± 0.03 d	3.02 ± 0.02 g
Distilled water (100 °C, 2 h) + MeOH 100%	M11	0.50	51.54 ± 0.95 b	102.23 ± 5.32 ab
Distilled water (100 °C, 3 h) + MeOH 100%	M12	0.50	21.78 ± 0.79 ef	45.65 ± 2.62 cdef
Distilled water (100 °C, 4 h) + MeOH 100%	M13	0.50	14.72 ± 0.19 gh	30.65 ± 1.38 ef
Dried (100 °C, 1 h) + MeOH 100%	M14	0.45	58.76 ± 3.75 a	104.43 ± 6.44 a
Dried (100 °C, 1 h) + MeOH 70%	M15	0.40	13.89 ± 0.62 gh	29.68 ± 1.89 ef
Dried (100 °C, 1 h) + MeOH 50%	M16	0.17	nd	nd
Dried (100 °C, 1 h) + MeOH 30%	M17	0.06	nd	nd
Dried (100 °C, 1 h) + MeOH 10%	M18	0.20	nd	nd

Dried (100 °C, 1 h) + distilled water (room temperature)	M19	0.16	nd	nd
Dried (100 °C, 1 h) + distilled water (100 °C)	M20	0.62	nd	nd
Dried (100 °C, 1 h), distilled water (100 °C, 1 h) + MeOH 100%	M21	0.31	15.03 ± 0.51 g	14.04 ± 0.33 f
Dried (100 °C, 2 h), distilled water (100 °C, 2 h) + MeOH 100%	M22	0.50	11.76 ± 0.64 hi	35.19 ± 2.43 def
Dried (100 °C, 3 h), distilled water (100 °C, 3 h) + MeOH 100%	M23	0.60	28.56 ± 0.09 d	53.03 ± 2.45 cde
Dried (100 °C, 4 h), distilled water (100 °C, 4 h) + MeOH 100%	M24	0.40	6.68 ± 0.34 i	20.26 ± 1.51 f
Distilled water (100 °C, 120 kPa) + MeOH 100%	M25	0.40	26.26 ± 1.44 de	40.78 ± 2.82 cdef
Dried (100 °C, 120 kPa) + MeOH 100%	M26	0.50	17.90 ± 0.18 fg	71.00 ± 6.14 bc

EtOAc: ethyl acetate; MA and MB: momilactones A and B, respectively; nd: not detected; Values with similar letters in each column are not significantly different ($p < 0.05$); Values are means ± SD (standard deviation)

3.2.7. Preparation of MA, MB, and Extracts for Quantification by HPLC

The stock solution of 1000 µg/mL ethyl acetate extract was prepared by dissolving the purified MA and MB in methanol. The prepared solution was sonicated for 5 min. Sample solutions of the extracts as showed in Table 5 were also prepared at 1000 µg/mL in methanol and analyzed. All the samples were filtered through 0.45 µm Millipore membranes before injected to HPLC for identification and quantification of MA and MB.

3.2.8. Statistical Analysis

The data were analyzed by one-way ANOVA using the Minitab 16.0 software for Window. Upon significant differences, means were separated using Tukey's test at $p < 0.05$ with three replications and expressed as the mean ± standard deviation (SD).

3.3. Results

Results in Table 5 show quantities of MA and MB in different extractions, and the relevant amount of the EtOAc extracts, of which the controls were the actual amounts of MA and MB purified by column chromatography in this study (1.20 and 0.70 µg/g DW, respectively).

It was showed that the use of dried (100 °C, 1 h) and distilled water (100 °C, 2 h) combined with MeOH 100% provided maximum amounts of MA and MB (58.76 and 104.43, 51.54 and 102.23 µg/g DW, respectively), as compared to other extracting methods. The use of pressure set at 120 kPa, 100 °C, and distilled water (100 °C, 30 min) combined with MeOH 100% produced the yields of MB from 63.80-71.00 µg/g DW. Other extracting methods showed 20.26-53.03 µg/g DW, while the combination of distilled water (100 °C, 1 h) with MeOH 100% caused the lowest quantity of MB (3.02 µg/g DW) (Table 5). For MA, when the sample was dried at 100 °C for 4 h then put in the distilled water (100 °C, 4 h), this resulted in the minimum amount of MA (6.68 µg/g DW), whilst other extracting methods provided 11.76-38.35 µg/g DW. The use of pressure was effectively enhanced yields of MA (17.90-26.26 µg/g DW) and MB (40.78-71.0 µg/g DW). In general, MB was enriched in greater quantities than MA. It was observed that the use of distilled water only and MeOH $\leq$ 50% at any temperature could not successfully purify either MA nor MB (Table 5, methods M3-M7, M16-M20).

3.4. Discussion

MA and MB were isolated from rice husk as plant growth inhibitors (Kato *et al.*, 1973, 1977; Takahashi *et al.*, 1976; Kato-Noguchi *et al.*, 2008a, b, 2010). The chemical structure of MB was determined as 3,20-epoxy-3α-hydroxy-synpimara-7,15-dien-19,6β-olide, $C_{20}H_{26}O_4$. Secretion of momilactone B was later confirmed for other rice cultivars as well (Kato-Noguchi *et al.*, 2013). Besides this, MA (3-oxo-syn-pimara-7,15-dien-19,6β-olide, $C_{20}H_{26}O_3$) was also found in the root exudates of rice (cv. Koshihikari) (Kato-Noguchi *et al.*, 2005). Both MA and MB were further found in various rice cultivars and origines (Chung *et al.*, 2005, 2006; Xuan *et al.*, 2016). To date, the biological activities of MA and MB have been limited to allelopathy, antioxidant, antifungal, and antimicrobial activities, although the cytotoxic and antitumor activity of MB on human colon cancer cell was reported (Kim *et al.*, 2007). The medicinal and pharmaceutical properties of MA and MB have not been much known, as the isolation and purification of MA and MB are complicated and laborious. There are only several laboratories worldwide have worked on MA and MB, and

thus no standard MA and MB can be purchased. This fact has prevented us from understanding the physiological roles of the two compounds in rice plants, and exploiting potential uses in medicines and pharmaceutics.

Table 6 shows the isolation and quantification of MA and MB which have been conducted so far by different analytical instruments, such as HPLC, LC-MS-MS, GC-MS, and GC-MS-SIM, which were applied to measure MA and MB in different parts of rice plants, seedlings, and root exudates. HPLC and TLC were useful in measuring contents of MA and MB in *H. plumaeforme* (Nozaki *et al.*, 2007; Kato-Noguchi *et al.*, 2009). It is not known why rice and *H. plumaeforme* have quite distinct taxonomies, but they possess both MA and MB (Nozaki *et al.*, 2007; Kato-Noguchi *et al.*, 2009). In this study, by column chromatography (CC), there were 1.2 and 0.7 µg/g dry weights (DWs) of MA and MB, respectively, which were purified. Comparing with other reports using CC to isolate MA and MB, they were similar to the yields conducted by Kato *et al*. (1973) (0.8 and 0.5 µg/g DW, respectively) and Takahashi *et al*. (1977) (1.0 and 0.8 µg/g DW, respectively). It is a fact that all of these Japanese researchers used similar rice varieties (cv. Koshihikari) as this study. In contrast, Chung *et al*. (2005) achieved much higher quantities of MA and MB (15.0 and 10.0 µg/g DW, respectively) (Table 6), because they apparently used the husks of different rice cultivars in South Korea. In addition, the amounts of MA and MB may also be varied in CC techniques. In this study, the yields of both MA and MB were enriched up to 58.8 and 104.4 µg/g DW (Tables 5 and 6). Apparently, the quantities of MA and MB were much increased, and methods that used either rice husk dried (100 °C, 1 h), or boiled (100 °C, 2 h), then combined with MeOH 100% provided the maximum quantities of both MA and MB (Table 5). By GC-MS, Chung *et al*. (2006) obtained yields of MA and MB in rice husk by 34.7 and 37.8 µg/g DW, respectively, but the actual amounts purified by column chromatography were only 4.6 and 3.1 µg/g DW, respectively (Chung *et al*., 2007), Thus, it can be seen that the authentic quantities of MA and MB were much less than their amounts estimated by GC-MS, approximately 7.5 and 12.2 folds. In comparison with the yields of MA and MB purified in this study (1.2 and 0.7 µg/g DW, respectively, Table 5), the theoretical volumes quantified by HPLC were 58.8 and 104.4 µg/g DW (Table 5), respectively, equivalent to approximately 50 and 150 folds, respectively. Thus, the certain extents of MA and MB after the enrichment which can be purified by CC may elevate to 5 and 15 folds.

Table 6 showed that, in previous research, different combinations among hexane, EtOAc, MeOH, and water were the most common solvents to extract MA and MB (Kato *et*

al., 1973; Cartwright *et al.*, 1977; Lee *et al.*, 1999; Kato-Noguchi *et al.*, 2003; Chung *et al.*, 2005; Chung *et al.*, 2006; Fukuta *et al.*, 2007; Nozaki *et al.*, 2007; Kato-Noguchi *et al.*, 2008), although benzene (Takahashi *et al.*, 1976) and chloroform (Kato *et al.*, 1973) were also used. However, the use of either benzene and chloroform is not encouraged because of their toxicity. Temperature and pressure have been effective in optimizing yields of natural products, especially in industrial scale (Mottaleb *et al.*, 2012). The replacement of MeOH by EtOH to reduce the hazardous possibility (Castro *et al.*, 2009) during extraction of MA and MB should be also considered. In general, MA was found to be greater in quantity than MB in both rice and *H. plumaeforme* (Table 6). It was reported that, although MA had a greater amount in rice plant parts than MB, the biological activities, including allelopathy, antimicrobial and antioxidant activities of MB were stronger than MA (Fukuta *et al.*, 2007; Xuan *et al.*, 2016). However, in this study, by the treatment of temperature combined with pressure and different dilutions of MeOH, MB was increased in much greater quantity than MA (Table 6).

The quantities of both MA and MB were enriched, although they varied among extractions (Table 5). Results in Table 5 noted that, although MeOH was an effective solvent in combination with EtOAc, temperature, and pressure, the use of either MeOH ≤ 50% or distilled water only, at any temperature, could not successfully purify both MA and MB (Table 5). Although quantities of both MA and MB were enriched to 58.8 and 104.4 μg/g DW, they were in much lower amounts than other phytochemicals present in rice husk, such as phenolic acids and fatty acids.

In this study, the use of temperature was shown to be effective in quantity enrichment of both MA and MB (Tables 5 and 6). The treatment of temperature at 100 °C up to 3 h may affect the stability of both MA and MB, and the degradation of natural products from plants can be influenced by high temperature, light, and microbes (Castro *et al.*, 2009). In this study, after treatment by temperatures, quantities of MA and MB were soon determined by HPLC and showed effective in optimizing yields of MA and MB (Table 5), but the stability of both MA and MB as well as other potent chemicals from rice husk should be further investigated.

Table 6. Contents of MA and MB in rice husk and rice plants

MA (µg/g DW)	MB (µg/g DW)	Materials	Instruments	Extraction Protocols	References
15.0	10.0	Rice husk	CC	Hexane:EtOAc (8:2)	(Chung *et al*., 2005)
4.6	3.1	Rice husk	CC	Hexane:EtOAc (8:2)	(Fukuta *et al*., 2007)
4.5	3.0	Rice straw	LC-MS-MS	EtOAc	(Lee *et al*., 1999)
4.9	2.9	Rice husk	GC-MS	EtOAc:H_2O (1:1)	(Chung *et al*., 2006)
140.0	95.0	Whole rice plants	HPLC	EtOAc	(Kato-Noguchi *et al*., 2008)
69.9-140.0	64.4-114.1	Seedling *	GC-MS	EtOAc	(Xuan *et al*., 2016)
80.6	nd	Rice seeding	HPLC	MeOH: H_2O (8:2)	(Kato-Noguchi *et al*., 2003)
1.0	0.8	Rice husk	CC	Benzene:EtOAc (10:1)	(Takahashi *et al*., 1976)
87.0	9.6	UV-irradiated rice leaves	GC-MS-SIM	MeOH	(Kodama *et al*., 1988)
34.7	37.8	Rice husk	GC-MS	EtOAc:H_2O (1:1)	(Chung *et al*., 2006)
58.7	23.4	*Hypnum plumaeforme* L.	HPLC	EtOAc	(Kato-Noguchi *et al*., 2009)
8.4	4.2	*Hypnum plumaeforme* L.	TLC	EtOAc	(Nozaki *et al*., 2007)
1.2	0.7	Rice husk	CC	Hexane:EtOAc (8:2)	This study; by CC
11.8-58.8	3.0-104.4	Rice husk **	HPLC	EtOAc; MeOH; Temperature; Pressure	This study

nd: not detected; DW: dry weight; CC: column chromatography; HPLC: high performance liquid chromatography; GC-MS: gas chromatography mass spectrometry; GC-MS-SIM: gas chromatography mass spectrometry—selected ion monitoring; LC-MS-MS: liquid chromatography tandem mass spectrometric; TLC: thin layer chromatography; * Quantities varied among rice origins and subtypes;
** Quantities of MA and MB were enriched and varied among extractions.

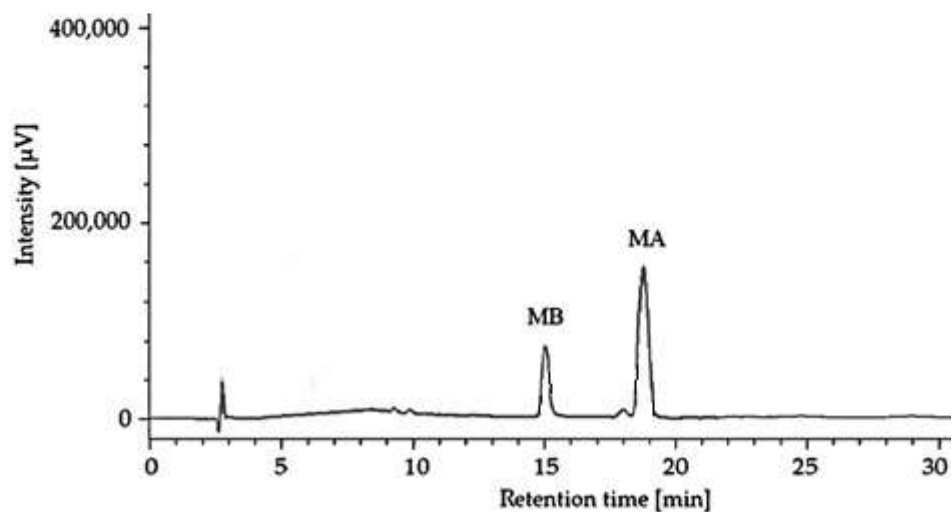
As shown in Table 6, although the endogenous amounts of MA were generally higher than MB in rice husk, exudates, and other plant parts, the treatments of temperature at 100 °C was shown to be more effective in optimizing yields of MB than MA (Table 5). The melting point of 240 °C of MB was higher than the 234-236 °C of MA, which might not be crucial to explain why the yield of MB was greater than MA in this study. The synthesis of momilactones required temperature > 150 °C to achieve high yields (Germain *et al.*, 2002). The chemical structure of MB differs from MA by an oxygen bridge in a lactone ring (Figure 4) which might be more relevant to the superior efficacy from using high temperature to isolate MB as compared to MA, but this needs further elaboration. This study highlighted that MeOH was among the most productive, but the concentrations should not be ≤ 50%, whilst water was not the ideal solvent to isolate and purify MA and MB (Table 5). It has been known that amounts of MA and MB largely varied among rice cultivars and origins (Xuan *et al.*, 2016), and thus the yields of MA and MB in husks of similar rice cultivar in Japan (cv. Koshihikari) in Kato *et al.* (1973) and Takahashi *et al.* (1976) and this study were compared. Firstly, by conventional protocol using column chromatography, we purified 1.2 and 0.7 µg/g, and showed closed values as compared with Kato *et al.* (1973) that successfully yielded 0.8 and 0.5 µg/g, whereas Takahashi *et al*. (1976) showed 1.0 and 0.8 µg/g MA and MB, respectively. However, by HPLC analysis, the quantities of MA and MB were theoretically optimized to 11.8-58.8 µg/g and 3.0-104.4 µg/g, respectively. The use of pressure did not approach the maximum yields of MA and MB in this study, but the optimized amounts were promising (MA: 17.90-26.26 µg/g DW; MB: 40.78-71.0 µg/g DW) (Table 5). This research provided practical information to efficiently purify greater quantities of MA and MB from rice husk to investigate further potent biological activities of the two compounds.

3.5. Conclusions

This study established a protocol to enrich and optimize quantities of MA and MB in rice husk by treatments with temperature and pressure, combined with extracting solvents of EtOAc and MeOH. Amounts of MA and MB were strongly productive by treatments of 100 °C, which may be due to their melting points of 234-236 °C and 240 °C, respectively, although the stability of MA and MB after temperature treatment should be further examined. The application of pressure was effective to promote yields of MA and MB. The findings of this study assisted the purification of MA and MB in rice husk more effectively

to exploit further biological activities of the two compounds, such as medicinal and pharmaceutical properties

3.6. Supplementary Data



Supplement Material, Figure S1. HPLC chromatogram of MA (18.696 ± 0.157 min) and MB (15.037 ± 0.120 min) measured at 210 nm.

CHAPTER IV.
ANTI-HYPERURICEMIC EFFECT THROUGH XANTHINE OXIDASE INHIBITOR OF MOMILACTONES A AND B

4.1. Introduction

Natural products have become a source of novel pharmaceuticals due to their potent efficacy with fewer side effects, which relies on the containing of complex bioactive compounds (Gordon *et al*., 2013). Quercetin isolated from Biota orientalis reduces UA in hyperuricemia mice caused by oxonate, which is partly due to its inhibition on XO activity in the liver. Ginkgo Folium suppresses XO activity and shows anti-inflammatory effects in the model of gout and arthritis induced by MSU crystals (Lanzhou *et al*., 2017).

Hyperuricemia is characterized by an abnormally high level of uric acid in the blood due to a metabolic disorder on its production or on its excretion (Johnson *et al*., 2007). Increasing clinical reports have shown that hyperuricemia is associated with an increasing risk of not only gout, but also cardiovascular disorders, renal dysfunction, hyperlipidemia, hypertension, cancer, diabetes and metabolic syndromes (Hayden *et al*., 2004). Lifestyle modifications such as weight reduction, decreased dietary purine intake and alcohol consumption may help to decrease blood uric acid, but many patients will still need medication to control their hyperuricemia (Roddy *et al*., 2007). Xanthine oxidase (XO) plays a key role in uric acid biosynthesis by converting hypoxanthine to xanthine and further converting xanthine to uric acids. Allopurinol, an XO inhibitor which is structurally related to xanthine, binds tightly to the active site of XO and thus causes XO inhibition. This drug has been used clinically for more than 40 years (Berry *et al*., 2004). Unfortunately, severe adverse effects in some patients, including fever, skin rashes, allergic reactions, hepatitis, and nephropathy limit the clinical use of allopurinol. For this reason, xanthine oxidase inhibitors from natural products have been explored as viable, harmless, and nontoxic alternatives for the treatment of hyperuricemia (Valko *et al*., 2007). Therefore, the discovery and application of a satisfactory dose of XO inhibitors is a valuable approach to high level of uric acid in the blood.

Momilactones A (MA) and B (MB) were first identified and isolated from rice husk and found that they caused inhibition on germination and root elongation of rice seedling (Chung *et al*., 2006). Momilactone is the combination of two words: *momi* means rice in

Japanese, with the plant growth inhibitors detected in rice husk belonged to lactone. They were also found in rice leaves and straw and reported as phytoalexins (Minh *et al*., 2018b).

To date, the biological activities of MA and MB have been limited to allelopathy, antioxidant, antifungal, and antimicrobial activities, although the cytotoxic and antitumor activity of MB on human colon cancer cell was reported (Li *et al*., 2009). The medicinal and pharmaceutical properties of MA and MB have not been much known, as the isolation and purification of MA and MB are complicated and laborious. There are only several laboratories worldwide have worked on MA and MB, and thus no standards of MA and MB can be purchased. This fact has prevented us from understanding the physiological roles of the two compounds in rice plants, and exploiting potential uses in medicines and pharmaceutics. Therefore, the present study reports at first assessment the XO inhibitory potential of individual MA, MB, and their combinations by *in vitro* studies. The obtained MA and MB were identified and confirmed by high performance liquid chromatography (HPLC), electrospray ionization mass spectrometry (ESI-MS), and ultra-performance liquid chromatography coupled with electrospray mass spectrometry (UPLC/ESI-MS).

4.2. Materials and Methods

4.2.1. Plant Material and Preparation of Extract

Rice husk (subtype: Japonica; var. Koshihikari) were collected from a rice mill in Saijo area, near Hiroshima University, Japan, in May 2017. The dried of rice husk were ground to fine powder and dried by an oven at 100 °C (SANYO Laboratory Convection Oven MOV-212F D, Sanyo Electric, Osaka, Japan) for 1 h (Figure 5), then immersed in 6 L of MeOH 100% for 2 weeks at room temperature (Minh *et al*., 2018b). The solution of MeOH was concentrated under vacuum to produce an extract for isolation and purification of MA and MB.

The crude extract was then suspended in distilled water (200 mL) and successively fractionated with hexane and EtOAc. The obtained amount of water, hexane and EtOAc extracts were 25, 23, and 50 g, respectively.

4.2.2. Fractions and Isolation of MA and MB

The ethyl acetate extract was subjected to normal phase column chromatography (CC) over silica gel (250 g) of 70-230 mesh ASTM and LiChroprep RP-18 (40-63 mm). All fractions were examined by thin-layer chromatography (TLC) (Merck, Darmstadt, Germany). In TLC analysis, TLC Silica gel 60 was used as a solid phase and a mixture of

solvents hexane: ethyl acetate 8:2 (v:v) was a mobile phase. There are 50 fractions was collected, of which fractions 1-10 in hexane, fractions 11-20 in hexane:EtOAc (9:1), fractions 21-40 in hexane:EtOAc (8:2), fractions 41-50 in hexane:EtOAc (7:3). After examining by TLC, fractions 21-35, were further subjected to a column chromatography over silica gel with chloroform:methanol (100%, 99:1) to yield a mixture of MA and MB. These mixture was further purified by column chromatography over silica gel with chloroform:methanol (99.9:0.1; 99.8:0.2; 99.7:0.3; 99.6:0.4; 99.5:0.5) to successfully purify MA (20 mg) and MB (12 mg).

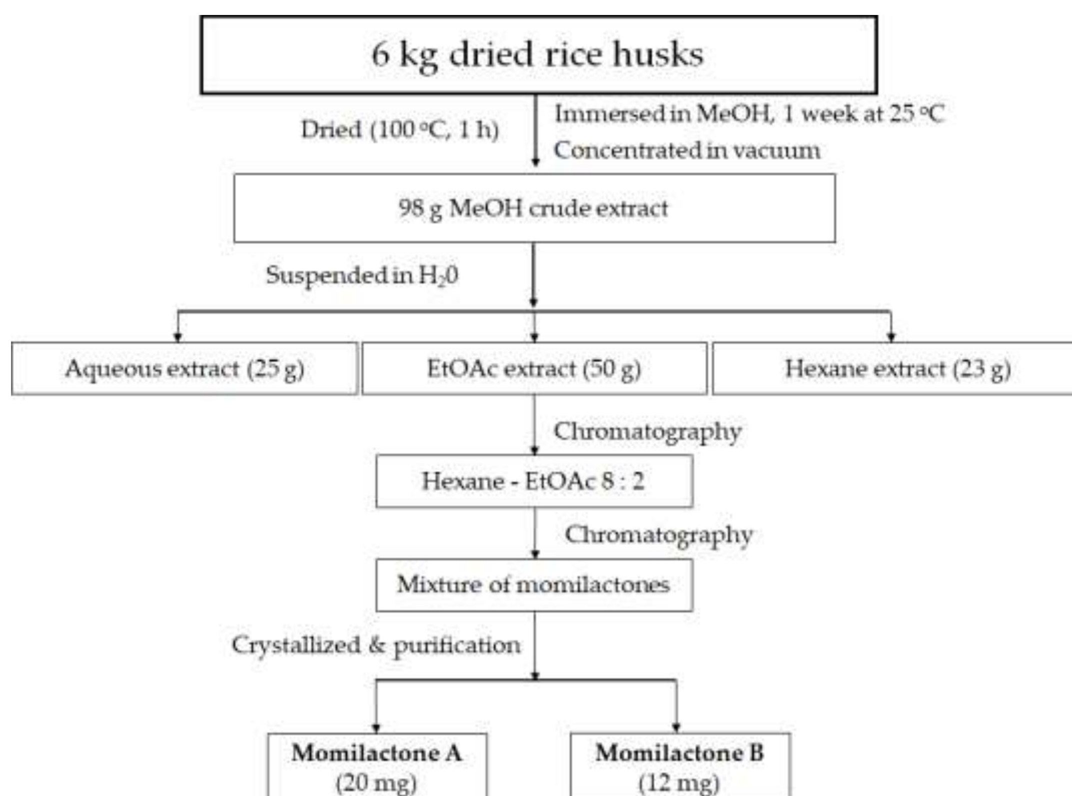


Figure 5. Procedure of isolation of momilactones A and B from rice husk.

4.2.3. Identification and Confirmation of Momilactones A and B by HPLC, ESI-MS, and UPLC/ESI-MS

The gradient liquid chromatographic system including HPLC (JASCO PU-2089 Plus, JASCO Engineering Co., Tokyo) equipped with a J-Pak Symphonia C18 (5 µm, 250 mm×4.6 mm i.d.) column (JASCO Engineering Co., Ltd, Tokyo, Japan). Mobile phase consisting of 0.1% TFA in acetonitrile:water (70:30, v/v). The flow rate was adjusted to 0.4 mL/min within 30 min. The detector was set at 210 nm. The injection volume was 10 µL. Data acquisition was executed on ChromNAV software (JASCO, Tokyo, Japan). Linearity was observed in the range of 10 to 200 µg/mL to determined LOD and LOQ of

momilactones A and B.

ESI-MS analysis was conducted on negative/positive ion mode. Mass spectral characterization was performed using a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, CA, USA) connected with an electrospray ionization (ESI) source in negative (between m/z 120 and 2000) and positive (between m/z 100 and 2000) ionization mode recording spectra. The instrumental conditions were as follows: spray voltage, 5.0 kV; sheath gas flow, 50 arb (arbitrary unit); aux gas flow rate, 10 arb; capillary temperature, 330 °C; capillary voltage, 50 V; tube lens, 80V (Banerjee *et al*., 2012).

The characterization of major component peaks of momilactones A and B were performed on the Waters Acquity UPLC instrument equipped with the Acquity HPLC BEH C18 1.7 µm (2.1x50 mm Column). The UPLC mobile phases were (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in acetonitrile (v/v). Isocratic elution was accomplished with a mixture of A 50% and B 50%. The flow rate was 0.3 mL/min, injection volume was 3.0 µL, and column temperature was 30 °C, with an ambient sample temperature. Mass spectral characterization was performed using a LTQ Orbitrap XL equipped with an electrospray ionization source in positive ionization mode recording spectra between m/z 100 and 1000. The instrumental conditions were as follows: spray voltage, 4.5 kV; sheath gas flow, 55 arb; aux gas flow rate, 15 arb; capillary temperature, 340 °C; capillary voltage, 50 V; tube lens, 80V (Prokudina *et al*., 2012).

4.2.4. Xanthine Oxidase Inhibitory Activity

The XO inhibitory activity was assayed spectrofotometrically *in vitro* under aerobic condition at 290 nm based on a method reported previousy (Nguyen *et al*., 2004). Briefly, a volume of 50 µL of tests solution was mixed with 35 µL of 70 mM phosphate buffer (pH=7.5), and 30 µL of fresh enzyme solution (0.1 units/mL in 70 mM phosphate buffer, pH=7.5). Reaction was initiated by adding 60 µL of substrate solution (150 µM xanthine in same buffer) after pre-incubation at 25 °C for 15 min. The assay mixture was then incubated at 25 °C for 30 min. A volume of 25 µL HCl (1 M) was added to stop reaction and the absorbance was measured at 290 nm with a microplate reader (MultiskanTM Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). For the blank, the assay mixture was prepared in its present condition, but the enzyme solution was added after adding HCl. One unit of XO was defined as the amount of enzyme required to produce 1 µmol of uric acid/min at 25 °C. The XO inhibitory activity was expressed as the

percentage inhibition of XO in above assay system and calculated by the following formula:

$$\% \text{ Inhibition} = \{\frac{(A-B)-(C-D)}{(A-B)}\} * 100$$

Where A was the activity of the enzyme without either test extract or fraction, B was the control of A without either test extract or fraction and enzyme, C and D were the activities of either the test extract or fraction with and without XO. Allopurinol (10-100 μg/mL) was used as a positive control. The IC_{50} values were calculated from the mean values of percentage inhibition data.

4.2.5. Lineweaver-Bruk Plot

To determine the mode of inhibition of momilactones A and B on xanthine oxidase, Lineweaver-Burk plot analysis was performed. This kinetics study was carried out in the absence of momilactones A and B, 0.5 of IC_{50}, and IC_{50} concentrations with varying concentrations of xanthine as substrate. The initial rates were determined on the basis of the rate of increase in absorbance at 290 nm between 0.5 and 3 min. The data represent the mean ± SD. of four determinations. The inhibition constants (Ki) were determined from the slopes of the Lineweaver-Burk plot for competitive inhibition, and intercept on vertical axis for noncompetitive inhibition

4.2.6. *Statistical Analysis*

The statistical analysis was performed by one-way ANOVA using Minitab® 16.2.3 (copyright © 2012 Minitab Inc., Philadelphia, USA). The results were reported as mean ± standard deviation values. Differences among treatment, control and standard data are considered significant at $p < 0.05$ using Tukey's test.

4.3. Results

4.3.1. Phytochemical Isolation of Momilactones A and B

MA (20 mg) and MB (12 mg) were purified from rice husk of *Oryza sativa* cv. Koshihikari by column chromatography (CC) (Figure 5). The presence of MA and MB was confirmed by HPLC, ESI-MS, and UPLC/ESI-MS.

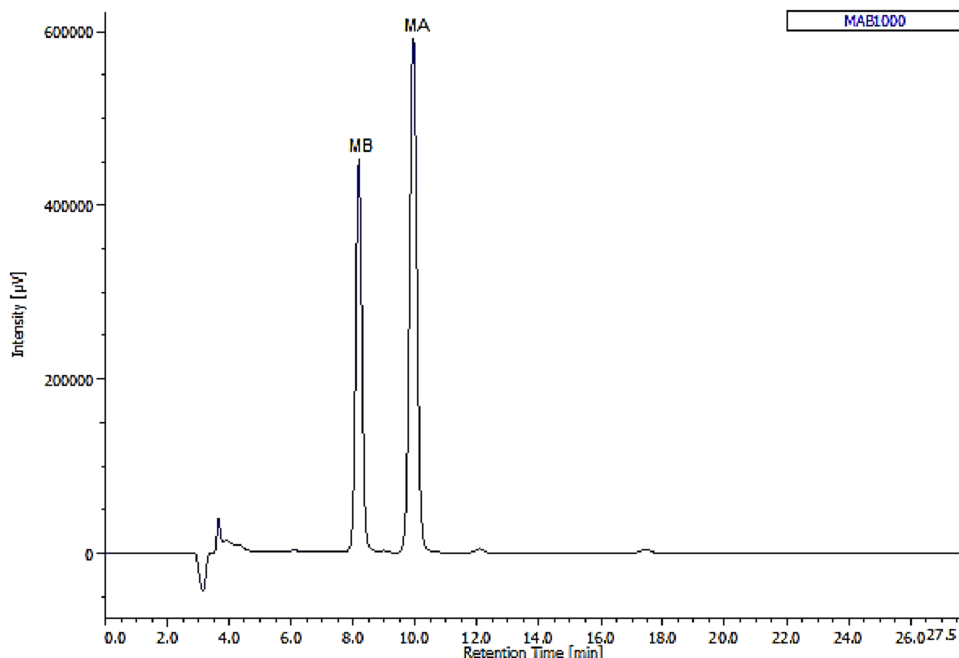


Figure 6. HPLC chromatogram of MA (10.01 ± 0.04 min) and MB (8.22 ± 0.03 min) measured at 210 nm.

The isolated MA and MB by HPLC appeared at 10.01 ± 0.04 min and 8.22 ± 0.03 min, respectively. Detection limit (LOD) of MA and MB by HPLC were 0.1988 and 0.1838 ng/mL, respectively; with regard to limits of quantitation (LOQ) were 0.6026 and 0.5569 ng/mL, respectively (Figure 6, Table 7).

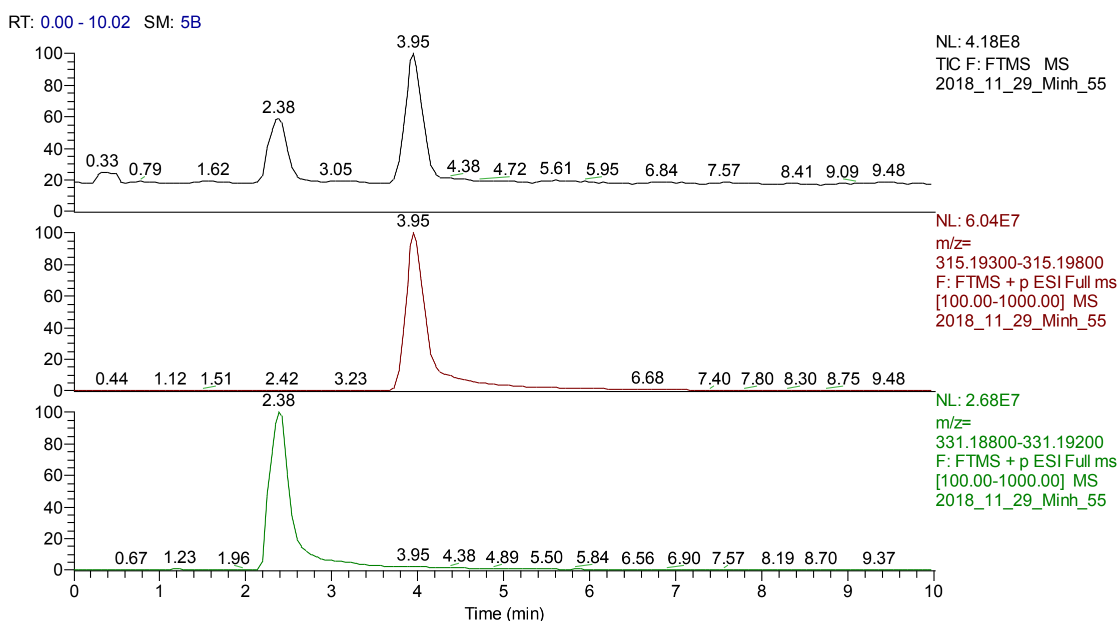


Figure 7. UPLC/ESI-MS chromatogram of MA (3.95 ± 0.03 min) and MB (2.38 ± 0.02 min).

By using UPLC/ESI-MS MA and MB were definite at 4.00 ± 0.03 min and 2.45 ± 0.02 min, respectively. Meanwhile, LOD of MA and MB were 0.1540 and 0.0904 ng/mL,

respectively; LOQ were calculated as 0.4666 ng/mL for MA and 0.2739 ng/mL for MB. (Figure 7, Table 7).

Table 7. Parameters of momilactones A and B detection limits

Index	**HPLC**		**UPLC/ESI-MS**	
	MA	**MB**	**MA**	**MB**
Retention time	*10.01 ± 0.04*	*8.22 ± 0.03*	*3.95 ± 0.04*	*2.38 ± 0.03*
LOD (ng/mL)	0.1988	0.1838	0.1540	0.0904
LOQ (ng/mL)	0.6026	0.5569	0.4666	0.2739

Values are means ± SD (standard deviation). (n = 3).

4.3.2. XOI Activity of Momilactones A and B

Inhibitory effects of momilactones A, B, and their mixtures on xanthine oxidase (XO) were presented in Table 8. Combination of MA and MB (4:1) was the most inhibitory (IC_{50} = 531.14 µg/mL) followed by MA and MB (1:1) (IC_{50} = 787.32 µg/mL), and MA (IC_{50} = 821.42 µg/mL). The inhibitory effects of momilactones A, B, and their combination mixtures were lower than allopurinol as reference.

Table 8. Xanthine oxidase inhibitory activity of momilactones A, B, and their mixtures

Sample	**XOI Levels, IC_{50} (µg/mL)**
MA	821.42 ± 2.50 e
MA:MB 4:1	531.14 ± 6.03 g
MA:MB 3:2	2008.88 ± 6.56 a
MA:MB 1:1	787.32 ± 5.04 f
MA:MB 2:3	1334.05 ± 5.77 b
MA:MB 1:4	1115.46 ± 4.62 c
MB	928.65 ± 2.76 d
Allopurinol*	21.37 ± 0.21 h

*Positive control. Data are presented as means ± SD. Mean values with different lowercase letters indicate significant differences in the same column ($p < 0.05$) (n = 3)

Lineweaver-Burk plot of momilactones A, B, and their mixtures on XO were presented in Figure 9. Although IC_{50} values of momilactones A, B, and their mixture were lower than allopurinol, based on lineweaver-Burk plot they exhibited competitive inhibitions. Inhibitory effects of momilactones A, B, and their mixture may be caused by lactone ring belonging momilactones A and B. Lactone ring been reported to have inhibitory activity on XO as reported by (Honda *et al*., 2014).

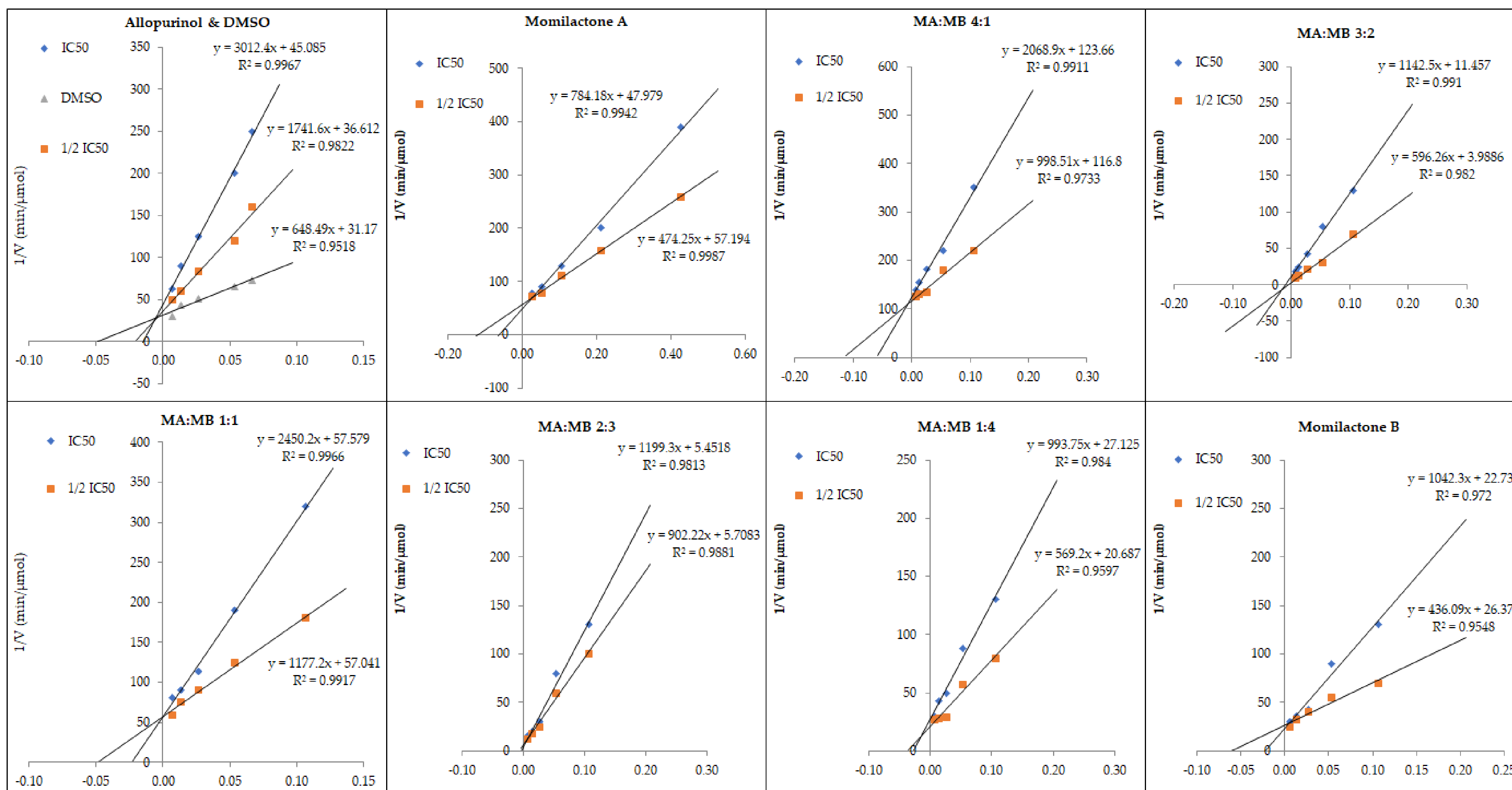


Figure 8. Lineweaver-Burk plots of XO inhibitions of compounds with concentrations of IC_{50} and 1/2 IC_{50} of Allopurinol, DMSO, MA, MB, and combinations of MA:MB.

4.4. Discussion

MA and MB were isolated from rice husk as plant growth inhibitors (Zhao *et al*., 2018). The chemical structure of MB was determined as 3,20-epoxy-3α-hydroxy-synpimara-7,15-dien-19,6β-olide, $C_{20}H_{26}O_4$. Secretion of momilactone B was later confirmed for other rice cultivars as well (Xuan *et al*., 2016). Moreover, MA (3-oxo-syn-pimara-7,15-dien-19,6β-olide, $C_{20}H_{26}O_3$) also was found in the root exudates of rice cv. Koshihikari (Xuan *et al*., 2016). Both MA and MB further were found from many other rice cultivars (Minh *et al*., 2018b). In this study, by column chromatography, there were 3.3 and 2.0 µg/g DW of MA and MB, respectively, were purified. Comparing with other reports using column chromatography to isolate MA and MB, it was higher to the yields conducted by previous study (1.2 and 0.7 µg/g DW, respectively) (Minh *et al*., 2018b), Kato *et al*. (1973) (0.8 and 0.5 µg/g DW, respectively) and Takahashi *et al*. (1976) (1.0 and 0.8 µg/g DW, respectively), as these Japanese researchers used similar rice variety (Koshihikari) as this study did. Finding of this study showed that, by treatments applied dried (100 °C, 1h), the amounts of both MA and MB were both enriched. Because the melting points of MA and MB were 234-236 °C and 240 °C, respectively, the treatment by 100 °C may thus effectively promote quantities of MA and MB.

Due to unhealthy diets, the morbidity of gout is increasing year by year worldwide. In patients with gout, high levels of serum UA cause MSU deposition in joints and other tissues (Terkeltaub *et al*., 2010), which induces the release of proinflammatory cytokines and further promotes inflammation (Kim *et al*., 2013). This study has limitations that will be addressed in our ongoing experiments.

Enzyme inhibition kinetic experiments were carried out to further characterize the inhibitory activities of the compounds isolated from active fractions. The inhibition IC_{50} of the all combination of MA and MB given in Table 8 were determined by analyzing the data by Lineweaver-Burk plots. For apigenin, a mixed inhibition mode was observed. The result suggested that apigenin inhibited XO activity not only by a competitive mode of action, but also by interaction with the enzyme at a site other than the active center which was accorded with the consequences in previous studies (Flemmig *et al*., 2011). All other combination of MA and MB and allopurinol exhibited competitive inhibitions. The Lineweaver-Burk plots in the absence or presence of compounds are shown in Figure 8. However, different types of inhibition by caffeic acid and its derivatives have been reported in the previous studies

(Nakanishi *et al*., 1990; Flemmig *et al*., 2011; Chen *et al*., 2011; Chiang *et al*., 1994). Under the experimental conditions, caffeic acid exhibited a competitive inhibition mode, which is consistent with the result reported by Chen *et al*. (2011).

4.5. Supplementary Data

Figure S2. ESI-MS spectrum of momilactone A.

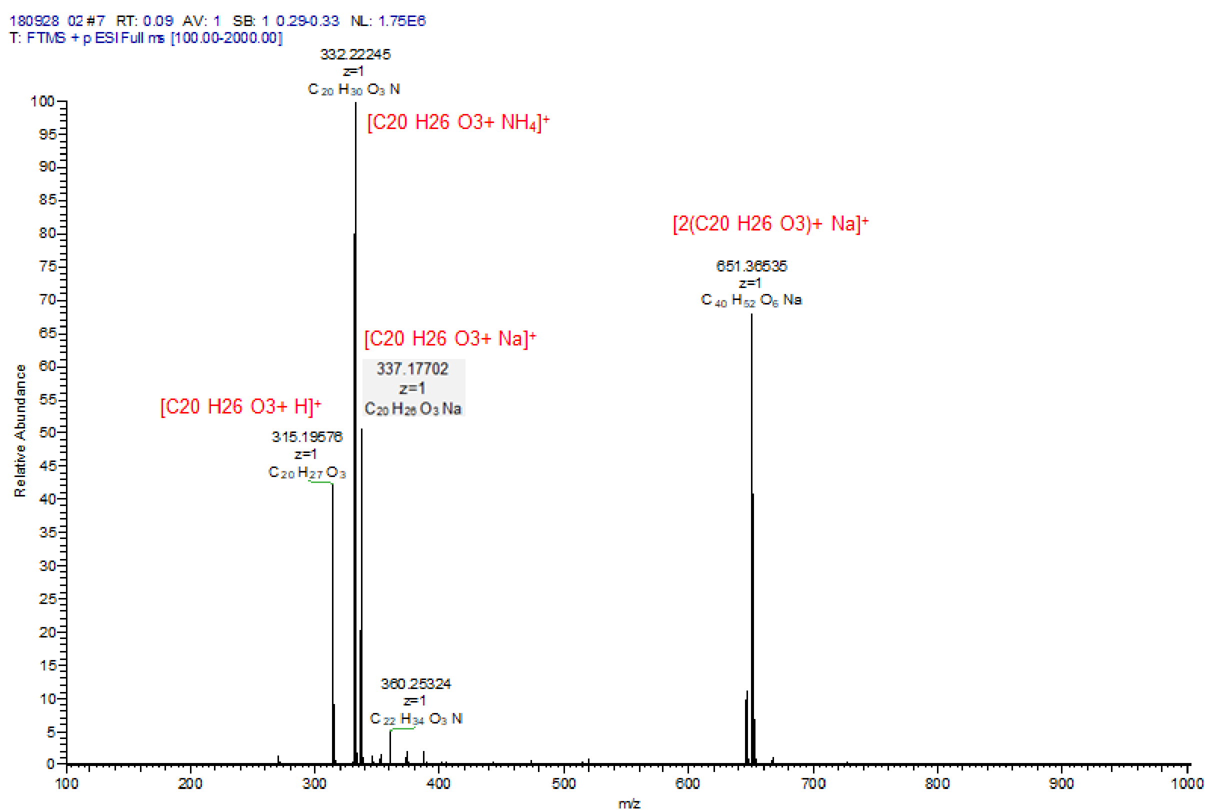
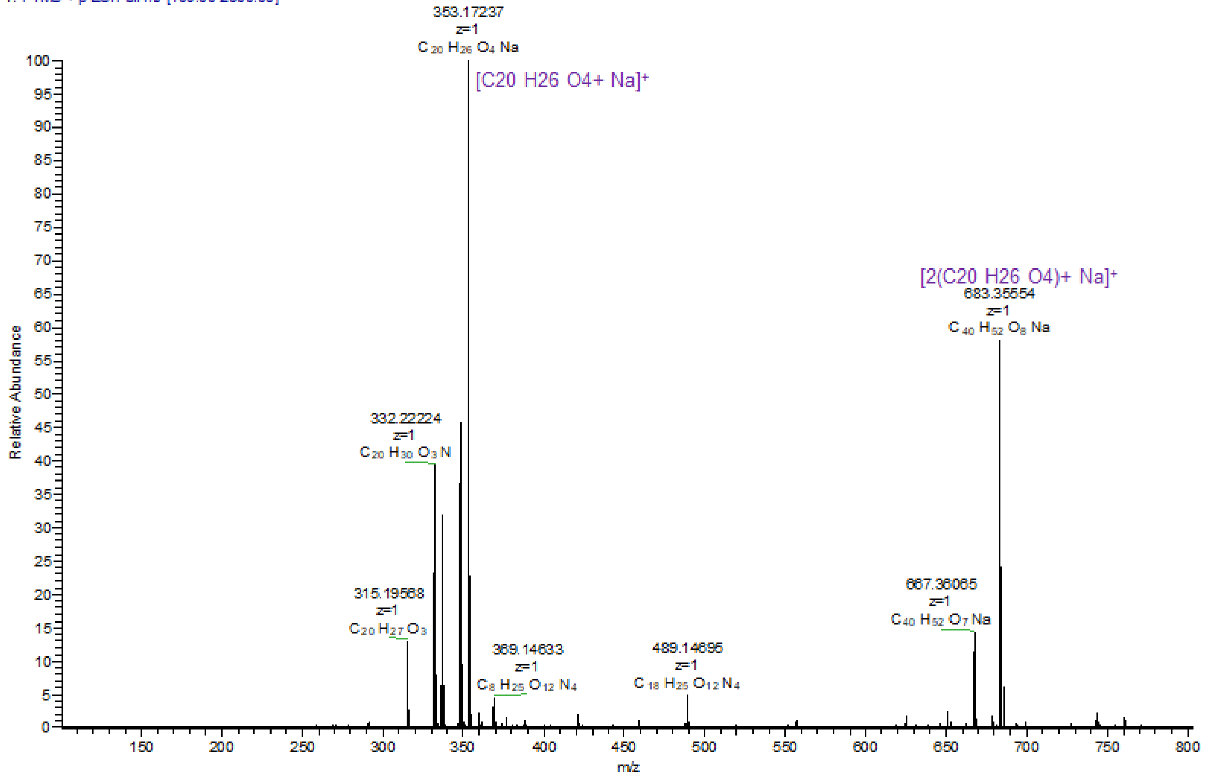


Figure S3. ESI-MS spectrum of momilactone B.



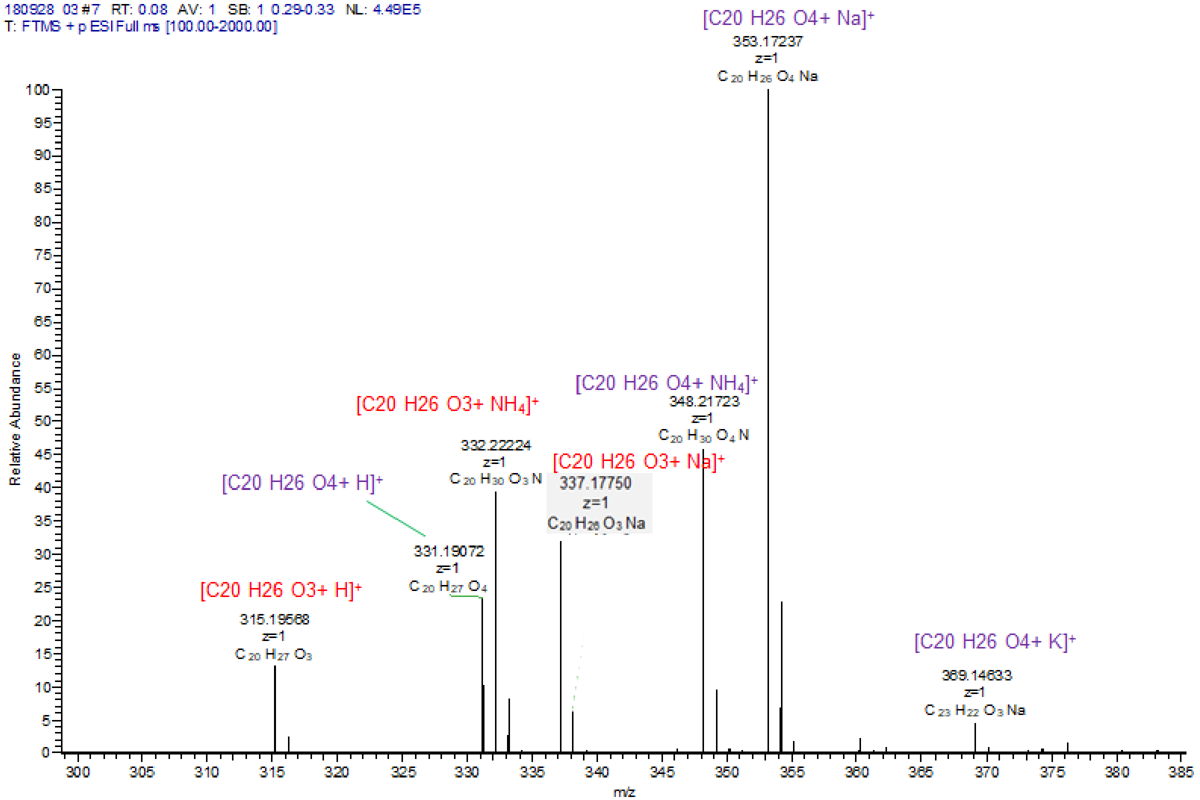
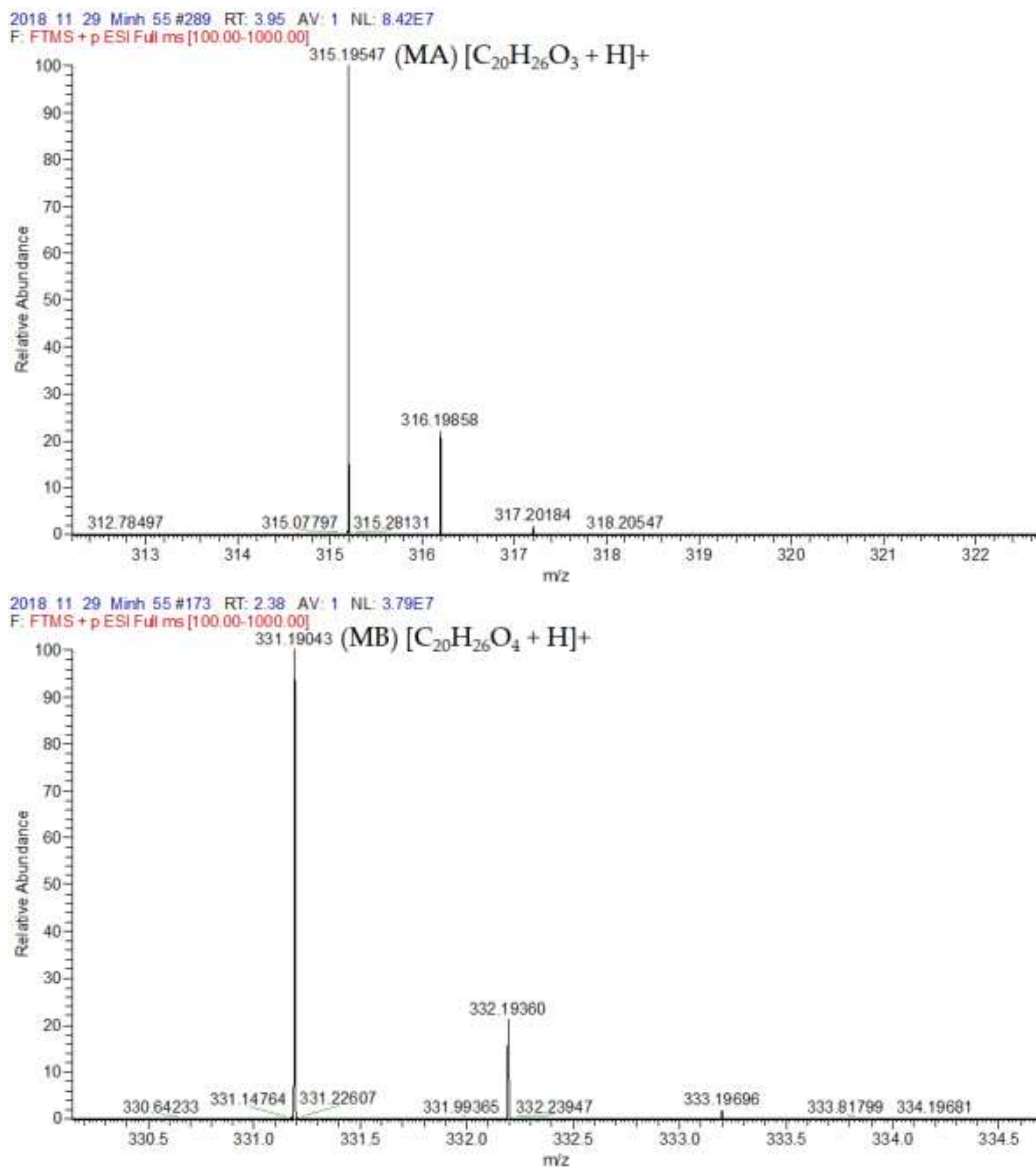


Figure S4. UPLC/ESI-MS spectrum of momilactones A and B.



CHAPTER V.
PHYTOCHEMICAL INVESTIGATION AND BIOLOGICAL EVALUATION OF ESSENTIAL OIL FROM RICE LEAF

5.1. Introduction

The plant kingdom abounds with natural products which are chemically diverse (Farmer *et al*., 2001). During growth and development, plants generate around 100,000 chemical products, of which, 1700 are volatile organic compounds (VOC) (Kask *et al*., 2013) presented in essential oils. These molecules are emitted by different plant organs including leaves, flowers, fruits, and roots (Kask *et al*., 2013; Villamar-Torres *et al*., 2018). Plant VOCs are chemically classified into different groups such as fatty acid derivatives, terpenes, indole and molecules from other chemical families. Biologically, volatiles compounds are not only to protect themselves from herbivores and microbial pathogens but also play an important role as release signals and messages to insects and neighboring plants. Due to having therapeutic benefits and high absorbability through the skin, VOCs and essential oils have been widely applied in different sectors including food, cosmetic and medicinal production (Djilani *et al*., 2012). From the agricultural point of view, such constituents play a notable role in enhancing crop protection (Pickett *et al*., 2016).

Rice (*Oryza sativa* L.), which belongs to the *Gramineae* family, has been consumed by humans for almost 5000 years. Rice is a major crop used by two thirds of population over the world as a staple food (Cha *et al*., 2012). Instrumental analyses have found over 200 volatile compounds presented in rice (Champagne *et al*., 2018) and usually distributed in rice leaves and rice bran (Wang *et al*., 2018). Among them, rice diterpenoids such as momilactones and oryzalexins play vital functions as phytohormones and phytoalexins. Most of previous studies have investigated on VOCs of rice grain of different varieties (Suzuki *et al*., 2018), the changes of VOCs during storage and cooked process (Lin *et al*., 2010; Yang *et al*., 2007, 2008; Sukhonthara *et al*., 2009; Liyanaarachchi *et al*., 2014; Yajima *et al*., 1978; Kongkiattikajorn *et al*., 2008; Kim *et al*., 2015; Sirisantimethakom *et al*., 2008; Lee *et al*., 2014). However, little information has been found concerning VOC releasing from rice leaves.

In rice production, abundant rice leaves are available but no potential value which may provide further income for rice farmers has been achieved. Thus, this study aimed at investigating the profile of essential oil (EO) in rice leaves and evaluate their potential biological properties including antioxidant, antibacterial, and anti-hyperuricemia activities *in vitro* assays. The obtained compounds were identified by gas chromatography-mass spectrometry (GC-MS), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and ultra-performance liquid chromatography coupled with electrospray mass spectrometry (UPLC/ESI-MS).

5.2. Materials and Methods

5.2.1. Plant Material

Fresh rice leaves were collected from rice (*Oryza sativa* L. cv. Koshihikari) paddy fields closed to Hiroshima University, Higashi Hiroshima City, Hiroshima Prefecture, Japan, in August 2017. The samples were kept in the sealed nylon bags, cleaned and sterilized at Laboratory of Plant Physiology and Biochemistry, Hiroshima University, for further analyses.

5.2.2. Preparation of Essential Oil

Extraction of EO from rice leaves was conducted by the steam distillation method described by Charles and Simon (1990) with minor modifications (Figure 9). The apparatus of extracting EO by steam distillation is made up of a heat source, a pear-shaped glass (PSG), and a spherical glass container (SGC) with upper and bottom entrances, a straight glass condenser, and a glass collector for separating and recovering the essential oil; oil appears on top of the water in the collector. A total amount of 1.5 kg fresh rice leaves mixed with 5% (*w*/*v*) sodium chloride solution was sonicated using an ultrasonic cleaner (Branson 5510R-MT Ultrasonic Cleaner, Branson Ultrasonics Corporation, CT 06813, Danbury, Connecticut, USA) for 30 min. The sterilized sample and 2L of water were then placed together in a pear-shaped glass container. The mixture was then heated at 100 °C for 2 days. Consequently, vapor drags the essential oil and is condensed and recovered in the glass collector. The mixture was then extracted using ethyl acetate (EtOAc) to separate EO from the water by a reparatory funnel. Finally, EtOAc extract was evaporated using a rotary evaporator at room temperature (25 °C) to produce 20 mg of rice leaf EO. In each test, an amount of 10 mg EO from fresh rice leaves was used (0.75 kg).

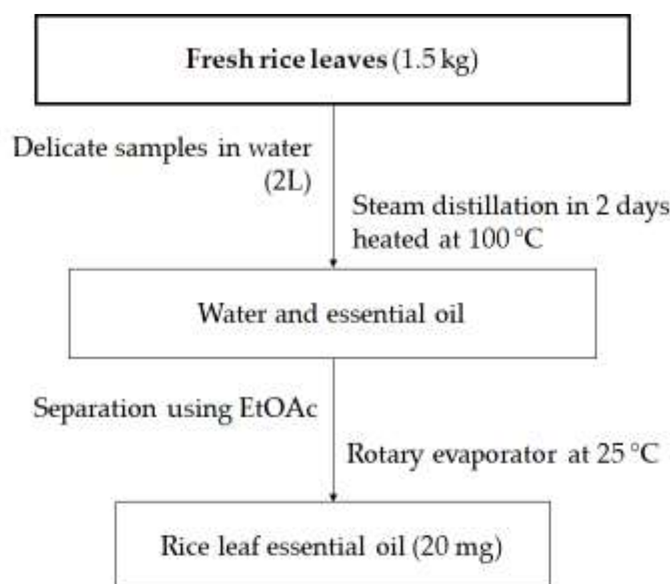


Figure 9. Process of rice leaf essential oil extraction.

5.2.3. Antioxidant Assays

5.2.3.1. DPPH Radical Scavenging Assay

The free radical scavenging activity was evaluated by using DPPH based on the method described previously by Minh *et al*. (2018a). The mixture assay consisted of 0.08 mL sample extracts and 0.04 mL 0.5 mM DPPH and 0.08 mL of 0.1 M acetate buffer (pH 5.5) was incubated in the dark at room temperature for 30 min. The DPPH radical scavenging activity was measured at absorbance at 517 nm using a microplate reader (MultiskanTM Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). BHT was used as a positive reference, while methanol was used as a control. The DPPH radical scavenging activity was expressed as the inhibition percentage and calculated by using followed formula:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of reaction without sample and A_{sample} is the absorbance of reaction with sample. The IC_{50} (inhibitory concentration) values were calculated as the concentration of sample required to scavenge 50% of DPPH. Lower IC_{50} values indicated higher antioxidant activity.

5.2.3.2. Reducing Power

The reducing power activity of samples was evaluated by using the method described previously by Minh *et al*. (2017). A volume of 0.1 mL of each sample or BHT (with

concentrations 25, 50, 100 and 250 μg/mL) was reacted with 0.25 mL phosphate buffer (0.2M, pH 6.6) and 0.25 mL potassium ferricyanide [$K_3Fe(CN)_6$] (10 g/L) and incubated at 50 °C for 30 min. Afterward, 0.25 mL trichloroacetic acid (100 g/L) was added to the mixture assay and then centrifuged at 4,000 rpm for 10 min. A volume of 0.075 mL of the supernatant was then diluted with 0.075 mL of distilled water and 0.015 mL $FeCl_3$ (1 g/L). The mixture was mixed thoroughly and was measured at 700 nm. The IC_{50} values were calculated at which the absorbance was 0.5. Lower IC_{50} indicated higher reducing power.

5.2.3.3. ABTS Radical Scavenging Assay

The ABTS radical scavenging activity was carried out following ABTS method described previously by Tuyen *et al*. (2017). The preparation of samples was in the same manner as the DPPH assay. The $ABTS^+$ radical cation was obtained by reacting 15 mL aqueous solution of ABTS 7mM with 2.45 mM potassium persulfate 15 mL. The mixture was kept in the dark at room temperature for 16 h before use. Prior to assay, the $ABTS^+$ was diluted with MeOH to obtain an absorbance of 0.70 ± 0.05 at 734 nm. The mixture assay was contained 0.15 mL of methanolic $ABTS^+$ solution and 0.018 mL of each sample and measured at 734 nm after incubated for 30 min in the dark at room temperature. BHT standard was used as positive control. The ABTS radical scavenging activity was expressed by following the equation:

$$\text{ABTS radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of reaction without sample; A_{sample} is the absorbance of ABTS radical with sample. The antioxidant activity of sample was expressedby IC_{50} as inhibitory concentration of the sample for 50% reduction of ABTS.

5.2.3.4. Antioxidant Activity using the β-Carotene Bleaching Test

The β-carotene bleaching assay was conducted by the method described previously by Minh *et al*. (2016) with some modifications. An amount of 2 mg of β-carotene/linoleic acid was initially prepared in 10 mL of chloroform. After that, 1 mL of the chloroform solution was added to 20 μL of linoleic acid and 200 mg of Tween-40. Chloroform was then evaporated under vacuum at 45 °C and an amount of 50 mL pure oxygenated water was added and shaken vigorously to form an emulsion. The mixture assay was consisted of 0.012 mL of sample extracts in methanol and 0.1 mL of the emulsion. The mixture assay solutions were incubated at 50 °C and measured at 492 nm using a microplate reader (MultiskanTM Microplate

Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). All sample were measured at 0 min and every 15 min up to 180 min. The assay was carried out in triplicate for each extract. Lipid peroxidation inhibition (LPI) was calculated using the following formula:

$$LPI (\%) = A_1/A_0 \times 100$$

Where A_0 is the absorbance value measured at zero time for the test sample, A_1 is the corresponding absorbance value measured after incubation for 180 min. Higher LPI value shows the higher antioxidant activity.

5.2.4. Growth Inhibitory Activity Bioassays

Growth suppressing potential of EO from rice leaf was assayed on *Oryza sativa* (var. Koshihikari), *Echinochloa crus-galli*, *Bidens pilosa* L., *Raphanus sativus* L., and *Lactuca sativa* L. seeds in a growth chamber (Biotron NC system, Nippon Medical & Chemical Instrument, Co. Ltd., Osaka, Japan). Photoperiodic was set up at day/night 12/12 hours with temperature 25/23 °C. EO sample was dissolve in water contained 0.2% of Tween 20 to obtain different concentrations (10, 100, 500, 1000, and 2000 µg/mL). The test solution (100 µL) was permeated filter papers lined in 12 well-plate (each well has 22 mm diameter x 18 mm height). Each healthy seed was then placed in a well, followed by the addition of 100 µL of distilled water. Plant germination monitoring was performed every 24 hours for seven days. This bioassay was replicated six times (n = 6). The growth parameters of radicle (root) and hypocotyl (shoot) length were measured. Concentration in reducing 50% shoot and root lengths (IC_{50}) was also calculated using a method described previously (Xuan *et al*., 2003).

5.2.5. Xanthine Oxidase Inhibition (XOI) Activity

The inhibitory effect on xanthine oxidase (XO) of rice leaf EO was measured spectrophotometrically according to the method reported previously (Nguyen *et al*., 2004) with minor modifications. The mixture assay was consisted of 50 µL sample solution (3000, 2000, 1000, 500, 250, 125, and 62.5 µg/mL), 30 µL phosphate buffer (70 mM, pH = 7.5), and 30 µL fresh enzyme solution (0.1 units/mL in the same buffer). The mixture assay then pre-incubated at 25 °C for 15 min before the main reaction conducted. The main reaction was initiated by adding 60 µL of substrate solution (150 µM xanthine in same buffer) and then incubated at 25 °C for 30 min. The reaction was stopped by adding 25 µL HCl (1 M) and the absorbance was recorded at 290 nm using a microplate reader. In this assay, allopurinol (6.25, 12.5, 25, 50 µg/µL) was used as a reference. A blank was prepared in the same manner, but

the enzyme solution was added after HCl. XO inhibitory activity was expressed as the percentage inhibition of XO and calculated as following the formula:

$$\% \text{ Inhibition} = \{\frac{(A-B)-(C-D)}{(A-B)}\} *100$$

Where A is the activity of the enzyme without tested samples, B is the control of A without enzyme. C and D are the activities of the test solutions with and without XO, respectively. The IC_{50} values were calculated from the mean values of the data.

5.2.6. Identification of Volatile Compounds from Rice Leaf Essential Oil

The compounds from EO of rice leaf were analyzed by GC-MS system (JMS-T100 GCV, JEOL Ltd., Tokyo, Japan) equipped with a DB-5MS capillary column, 30 m in length, 0.25 mm internal diameter, and 0.25 µm in thickness (Agilent Technologies, J & W Scientific Products, Folsom, CA, USA). The column temperature was setup initially at 50 °C without hold time, followed by an increase to 300 °C with the gradients of 10 °C/min and hold for 20 min. The temperature of injector and detector were programmed respectively at 300 °C and 320 °C, with a mass scan range 29-800 amu. The compounds were determined by the comparison between their mass spectral fragmentation pattern with the mass spectral libraries of JEOL's GC-MS Mass Center System Version 2.65a. The compounds with the high purity were selected for further spectroscopic techniques to structure elucidation (Van *et al*., 2018).

ESI-MS analysis was conducted on negative/positive ion mode. Mass spectral characterization was performed using a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) connected with an ESI source in negative (between m/z 120 and 2000) and positive (between m/z 100 and 2000) ionization mode recording spectra. The instrumental conditions were as follows: spray voltage, 5.0 kV; sheath gas flow, 50 arb (arbitrary unit); aux gas flow rate, 10 arb; capillary temperature, 330 °C; capillary voltage, 50 V; tube lens, 80V (Banerjee *et al*., 2012).

APCI-MS analysis was implemented using mass spectrometer with electrospray ion source. Mass spectral characterization was also performed using a LTQ Orbitrap XL mass spectrometer. The measurements were conducted on the positive mode (*m/z* 100-2000) FTMS at a resolution of 60,000. The instrumental conditions were as follows: source voltage, 3.5 kV; source current, 6.12 uA; vaporizer temperature 401 °C; sheath gas flow, 20 arb; aux gas flow rate, 10 arb; capillary temperature, 250 °C; capillary voltage, 30 V (Kim *et al*., 2010).

5.2.7. Identification and Quantification of Momilactones A and B from Essential Oil of Rice Leaf by UPLC/ESI-MS

The characterization of major component peaks of EO from rice leaf was performed on the Waters Acquity UPLC instrument equipped with the Acquity HPLC BEH C18 1.7 µm (2.1x50 mm Column). The UPLC mobile phases were (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in acetonitrile (v/v). Isocratic elution was accomplished with a mixture of A 50% and B 50%. The flow rate was 0.3 mL/min, injection volume was 3.0 µL, and column temperature was 30 °C, with an ambient sample temperature. Mass spectral characterization was performed using a LTQ Orbitrap XL equipped with an electrospray ionization source in positive ionization mode recording spectra between m/z 100 and 1000. The instrumental conditions were as follows: spray voltage, 4.5 kV; sheath gas flow, 55 arb; aux gas flow rate, 15 arb; capillary temperature, 340 °C; capillary voltage, 50 V; tube lens, 80V (Prokudina *et al*., 2012).

5.3. Results

5.3.1. Antioxidant and Xanthine Oxidase Inhibitory (XOI) Activities of Rice Leaf Essential Oil

The antioxidant potential of rice leaf EO was evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging, ferric reducing antioxidant power (FRAP), and β-carotene linoleate assays. The inhibitory effect of EO was compared with a positive standard butylated hydroxytoluene (BHT) (Table 9). The result of β-carotene oxidation assay was expressed as percent inhibition of β-carotene bleaching after 180 min of reaction (79.0%). Whilst that of other tests were presented as IC_{50} values at which concentration requires to inhibit 50% of scavenging free radicals (DPPH (73.1 µg/mL) and ABTS (193.3 µg/mL)) and reducing of ferric iron Fe III in the complex to ferrous iron Fe II (700.8 µg/mL). A small IC_{50} value indicates a superior activity. The obtained data showed that the EO from rice leaf exhibited a strong antioxidant property in all assays, although the synthetic BHT exerted stronger antioxidant level.

Table 9. Antioxidant activity measured by DPPH, ABTS, reducing power (FRAP), β-carotene bleaching assay, and XOI activity of rice leaf essential oil in terms of IC_{50} values

Sample	IC_{50} (μg/mL)			LPI (%)	IC_{50} (μg/mL)
	DPPH	ABTS	FRAP	β-carotene	XOI
Rice leaf EO	73.1 ± 1.4	198.3 ± 2.2	700.8 ± 5.7	79.0%	526.0 ± 2.3
BHT*	9.3 ± 1.1	45.7 ± 1.4	426.7 ± 3.8	90.0%	-
Allopurinol*	-	-	-	-	21.5 ± 0.2

*Positive control. -: measurements were not conducted. Data are means ± SD (standard deviation) (n = 3).

The inhibitory effect of rice leaf EO on xanthine oxidase was presented in Table 9. The result showed that the EO from rice leaf exhibited a considerable activity against xanthine oxidase enzyme (IC_{50} = 526.0 μg/mL). Although the anti-hyperuricemia property of allopurinol was superior than that of the tested EO, further examination on the xanthine oxidase inhibition of individual volatile constituents in EO should be elaborated to search for more potent volatiles in rice EO.

5.3.2. Growth Inhibitory Activities of Rice Leaf Essential Oil

The levels of growth inhibitory effects were varied among *Oryza sativa* (Koshihikari), *Echinochloa crus-galli, Bidens pilosa*, *Raphanus sativus*, and *Lactuca sativa*. Table 10 showed that the EO reduced the shoot and root elongation of the two indicator plants *R. sativus* and *L. sativa*, and the two noxious weeds *E. crus-galli* and *B. pilosa*. The inhibition was proportional to the applied doses of EO (Table 10). In contrast, all applied doses of EO stimulated slightly (5–13%) the growth of the rice.

The IC_{50} parameter was used to compare the inhibitory levels among the examined species, of which the smaller IC_{50} value presented the higher suppressing level. Among the tested plants, the maximum inhibition was observed on the root growth of weed *E. crus-galli* (IC_{50} = 455.6 μg/mL) (Table 10). Statistically, the suppressing capacity on root growth of *B. pilosa*, *R. sativus*, and *L. sativa* was similar as their IC_{50} values were 912.5, 916.3, and 926.7 μg/mL, respectively. In general, the strongest inhibition was found on the shoot of R. sativus, followed by *B. pilosa*, *L. sativa*, and *E. crus-galli*. Their IC_{50} values were 866.2, 869.2, 908.0, and 964.3 μg/mL, respectively.

Table 10. Inhibitory effects of rice leaf essential oil on growth of *O. sativa, E. crus-galli, B. pilosa, R. sativus,* and *L. sativa*

Plant Species	**Inhibition Percentage (%)**						**IC_{50} (µg/mL)**	
	Root			**Shoot**				
	100 *	500 *	1000 *	100 *	500 *	1000 *	**Root**	**Shoot**
Oryza sativa L.	−10.0	−12.0	−3.0	−5.0	−7.0	−11.0	St **	St **
Echinochloa crus-galli	38.0	51.0	63.0	18.0	25.0	51.0	455.6 ± 11.5 b	964.3 ± 12.1 a
Bidens pilosa L.	12.0	20.0	52.0	16.0	19.0	56.0	912.5 ± 10.3 a	869.2 ± 6.1 bc
Raphanus sativus L.	4.0	21.0	60.0	7.0	20.0	60.0	916.3 ± 10.0 a	866.2 ± 26.1 c
Lactuca sativa L.	6.0	34.0	55.0	12.0	24.0	55.0	926.7 ± 11.6 a	908.0 ± 11.1 b

* Concentration of EO (µg/mL). ** Stimulation. Data are presented as means ± SD. Mean values with different lowercase letters indicate significant differences in the same column ($p < 0.05$) ($n = 3$).

5.3.3. Identification of Phytochemicals in Rice Leaf Essential Oil

Table 11. Identification of phytochemicals in essential oil of rice leaf by GC-MS, confirmation by ESI-MS and APCI-MS

Chemical Formula	**Compounds**	**Molecular weight**	**Retention time (min)**	**Peak area [% of Total]**
C_8H_8O	Coumaran	120	8.22	4.02
$C_9H_{10}O_2$	Benzyl acetate	150	9.58	2.54
$C_8H_8O_3$	Vanillin	152	10.73	8.22
$C_{11}H_{22}O_2$	Undecanoic acid	186	12.73	2.56
$C_{13}H_{18}O$	Megastigmatrienone	190	13.02	3.20
$C_{14}H_{28}O_2$	Myristic acid	228	15.00	3.26
$C_{18}H_{36}O$	2-Pentadecanone	268	15.89	2.13
$C_{10}H_{20}O_2$	Capric acid	172	16.05	2.17
$C_{16}H_{32}O_2$	Palmitic acid	256	17.13	17.34
$C_{18}H_{30}O_2$	Linolenic acid	278	18.80	11.16
$C_{19}H_{36}O_3$	Methyl ricinoleate	312	19.46	27.86
$C_{16}H_{34}$	Hexadecane	226	20.22	3.82

The GC-MS analysis had quantitatively identified twelve VOCs as they accounted for 88.28 % in the rice leaf EO (Table 11). The structure formulas of those compounds were further confirmed by ESI-MS and APCI-MS (Supplementary Materials Figures S5-S8). Based on the percent peak area of each components, methyl ricinoleate (27.86%) was the most dominant compound among volatile oil in rice leaves, followed by palmitic acid (17.34%), and linolenic acid (11.16%), while 2-pentadecanone was the least (2.13%).

5.3.4. Identification and Quantification of MA and MB from Rice Leaf Essential Oil by UPLC/ESI-MS Analysis

Table 12. Identification and quantification of MA and MB from rice leaf essential oil by UPLC/ESI-MS

Rice leaf EO	UPLC	
	MA	MB
Retention time (min)	*4.00 ± 0.04*	*2.45 ± 0.06*
LOD (ng/mL)	0.097	0.157
LOQ (ng/mL)	0.293	0.476
Yield (ng/g DW)	9.80 ± 0.22	4.93 ± 0.13
% of Total EO	7.35	3.70

FW: Fresh weigh; Data are means ± SD (standard deviation) (n = 3); MA: momilactone A; MB: momilactone B

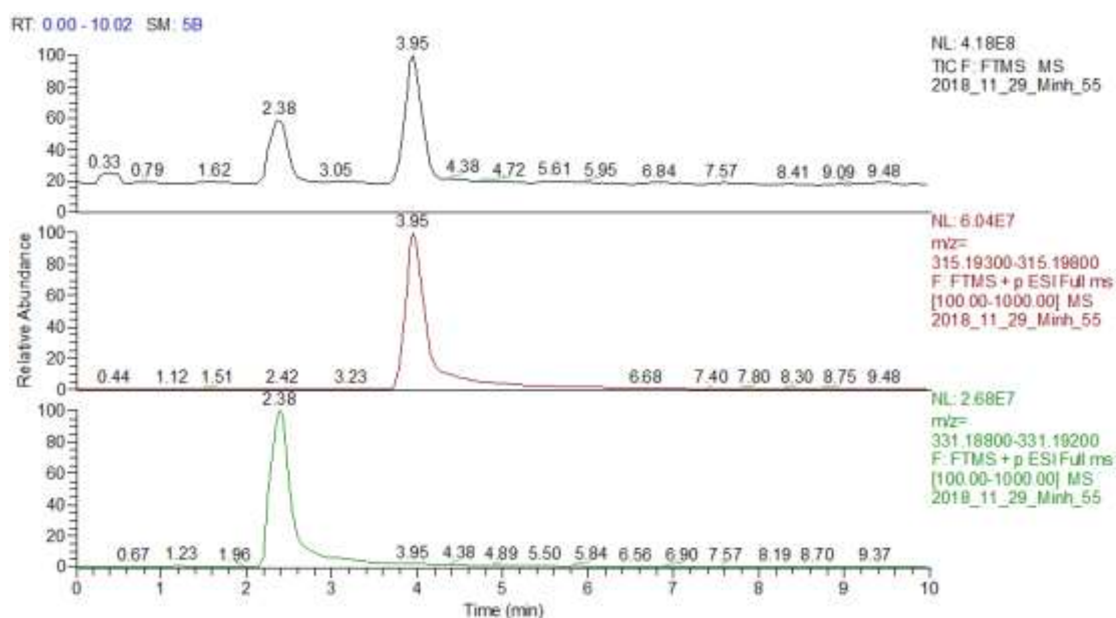


Figure 10a. UPLC/ESI-MS chromatogram of MA and MB (standard).

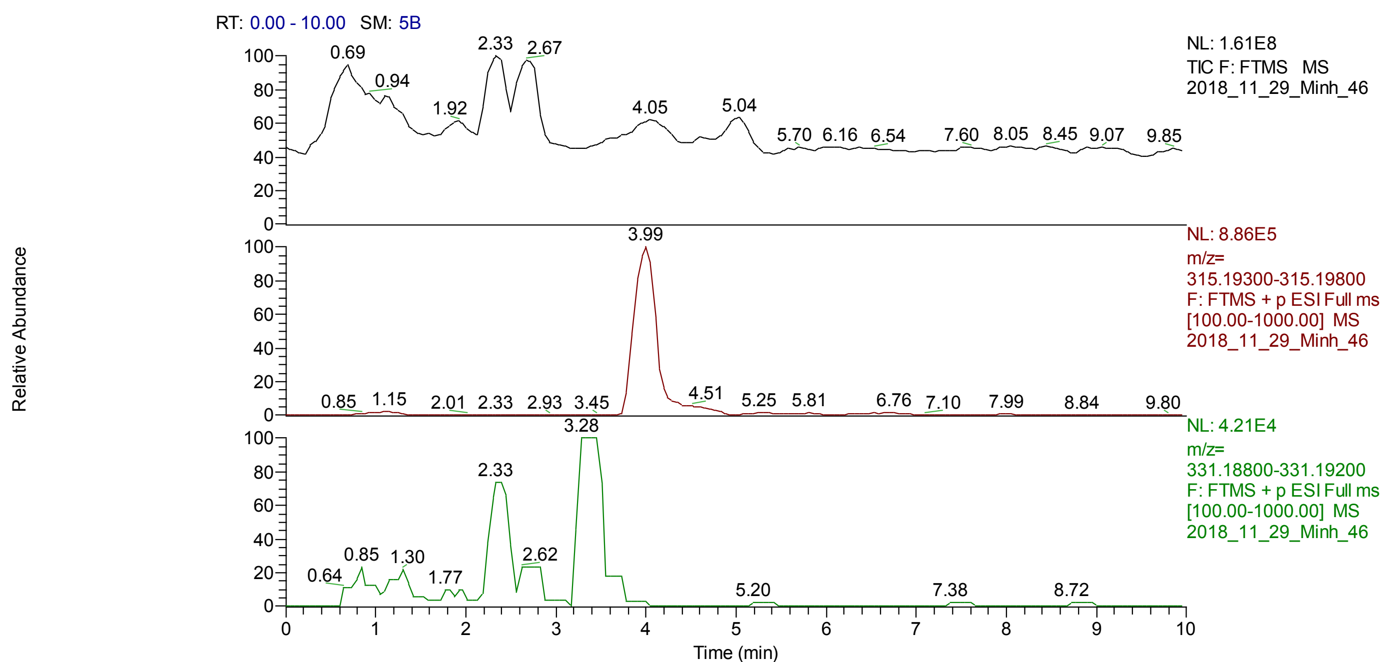


Figure 10b. UPLC/ESI-MS chromatogram of MA and MB detected in rice leaf essential oil.

The presence of two momilactones MA and MB was identified and confirmed by UPLC/ESI-MS analysis (Supplementary Materials Figures S9a, b), compared with the standard MA and MB isolated in our laboratories as described previously. From 20 mg of rice leaf EO, the amounts of MA and MB detected by this method were 9.80 and 4.93 ng/g fresh weight (FW), respectively. Furthermore, limit of detection and quantification (LOD and LOQ) parameters were also determined as 0.097, 0.293 ng/mL for MA and 0.157, 0.476 ng/mL for MB, respectively (Table 12, Figures 10a, b).

5.4. Discussion

In this study, the obtained yield of rice leaf EO was 13.33 µg/g fresh weight (FW). This yield is higher than essential oil extracted from *Tagetes erecta* L. leaves (0.72 µg/g FW) (Laosinwattana *et al.*, 2018) but lower than common EO such as *Ocimum basilicum* L. (170–2100 µg/g FW) (Tsasi *et al.*, 2017). EtOAc is the common solvent to extract essential oils from plants (Ahmad *et al.*, 2019); therefore it was used to extract the EO in our research. However, the use of room temperature (25 °C) during the evaporation might result in the yield loss of EO. The yield of EO depends on EO contents in plant tissues and method of extractions (Sefidkon *et al.*, 2006). Steam distillation is the most prevalent method of EO extraction (Božović *et al.*, 2017; Giacometti *et al.*, 2018). The use of lower temperature during

evaporation should be examined to enhance the yield of EO in rice leaves. Because rice EO showed potent biological activities as observed by this study, the breeding of new rice cultivars rich with EO may be beneficial to exploit the potent use of rice leaves.

Biological activities of the EO obtained from rice leaves and chemical components were investigated in this study by *in vitro* assays and spectroscopic analyses. Present results showed that the EO inhibited xanthine oxidase activity by 50% at 526 µg/mL. Additionally, the antioxidant property of the EO were compared with the positive standard BHT in assays DPPH, ABTS, FRAP and β-carotene oxidation (Table 9). It was found that rice leaf EO was a potent anti-hyperuricemia source and an effective scavenger of superoxidase radicals, although its potency was lower than that of the reference standards (allopurinol and BHT, respectively). Moreover, having both anti-hyperuricemia and antioxidant properties, the rice leaf EO may be well developed as a promising treatment for gout arthritis (Amic *et al*., 2017).

The inhibitory activity on growth of *O. sativa*, *E. crus-galli*, *B. pilosa*, *R. sativus*, and *L. sativa* was also investigated. The reduction observed in shoot and root lengths of *E. crus-galli*, *B. pilosa*, *R. sativus*, and *L. sativa (*as compared with controls) suggested that the EO exerted toxicity effects on the growth of those tested plants. Among them, the inhibition of the EO on root elongation of *E. crus-galli* was about two-fold stronger than that of *B. pilosa*, *R. sativus*, and *L. sativa*. Interestingly, the EO obtained from rice leaves showed a slight promotion on its own growth. By treating with the EO 1000 µg/mL, the root and shoot of rice plants were increased 13% and 11%, respectively. The results indicated that the rice leaf EO might be highly applicable not only to manage the problematic weed *E. crus-galli* but also to enhance the growth of rice plants in crop production.

EOs are aromatic oily liquids obtained from plant materials. The chemical constituents of EOs are VOCs which can be classified into two major biosynthetic groups including terpenes/terpenoids and aromatic/aliphatic molecules (Bassolé *et al*., 2012). In this work, twelve VOCs of the EO from rice leaves were identified by GC-MS, ESI-MS, and APCI-MS analyses. Although methyl ricinoleate was found in relatively high concentration (27.86%) of the EO, fatty acids were the most abundant with five components accounting for 36.49%. Among identified volatile acids, palmitic acid was detected in the highest amount (17.34%). These compounds also encountered in the chemical profile of EOs originated from rice straw (Miyazawa *et al*., 2017), black and red rice bran (Sukhonthara *et al*., 2009). In literature, methyl ricinoleate was reported as an antioxidant compound which supports the

ethno-medicinal application of *Ricinus communis* seeds extracts in medicine. Additionally, this active metabolite has been considered as an important ingredient in cosmetic industry, which is used as a plasticizer, lubricant, emollients and in skin conditioning or as a fragrance (Oloyede *et al*., 2012). The compound palmitic acid was previously reported to attribute to antioxidative and antibacterial properties of *Labisia pumia* Benth leaf (Karimi *et al*., 2015). As a consequence, these substances might be contributing to the observed antioxidant activity of the rice leaf EO.

Momilactones A and B have been known as phytoalexins and allelochemicals found in rice and the moss *Hypnum plumaeforme* (Kato-Noguchi *et al*., 2011; Xuan *et al*., 2016). Due to the fact that isolation and purification of the two phytoalexins are laborious and complicated, only few laboratories worldwide have worked with momilactones A and B so far (Minh *et al*., 2018a, b). Therefore, those compounds might not be detectable by common analytical instruments such as GC-MS, ESI-MS and APIC-MS methods. Because the boiling points of momilactones A and B were 460.1 °C and 504.5 °C, respectively, which exceeded the limited operating temperature of GC (300 °C) that could be detected by the GC-MS. As a consequence, ionizing electrons were not achieved, and fragments could not be produced by molecular ion. By contrast, the replacement of those common spectrometric techniques by UPLC/ESI-MS analysis led to successfully confirming the presence of momilactones A and B in EO of rice leaves. It can be explained by the separation and quantification in UPLC coupled with ESI was performed under very high pressure (up to 15,000 psi, or double as compared with HPLC) in the mobile phase and column design of small material particle size (1.7 μm) in the stationary phase. This analytical technique might be helpful to acquire better resolution, speed, and sensitivity in the analytical process. The presence of momilactones A and B may be responsible for the inhibitory effects of the rice leaf EO on the growth of examined species in this study including *L. sativa*, *R. sativus*, and the two noxious weeds *E. crus-galli* and *B. pilosa*, although they presented in low quantities (9.80 and 4.93 ng/g fresh weight, respectively) (Table 12). This finding was in agreement with previous reports, which documented that amounts of MA in rice plant parts and root exudates was generally greater than MB (Kato-Noguchi *et al*., 2011).

5.5. Conclusions

Findings of this study not only revealed the chemical profile but also revealed the potential antioxidant, anti-hyperuricemia, and plant growth inhibitory activities of EO from rice leaves.

There were twelve volatile constituents identified, of which methyl ricinoleate (27.86%) and palmitic acid (17.34%) were the principal compounds. This study was the first to successfully identify the presence of momilactones A and B by ultra-performance liquid chromatography coupled with electrospray mass spectrometry (UPLC/ESI-MS), suggesting that they may be involved in plant growth inhibition as well as other biological activities of rice EO. Because rice EO exhibited promising inhibition on the two noxious weeds of *E. crus-galli* anb *B. pilosa*, further trials on examining individual volatile compounds as well as momilactones A and B on the two weed species should be further elaborated. It was observed that the EO of rice leaf was beneficial as a source of antioxidants and a reduction of gout disease, and thus provided extra benefits for rice farmers in developing countries. However, the use of lower temperatures during evaporation should be further conducted to enhance the actual yield of EO in rice leaves.

5.6. Supplementary Data

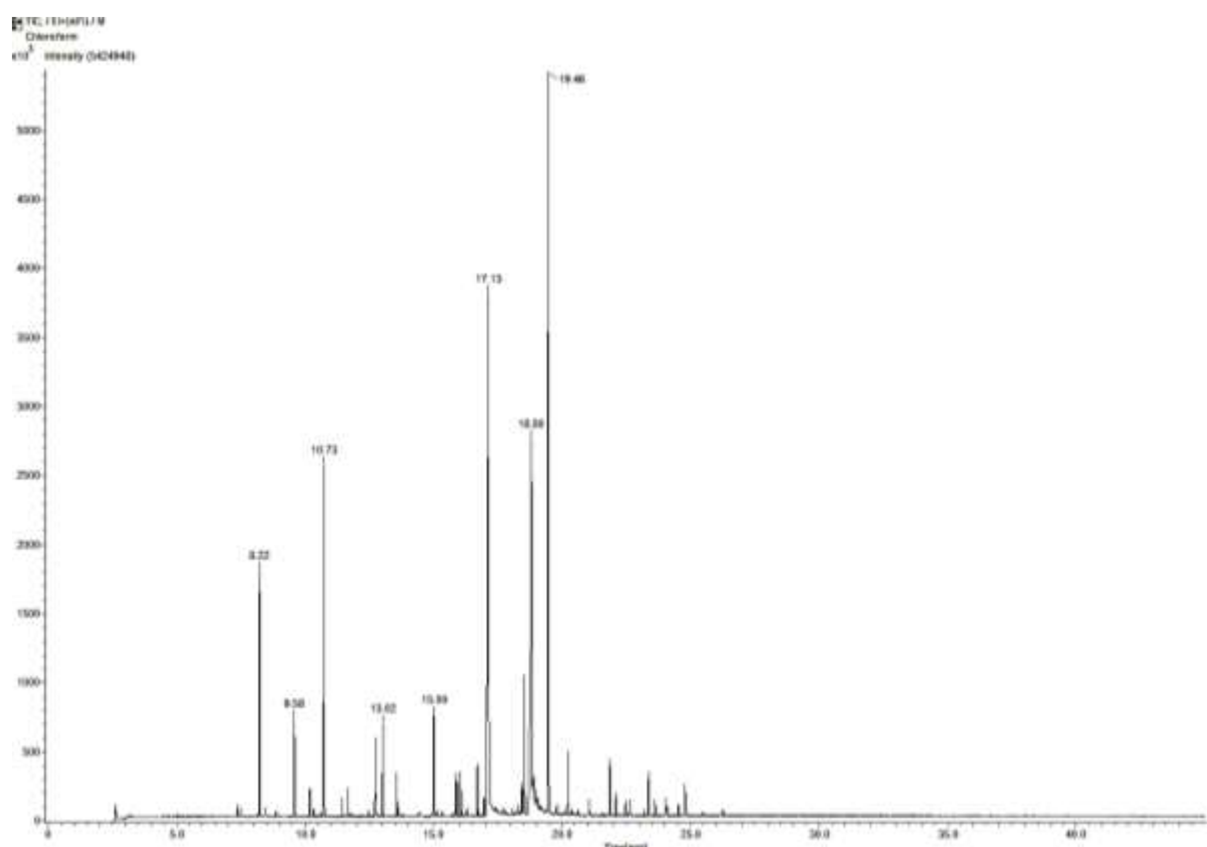


Figure S5. GC-MS chromatogram of rice leaf essential oil.

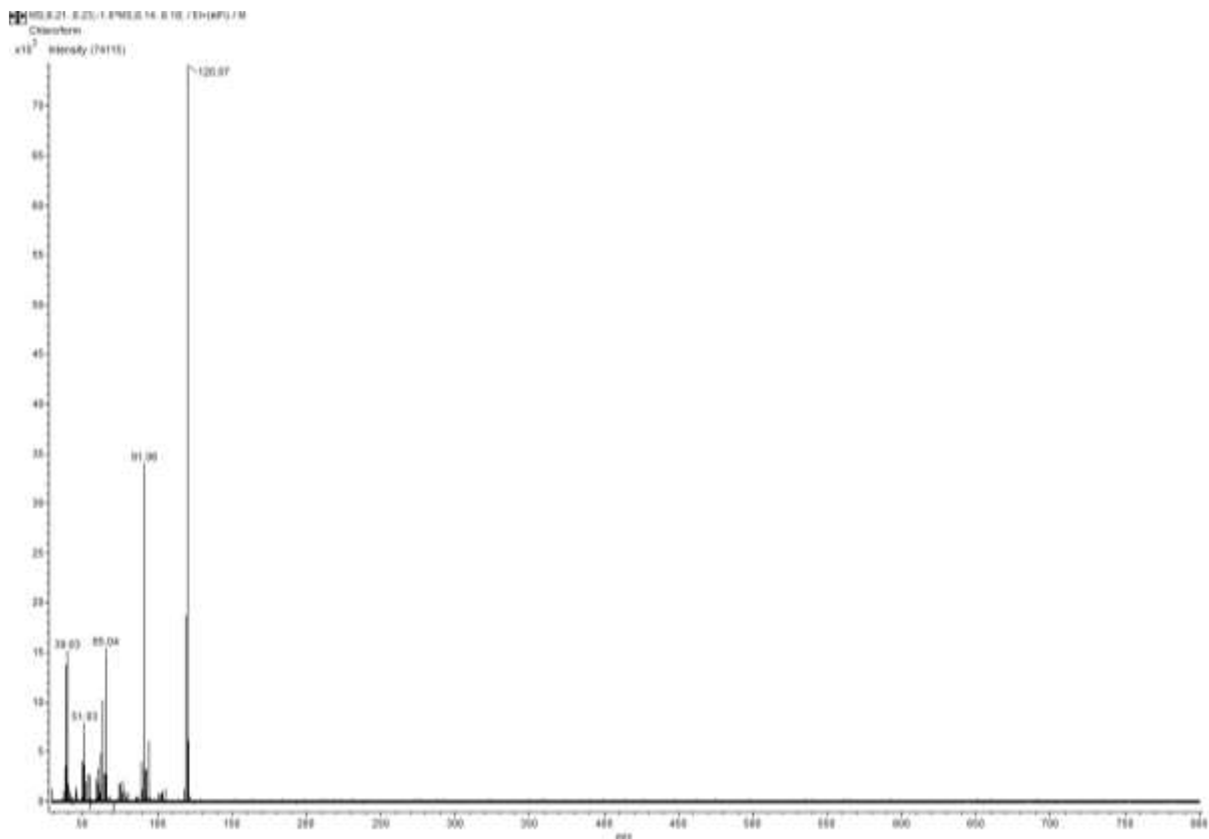


Figure S6a. GC-MS spectrum of rice leaf essential oil.

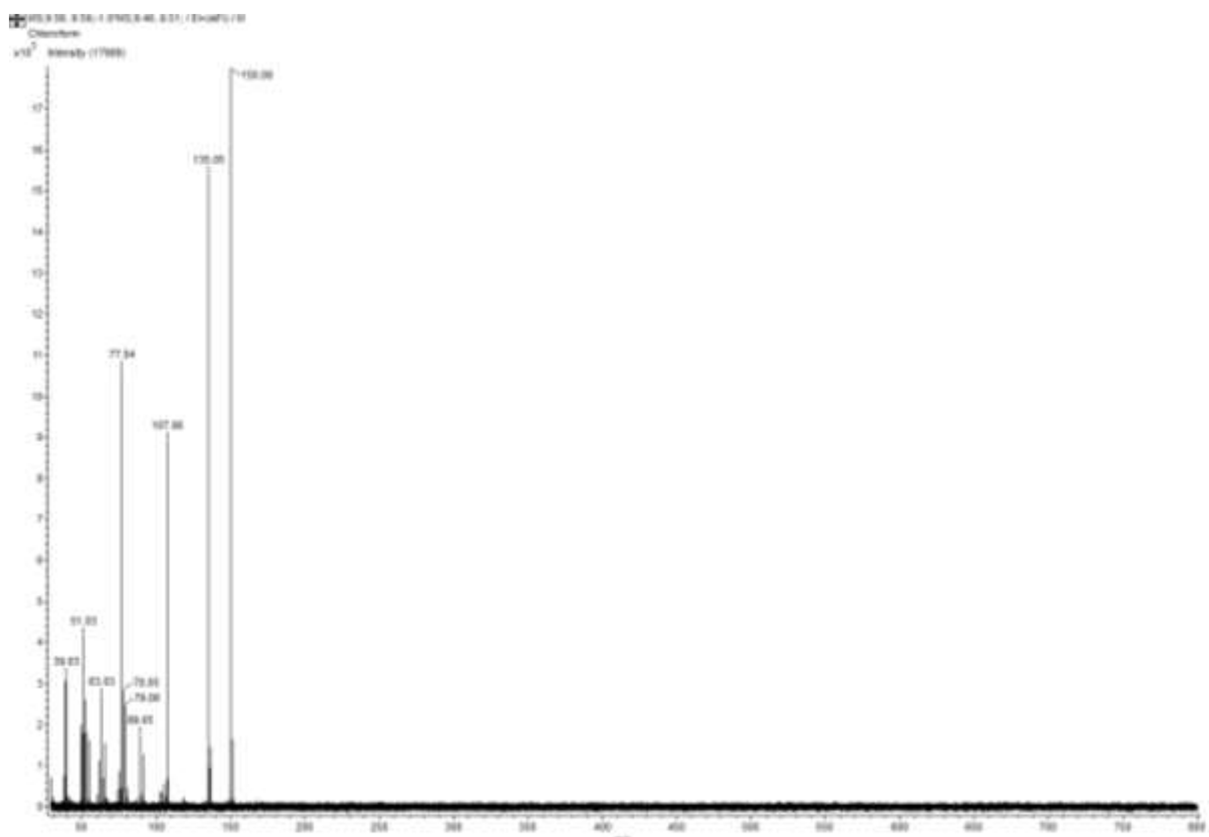


Figure S6b. GC-MS spectrum of rice leaf essential oil.

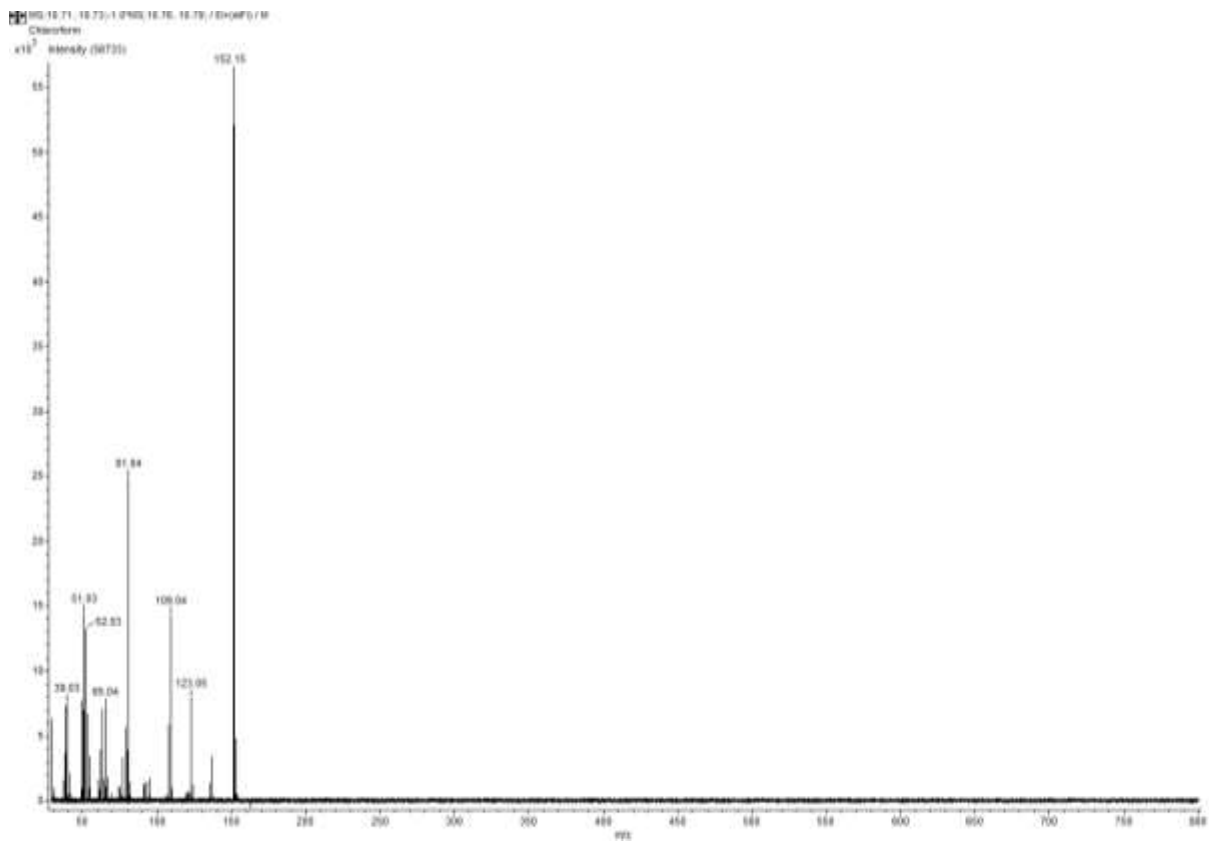


Figure S6c. GC-MS spectrum of rice leaf essential oil.

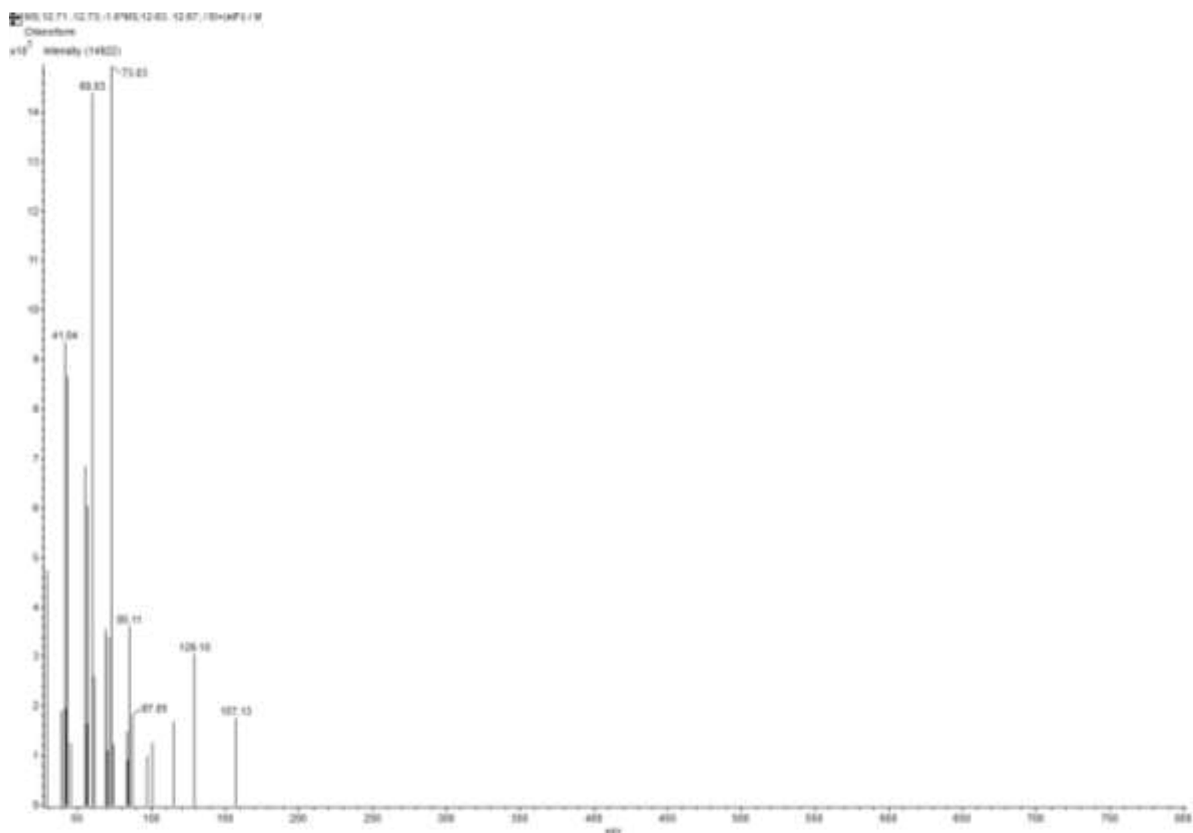


Figure S6d. GC-MS spectrum of rice leaf essential oil.

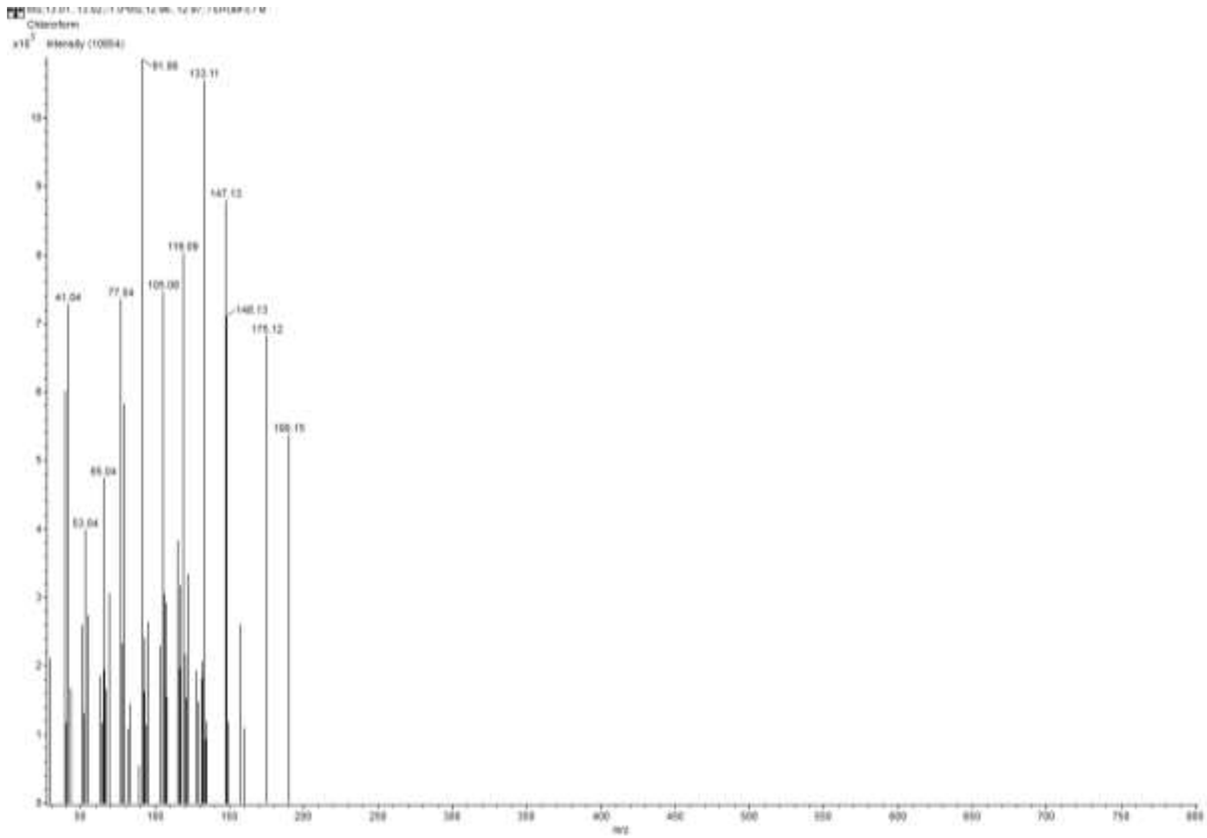


Figure S6e. GC-MS spectrum of rice leaf essential oil.

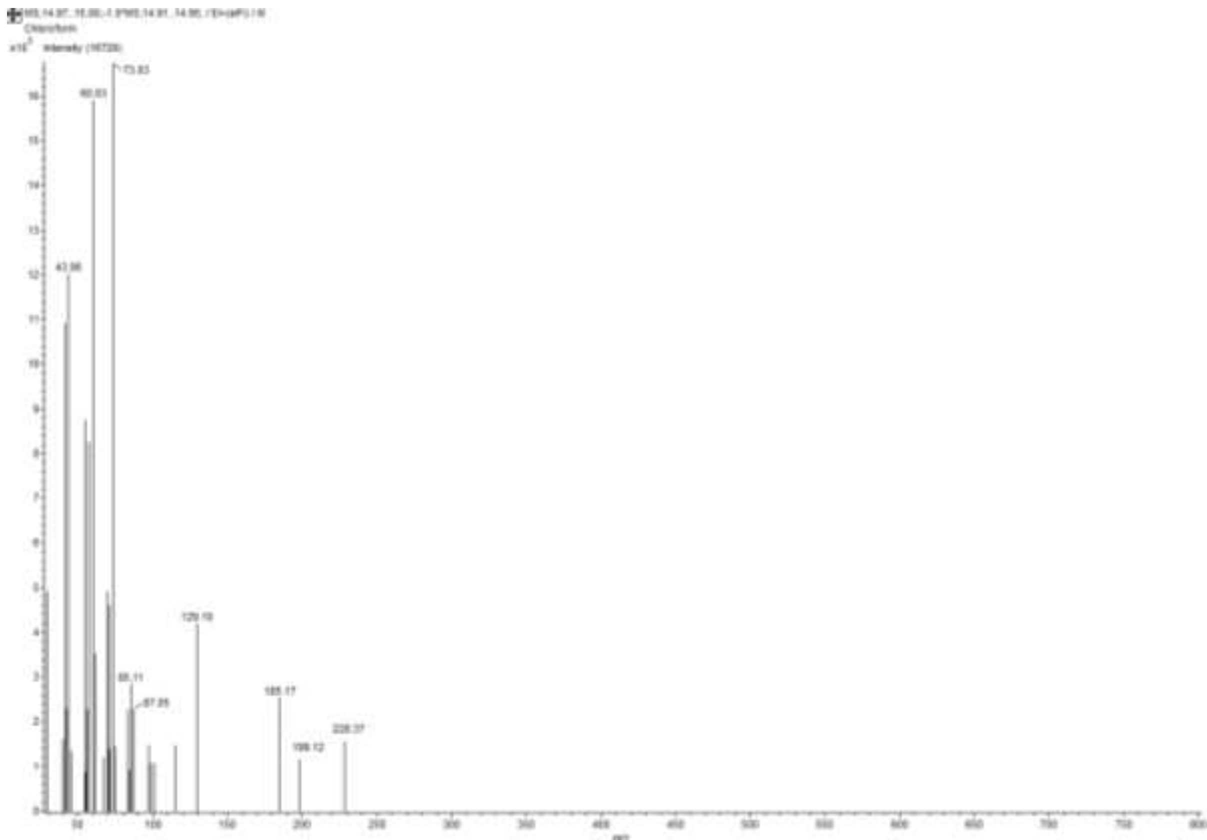


Figure S6f. GC-MS spectrum of rice leaf essential oil.

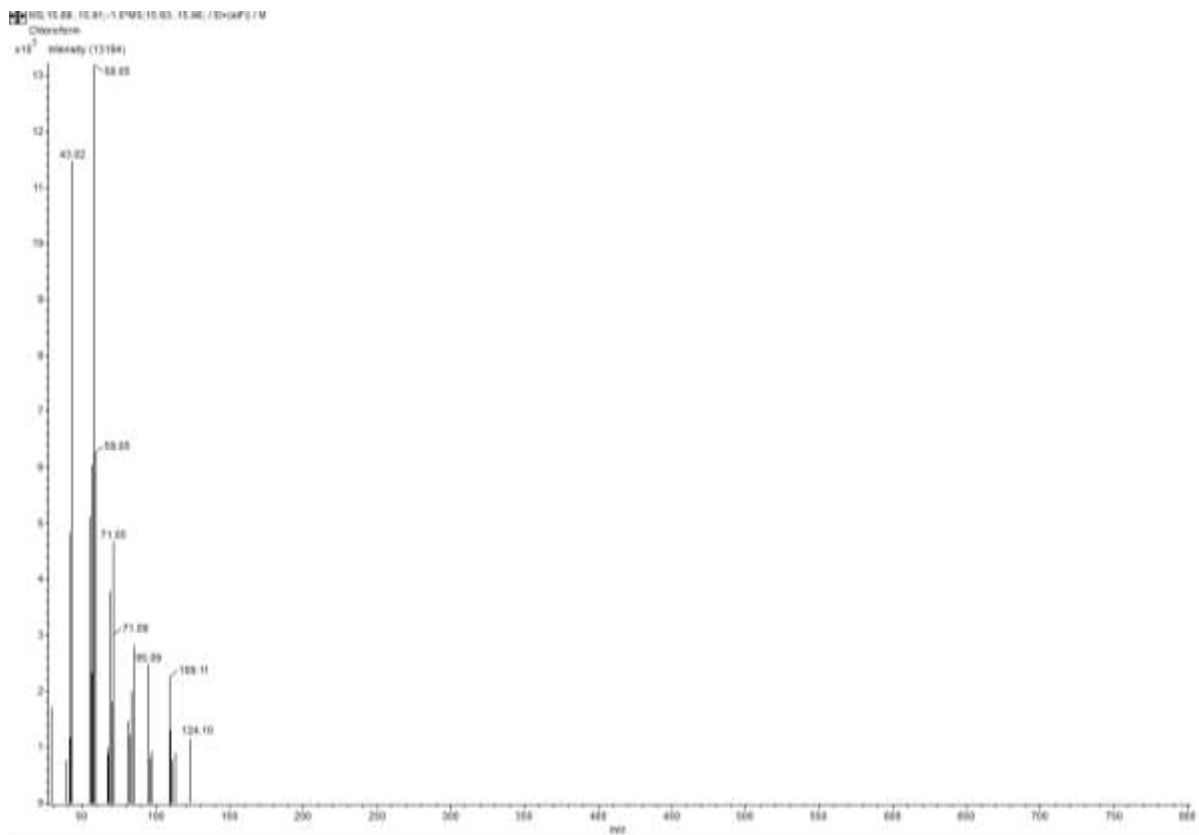


Figure S6g. GC-MS spectrum of rice leaf essential oil.

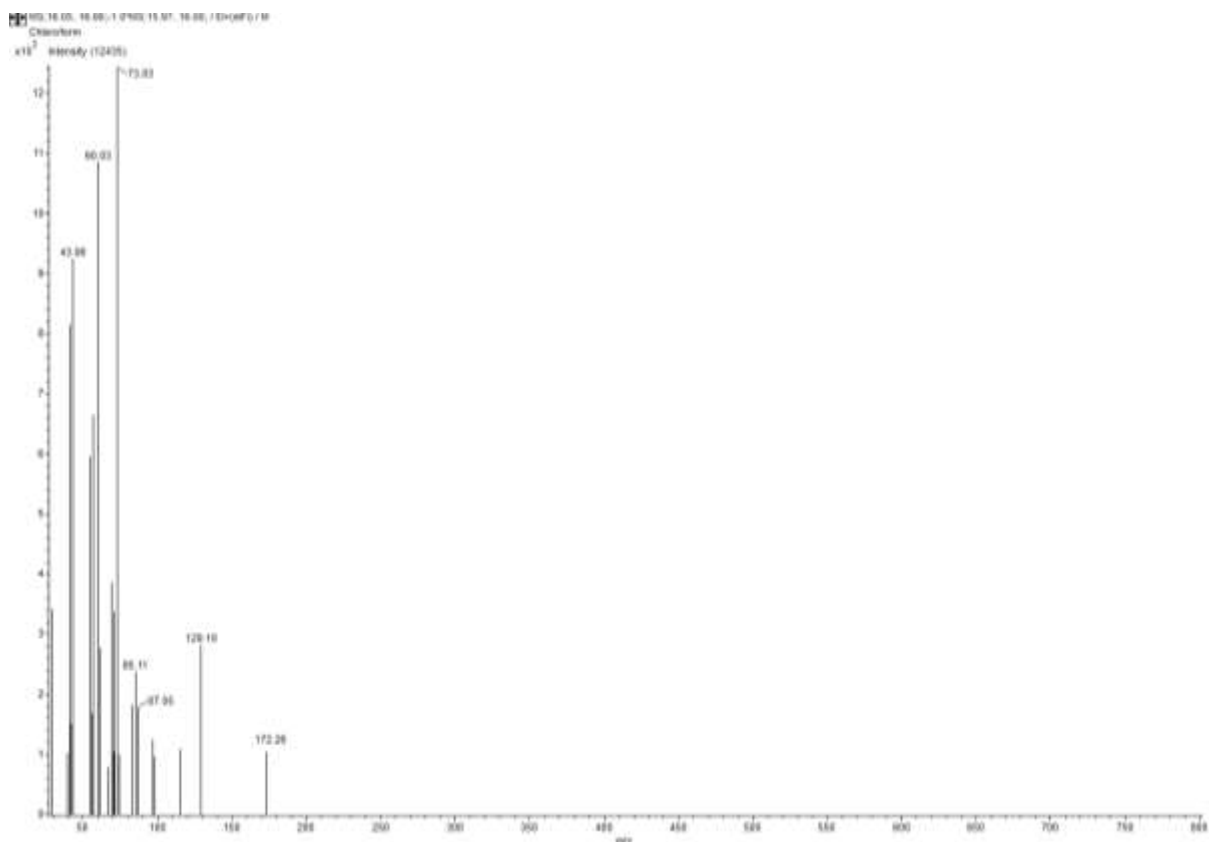


Figure S6h. GC-MS spectrum of rice leaf essential oil.

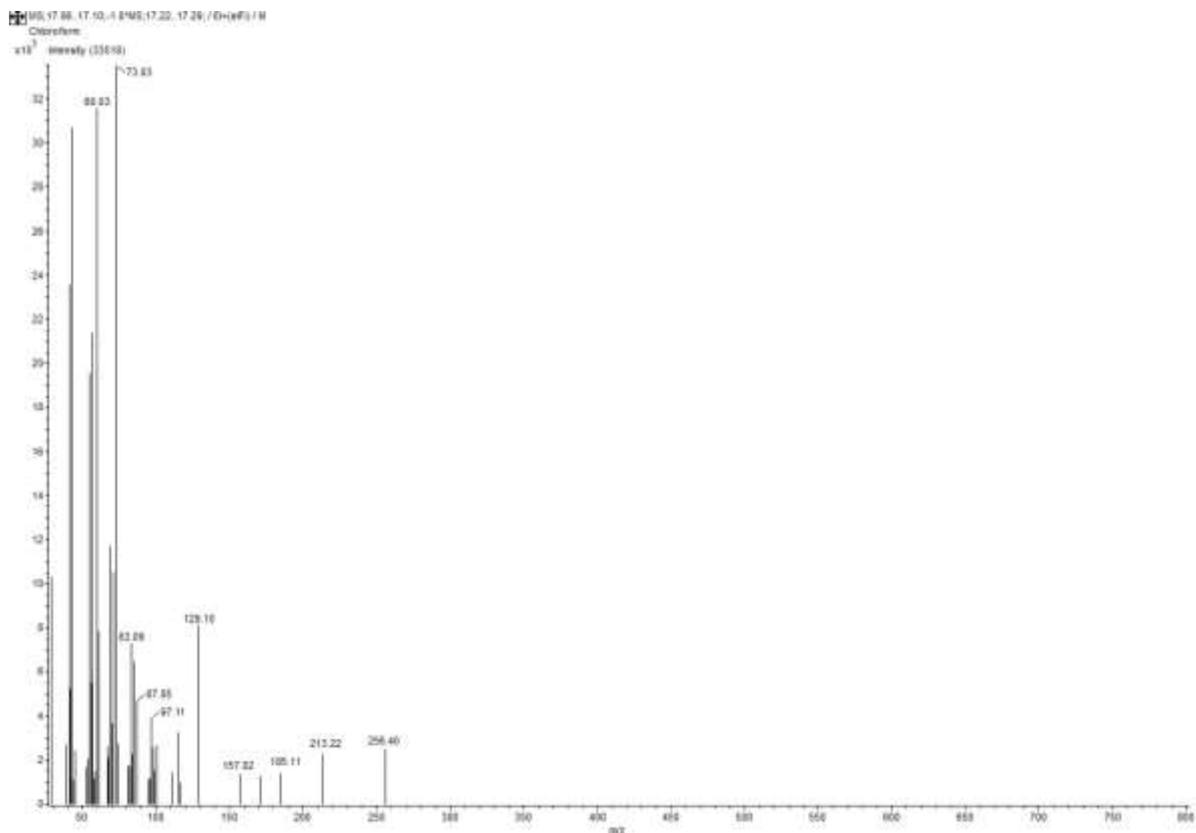


Figure S6i. GC-MS spectrum of rice leaf essential oil.

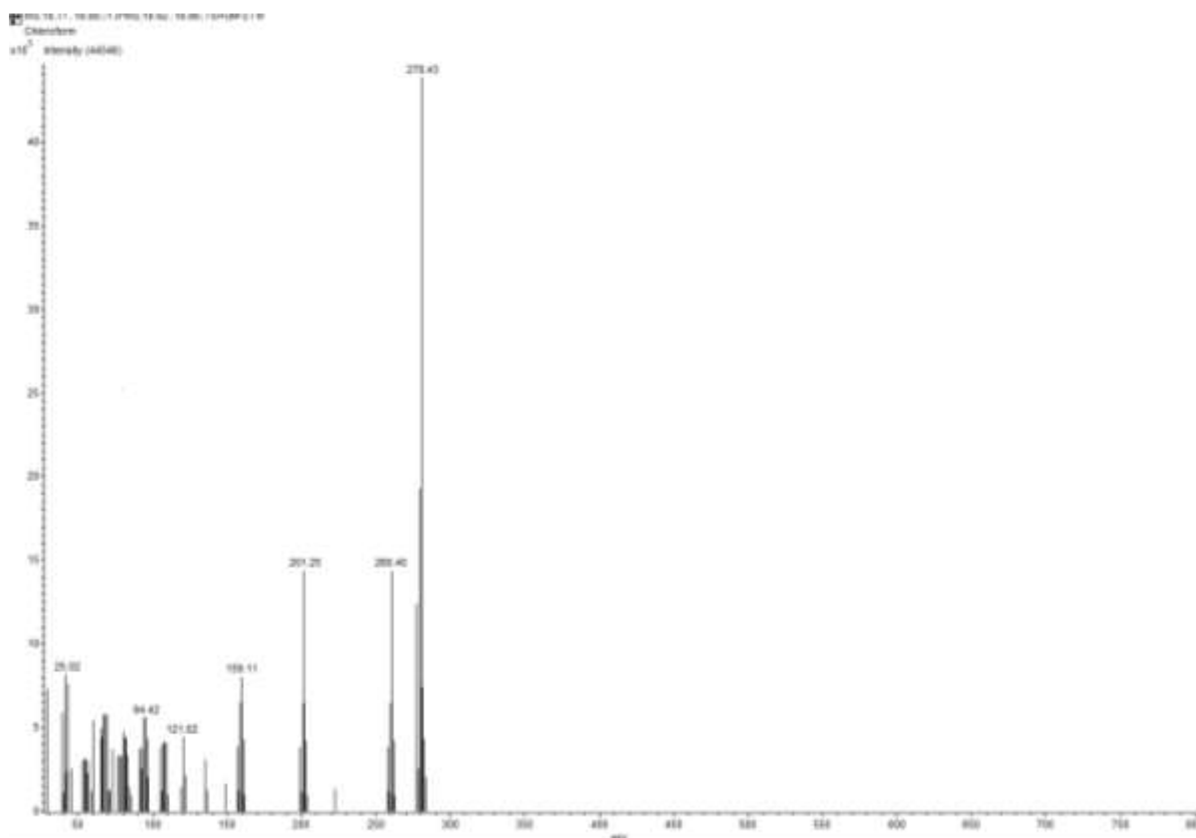


Figure S6k. GC-MS spectrum of rice leaf essential oil.

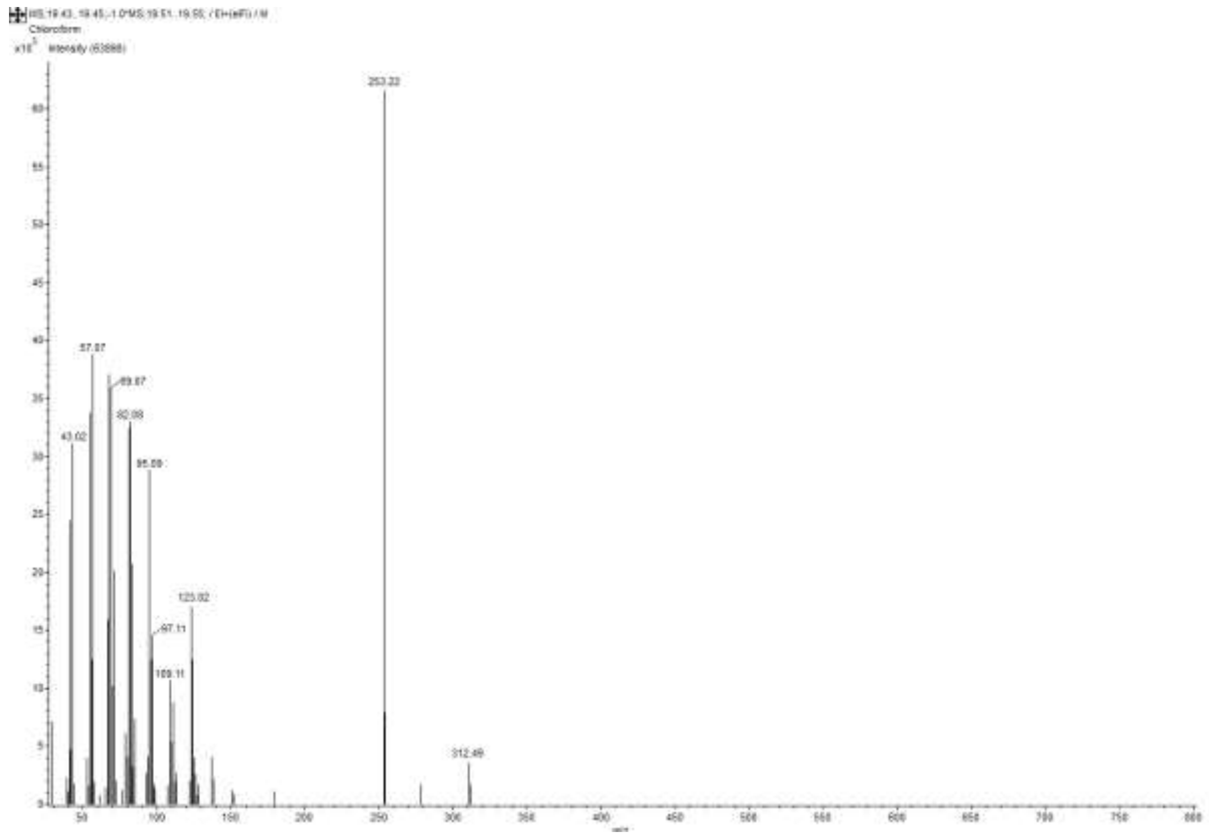


Figure S6l. GC-MS spectrum of rice leaf essential oil.

Figure S6m. GC-MS spectrum of rice leaf essential oil.

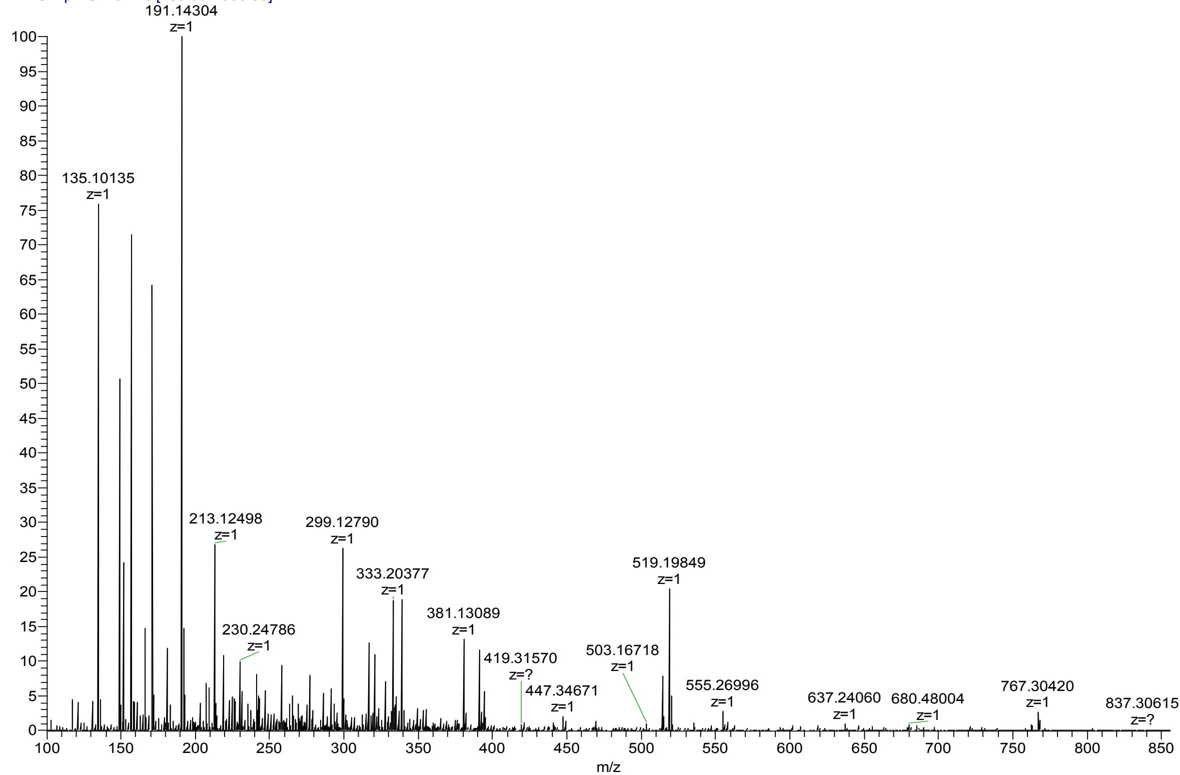


Figure S7a. ESI-MS spectrum of rice leaf essential oil.

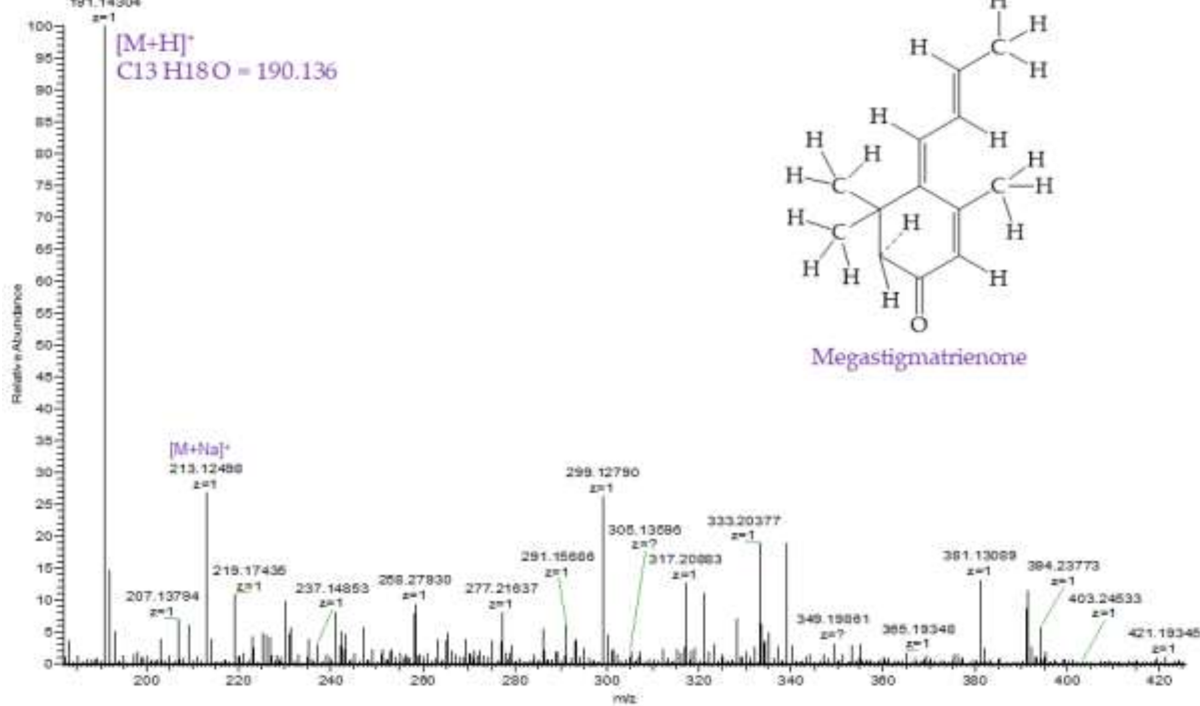


Figure S7b. ESI-MS spectrum of rice leaf essential oil.

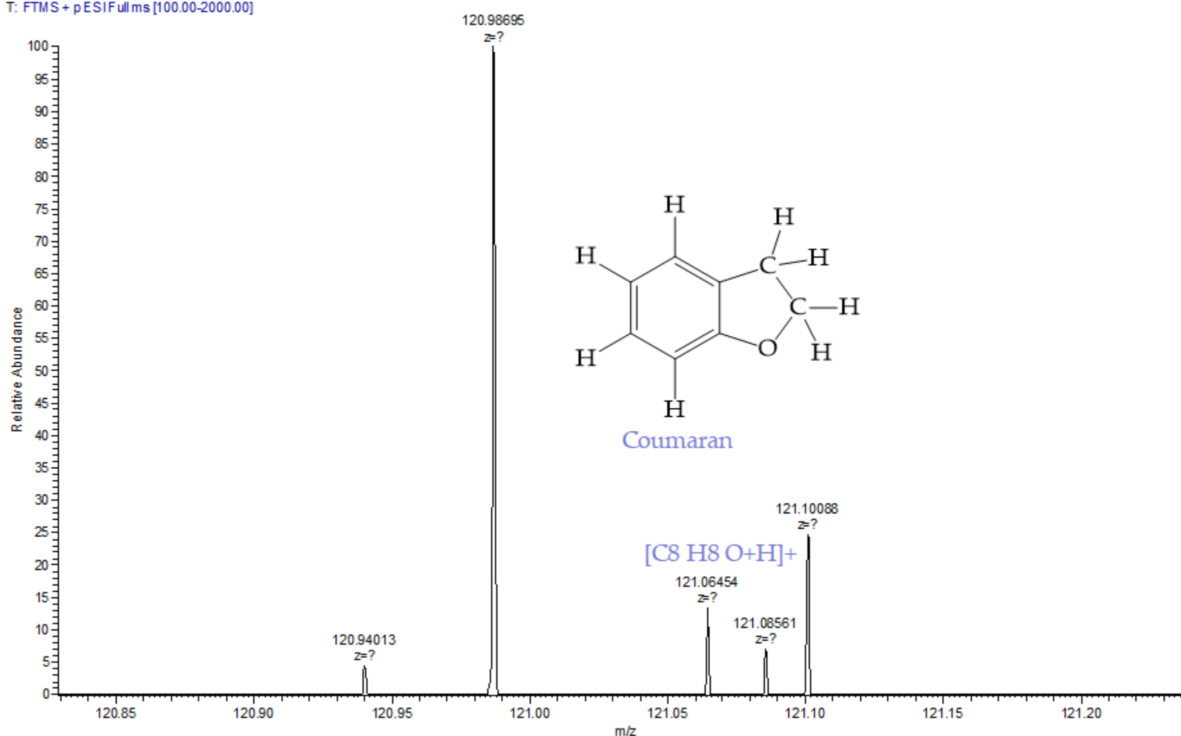


Figure S7c. ESI-MS spectrum of rice leaf essential oil.

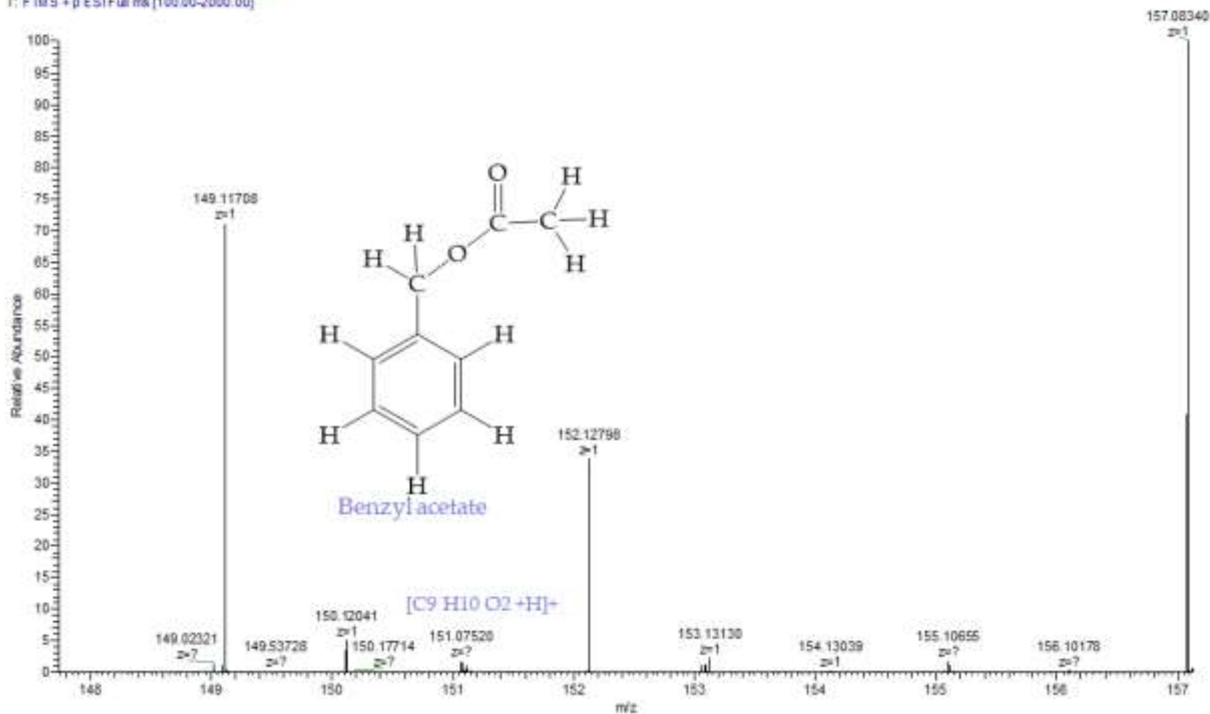


Figure S7d. ESI-MS spectrum of rice leaf essential oil.

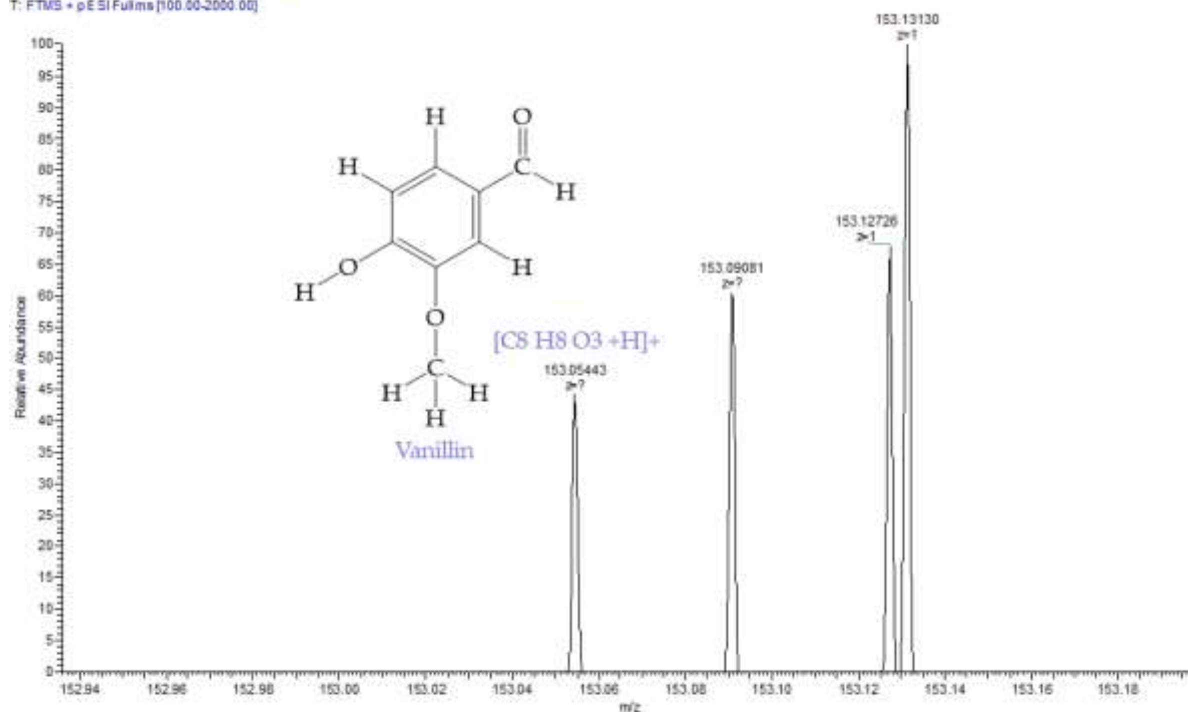


Figure S7e. ESI-MS spectrum of rice leaf essential oil.

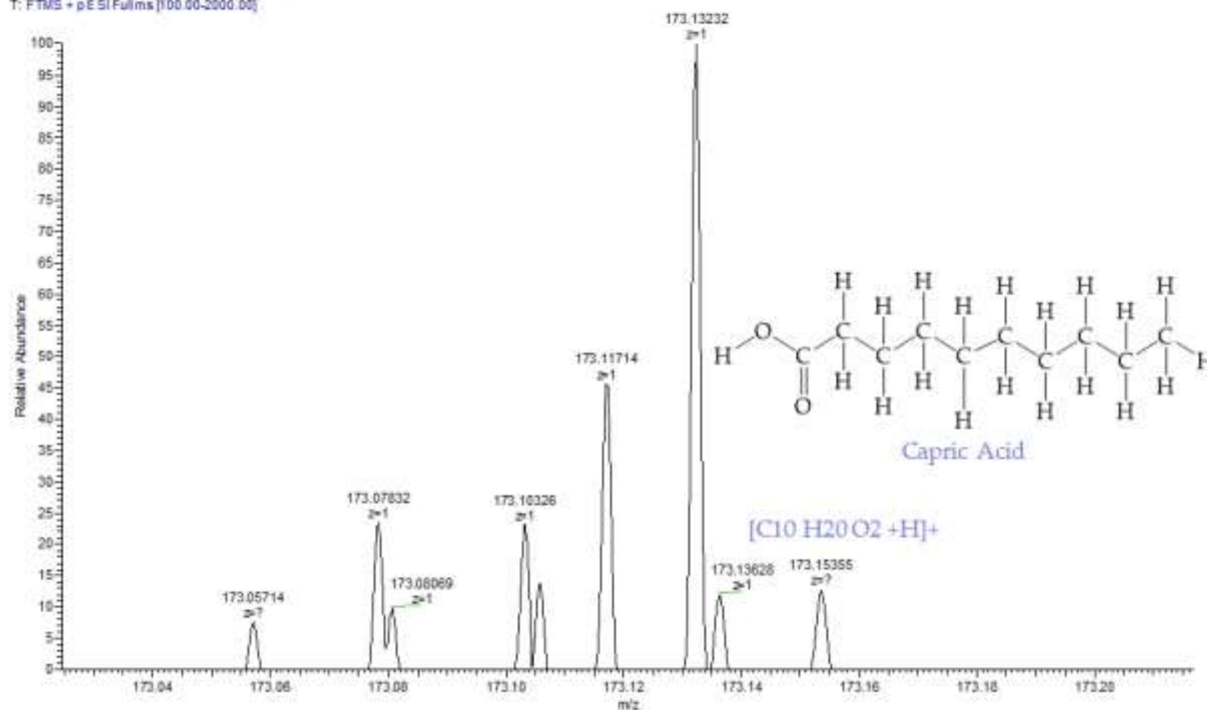


Figure S7f. ESI-MS spectrum of rice leaf essential oil.

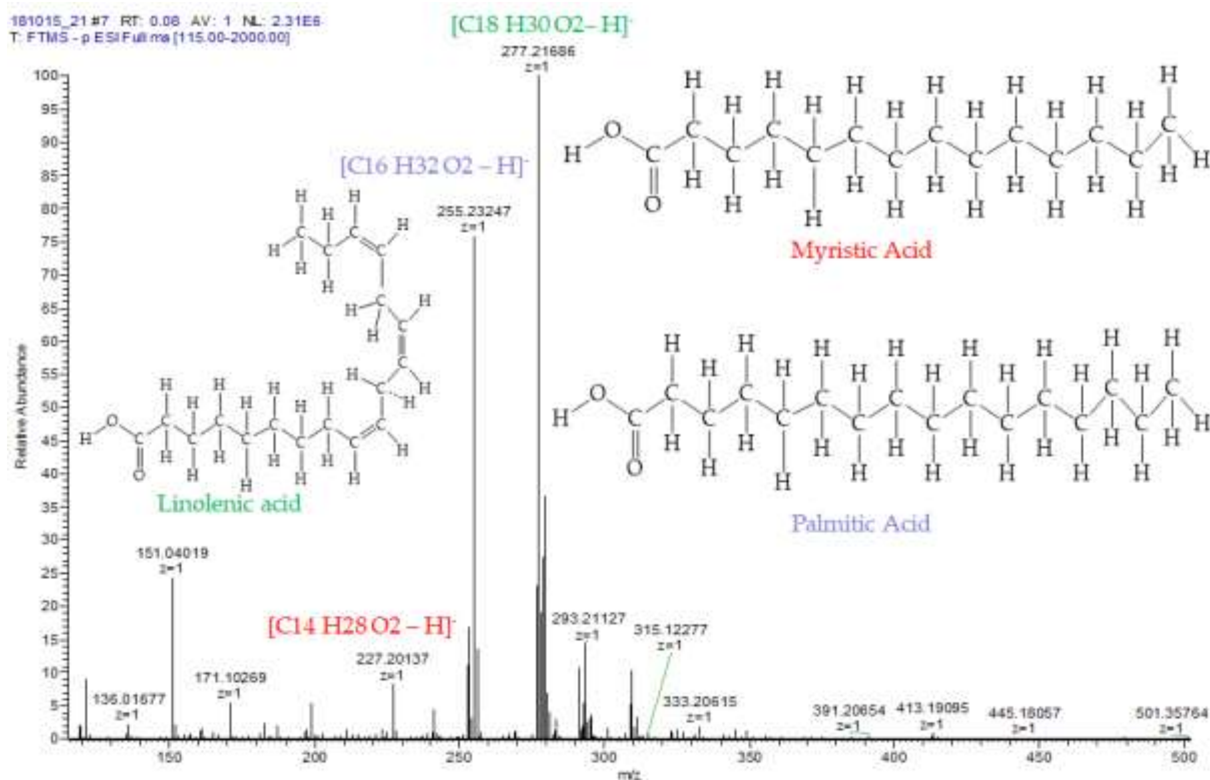


Figure S7g. ESI-MS spectrum of rice leaf essential oil.

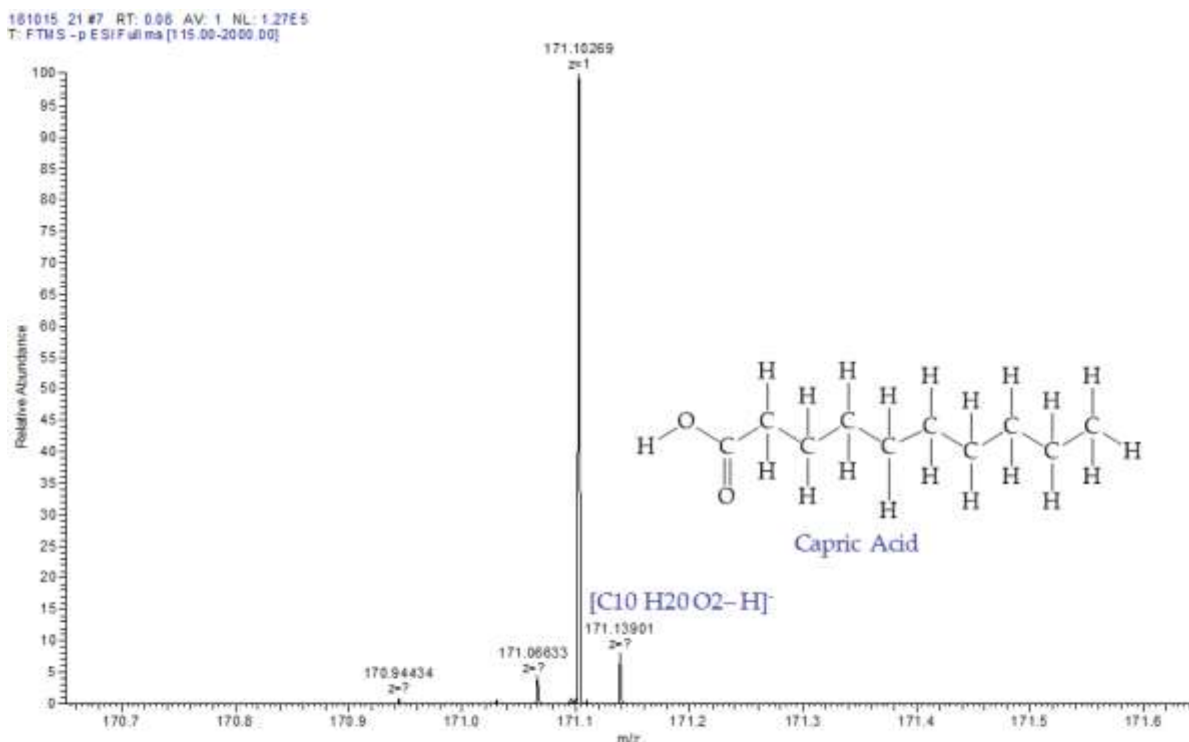


Figure S7h. ESI-MS spectrum of rice leaf essential oil.

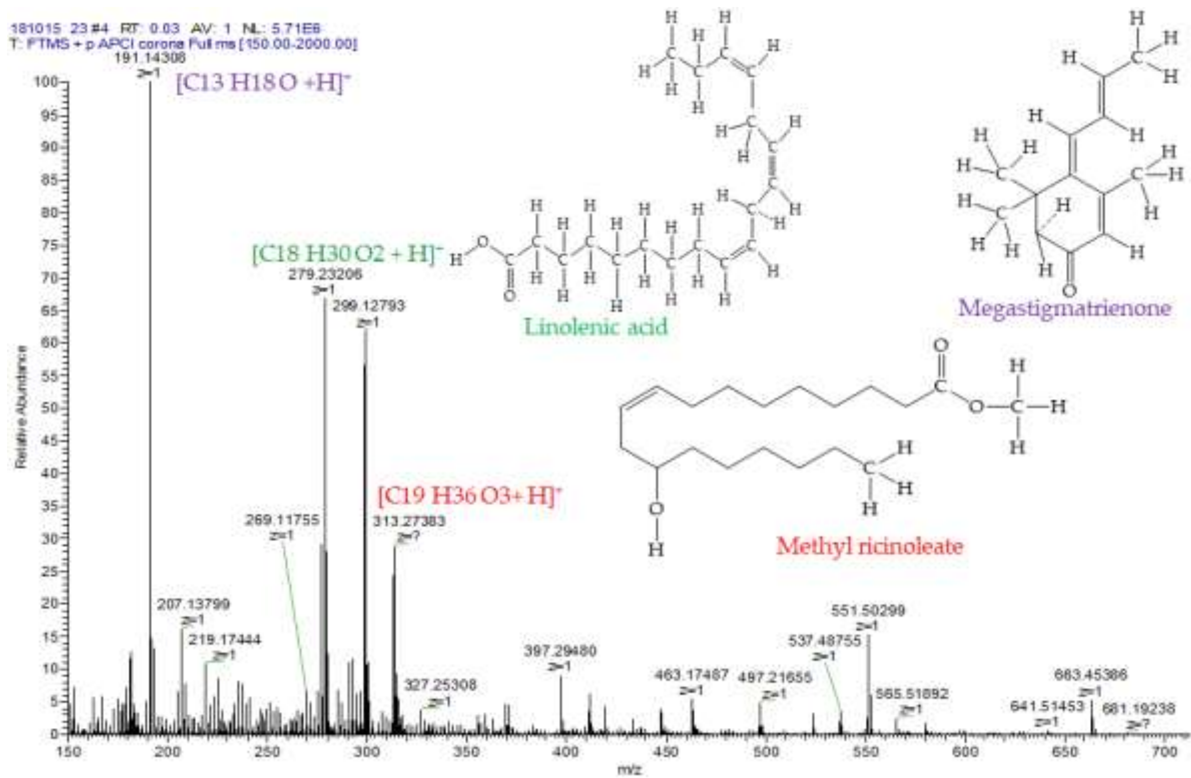


Figure S8. APCI-MS spectrum of rice leaf essential oil.

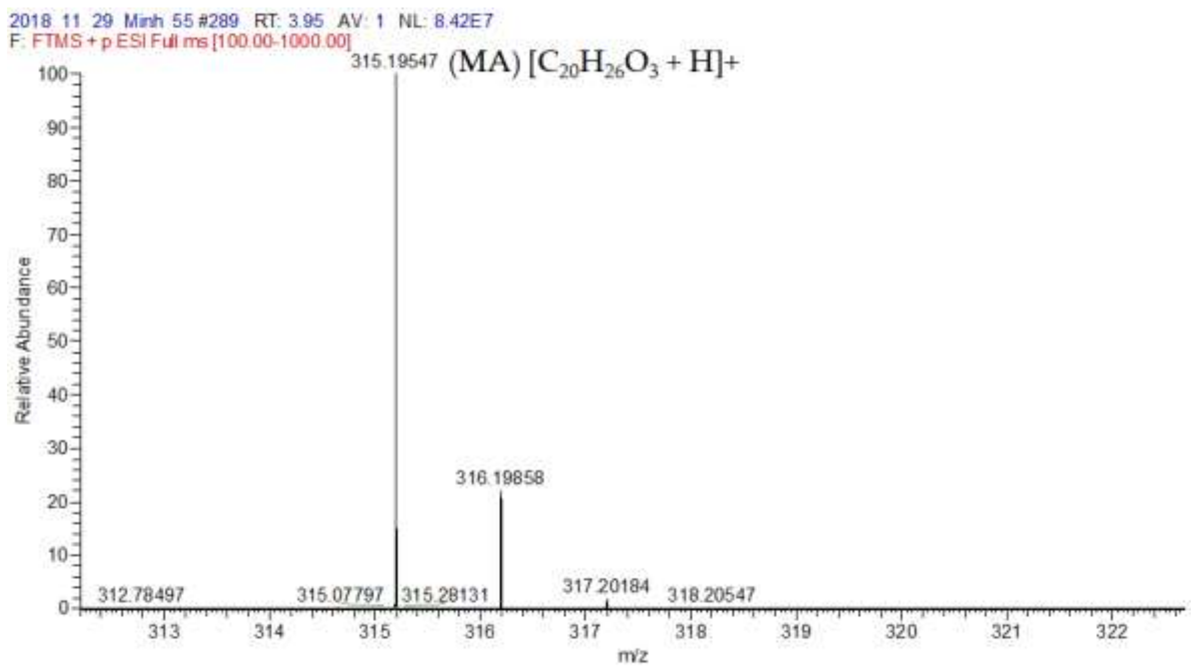


Figure S9a. UPLC/ESI-MS spectrum of MA (standard).

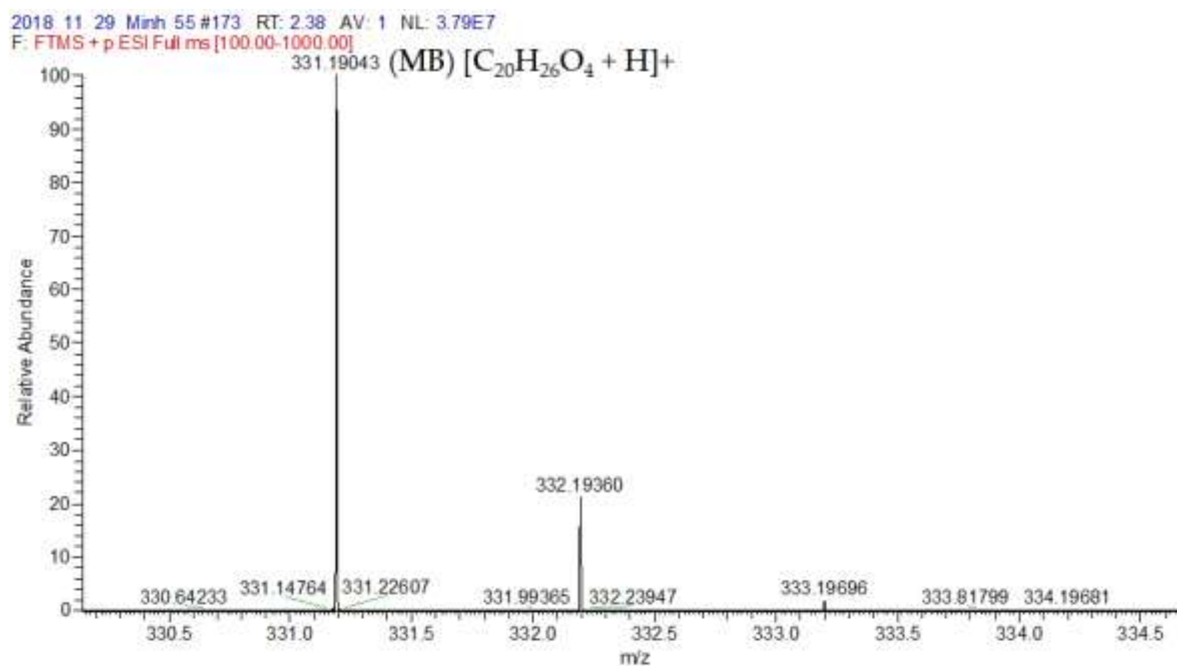


Figure S9b. UPLC/ESI-MS spectrum of MB (standard).

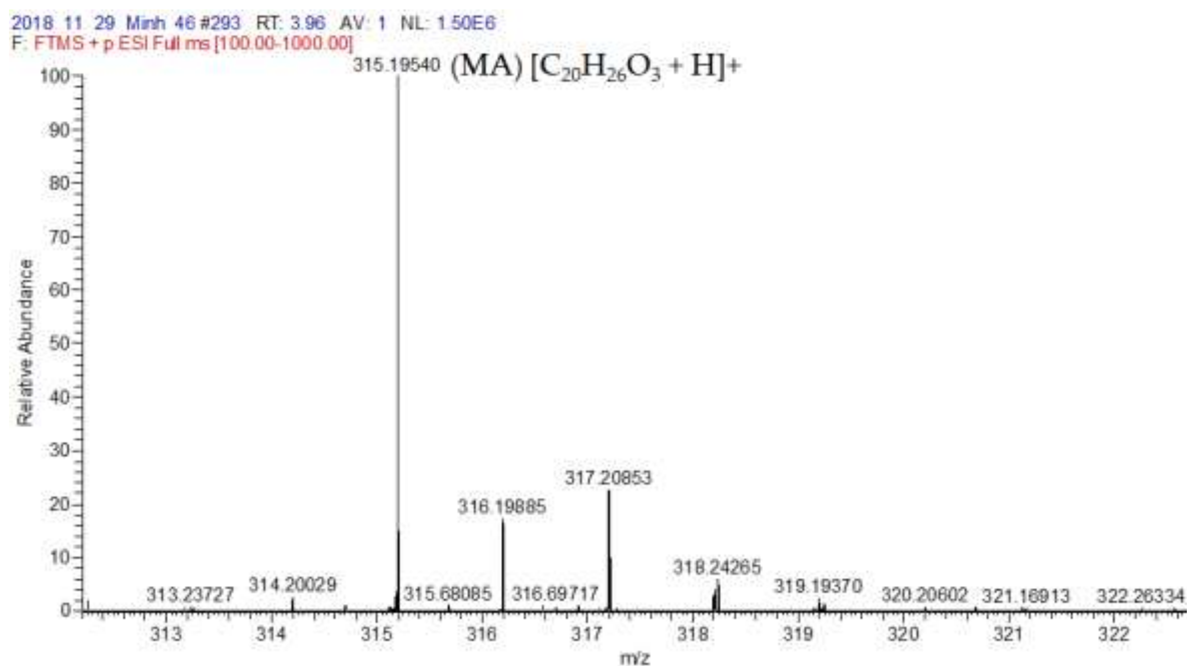


Figure S10a. UPLC/ESI-MS spectrum of MA detected in rice leaf essential oil.

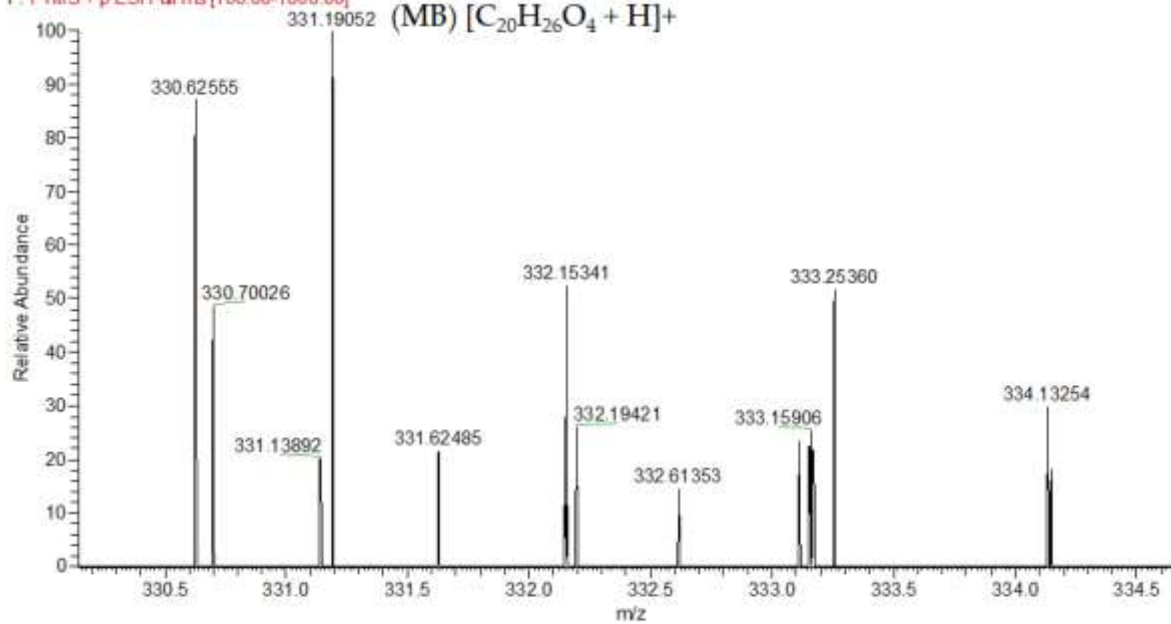


Figure S10b. UPLC/ESI-MS spectrum of MB detected in rice leaf essential oil.

CHAPTER VI.
ENVIRONMENTAL INTERACTIONS OF MOMILACTONES AND PHYTOCHEMICALS BETWEEN RICE AND WEEDS IN FIELDS

6.1. Introduction

A plant may interfere with its neighbouring plant growth directly through competition or allelopathy (Burgess *et al*., 1998; Jackson *et al*., 2000; Caldwell *et al*., 1998). Allelopathy is an interference mechanism in which a living or dead plant releases allelochemicals exerting an effect on the associated plants, which plays an important role in natural and managed ecosystems (Aiyaz *et al*., 2015; Alsaadawi *et al*., 2001). In-depth understanding of the mechanisms of allelopathic interference in cropping systems can develop new, environmentally safe strategies for sustainable agriculture (Sutherland *et al*., 2004; Ward *et al*., 2008; Rasmussen *et al*., 2000).

Rice is one of the principal food crops in the world (Nagato *et al*., 1957). A range of secondary metabolites, such as phenolic acids, flavonoids and momilactones have been identified to be potent allelochemicals from rice root exudates (Hartmann *et al*., 2005). Allelopathic interference of rice with weeds may be demonstrated by plants in the rice fields, but which allelochemicals are predominantly involved in rice allelopathy emit signals and the interactions of plant originated chemicals remain obscure (Mattice *et al*., 2001).

Weed interference causes serious loss in agricultural production. Weeds reduce crop yield by 5% in the most highly developed countries, 10% in the less developed countries and 25% in the least developed countries (Akobundu 1987). Weeds compete with cultivated crops for growth factors (water, light, nutrients and spaces), and harbour pests and plant pathogens (Qasem and Foy 2001). In developing countries, urbanization decreases labour force in the agricultural sector and farmers also tend to spend more time outside agricultural work to earn extra money. Thus, the application of pesticides and herbicides has increased rapidly. The overuse of synthetic agrochemicals for pest and weed control has increased environmental pollution, unsafe agricultural products and human health concerns (Xuan *et al*. 2004). In Japan, *Monochoria vaginalis* and *Eleocharis* are the most common noxious weeds. *Monochoria vaginalis* and *Eleocharis* have damage to farmer for about 12% losses of rice

grain yield from paddy fields (Chisaka 1966). It often remains in the field, even after one shot herbicide applications, which were developed to be effective against annual and perennial paddy weeds. A biotype resistant to sulfonylurea herbicides has been reported (Koarai & Morita 2002).

The objectives of this study were thus to clarify the allelochemicals released by plants to field wet soil and how the production of these allelochemicals changed by the interference of neighboring plants, with an attempt to explain how allelopathic rice interferes with weed in paddy.

6.2. Materials and Methods

6.2.1. Material

Rice plant samples and wet soil were collected from 5 type of rice paddy fields closed to Hiroshima University, Higashi Hiroshima City, Hiroshima Prefecture, Japan, in July 2017 (Table 13). The samples were kept in the sealed nylon bags and sterilized at Laboratory of Plant Physiology and Biochemistry, Hiroshima University, for further analyses.

Table 13. Characteristics of paddy field samples

Sample	Characteristics
Field 1	*Oryza sativa* (Koshihikari) + Herbicide application
Field 2	*Oryza sativa* (Koshihikari) + *Monochoria vaginalis*
Field 3	*Monochoria vaginalis*
Field 4	*Monochoria vaginalis* + *Eleocharis*
Field 5	Abandoned field/ empty field

6.2.2. Preparation of Extract

The powder of rice plant and fresh wet soil was immersed in methanol for seven days at room teperature. After filtration, the filtrate was then evaporated under vacuum at 45 ^{o}C using a rotary evaporator (SB-350-EYELA, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to obtain MeOH crude extract. This crude extract was then subsequently extracted with hexane, ethyl acetate, and water.

6.2.3. Determination of Total Phenolic Contents

The total phenolic contents of samples were measured by the Folin Ciocalteu (FC) reagent following a previously reported method (Minh *et al*., 2016) with some modifications. Briefly, a volume of 20 µL of either samples solution (1.0 mg/mL) or gallic acid standard solution (5-25 µg/mL) was pipetted into separate wells of a 96-well microplate. Then a volume of 100 µL of the FC reagent (10% v/v in MeOH) was added to each well, thoroughly mixed, and an aliquot of 80 µL sodium carbonate (5% w/v in water) was then added. The reaction was carried out for 30 minutes at room temperature. The absorbance was read at 765 nm using a microplate reader (MultiskanTM Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). The total phenolic contents were expressed as mg gallic acid equivalent (GAE) per gram of extract or fraction (r^2= 0.996).

6.2.4. Determination of Total Flavonoid Contents

The total flavonoid contents were assessed by a colorimetric assay as described, with some modifications (Minh *et al*., 2017). Briefly, a volume of either 100 µL sample (1 mg/mL) or rutin standard (5-25 µg/mL) was mixed with 100 µL aluminum (III) chloride hexahydrate (2% w/v in MeOH) in a 96-well microplate. After a 15-minute incubation at room temperature, the absorbance of the reaction mixture was measured at 430 nm using a microplate reader (MultiskanTM Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). The total flavonoid contents were expressed as mg rutin equivalent (RE) per gram of extract or fraction (r^2 = 0.999).

6.2.5. Germination and Growth Bioassay

Growth suppressing potential of isolated fractions was assayed on *Oryza sativa* (var. Koshihikari), *Echinochloa crus-galli*, *Bidens pilosa* L., *Raphanus sativus* L., and *Lactuca sativa* L. seeds in an incubator (Biotron NC system, Nippon Medical & Chemical Instrument, Co. Ltd). Photoperiodic was set up at day/night 12/12 hours with temperature 25/23 °C. Each sample was diluted in methanol (MeOH) to obtain different concentrations (10, 100, 500, 1000 and 2000 µg/mL). The test solution (100 µL) was permeated filter papers lined in 96 well-plate (each well has 22 mm diameter x 18 mm height). After MeOH evaporation at room temperature, each healthy seed was placed in a well, followed by the addition of 100 µL of distilled water. Plant germination monitoring was performed every 24 hours for seven days. This bioassay was replicated ten times (n = 10). The growth parameters of radicle (root) and

hypocotyl (shoot) length were measured. Concentration in reducing 50% shoot and root lengths (IC_{50}) was also calculated Xuan *et al*. (2003).

6.2.6. Identification of Phytochemicals in Wet Soils by GC-MS

The compounds in wet soils were analyzed by GC-MS system (JMS-T100 GCV, JEOL Ltd., Tokyo, Japan) equipped with a DB-5MS capillary column, 30 m in length, 0.25 mm internal diameter, and 0.25 µm in thickness (Agilent Technologies, J & W Scientific Products, Folsom, CA, USA). The column temperature was setup initially at 50 °C without hold time, followed by an increase to 300 °C with the gradients of 10 °C/min and hold for 20 min. The temperature of injector and detector were programmed respectively at 300 °C and 320 °C, with a mass scan range 29 -800 amu. The compounds were determined by the comparison between their mass spectral fragmentation pattern with the mass spectral libraries of JEOL's GC-MS Mass Center System Version 2.65a. The compounds with the high purity were selected for further spectroscopic techniques to structure elucidation (Van *et al*., 2018).

6.2.7. Identification and Quantification of Momilactones A and B by HPLC and UPLC/ESI-MS

The HPLC system including PU-4180 RHPLC pump, LC-Net II/ADC controller, and UV-4075 UV/Vis detector (Jasco, Tokyo, Japan) equipped with a Waters Spherisorb ODS2 (10 µm, 250 mm×4.6 mm i.d.) column (Waters Cooperation, MA, USA). Mobile phase comprised 0.1% trifluoroacetic acid in 70% acetonitrile. The flow rate was adjusted to 0.4 mL/min within 30 min. The detector was set at 210 nm. The injection volume was 10 µL. Data acquisition was executed on ChromNAV software (JASCO, Tokyo, Japan). The sensitivity of HPLC system was determined and expressed as limits of detection (LOD) and limits of quantitation (LOQ) by linear regression analyses of peak areas against concentrations of individual momilactones A and B.

The characterization of major component peaks sample extracts was performed on the Waters Acquity UPLC instrument equipped with the Acquity HPLC BEH C18 1.7 µm (2.1x50 mm Column). The UPLC mobile phases were (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in acetonitrile (v/v). Isocratic elution was accomplished with a mixture of A 50% and B 50%. The flow rate was 0.3 mL/min, injection volume was 3.0 µL, and column temperature was 30 °C, with an ambient sample temperature. Mass spectral characterization was performed using a LTQ Orbitrap XL equipped with an electrospray ionization source in

positive ionization mode recording spectra between m/z 100 and 1,000. The instrumental conditions were as follows: spray voltage, 4.5 kV; sheath gas flow, 55 arb (arbitrary unit); aux gas flow rate, 15 arb; capillary temperature, 340°C; capillary voltage, 50 V; tube lens, 80V.

6.2.7. Statistical Analysis

The statistical analysis was performed by one-way ANOVA using Minitab® 16.2.3 (copyright © 2012 Minitab Inc., Philadelphia, USA). The results were reported as mean ± standard deviation values. Differences among treatment, control and standard data are considered significant at $p < 0.05$ using Tukey's test.

6.3. Results and Discussion

6.3.1. Plant Growth Parameters Profiles

In the fields, the emergence of studied plant species was recorded as shown in Table 14. In comparison with field 1, the interference of *M. vaginalis* in field 2 reduced the growth of *O. sativa*. The percentage inhibition of root, shoot and DW of *O. sativa* was 2.47, 14.00 and 39.78%, respectively. By contrast, the magnitude of *M. vaginalis* was strongly inhibited by *O. sativa* in field 2 as inhibitory percentages of root, shoot and DW were 21.99, 42.22, 19.05%, respectively. In the field 4, the *M. vaginalis* was also competed by *Eleocharis*, the corresponding inhibitory parameters of root, shoot and DW were 15.55, 12.07, and 1.59%.

6.3.2. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of Rice and two Types of Weed Species

In this study, the TPC and TFC of EtOAc extract from rice plants (field 1) were increased when crop infested by weed (field 2) 43% and 9%, while that of hexane and water were reduced. The TPC and TFC of extracts from weed (field 3) were reduced by interference of rice plants in field 2 (Hexane extract: 95%, EtOAC extract: 39%, Aqueous extract: 79%). The presence of weed 2 in field 4 reduced the TPC and TFC of hexane and water extracts from weed 1, whilst that of EtOAc was slightly increased.

Table 14. Plant growth parameters (root, shoot, and dry weight)

Sample	*Oryza sativa* (Koshihikari)			*Monochoria vaginalis*			*Eleocharis*		
	Root (cm)	Shoot (cm)	DW (g)	Root (cm)	Shoot (cm)	DW (g)	Root (cm)	Shoot (cm)	DW (g)
Field 1	14.57 ± 0.04	59.57 ± 0.42	1.86 ± 0.04	-	-	-	-	-	-
Field 2	14.21 ± 0.26	51.23 ± 0.51	1.12 ± 0.25	5.57 ± 0.35b	10.10 ± 0.50c	0.51 ± 0.01b	-	-	-
Field 3	-	-	-	7.14 ± 0.09a	17.48 ± 0.42a	0.61 ± 0.03a	-	-	-
Field 4	-	-	-	6.03 ± 0.13b	15.37 ± 0.15b	0.62 ± 0.06a	8.64 ± 0.08	37.53 ± 0.25	0.08 ± 0.01
Field 5	-	-	-	-	-	-	-	-	-

Values are means ± SD. Mean values with different lowercase letters indicate significant differences in the same column ($p < 0.05$) ($n = 3$)

Table 15. Total phenolic content (TPC) and total flavonoid content (TFC) of rice and two types of weed strains in different extraction

Sample		TPC (mg GAE/g DW)			TFC (mg RE/g DW)		
		O. sativa	***M. vaginalis***	***Eleocharis***	***O. sativa***	***M. vaginalis***	***Eleocharis***
Field 1	Hexane	2.74 ± 0.22d	-	-	25.78 ± 1.71ab	-	-
	EtOAc	47.62 ± 2.38c	-	-	23.54 ± 2.33ab	-	-
	Aqueous	91.90 ± 3.08a	-	-	20.73 ± 1.79b	-	-
Field 2	Hexane	0.38 ± 0.03d	0.41 ± 0.05f	-	12.69 ± 1.31c	14.98 ± 2.31c	-
	EtOAc	81.79 ± 1.42b	34.71 ± 1.65c	-	27.05 ± 3.73a	31.12 ± 3.60b	-
	Aqueous	50.48 ± 0.74c	14.90 ± 0.86d	-	20.55 ± 0.92b	4.17 ± 0.43d	-
Field 3	Hexane	-	8.43 ± 0.54e	-	-	33.78 ± 2.01b	-
	EtOAc	-	56.90 ± 1.15b	-	-	45.78 ± 6.53a	-
	Aqueous	-	68.98 ± 2.07a	-	-	10.76 ± 0.69cd	-
Field 4	Hexane	-	7.50 ± 1.24e	2.67 ± 0.54b	-	25.22 ± 1.78b	22.20 ± 2.27a
	EtOAc	-	67.98 ± 1.44a	54.36 ± 0.99a	-	45.89 ± 3.98a	20.73 ± 1.52a
	Aqueous	-	53.62 ± 2.21b	53.74 ± 0.79a	-	9.75 ± 0.90cd	12.87 ± 0.92b
Field 5	Hexane	-	-	-	-	-	-
	EtOAc	-	-	-	-	-	-
	Aqueous	-	-	-	-	-	-

Values are means ± SD. Mean values with different lowercase letters indicate significant differences in the same column ($p < 0.05$) ($n = 3$)

6.3.3. Effects of Wet Soil Extracts on the Growth of Several Plant Species

The influence of the various wet soil extracts against the emergence of *Oryza sativa* (Koshihikari), *Lactuca sativa, Raphanus sativus, Echinochloa crus-galli,* and *Bidens pilosa* is shown in Table 16. EtOAc and hexane extracts of soil from fields 1 and 2 (containing rice plants) exerted the strong inhibition against the growth of test plants, except for rice itself was not much influenced. The emergence of rice was reduced by EtOAc and hexane extracts of weed-infested soils from fields 3 and 4. EtOAc and hexane extracts obtained markedly higher suppression against the growth of *Raphanus sativus* and *Lactuca sativa* (field 1, 2, 3 and 4). All extracts of abandoned field (field 5) showed negligible inhibitory effect to all plants.

6.3.4. Phytochemicals in Wet Soils

The GC-MS analysis had quantitatively identified 14 compounds were detected and identified in extracts from wet soils by GC-MS (Table 17). The majority of compounds were long-chain fatty acids, phenolic acids, terpenes. Most of identified compounds were comprised in all EtOAc extracts and hexane extracts of field 1, 2.

Table 16. Effects of different extraction from wet soil on growth of *Oryza sativa* (Koshihikari), *Lactuca sativa, Raphanus sativus, Echinochloa crus-galli,* and *Bidens pilosa*

Sample (Wet soil)		**IC_{50} µg/mL**									
		Oryza sativa (Koshihikari)		*Lactuca sativa*		*Raphanus sativus*		*Echinochloa crus-galli*		*Bidens pilosa*	
		Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Field 1	Hexane	880.8 ± 9.5 g	1735.0 ± 7.2 b	139.2 ± 9.6 e	37.5 ± 2.0 hi	189.2 ± 9.3 hi	87.8 ± 2.0 i	464.8 ± 9.8 h	132.4 ± 7.1 j	245.4 ± 10.4 j	511.3 ± 11.8 i
	EtOAc	1670.2 ± 15.3 c	854.5 ± 10.5 g	191.2 ± 8.5 c	25.2 ± 7.2 i	291.2 ± 8.8 e	125.2 ± 7.2 h	1848.4 ± 8.4 a	239.6 ± 8.5 hij	354.9 ± 7.4 hi	337.5 ± 5.3 j
	Aqueous	1610.0 ± 13.2 d	761.5 ± 9.0 h	769.7 ± 13.1 a	1572.0 ± 9.0 a	799.6 ± 13.2 c	1573.3 ± 9.3 a	843.6 ± 13.2 d	1641.7 ± 11.7 a	445.4 ± 7.0 fg	928.0 ± 10.5 fg
Field 2	Hexane	1655.3 ± 15.3 c	1888.0 ± 4.4 a	142.12 ± 13.1 de	286.4 ± 4.6 d	162.1 ± 13.9 i	276.4 ± 17.1 e	143.5 ± 13.9 j	287.8 ± 4.6 gh	413.4 ± 40.0 g	861.3 ± 8.3 g
	EtOAc	2590.8 ± 11.5 a	1352.3 ± 10.0 c	158.7 ± 9.2 de	44.4 ± 6.0 h	218.6 ± 9.9 fgh	104.8 ± 6.0 hi	1564.6 ± 46.2 b	425.2 ± 7.3 f	450.6 ± 13.3 efg	938.7 ± 7.7 efg
	Aqueous	2088.3 ± 11.5 b	1154.3 ± 12.6 d	678.7 ± 15.1 b	905.0 ± 4.0 c	678.7 ± 15.2 d	907.7 ± 6.1 c	752.7 ± 15.2 e	979.0 ± 4.0 c	469.7 ± 9.5 def	978.5 ± 9.7 def
Field 3	Hexane	1125.6 ± 15.3 e	649.1 ± 6.7 j	137.9 ± 5.4 e	85.86 ± 4.3 g	227.9 ± 5.5 f	175.6 ± 4.3 g	1310.2 ± 51.9 c	813.3 ± 11.1 d	503.1 ± 19.9 de	1048.1 ± 9.5 de
	EtOAc	1580.0 ± 15.3 d	717.7 ± 5.1 i	99.6 ± 0.5 f	54.3 ± 5.9 h	219.6 ± 2.5 fg	174.0 ± 5.9 g	647.3 ± 3.5 f	351.3 ± 8.2 fg	341.8 ± 1.8 i	712.1 ± 3.8 h
	Aqueous	St*	St*	190.7 ± 9.0 c	1195.5 ± 4.4 b	190.7 ± 9.7 ghi	1195.5 ± 4.4 b	224.7 ± 9.0 i	1229.5 ± 4.4 b	647.0 ± 26.0 b	1347.9 ± 8.2 b
Field 4	Hexane	951.3 ± 15.3 f	1045.3 ± 12.8 e	170.1 ± 8.4 cd	108.9 ± 0.1 f	170.1 ± 8.8 i	118.9 ± 17.3 h	425.3 ± 21.1 h	272.3 ± 5.0 ghi	408.3 ± 20.3 gh	850.6 ± 9.2 g
	EtOAc	711.0 ± 17.3 h	356.3 ± 11.5 k	170.0 ± 9.6 cd	52.1 ± 7.6 h	240.0 ± 9.7 f	122.8 ± 7.6 h	544.1 ± 31.0 g	168.8 ± 7.2 ij	522.4 ± 29.7 cd	1088.3 ± 12.0 cd
	Aqueous	St*	991.6 ± 8.7 f	656.9 ± 5.7 b	216.5 ± 9.4 e	656.9 ± 5.8 d	229.5 ± 14.0 f	788.3 ± 6.9 de	259.4 ± 11.2	756.8 ± 6.7 a	1576.6 ± 11.9 a
Field 5	Hexane	2580.1 ± 13.2 a	1748.4 ± 11.8 b	755.2 ± 12.9 a	1192.7 ± 10.1 b	1138.8 ± 12.4 a	1188.8 ± 12.4 b	1307.7 ± 13.6 c	1315.1 ± 4.8 b	564.9 ± 5.9 c	1176.9 ± 12.2 c
	EtOAc	2152.5 ± 12.9 b	1361.1 ± 14.1 c	657.8 ± 7.4 b	920.1 ± 6.2 c	878.0 ± 7.2 b	742.8 ± 7.6 d	811.8 ± 7.9 de	580.3 ± 8.3 e	350.7 ± 3.4 i	730.6 ± 7.1 h
	Aqueous	St*	St*	St*	1598.4 ± 12.7 a	St*	1588.2 ± 8.9 a	St*	St*	St*	St*

*Stimulation

Table 17. Principal compounds identified in wet soils by GC-MS

Compounds	Formula	Field 1			Field 2			Field 3			Field 4		
		H	E	A	H	E	A	H	E	A	H	E	A
1,2-Ethanediol	$C_2H_6O_2$	-	-	-	-	-	-	-	-	-	-	-	+
Pentanoic acid, 3-hydroxy-, methyl ester	$C_6H_{12}O_3$	+	+	+	+	+	+	-	+	+	-	+	-
Hexanoic acid	$C_6H_{12}O_2$	-	-	-	-	-	-	-	+	-	-	-	-
Pentanoic acid, 3-hydroxy-2-methyl-, methyl ester	$C_7H_{14}O_3$	-	-	-	-	+	-	-	+	-	-	-	-
Hexanoic acid, 3-hydroxy-, methyl ester	$C_7H_{14}O_3$	-	-	-	-	+	-	-	-	-	-	-	-
Pentanoic acid, 3-hydroxy-, methyl ester	$C_6H_{12}O_3$	+	+	-	+	+	-	-	+	-	-	+	-
Furan, tetrahydro-2,2,5,5-tetramethyl-	$C_8H_{16}O$	+	+	+	-	+	-	-	-	-	-	-	-
2-Pentenoic acid, 2-methoxy-4-methyl-, methyl ester	$C_8H_{14}O_3$	+	+	-	+	+	-	+	+	-	+	+	-
1,3-Dioxan-4-one, 2-(1-methylethyl)-5-methyl	$C_8H_{14}O_3$	+	-	-	-	-	-	-	-	-	-	-	-
Pentanoic acid, 3-hydroxy-, methyl ester	$C_6H_{12}O_3$	+	+	+	+	+	+	-	-	+	+	+	-
Pentanedioic acid, 2,2-dimethyl-, dimethyl ester	$C_9H_{16}O_4$	+	-	-	-	-	-	-	-	-	-	-	-
Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	-	-	-	+	-	-	-	-	-	-	-	-

H: Hexane, E: Ethyl acetate, A: Aqueous. +: detected, –: not detected

6.3.5. Identification of Momilactones A and B in Wet Soil Extracts

The presence of two momilactones MA and MB in different wet soil extracts from five studied fields was identified by HPLC and UPLC analyses. The isolated momilactones MA and MB of our laboratory were used as standards. Among studied fields, the highest amounts of MA and MB were detected in field 1. The obtained results show that the phytoalexins MA and MB were the most concentrated in EtOAc fractions, followed by hexane, whilst that of aqueous fractions was negligible. Of them, the content of MB was in a greater amount than MA in all tested extracts, except for HPLC analysis of filed 1. The analysis results reveal that the use of UPLC detected significantly higher the amounts of MA and MB as compared with HPLC. In the field 5, the constituent MA was only observed in hexane and EtOAc fractions by UPLC method. Furthermore, limit of detection and quantification parameters measured by HPLC and UPLC were also determined as (LOD = 0.199, 0.097; LOQ = 0.603, 0.293 ng/mL for MA) and (LOD = 0.184, 0.157; LOQ = 0.557, 0.476 ng/mL for MB, respectively (Table 18)

Table 18. MA and MB in different extractions from wet soil

Sample (Wet soil)		HPLC (ng/g FW)		UPLC (ng/g FW)	
		MA	**MB**	**MA**	**MB**
Field 1	Hexane	64.91 ± 1.92 a	12.92 ± 1.22 a	73.48 ± 1.01 b	136.73 ± 1.29 b
	EtOAc	13.38 ± 0.84 b	12.41 ± 1.28 a	103.76 ± 0.99 a	185.97 ± 1.14 a
	Aqueous	-	-	-	-
Field 2	Hexane	6.92 ± 0.20 c	9.87 ± 1.07 b	70.44 ± 0.21 c	125.09 ± 0.25 c
	EtOAc	5.52 ± 0.11 cd	6.96 ± 0.86 cd	61.19 ± 0.58 d	100.36 ± 0.83 d
	Aqueous	-	-	-	-
Field 3	Hexane	1.48 ± 0.05 e	4.77 ± 0.44 d	34.57 ± 0.05 h	61.11 ± 0.18 h
	EtOAc	5.95 ± 0.16 c	1.29 ± 0.03 e	40.88 ± 0.98 f	69.50 ± 1.42 f
	Aqueous	-	-	-	-
Field 4	Hexane	1.56 ± 0.18 e	3.56 ± 0.62 de	37.65 ± 0.64 g	63.83 ± 0.13 g
	EtOAc	3.56 ± 0.62 de	1.65 ± 0.22 e	47.02 ± 0.27 e	86.16 ± 0.32 e
	Aqueous	-	-	-	-
Field 5	Hexane	-	-	34.40 ± 0.03 h	-
	EtOAc	-	-	41.21 ± 0.31 f	-
	Aqueous	-	-	-	-
LOD (ng/mL)		0.199	0.184	0.097	0.157
LOQ (ng/mL)		0.603	0.557	0.293	0.476

Values are means ± SD. Mean values with different lowercase letters indicate significant differences in the same column ($p < 0.05$) ($n = 3$)

6.3.6. Quantification of Momilactones in Rice Plants

The contents of MA and MB of rice plants varied between fractions, fields and analysis methods (Table 19). The quantification of MA and MB detected by UPLC (from 309.8 - 2560.8 ng/g DW) was significantly superior than that of HPLC (from 103.2 - 881.2 ng/g DW). By using UPLC, MA was greater in quantity than MB. Additionally, the two momilactones were enriched in EtOAc fractions in both studied fields, followed by hexane. But in contrast, higher amounts of MB were identified than MA by HPLC. The highest contents of these phytoalexins were found from hexane fractions in HPLC analysis. Momilactones MA and MB were not detected in the aqueous fractions.

Table 19. MA and MB in different extractions from rice

Sample (Rice-whole plant)		HPLC (ng/g DW)		UPLC (ng/g DW)	
		MA	**MB**	**MA**	**MB**
Field 1	Hexane	104.6 ± 4.8	881.2 ± 9.9 a	1268.2 ± 1.1 b	1219.6 ± 0.9 b
	EtOAc	509.8 ± 9.2	766.3 ± 5.7 b	2560.8 ± 2.5 a	2529.6 ± 1.7 a
	Aqueous	-	-	-	-
Field 2	Hexane	68.3 ± 2.2	244.0 ± 6.8 c	1162.1 ± 1.3 c	1155.6 ± 1.4 c
	EtOAc	13.1 ± 1.0	103.2 ± 2.7 d	310.1 ± 1.5 d	309.8 ± 0.6 d
	Aqueous	-	-	-	-

Values are means ± SD. Mean values with different lowercase letters indicate significant differences in the same column ($p < 0.05$) ($n = 3$)

6.4. Discussion

The result of field study showed that rice and weed exerted strong mutual inhibition as their growth parameters were reduced 2.47-42.22%. The EtOAc and hexane extracts from paddy soils strongly inhibited the growth of tested plants, whereas that of rice itself (in field 1 and 2) was not much influenced. The identified compounds mostly belong to EtOAc and hexane extracts of plants, which suggest that they might be secreted by roots and potentially involved in the suppressing activity of soil extracts. Competitive stress can increase the production of allelochemical (Trezzi *et al*., 2018). In this study, the TPC and TFC of EtOAc extract from rice plants (field 1) were increased when crop infested by weed (field 2). This suggests that EtOAc extract contains high concentration of phenolic acids and flavonoids which were potentially involved in herbicide activity of rice plants. Some of those compounds might be identified (Table 17). Further isolation of allelochemicals from EtOAc extract of rice should be conducted. Those compounds might possess high potential to be used for herbicide synthesis.

The presence of phytoalexins MA and MB were only identified in rice plants. These compounds were not found in weed extracts. The released levels of MA and MB from rice roots into soil measured by UPLC/ESI-MS were 5-9% (field 1) and 10-60% in the field 2. This suggests that the interference of weed *Monochoria vaginalis* in the field 2 as a competitor which significantly boosted exudation of MA and MB into soil. The small amounts of MA and MB were detected in non-rice fields (including abandoned field), which suggest that the

retention of MA and MB in soil was in a long time. However, they were all in low doses in the soils, which is not sufficient to exhibit inhibition against tested plants.

6.5. Conclusions

It has long been known that allelopathic rice seedlings may suppress the growth of neighboring or successive plants through production and release of allelochemicals to environment. However, the identification of allelochemicals involved in rice allelopathic still remains as a controversial issue. This study showed that momilactones A and B and phenols may act as potent allelochemicals participating in defences of rice against *Oryza sativa* (Koshihikari), *Lactuca sativa, Raphanus sativus, Echinochloa crus-galli,* and *Bidens pilosa.*

CHAPTER VII. GENERAL DISCUSSION

7.1. Discussion

Natural compounds are considered to be more environmentally benign than most synthetic herbicides (Duke *et al*., 2000). In many cases, natural compounds are also highly active at a molecular target site (Streibig *et al*., 1999). Momilactones A and B (MA and MB) may have potential as a template for the development of new plant control substances due to their selective inhibitory activities for weed plant species. More importantly, identification of momilactones A and B as allelochemicals in rice provides a molecular marker for breeding and/or engineering efforts directed at increasing allelopathic activity of this critical staple food crop (Kato-Noguchi *et al*., 2013).

To date, the biological activities of MA and MB have been limited to allelopathy, antioxidant, antifungal, antimicrobial, and antitumor activities. The medicinal and pharmaceutical properties of MA and MB are not well known because isolation and purification of MA and MB are complicated and laborious. This has prevented us from understanding the physiological roles of these phytochemicals and exploiting their potential value. In this study, a protocol of isolation and purification was established to enrich and optimize quantities of MA and MB in rice husks. The results highlight that temperature treatment at 100 °C combined with the extracting solvents EtOAc and MeOH improve yields of MA and MB by 10-fold. Pure MA and MB separation was achieved by column chromatography, as shown in the experimental part. The findings of this study provide an effective and practical method, which will further aid exploitation of biological activities of MA and MB in the future. The antihyperuricemic property of MA and MB was also examined in this study. By combination of MA and MB at a ratio of 4:1, the inhibitory effect on the growth of xanthine oxidase enhanced 1.5–2 fold. The presence of these compounds might also involve in antigout capacity and natural herbicidal property of rice leaf essential oil (EO).

The release level of MA and MB from rice cultivars probably affects the allelopathic potential of the rice plants because MA and MB may act as a potent allelochemical (Kato-Noguchi *et al*., 2002; Kato-Noguchi and Ino, 2003a, b). In fact, the release levels of MA and MB from rice and allelopathic potential (Kato-Noguchi and Ino, 2001) seem to be closely correlated. Many researchers have observed differences in allelopathic potential

between rice cultivars (Dilday *et al*., 1998; Kim *et al*., 1999; Olofsdotter *et al*., 1999; Azmi *et al*., 2000). It is possible that the release level of MA and MB into culture solution or the neighboring environment accounts for the allelopathic action of rice. Although the mechanisms of exudation are not well understood, plants may secrete a wide variety of compounds from root cells by plasmalemma-derived exudation, endoplasmic-derived exudation, and proton-pumping mechanisms (Hawes *et al*., 2000, Bais *et al*., 2004, Badri and Vivanco, 2009). Through the secretion of compounds, plants are able to change the chemical and physical properties that regulate the soil microbial community and inhibit the growth of other plants (McCully, 1999, Hawes *et al*., 2000, Bais et al., 2004, Badri *et al*., 2009). As MA and MB are phytoalexin and allelopathic, the increasing secretion of MA and MB may be associated with the activation of the defense responses of rice in the rhizosphere, where plants must compete with invading root systems of neighboring plants, prevent bacterial and fungal infections. Thus, MA and MB may provide a competitive advantage for rice to form a pure colony through the prevention of bacterial and fungal infections, and by inhibiting the growth of competitive plant species.

This study provides practical methods to exploit additional value from the waste of rice production, which may provide extra benefits for rice farmers. The presence of MA and MB in various parts of the rice plant might partly contribute to its antioxidant, antibacterial and antigout properties, as well as weed management.

7.2. Conclusions

The findings of this study suggest that the quantity of waste generated as a byproduct of paddy rice production can be reduced when rice husk is exploited for functional food and pharmaceutical purposes, as it has been shown to possess rich and safe antioxidants, and antimicrobials. Momilactones A and B from rice husk have the potential to be used as a natural xanthine oxidase inhibitor in dietary supplements for hyperuricemia and gout. Rice leaves and rice husk may be a promising material in the pharmaceutical industry, particularly through have advantages for antioxidant and antihyperuricemia. Especially in the context of concerns about sustainable development, this research provides biological evidence for practical applications and further help to promote the high economic value for this abundant agricultural waste. However, future studies are needed to elucidate the capacity of each component, as well as the molecular interactions within the rice leaf essential oil to determine the mode of actions.

7.3. New Findings

- ✓ The use of temperature at 100 °C for >2 h, combined with either EtOAc or 10% MeOH can optimize the chemical components and biological activity of rice husk.
- ✓ Momilactones A and B cannot be isolated or purified without using MeOH.
- ✓ The use of dried (100 °C, 1 h) rice husk combined with MeOH 100% provided maximum yields of MA and MB.
- ✓ EO from rice leaves contained rich phytochemicals which were potent in antioxidants and suitable for treatment of gout, as well as weed management
- ✓ Two phytoalexin MA and MB were identified for the first time in EO using UPLC/ESI-MS
- ✓ This is the first study to report xanthine oxidase inhibitory activity of MA and MB. A combination of MA and MB (4:1) was the most useful for gout treatment.
- ✓ The exudation levels of MA and MB in soil were measured for the first time. These phytoalexins can be stored in soil for a long time, and reduce the amount of *Echinochloa crus-galli, Bidens pilosa, Raphanus sativus,* and *Lactuca sativa,* of which the root of *E. crus-galli* was most strongly inhibited (IC_{50} = 455.6 ppm). But, the growth of rice seedlings was slightly promoted by treating with its own EO.
- ✓ The EO of rice leaf was beneficial as a source of antioxidants and reduced gout disease, and thus provides extra benefits for rice farmers in developing countries.

REFERENCES

Adam, F.; Iqbal, A. The oxidation of styrene by chromium-silica heterogeneous catalyst prepared from rice husk. *Chem. Eng. J.* **2010**, *160*, 742-750.

Aiyaz, M.; Divakara, S.T.; Chandranayaka, S.; Niranjana, S.R. Efficacy of seed hydropriming with phytoextracts on plant growth promotion and antifungal activity in maize. *Int. J. Pest Manag.* **2015**, *2*, 153-160.

Akobundu, I.O. *Weed Science in the Tropics: Principles and Practices*. Wiley and Sons, Chichester, UK. 1987.

Ali, S.Z.; Bhattacharya, K.R. Changes in sugars and amino acids during parboiling of rice. *J. Food Biochem.* **1980a**, *4*, 169-179.

Ali, S.Z.; Bhattacharya, K.R. Comparative properties of beaten rice and parboiled rice. *Lebensmit. Wiss. Technol.* **1976**, *9*, 11-13.

Ali, S.Z.; Bhattacharya, K.R. High-temperature-drying-cum-par-boiling of paddy. *J. Food Process Engr.* **1980b**, *4*,123-126.

Ali, S.Z.; Bhattacharya, K.R. Pasting behavior of parboiled rice. *J. Texture Stud.* 1980c, *11*, 239-245.

Ali, S.Z.; Bhattacharya, K.R. Studies on pressure parboiling of rice. *J. Food Sci. Technol*. **1982**, *19*, 236-242.

Alsaadawi, I.S. Allelopathic influence of decomposing wheat residues in agroecosystem. *J. Crop Prod.* **2001**, *4*, 185-196.

Amic, D.; Davidovic-Amic, D.; Beslo, D.; Rastija, V.; Lucic, B.; Trinajstic, N. SAR and QSAR of the antioxidant activity of flavonoids. *Curr. Med. Chem.* **2007**, *14*, 827-845.

Andreoli, M.; Luca, G.T.; Seo, E.S.M. Characteristics of rice husk for chlorination reaction. *Mater. Lett.* **2000**, *44*, 294-298.

Anitha, A.; Rabeeth, M. Degradation of fungal cell walls of phytopathogenic fungi by lytic enzyme of Streptomyces griseus. *Afr. J. Plant Sci.* **2010**, *4*, 61-66.

Ansaldi, M.; Marolt, D.; Stebe, T.; Mandic-Mulec, I.; Dubnau, D. Specific activation of the *Bacillus* quorum-sensing systems by isoprenylated pheromone variants. *Mol. Microbiol.* **2002**, *44*, 1561-1573.

Anthoni, R.S.; Singaravadivel, K. Reduction in free fatty acids due to parboiling of paddy. *J.*

Food Sci. **1982**, *47*, 692-693.

Appanna, V.D.; Whitmore, L. Biotransformation of zinc by Pseudomonas fluorescens. *Microbios* **1995**, *82*, 149-155.

Applewaite, T.W. *Bailey's industrial oil and fat products, new york: Wiley-interscience*. 1985. Vol. 3.

Arora, N.K.; Kang, S.C.; Maheshwari, D.K. Isolation of siderophore producing strain of *Rhizobium meliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of groundnut. *Curr. Sci.* **2001**, *25*, 674-677.

Badri, D.V.; Vivanco, J.M. Regulation and function of root exudates. *Plant Cell Environ.* **2009**, *32*, 666-81.

Bais, H.P.; Park, S.W.; Weir, T.L.; Callaway, R.M.; Vivanco, J.M. How plants communicate using the underground information superhighway. *Trends Plant Sci.* **2004**, *9*, 26-32.

Banerjee, S.; Mazumdar, S. Electrospray ionization mass spectrometry: A technique to access the information beyond the molecular weight of the analyte. *Int. J. Anal. Chem.* **2012**, *2012*, 40 pages.

Bansal, M.; Garg, U.; Singh, D.; Garg, V.K. Removal of Cr (VI) from aqueous solutions using pre-consumer processing agricultural waste: a case study of rice husk. *J. Hazard. Mater.* 2**009**, *162*, 312-320.

Bartik, K.; Braekman, J.-C.; Daloze, D.; Stoller, C.; Huysecom, J.; Vandevyer, G.; Ottinger, R. Topsentins, new toxic bis-indole alkaloids from the marine sponge *Topsentia genitrix*. *Can. J. Chem.* **1987**, *65*, 2118-2121.

Bassolé, I.H.N.; Juliani, H.R. Essential oils in combination and their antimicrobial properties. *Molecules* **2012**, *17*, 3989-4006.

Bauer, A.W.; Kirby, W.M.M.; Sherris, J.C.; Turck, M. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clinical Pathol.* **1966**, *45*, 493-496.

Beauchemin, K.A.; McGinn, S. Methane emissions from beef cattle: effects of fumaric acid essential oil and canola oil. *J. Anim. Sci.* **2006**, *84*, 1489-1592.

Belter, P.A.; Cussler, E.L.; Hu.; W.S. *Bioseparations.* Wiley, New York. 1988.

Bi, H.H.; Zeng, R.S.; Su, L.M.; An, M.; Luo, S.M. Rice allelopathy induced by methyl jasmonate and methyl salicylate. *J. Chem. Ecol.* **2007**, *33*, 1089-1103.

Bridgwater, A.V. The technical and economic feasibility of biomass gasification for power

generation. *Fuel* **1995**, *74*, 631-653.

Bridgwater, A.V.; Meier, D.; Radlein, D. An overview of fast pyrolysis of biomass. *Org. Geochem*. **1999**, *30*, 1479-1493.

Bristow, P.A. *LC in iractice, hetp*. Cheshire, UK. 1976.

Burgess, S.S.O.; Adams, M.A.; Turner, N.C.; Ong, C.K. The redistribution of soil water by tree root systems. *Oecologia* **1998**, *115*, 306-311.

Busca, G.; Berardinelli, S.; Resini, C.; Arrighi, L. Technologies for the removal of phenol from fluid streams: a short review of recent developments. *J. Hazard. Mater.* 2**008**, *160*, 265-288.

Butler, M.S.; Robertson, A.A.B.; Cooper, M.A. Natural product and natural product derived drugs in clinical trials. *Nat. Prod. Rep.* **2014**, 31, 1612-1661.

Caccamese, S.; Azzolina, R. Screening for antimicrobial activities in marine algae from eastern Sicily. *Planta Med.* **1979**, *37*, 333-339.

Caillat, S.; Vakkilainen, E. Large-scale biomass combustion plants: an overview. Biomass Combustion Science. *Tech. Eng*. **2013**, *2013*, 189-224.

Caldwell, M.M.; Dawson, T.E.; Richards, J.H. Hydraulic lift: Consequences of water efflux from the roots of plants. *Oecologia* **1998**, *113*, 151-161.

Cha, H.M.; Han, G.; Chung, H.J. A study on the trend analysis regarding the rice consumption of Korean adults using Korean National Health and Nutrition Examination Survey data from 1998, 2001 and 2005. *Nutr. Res. Pract.* **2012**, *6*, 254-262.

Champagne, E.T. Rice aroma and flavor: a literature review. *Cereal Chem.* **2008**, *85*, 445-454.

Chandler, R.F. *Rice in the tropics: a guide to the development of national programs*. Colorado, Western United States, 1979.

Chang, F.-W.; Hsiao, T.-J.; Chung, S.-W.; Lo, J.-J. Nickel supported on rice husk ash—activity and selectivity in CO2 methanation. *Appl. Catal. A Gen.* **1997**, *164*, 225-236.

Charles, D.J.; Simon, J.E. Comparison of extraction methods for the rapid determination of essential oil content and composition of basil. *J. Am. Soc. Hortic. Sci.* **1990**, *115*, 458–462.

Chatterjee, A.; Tandon, S.; Ahmad, A. Comparative extraction and downstream processing techniques for quantitative analysis of rosmarinic acid in Rosmarinus officinalis.

Asian J. Chem. **2014**, *26*, 4313-4318.

Chen, L.Y.; Yin, H.F.; Lan, Z.; Ma, S.W.; Zhang, C.F.; Yang, C.L.; Li, P.; Lin, B.Q. Anti-hyperuricemic and nephroprotective effects of *Smilax china* L. *J. Ethnopharmacol*. **2011**, *135*, 399-405.

Chiang, H.C.; Lo, Y.J.; Lu, F.J. Xanthine-oxidase inhibitors from the leaves of *Alsophila spinulosa* (hook) *tryon*. *J. Enzym. Inhib*. **1994**, *8*, 61-71.

Chin, Y.W.; Kinghorn, A.D. *Natural products*. In Schwab M (ed) Encyclopedia of cancer, 2nd edn. Springer, Heidelberg, 2008, Part 14 (N), pp 2032-2034.

Chisaka, H. Competition between rice plants and weeds. *Weed Res. Jpn.* **1966**, *5*, 16-22.

Chou, C-H.; Lin, H-J. Autointoxication mechanism of *Oryza sativa* L. phytotoxic effects of decomposing rice residues in soil. *J. Chem. Ecol.* **1976**, *2*, 353-367.

Chung, I.M.; Ahn, J.K.; Yun, S.J. Identification of allelopathic compounds from rice (*Oryza sativa* L.) straw and their biological activity. *Can. J. Plant Sci.* **2001**, *81*, 815-819.

Colegate, S.M.; Molyneux, R.J. *Bioactive natural products: Detection, isolation and structural determination*. CRC, Boca Raton, FL. eds. 1993.

Cragg, G.M.; Kingston, D.G.I.; Newman, D.J. *Anticancer agents from natural products*. 2nd edn. CRC Press, Boca Raton. 2012.

Demirbaş, A. Biomass resource facilities and biomass conversion processing for fuels and chemicals. *Energy Convers. Manag*. **2001**, *42*, 1357-1378.

Djilani, A.; Dicko, A. The therapeutic benefits of essential oils. *In Nutrition, well-being and health*; Edited by Dr. Jaouad Bouayed; Publisher InTech, China, **2012**; 224 pages.

Duke, S.O.; Dayan, F.E.; Romagni, J.G.; Rimando, A.M. Natural products as sources of herbicide, current status and future trends. *Weed Res.* **2000**, *40*, 99-111.

Ebana, K.; Yan, W.G.; Dilday, R.H.; Namai, H.; Okuno, K. Analysis of QTL associated with the allelopathic effect of rice using water-soluble extracts. *Breed Sci*. **2001**, *51*, 47-51.

Farmer, E.E. Surface-to-air signals. *Nature* **2001**, *411*, 854.

Faulkner, D.J. Marine natural products, *Nat. Prod. Rep.* **1986**, *3*, 1-33.

Faulkner, D.J. Marine natural products: Metabolites of marine algae and herbivorous marine molluscs, *Nat. Prod. Rep.* **1984a**, *1*, 251-280.

Faulkner, D.J. Marine natural products: metabolites of marine marine invertebrates. *Nat. Prod. Rep.* **1984b**, *1*, 551-598.

Feng, Y.; Shen, D.; Song, W. Rice endophyte *Pantoea agglomerans* YS19 promotes host plant

growth and affects allocation of host photosynthesis. *J. Appl. Microbiol.* **2006**, *100*, 938-945.

Ferme, D.; Banjac, M.; Calsamiglia, S. The effects of plant extracts on microbial community structure in a rumen-simulating continuous-culture system as revealed by molecular profiling. *Folia Microbiol. 2004*, *49*, 151-155.

Flemmig, J.; Kuchta, K.; Arnhold, J.; Rauwald, H.W. *Olea europaea* leaf (Ph. Eur.) extract as well as several of its isolated phenolics inhibit the gout-related enzyme xanthine oxidase. *Phytomedicine* **2011**, *18*, 561-566.

Gandhi, K.S. Stabilization of expansive soil of surat region using rice husk ash & marble dust. *IJCET*. **2013**, *3*, 4.

Gariboldi, F. *Rice parboiling*. Food and Agriculture Organization of the United Nations, 1984.

Garrity, D.P.; Movillon, M.; Moody, K. Differential weed suppression ability in upland rice cultivars. *Agron. J.* **1992**, *84*, 586-591.

Grassmann, J. Terpenoids as plant antioxidants. *Vitam. Horm.* **2005**, *72*, 505-535.

Harada, H.; Kamei, Y. Selective cytotoxicity of marine algae extracts to several human leukemic cell lines. *Cytotechnology* **1997**, *25*, 213-219.

Hardt, I.H.; Fenical, W.; Cronin, G.; Hay, M.E. Acutilols, potent herbivore feeding deterrents from the tropical brown alga, *Dictyota acutiloba*. *Phytochemistry* **1996**, *43*, 71-73.

Hartmann, T.; Kutchan, T.M.; Strack, D. Evolution of metabolic diversity. *Phytochemistry* **2005**, *66*, 1198-1199.

Harvey, A.L.; Edrada-Ebel, R.; Quinn, R. The re-emergence of natural products for drug discovery in the genomics era. *Nat. Rev. Drug Discov.* **2015**, *14*, 111-129.

Hawes, M.C.; Gunawardena, U.; Miyasaka, S.; Zhao, X. The role of root border cells in plant defense. *Trends Plant Sci.* **2000**, *5*, 128-33.

He, H.Q.; Shen, L.H.; Xiong, J.; Jia, X.L.; Lin, W.X.; Wu, H. Conditional genetic effect of allelopathy in rice (*Oryza sativa* L.) under different environmental conditions. *Plant Growth Regul.* **2004**, *44*, 211-218.

Heath, H.B.; Reineccius, G. *Flavor chemistry and technology*. AVI-Van Nostrand Reinhold Co. New York. **1986**.

Honda, S.; Miura Y.; Masuda, A.; Masuda, T. Identification of crypto- and neochlorogenic lactones as potent xanthine oxidase inhibitors in roasted coffee beans. *Biosci. Biotechnol. Biochem*. **2014**, *78*, 2110-2116.

Horikawa, M.; Noro, T.; Kamei, Y. *In vitro* anti methicillin resistant *Staphylococcus aureus* activity found in extract of marine algae indigenous to the coastline of Japan. *J. Antibiotics* **1999**, *52*, 186-189.

Huang, S.B.; Dai, Q.J.; Peng, S.B.; Chavez, A.Q.; Miranda, L.L.; Visperas, R.M.; Vergara, B.S. Influence of supplemental ultraviolet-B on indoleacetic acid and calmodulin in the leaves of rice (*Oryza sativa* L). *Plant Growth Regul.* **1997**, *21*, 59-64.

Ikan, R. *Natural products: A laboratory guide*. Academic, San Diego, CA. 1991.

Jackson, R.B.; Sperry, J.S.; Dawson, T.E. Root water uptake and transport: using physiological processes in global predictions. *Trends Plant Sci.* **2000**, *5*, 482-488.

Jebakumar, S.R.D.; Kannan, N.; Nithyakalyani, R.; Jayaraman, J. A method for multiple residue analysis using HPLC with an isocratic system. *Sci. Tools.* **1984**, *31*, 3-4.

Juliano, B.O. *Rice in human nutrition*. Brisbane, Australia, 1991.

Kamra, N.D.; Agarwal, N.; Chaudhary, L.C. Inhibition of ruminal methanogenesis by tropical plants containing secondary compounds. *Int. Congr. Ser.* **2006**, *1293*, 156-163.

Karimi, E.; Jaafar, H.Z.; Ghasemzadeh, A.; Ebrahimi, M. Fatty acid composition, antioxidant and antibacterial properties of the microwave aqueous extract of three varieties of *Labisia pumila* Benth. *Biol. Res.* **2015**, *48*, 9.

Kask, K.; Kännaste, A.; Niinemets, Ü. Emission of volatile organic compounds as a signal of plant stress. *Sci. Bull. ESCORENA* **2013**, *8*, 79-92.

Kato-Noguchi, H. Convergent or parallel molecular evolution of momilactone A and B: potent allelochemicals, momilactones have been found only in rice and the moss *Hypnum plumaeforme*. *J, Plant Physiol.* **2011**, *168*, 1511-1516.

Kato-Noguchi, H.; Ota, K. Biological activities of rice allelochemicals momilactone A and B. *J. Rice Res.* **2013**, *1*, 2.

Kennedy, J.F.; Cabral, J.M.S. *Recovery processes for biological materials*. Wiley, Chicester, UK. 1993.

Kim, H.R.; Kim, K.M.; Woo, K.; Jeong, H.S.; Kim, K.O. Changes in volatile compounds of waxy rice and *gangjeong* (a traditional Korean oil-puffed snack) under different steeping conditions. *Food Sci. Biotechnol.* **2015**, *24*, 1565-1572.

Kim, K.; Harvell, C.D.; Kim, P.D.; Smith, G.W.; Merkel, S.M. Fungal disease resistance of Caribbean Sea fan corals (*Gorgonia* spp.). *Mar. Biol.* **2000**, *136*, 259-267.

Kim, K.Y.; Schumacher, H.R.; Hunsche, E. Wertheimer, A.I.; Kong, S.X. A literature review

of the epidemiology and treatment of acute gout. *Clin. Ther*. **2003**, *25(6)*, 1593-1617.

Kim, Y.H.; Kim, S. Improved abundance sensitivity of molecular ions in positive-ion APCI MS analysis of petroleum in toluene. *J. Am. Soc. Mass Spectr*. **2010**, *21*, 386-392.

Koarai, A.; Morita H. Sulfonylurea‑resistant biotypes of *Monochoria vaginalis* in paddy fields of Akita and Ibaraki Prefectures, eastern Japan. *J. Weed Sci. Technol.* **2002**, *47*, 20-28.

Kongkiattikajorn, J. Effect of storage time and temperature on volatile aroma compounds and physicochemical properties of rice. *Kasetsart J. Nat. Sci.* **2008**, *42*, 111-117.

Kubanek, J.; Jensen, P.R.; Keifer, P.A.; Sullards, M.C.; Collins, D.O.; Fenical, W. Seaweed resistance to microbial attack: A targeted chemical defense against marine fungi. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6916-6921.

Lee, S.M.; Han, H.Y.; Lee, S.J. Volatile compounds in takju (rice wine) using different types of fermentation starters. *Food Eng. Prog.* **2014**, *18*, 348-354.

Lim, J.S.; Manan, Z.A.; Alwi, S.R.W.; Hashim, H. A review on utilization of biomass from rice industry as a source of renewable energy. *Renew. Sust. Energ. Rev.* **2012**, *16(5)*, 3084-3094.

Lin, J.Y.; Fan, W.; Gao, Y.N.; Wu, S.F.; Wang, S.X. Study on volatile compounds in rice by HS-SPME and GC-MS. *Julius-Kühn-Archiv*. **2010**, *425*, 125.

Liyanaarachchi, G.D.; Kottearachchi, N.S.; Samarasekera, R. Volatile profiles of traditional aromatic rice varieties in Sri Lanka. *J. Natl. Sci. Found. Sri.* **2014**, *42*, 87-93.

M.; Ruiz-Pérez, N.J. Antimutagenic and antioxidant activity of the essential oils of *Citrus sinensis* and *Citrus latifolia*. *Sci. Rep.* **2017**, *7*, 11479.

Makkar, H.P.S.; Becker, K.; Sidhuraju, P. *Plant secondary metabolites methods in molecular biology*. Humana Press, Totowa, 2007, p 129.

Marston, A.; Hostettmann, K. Modern separation methods. *Nat. Prod. Rep.* **1991**, *8*, 391-413.

Mathieu, F.; Jouany, J.P.; Senaud, J. The effect of Saccharomyces cerevisiae and Aspergillus oryzae on fermentation in the rumen of faunated and defaunated sheep: protozoal and probiotic interaction. *Reprod. Nutr. Dev.* **1996**, *36*, 271-287.

Mattice, J.D.; Dilday, R.H.; Gbur, E.E.; Skulman, B.W. Barnyardgrass growth inhibition with rice using high performance liquid chromatography to identify rice accession activity. *Agron. J.* **2001**, *93*, 8-11.

McCully, E. Roots in soil: unearthing the complexities of roots and their rhizospheres. *Annu.*

Rev. Plant Physiol. Plant Mol. Biol. 1999, *50*, 695-718.

McGee, P. Natural products re-emerge. *Drug Disc. Develop.* **2006**, *9*, 18-26.

Minh, T.N.; Khang, D.T.; Tuyen, P.T.; Minh, L.T.; Anh, L.H.; Quan, N.V.; Ha, P.T.T.; Quan, N.T.; Toan, N.P.; Elzaawely, A.A.; *et al*. Phenolic compounds and antioxidant activity of *Phalaenopsis* orchid hybrids. *Antioxidants* **2016**, *5*, 31.

Minh, T.N.; Tuyen, P.T.; Khang, D.T.; Quan, N.V.; Ha, P.T.T.; Quan, N.T.; Yusuf, A.; Fan, X.; Van, T.M.; Khanh, T.D.; *et al*. Potential use of plant wastes of moth orchid (*Phalaenopsis* Sogo Yukidian 'V3') as an antioxidant source. *Foods* **2017**, *6*, 85.

Minh, T.N.; Xuan, T.D.; Ahmad, A.; Elzaawely, A.A.; Teschke, R.; Van, T.M. Momilactones A and B: Optimization of yields from isolation and purification. *Separations* **2018**, *5*, 28.

Minh, T.N.; Xuan, T.D.; Ahmad, A.; Elzaawely, A.A.; Teschke, R.; Van, T.M. Efficacy from different extractions for chemical profile and biological activities of rice husk. *Sustainability* **2018**, *10*, 1356.

Miyazawa, M.; Nagai, S.; Oshima, T. Volatile components of the straw of *Oryza sativa* L. *J. Oleo. Sci.* **2008**, *57*, 139-143.

Nagato, K.; Y. Kobayashi. Studies on the occurrence of notched-belly kernels (Dogire-mai) in rice plants. *Proc. Crop Sci. Soc. Japan* **1957**, *26*, 13-14.

Nakanishi, T.; Nishi, M.; Inada, H.; Obata, H.; Abe, S.; Wakashiro, M. Two new potent inhibitors of xanthine oxidase from leaves of *Perilla frutescens* Britton var. *acuta* kudo. *Chem*. *Pharm*. *Bull*. **1990**, *38*, 1772-1774.

Natarajan, E.; Nordin, A.; Rao, A.N. Overview of combustion and gasification of rice husk in fluidized bed reactors. *Biomass Bioenergy*. **1998**, *14*, 533-546.

Natori, S.; Ikekawa, N.; Suzuki, M. *Advances in Natural Products Chemistry. Extraction and Isolation of Biologically Active Compounds*. Halstead, Wiley, New York. 1981.

Newbold, C.J.; McIntosh, M.; Williams, P. Effects of a specific blend of essential oil compounds on rumen fermentation. *Anim. Feed Sci. Technol.* **2004**, *114*, 105-112.

Nguyen, M.T.T., Awale, S., Tezuka, Y., Tran, Q. Le, Watanabe, H., Kadota, S. Xanthine oxidase inhibitory activity of Vietnamese medicinal plants. *Biol. Pharm. Bull*. **2004**, *27*, 1414-1421.

Oloyede, G.K. Antioxidant activities of methyl ricinoleate and ricinoleic acid dominated *Ricinus communis* seeds extract using lipid peroxidation and free radical scavenging

methods. *Res. J. Med. Plant* **2012**, *6*, 511-20.

Patra, A.K.; Kamra, D.N.; Agarwal, N. Effect of plant extracts on *in vitro* methanogenesis enzyme activities and fermentation of feed in rumen liquor of buffalo. *Anim. Feed Sci. Technol.* **2006**, *128*, 276-291.

Pereira, H.S.; Leão-Ferreira, L.R.; Moussatché, N.; Teixeira, V.L.; Cavalcanti, D.N.; Costa, L.J.; Diaz, R.; Frugulhetti, I.C. Antiviral activity of diterpenes isolated from the Brazilian marine alga Dictyota menstrualis against human immunodeficiency virus type 1 (HIV-1). *Antiviral Res.* **2004**, *64*, 69-76.

Perez, R.M.; Avila, J.G.; Perez, S.; Martinez, A.; Martinez, G. Antimicrobial activity of some American algae. *J. Ethnopharmacol.* **1990**, *29*, 111-116.

Pickett, J.A.; Khan, Z.R. Plant volatile‐mediated signalling and its application in agriculture: successes and challenges. *New Phytol.* **2016**, *212*, 856-870.

Poole, C.K.; Poole, S.K. *Chromatography Today*. Elsevier, Amsterdam. 1991.

Prokudina, E.A.; Havlíček, L. Al-Maharik, N.; Lapčík, O.; Strnad, M.; Gruz. J. Rapid UPLC-ESI-MS/MS method for the analysis of isoflavonoids and other phenylpropanoids. *J. Food Compos. Anal.* **2012**, *26*, 36-42.

Qasem, J.R.; Foy, C.L. Weed allelopathy, its ecological impacts and future prospect: a review. *J. Crop Prod.* **2001**, *4*, 43-120.

Rasmussen, K.; Rasmussen. J. Barley seed vigour and mechanical weed control. *Weed Res.* **2000**, *40*, 219-230.

Rojas, J.J.; Ochoa, V.J.; Ocampo, S.A.; Muñoz, J.F. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. *BMC Complement Altern. Med.* **2006**, *6*, 2.

Ryan, E.P. Bioactive food components and health properties of rice bran. *J. Am. Vet. Med. Assoc*. **2011**, *238*, 593-600.

Santra, A.; Karim, S.A. Rumen manipulation to improve animal productivity. *Asian-Aust. J. Anim. Sci.* **2003**, *16*, 748-763.

Sewell, P.A.; Clarke, B. *Chromatographic separations*. Wiley, Chichester, U.K. 1987.

Silvia, A.T.; Wallace, R.J.; Ørskov, E.R. Use of particle bound microbial activity to predict the rate and extent of fibre degradation in the rumen. *Br. J. Nutr.* **1987**, *57*,407-415.

Sirisantimethakom, L.; Laopaiboon, L.; Danvirutai, P.; Laopaiboon, P. Volatile compounds of a traditional Thai rice wine. *Biotechnology* **2008**, *7*, 505-513.

Streibig, J.C.; Dayan, F.E.; Rimando, A.M.; Duke, S.O. Joint action of natural and synthetic photosystem II inhibitors. *Pest Sci.* **1999**, *55*, 137-146.

Sukhonthara, S.; Theerakulkait, C.; Miyazawa, M. Characterization of volatile aroma compounds from red and black rice bran. *J. Oleo Sci.* **2009**, *58*, 155-161.

Sutherland, S. What makes a weed a weed: life history traits of native and exotic plants in the USA. *Oecologia* **2004**, *141*, 24-39.

Suzuki, Y.; Ise, K.; Li, C.; Honda, I.; Iwai, Y.; Matsukura, U. Volatile components in stored rice [*Oryza sativa* (L.)] of varieties with and without lipoxygenase-3 in seeds. *J. Agric. Food Chem.* **1999**, *47*, 1119-1124.

Tandon, S. Pilot scale processing technology for extraction of Cliv-92: a combination of three coumari-nolignoids cleomiscosins A, B and C from Cleome viscosa. *Ind. Crops Prod.* **2010**, *31*, 335-343.

Terkeltaub, R. Update on gout: New therapeutic strategies and options. *Nat. Rev. Rheumatol.* **2010**, *6*, 30-38.

Tiwary, A.; Sharma, G. Gupta, P.K. Quantification of the reduced environmental Impacts with use of co-processing in Cement Kilns in India. *Environ. Res. Eng. Manag*. **2014**, *69*, 3.

Tsujii, S.; Rinehart, K.L.; Gunasekera, S.; Kashman, Y.; Cross, S.; Lui, M.; Pomponi, S.; Diaz, M.C. Topsentin, bromotopsentin, dihydro-deoxybromotopsentin: antiviral and antitumor bis(indolyl) imidazoles from Caribbean deep-sea sponges of the family Halichondriidae. Structural and synthetic studies. *J. Org. Chem.* **1988**, *53*, 5446-5453.

Tuyen, P.T.; Xuan, T.D.; Khang, D.T.; Ahmad, A.; Quan, N.V.; Anh, T.T.T.; Anh, L.H.; Minh, T.N. Phenolic compositions and antioxidant properties in bark, flower, inner skin, kernel and leaf extracts of *Castanea crenata* Sieb. et Zucc. *Antioxidants* **2017**, *6*, 31.

Ushida, K.; Tanaka, H.; Kjima, Y. Effect of phenolic acids on gas and volatile fatty acid production by mixed rumen population with or without protozoa. *Jpn. J. Zootech. Sci.* **1989**, *60*, 1135-1142.

Van, T.M.; Xuan, T.D.; Minh, T.N.; Quan, N.V. Isolation and purification of potent growth inhibitors from *Piper methysticum* root. *Molecules* **2018**, *23*, 1907.

Verrall, M.S. *Downstream processing of natural products*. Wiley, Chichester, U.K. **1996**.

Verrall, M.S.; Hudson, M.J. *Separations for biotechnology*. Ellis Horwood, Chicester, UK.

1987.

Villamar-Torres, R.; Mehdi, S.; Liuba-Delfini, G.; García, L.; Viot, C.R. Volatile organic compounds: plant natural defense mechanisms against herbivorous arthropods and an opportunity for plant breeding of cotton. *Sci. Agric.* **2018**, *9*, 287-297.

Wagman, G.H.; Cooper, R. Natural products isolation: Separation methods for antimicrobials, antiviral and enzyme inhibitors. *J. Chromatogr. Libr*. Elsevier, Amsterdam. **1989**, 43.

Wallace, R.J.; Arthaud, L.; Newbold, C.J. Influence of Yucca schidigera extract on ruminal ammonia concentration and ruminal micro-organisms. *Appl. Environ. Microbiol.* **1994**, *60*, 1762-1767.

Wallace, R.J.; McEwan, N.R.; McIntosh, F.M. Natural products as manipulators of rumen fermentation. *Asian-Aust. J. Anim. Sci.* **2002**, *15*, 1458-1468.

Wang, W.; Li, Y.; Dang, P.; Zhao, S.; Lai, D.; Zhou, L. Rice secondary metabolites: structures, roles, biosynthesis, and metabolic regulation. *Molecules* **2018**, *23*, 3098.

Ward, S.M.; Gaskin, J.F.; Wilson, L.M. Ecological genetics of plant invasion: what do we know? *Invasive Plant. Sci. Manage.* **2008**, *1*, 98-109.

Weinstein, M.J.; Wagman, G.H. *Antibiotics: isolation, separation, purification. J. Chromatogr. Libr.* Amsterdam. eds. **1978**, 15.

Wright, A.E.; Pomponi, S.A.; Cross, S.S.; McCarthy, P.M. A new bis(indole) alkaloid from a deep-water marine sponge of the genus *Spongosorites*. *J. Org. Chem.* **1992**, *57*, 4772-4775.

Xuan, T.D.; Minh, T.N.; Anh, L.H.; Khanh, T.D. Allelopathic momilactones A and B are implied in rice drought and salinity tolerance, not weed resistance. *Agron. Sustain. Dev*. **2016**, *36*, 52.

Xuan, T.D.; Tsuzuki, E.; Tawata, S.; Khanh, T.D. Methods to determine allelopathic potential of crop plants for weed control. *Allelopathy J.* **2004**, *13*, 149-164.

Xuan, T.D.; Tsuzuki, E.; Terao, H.; Matstuo, M.; Khanh, T.D.; Murayama, S.; Hong, N.H. Alfalfa, rice by-products and their incorporation for weed control in rice. *Weed Biol. Manag.* **2003**, *3*, 137-144.

Yajima, I.; Yanai, T.; Nakamura, M.; Sakakibara, H.; Habu, T. Volatile flavor components of cooked rice. *Agric. Biol. Chem.* **1978**, *42*, 1229-1233.

Yang, D.S.; Lee, K.S.; Jeong, O.Y.; Kim, K.J.; Kays, S.J. Characterization of volatile aroma

compounds in cooked black rice. *J. Agric. Food Chem.* **2007**, *56*, 235-240.
Yang, D.S.; Shewfelt, R.L.; Lee, K.S.; Kays, S.J. Comparison of odor-active compounds from six distinctly different rice flavor types. *J. Agric. Food Chem.* **2008**, *56*, 2780-2787.